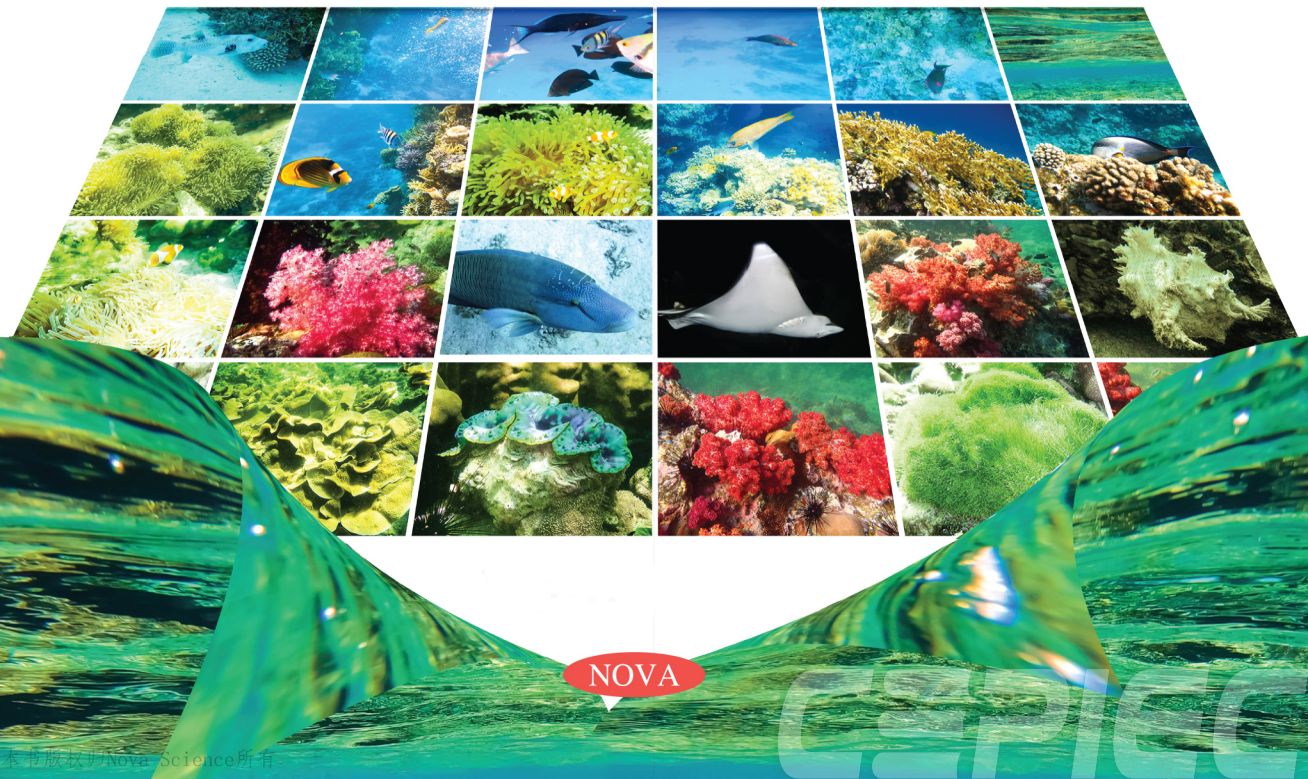
Marine and Freshwater Biology

Encyclopedia of Marine Biology

(12 Volume Set)

Steffen Fischer • Jonas Abend
Editors



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MARINE AND FRESHWATER BIOLOGY

ENCYCLOPEDIA OF MARINE BIOLOGY

VOLUME 1

(12 VOLUME SET)

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VOLUME 1

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STEFFEN FISCHER
AND
JONAS ABEND
EDITORS

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PREFACE

This 12-volume encyclopedia contains 160 chapters covering a broad range of topics related to marine biology. Subjects discussed in this encyclopedia include the ecotoxicology, genetics, and conservation of sea turtles; the ecological health of coral reefs and the species of plants and animals that inhabit them; the interaction of aquatic organisms with harmful algal blooms; the utilization of algae and seaweed for commodity chemicals, feeds, high value products, biofuels, cosmetics, fertilizers, and materials production; the biology and global distribution of tropical and subtropical copepods; the health, behavior, and factors affecting the meat quality of common carp; the prevalence of toxic metals, microplastics, and other pollutants among marine life and its environmental and ecological impact, as well as strategies for mitigation of pollution and cleanup; the distribution, ecophysiology, toxicology, and ecological impact of dinoflagellates; and the impact of climate change and fisheries on the overall marine ecosystem.

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Chapter 1

FUNCTIONAL MORPHOLOGY IN SEA TURTLE SKULLS

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ABSTRACT

The relationship between morphology, performance and ecology is critical to understand the evolutionary and ecological processes that lead to phenotype evolution. Despite the existence of a generalized life history model for sea turtles based on the biology of green turtles (*Chelonia mydas*), it should be noted that the seven species of sea turtles differ in their ecology and this in turn is reflected in their diet. Differential diets have even been reported between turtles from different populations of the same species present on the same foraging ground. Additionally, as they grow, most sea turtles present ontogenetic changes in habitat and diet, closely related to the shape and function of their skull. Geometric morphometrics have proven highly effectiveness to discriminate closely related species, even in the case of sub-species that are differentiated on the basis of karyotypic differences. The use of this technique leads to the possibility of a deeper understanding of relationships between species, through the identification of landmarks in biologically definable and comparable locations on different specimens which allow the identification of differences due to the shape. Sea turtles are an excellent example of a taxonomic group in which aspects of ecological and environmental adaptations are reflected in morphology. There is a relatively extensive knowledge of food components for these organisms covering a wide range of groups: sponges, jellyfish, corals,

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crustaceans, mollusks, tunicates, fish, certain algae and seagrasses. However, to date few studies have analyzed variations in turtle skull shape while taking into account that the sources of variation could be: inter-specific, intra-specific, geographic, etc. Considering the above, this chapter addresses the application of geometric morphometrics as an approach to assess specific structure variations in cranial anatomy of sea turtles, and the relationship of these variations with these organisms with their ecology.

INTRODUCTION

Basic Terminology

Below are some of the most important concepts included in this chapter with their respective definitions.

- **Bending Energy:** Energy necessary to fit a thin straight plate to its new shape. Measuring the amount of difference in the shape, based on the metaphor of the thin-plate splines; so the bending energy corresponds to the amount of energy needed to set an infinitely thin metal plate at given amplitude between selected landmarks (Zelditch et al. 2004).
- **Centroid:** It is the center of gravity of a specimen, according to the configuration of given landmarks (Slice et al. 1998); mean x, y of all coordinates considering all landmarks (Polly 2012). It does not respond to physical properties of the material but the shape, and for this and because of this reason does not change through the analysis (Zelditch et al. 2004).
- **Centroid Size:** It is calculated as the square root of the sum of the squared distances of a group of landmarks to its centroid (Slice et al. 1998). It is a measure of size used to scale a set of landmarks.
- **Consensus Configuration:** Group of landmarks representing the central tendency of a sample for the process of superimposition (Slice et al. 1998).
- **Geometric Morphometrics:** Morphometric method based on the Cartesian coordinates of landmarks that retain all the information on geometric shape throughout the analysis (Slice 2007)
- **Homology:** Equivalence or similarity between derived attributes that are shared by two or more species, which may or may not have changed and have a common ancestor. The criteria of homology is the primary consideration when selecting landmarks, since its location only makes sense if a match occurs and has a biological meaning (Zelditch et al. 2004).
- **Landmarks:** Specific markers located on a biological shape according to scale (Slice et al. 1998), taking into account the criteria of homology in which they develop, they are biologically definable and comparable between specimens.
- **Morphometry:** Consists in measuring the shape of an organism or their constituent parts. The study of shape from a morphometric perspective examines central tendencies, variation, differences between groups and associations in relation to extrinsic groups (according to the comparisons desired).

- **Position:** One of the removed effects via Procrustes analysis. The centroid of the object is displaced to the origin of the coordinate plane (0, 0) and the relative positions of the points remain the same, but their coordinates vary (Rohlf & Slice 1990).
- **Procrustes Analysis:** The analysis of the shape coordinates from the superimposition of corresponding landmarks, based on the estimation of minimum squares of the position, rotation and scale which allow landmarks groups to be aligned in pairs of specimens (Slice 2007).
- **Procrustes Distances:** Approximately, the square root of the sum of squared differences between the positions of the landmarks in two optimally superimposed configurations at centroid size (Rohlf & Slice 1990).
- **Relative Warps:** Refers to the principal component analysis of shape distribution, in terms of Procrustes distances (Zelditch et al. 2004).
- **Rotation:** Another effect removed by means of Procrustes analysis. Points are aligned under the minimal squares criteria eliminating the movement of the object around an axis. The relative location of points is constant, although the coordinates vary (Rohlf & Slice 1990).
- **Scale:** One of the effects removed by means of Procrustes analysis. The effect of the size of the objects is removed by scaling the points to a centroid size equal to one (Rohlf & Slice 1990).
- **Shape:** Defined as all resulting geometric information once all effects of position, scale and rotation have been removed from an object (Kendall 1989).
- **Shape Variables:** Geometric variables that do not vary according to position, rotation or size of a specimen. The superimposed coordinates of the corresponding landmarks are variable in shape (Slice 2007).
- **Thin-plate Splines:** Visualization tools based on the interpolation of the differences between the locations of a set of landmarks from their equivalents (Slice 2007).

The function of any physical attribute of an organism is its action from a morphological perspective, and refers to how the structure works (Linde-Medina 2006). Refers to the emergent properties of the shape of a characteristic; different to the use given by an organism of the uses that a trait offers which defines its "biological role" or "biological function" (Linde-Medina 2006).

Functional morphology is the study of the relationship between the shape of structures in an organism and its functions (Alexander 1967). In the real world which we all study –living organisms- form and function are never separate. Form does not exist in any way that is not a result of function. Form does not exist without function and function does not exist without a formal cause and a context. Our minds take form and function as independent as an analysis exercise: in our reductionist world, we take things apart to understand how they work (Wainwright 1988). Therefore, mechanical design of animal systems is a fundamental subject within functional morphology (Westneat 2003).

The link between form-function and ecology is analyzed through the concept of ecomorphology, which is an approximation to understand the ecological relationships between species that is based on the supposed functional relationship that exists between morphological adaptations of an organism and environmental characteristics in which it is

found (e.g., the shape of structures associated with feeding is related to the characteristics and size of the prey) (Wainwright 1994). The relationship between an organism morphology and its ecological function is subject to the limitations imposed by different selective pressures and changes occurring in ecosystems (Bulté et al. 2010; Brecko et al. 2008). Conceptually, ecomorphology is the "study of the relationship between the morphology of the organism and its environment" (Wainwright & Reilly 1994). Essentially it deals with ecological questions through the phenotypic analysis of organisms (Schwenk & Rubega 2005).

Therefore, the need to know the relationship between morphology, performance and ecology of species and its effect on morphological plasticity, to help us to understand the ecological processes that lead to the evolution of phenotype; the correspondence between the anatomical features that influence the performance of associated species serves as a benchmark to represent the variation of their ecological role. Phenotypic variability present between individuals and species is directly related to ecological differences; so that variants in shape determine ecological strategies within which is found different ways of resource use (Wainwright 1994).

For example, muscle and bone structure in vertebrates jaw reflects the way they capture food (Westneat 2003). The variation in the structures involved in this process may reflect differences in how prey is captured and indirect ecological factors affecting these characteristics such as competition (Hulsey et al. 2005), predation (Ruehl & Dewitt 2005) and environmental conditions (Robinson et al. 1996; Ruehl & Dewitt 2005). Also the conditions that affect feeding morphology may affect non-trophic traits through correlated effects with other morphological components such as body shape; therefore a common goal in the analysis of trophic ecology is to predict the composition of individuals diet, taking into account that predators can consume either the prey that is in greatest abundance around them, or can choose to selectively feed on specific prey (Scharf et al. 2000).

Hence, the consumption patterns of prey depend on several factors and among them, one of the most important is predator's efficiency, since only prey that are located, captured, transported and digested can be included within the diet (Franco-Moreno 2011). This efficiency is controlled by the intrinsic properties that are product of the feeding system design of each predator (Wainwright 1994).

Sea turtles in particular are an excellent biological model which aspects of the ecological and environmental adaptations are reflected in morphology. There is a relatively extensive knowledge of the food components consumed by sea turtles that include a wide range of groups: sponges, jellyfish, corals, crustaceans, mollusks, tunicates, fish, algae and seagrasses. However, to date few studies have analyzed the variations in shape of the craniomandibular system of turtles taking into account sources of variation: inter-specific, intra-specific, geographic, etc.

SKULL IN SEA TURTLES

In general, the skull of vertebrates is characterized by a complex anatomical integration in which features such as kinetic union, dentition and mandibular mobility interact collectively, influencing feeding performance parameters (bite force or jaw force).

In general, vertebrate exhibit positive allometry in feeding performance (bite force and velocity) during ontogeny, so that the adults have a relatively higher performance than

juveniles; these allometric growth patterns have often been associated with dietary changes and niche distribution, because an increased performance facilitates consumption of functionally more complex prey (e.g., larger size, hardness and resistance), that younger members of the same species would be unable to consume (Hernández & Motta 1997; Herrel et al. 2002).

Most relevant skeletal components found in skulls of this group are derived from ancestral splanchnocranium; significant ancestral trait in the origin of vertebrates (and more specifically tetrapods), present in the head of practically all of these organisms under any variety in the form-function complex (Schwenk 2000).

The turtle skull is composed of an interior braincase –neurocranium- which contains the brain and an external bone structure—splanchnocranium—whose anterior part and mandibular bones form the jaws. The splanchnocranium also contains the sensory organs and provides attachment points for the jaw, throat and neck muscles. For chelonians this structure consists of strongly articulated bones; a secondary palate is presented and general shape of the skull and palate present differences between species. The following refers to the main anatomical differences for four species of sea turtles found in the west coast of Baja California Sur and Gulf of California, Mexico (*Chelonia mydas*, *Caretta caretta*, *Lepidochelys olivacea* and *Eretmochelys imbricata*). Dimensioning is because the morphometric exercise shown hereinafter will focus on the differences of the shape of the lower jaw of these species.

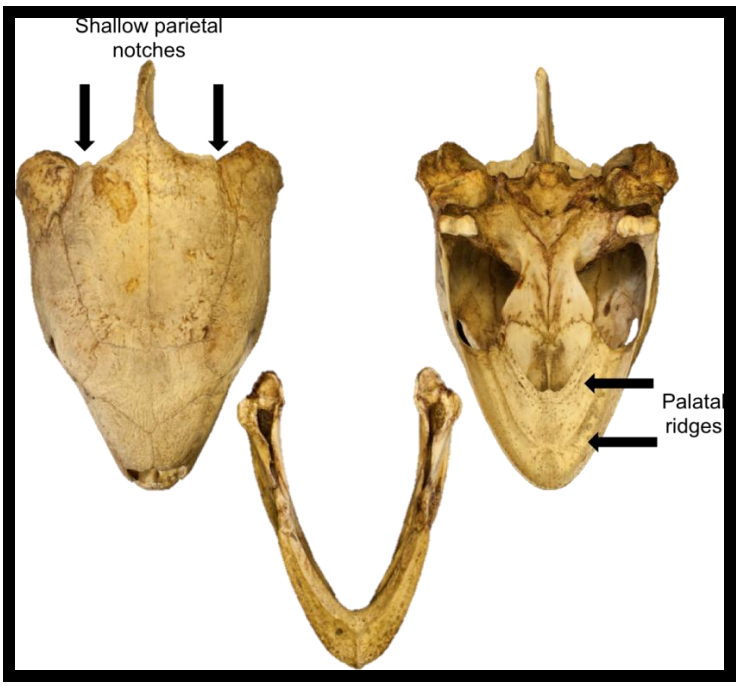

Figure 1. Skull of green turtle (*Chelonia mydas*) in dorsal view (top left), ventral view (top right) and dentary (below).

In the case of the green turtle *C. mydas* (Figure 1) the skull is rounded with a short snout and shallow parietal notches; the palate presents a pair of ridges that run parallel to the outer

edge of the mandible, between the margins of the upper jaw and internal nostrils. Dentary may have many small cusps in young individuals and these are usually reduced or absent in adults (Lutz et al. 1996).

The skulls of the loggerhead turtle, *C. Caretta* (Figure 2) and olive ridley turtle, *L. olivacea* (Figure 3) are relatively large, wide at the back and with the snout tapering towards the orbits. Parietal notches are presented along the rear edges of squamosal, parietal and supraoccipital bones; mandibles are robust and buccal canal ends sharpened.

For *C caretta* the two maxillary bones are articulated on the midline of the palate before the vomer (Figure 2), in contrast to *L. olivacea* (Figure 3) which has two maxillary bones separated by this structure, which extends to the front and articulates with the premaxillary bones; in both species the secondary palate is long and no alveolar ridges are presented. Particularly, the pterygoid in *L. olivacea* is proportional and its processes are very pronounced (Wyneken 2004).

The skull of the hawksbill turtle, *E. imbricata* (Figure 4) is elongated, narrow and has a length approximately equal to twice its width. This species I characterized by a deep parietal notches and well-developed secondary palate, resulting in the internal nares being located further back compared to other chelonians.

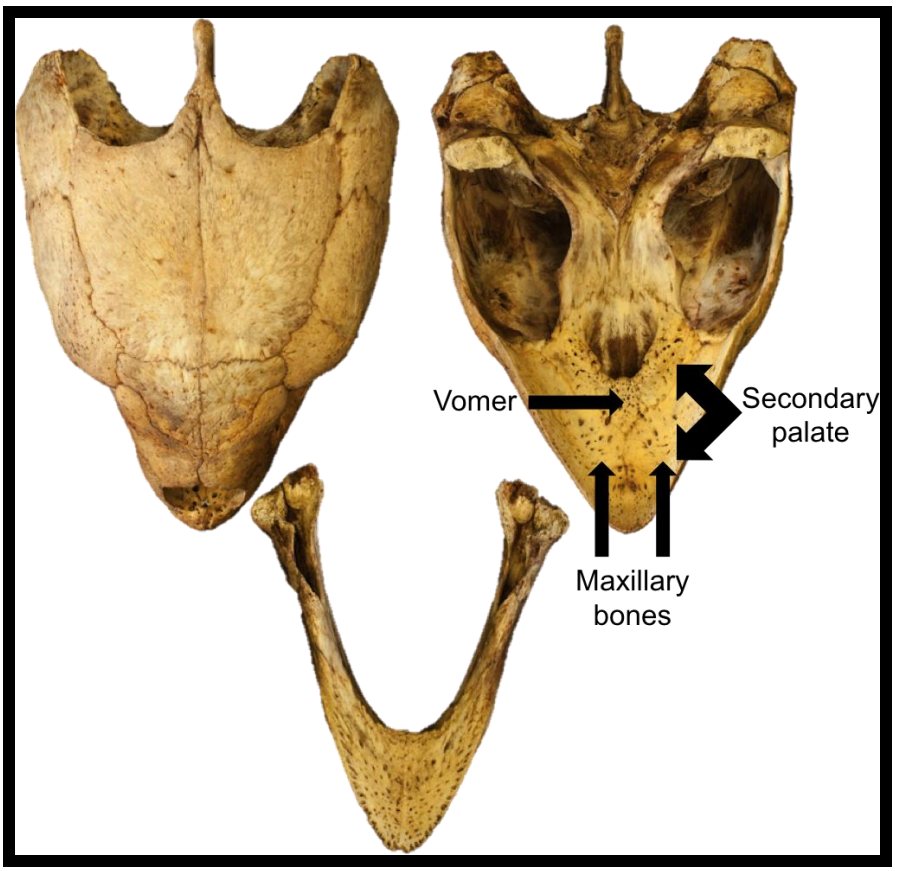

Figure 2. Skull of loggerhead (*Caretta caretta*) in dorsal (top left) view, ventral (top right) view and dentary (below).

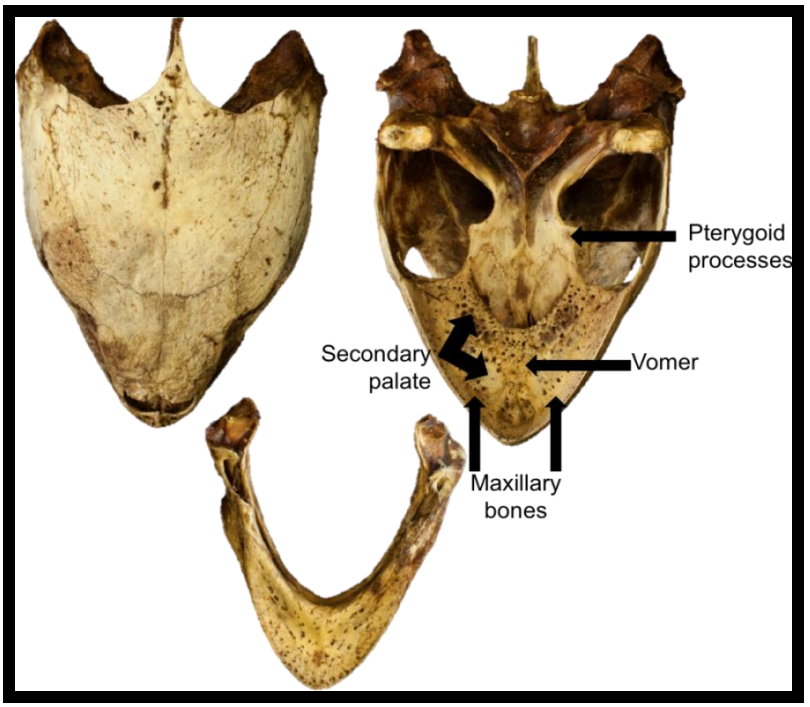

Figure 3. Skull of olive ridley (*Lepidochelys olivacea*) in dorsal (right) view (left) and ventral view.

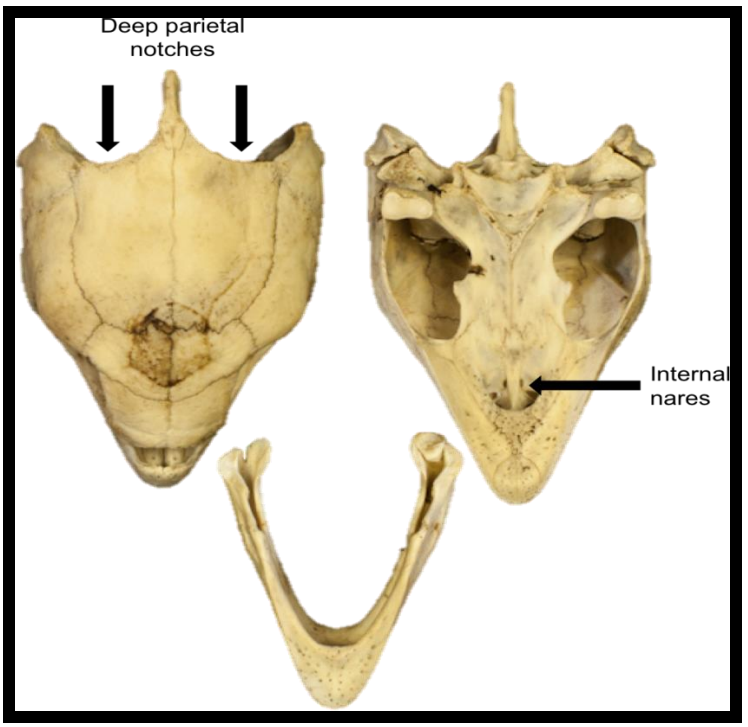

Figure 4. Skull of hawksbill (*Eretmochelys imbricata*) in dorsal (top left) view, ventral (top right) view and dentary (below).

Characteristically, turtles have lost their dentition and this is a derived condition (Schwenk 2000); these organisms have completely lost their teeth which have been replaced by a horny structure or ramphoteca. This covers the maxillary, premaxillary and vomer bones in the upper jaw and the dentary bone in the lower jaw (Wyneken 2004) (Figure 5).

The ramphoteca is derived from the outer layer of dead keratinized cells, typical of the integument of all tetrapods; which covers the mandible edges and extends to the palate and ventral part of the mouth, forming a grinding surface to process food. It is characterized by a very sharp apical edge; when the lower jaw is raised, it fits tightly within the upper jaw so that the lateral surface of the lower beak slides over the inner surface of the upper part, simulating the movement of a pair of scissors (Schwenk 2000).

As for the musculature, special attention is paid to those structures closely involved in the feeding process and which perform the following functions: *abduction*, in which a portion of the ventral surface moves away and *adduction*, which brings it towards the ventral surface. In the case of sea turtles these two functions are special because the depressor muscles open the jaws, while the levators close them (Wyneken 2004). Unlike what happens for other vertebrates in which the adductor mandibular muscles are those that are responsible for allowing the mandibular opening and closing.

FEEDING PROCESS AND FEEDING SYSTEM

One of the main functions of any organism is feeding. This process requires the anatomical and functional integration of a large number of components; one of the highlights of the feeding function in sea turtles, as a group, is its diversity (Schwenk 2000). The relative functionality of the feeding system has a major impact on individual survival and of course, in reproductive success, therefore it is reasonable that this system is under strong selection and that variations in its morphology significantly affect performance (Schwenk 2000). The importance of this system for survival and adaptation stands out due to its great impact on the body plan of tetrapods, mainly through its influence on cranial shape (Schwenk 2000).

For practical reasons, it is considered that the feeding type of sea turtles would consist of two phases: the first involves prey capture and it is defined mainly by mandibular prehension, with two variants: tearing and piercing. Specifically piercing would be the mechanism presented by these organisms, because it is typical in homodont or toothless species which trap and transport their prey with their jaws. The adjustment generated between the upper and lower beak creates a cutting action and additionally can generate a grinding action toward the lateral side, between the upper and lower surfaces.

The second phase, ingestion, would take place by suction; sea turtles are characterized by a large, articulated and ossified hyoid apparatus that is associated with the ability to generate significant amount of force that allows them to move large amounts of water during ingestion (Figure 6).

Figure 5. Skull of green turtle (*Chelonia mydas*), highlighting the horny structure or ramphoteca.

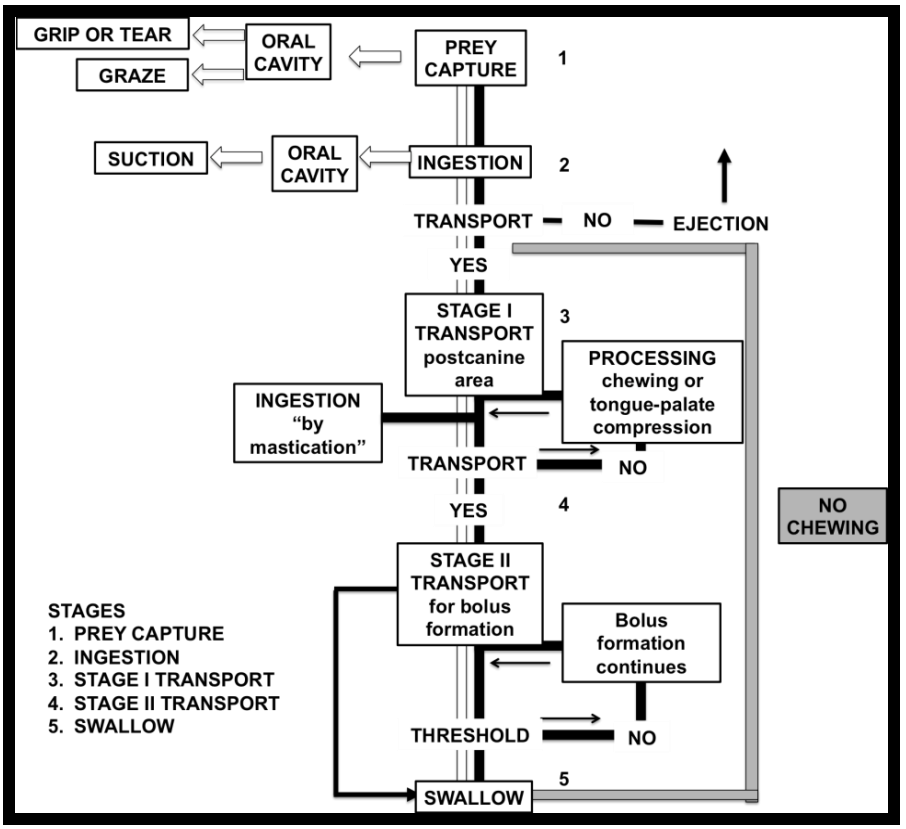

Figure 6. Feeding process for vertebrates. Sea turtles have phase 1 and 2 separated and not chewing presents, so after ingestion step into the esophagus occurs. Taken and modified: Hiiemae (1967).

FEEDING HABITS OF EACH SPECIES

Although sea turtle populations present different diets depending on the availability of characteristic sources within their habitats, in this chapter we will pay special attention to populations of the eastern Pacific. Particularly those living in the waters of Baja California Peninsula and the Gulf of California.

Green Turtle (*Chelonia mydas*)

The East Pacific green turtle –known regionally as the black turtle– has a distribution ranging from southern Canada to Chile (Parker & Wing 2000; Márquez 2000), with important nesting areas in Mexico, Panama and Ecuador (Cliffton et al. 1982). The coastal lagoons of Baja California Peninsula are considered important areas for development and supply of juveniles and sub-adults of this species (Márquez 1990; Gartner & Nichols 2001), which recruit the ±35 cm curved carapace length (CCL) (Seminoff et al. 2002a, López-Castro et al. 2010) and can inhabit lagoon channels for over 20 years, while reaching sexual maturity (Koch et al. 2007).

It is the only one of the seven species of sea turtles, -which in most of its populations- in subadult and adult stages presents an herbivorous diet (Balazs 1982; Bjorndal & Bolten 1988), so it has been considered to occupy a unique ecological niche among this group of reptiles (Mortimer 1982). Its ontogenetic habitat changes, in turn, have a strong influence in modifying its diet (Bjorndal 1997). It is considered the most carnivorous of the *C. mydas* populations, since its diet plus algae and seagrasses include lots of invertebrates throughout its life (Seminoff et al. 2002b; López-Mendilaharsu et al. 2005; Amorocho & Reina 2007; Carrión-Cortez et al. 2010; Rguez-Baron et al. 2011, un-publishing data) (Table 1).

Loggerhead (*Caretta caretta*)

The west coast of the Baja California Peninsula and to a lesser extent the Gulf of California, represent one of the most important feeding areas for North Pacific loggerheads (Bowen et al. 1995). These turtles which are born exclusively on the coast of Japan, travel across the North Pacific to find the productive waters of the Gulf of Ulloa (Baja California) and the Gulf of California (Nichols et al. 2000). It has been estimated that they can stay there up to 30 years until they reach sexual maturity and return to Japanese waters to reproduce (Nichols et al. 2000; Peckham et al. 2011).

The diet of juveniles that inhabit Mexican waters is mainly composed of red crab (*Pleuroncodes planipes*), demersal fish (discarded from fishing operations) and jellyfish (Peckham et al. 2011) and differs significantly from that reported by Parker et al. (2005) for the central North Pacific (Gastropoda, Hydrozoa, Maxillopoda, Malacostraca, Thaliacea, Cephalopoda, Actinopterygii, Actinopterygii eggs, Polychaeta and algae) (Table 1). This is interesting as these juvenile turtles are from the same reproductive stock (Bowen et al. 1995).

Olive Ridley (*Lepidochelys olivacea*)

Unlike most species of the Cheloniidae family, the olive ridley has a mainly oceanic behavior (Bolten 2003). Its great mandibular strength allows it to access prey with hard bodies such as snails and crustaceans; it also feeds regularly on benthic fish, sea urchins and jellyfish (Carr 1961; Mortimer 1982; Márquez 1990) (Table 1).

The nesting beaches of the Baja California Peninsula represent the northern limit of the north Pacific olive ridley reproductive stock. In the Gulf of Ulloa, juveniles and adults of this species are sympatric to juvenile *C. caretta* (Peckham unpublished data). This behavior has already been reported in Hawaii by Polovina et al. (2004); however, their diets differ significantly, which can be attributed to differences in patterns of diving behavior: olive ridleys spend 20% of their time on the surface and present deeper dives than those of the loggerhead turtles, which spend 40% of time on the surface (Polovina et al. 2004).

Hawksbill (*Eretmochelys imbricata*)

This species is considered to be more faithful to its habitats once it recruits to neritic areas after its oceanic phase. It is characterized by a considerably smaller range of distribution when compared with other sea turtle species (Bolten 2003). Most research on hawksbill ecology have focused on studies about coral reef systems and to a lesser extent on sea-grass beds, where until it was recently believed to be the limit of their distribution (Bjorndal 1997; Bjorndal & Bolten 1988, 2010).

Since in the eastern Pacific there was no evidence of their presence in these ecosystems, hawksbills were considered functionally extinct (Bjorndal 1997). However, Gaos et al. (2012) demonstrated by means of satellite telemetry, that adult hawksbill turtles gather in mangrove systems during certain periods, giving important information about their life history.

Their diet in the Caribbean is mainly spongivourous (Meylan 1988), although depending on the foraging area, cnidarians (Leon & Bjornadal 2002), false corals (Rincón-Díaz et al. 2011), algae (Berube et al. 2012) and sea-grass (Bjornadal & Bolten 2010) have been identified as major components in their diet. Even though studies have not been undertaken on East Pacific hawksbill diet, due to its use of habitat, it is thought to feed mainly on mangrove structures, sponges and tunicates (Gaos et al. 2012) (Table 1).

METHODS

Morphological Analysis through Geometric Morphometrics

Any quantitative measure or numerical analysis of morphological features of an organism is called Morphometry; the quantitative representation and analysis of shape using geometric coordinates instead of measurements is known as Geometric Morphometrics (Polly 2012).

Table 1. Feeding components of the diet of four species of sea turtles (*Chelonia mydas, Caretta caretta, Lepidochelys olivacea* and *Eretmochelys imbricata*)

Species	**Dietary** components	Source
Chelonia mydas	Seaweed	Seminoff et al., 2002b
	Cyanobacteria	López-Mendilaharsu et al., 2005, 2008
	Seagrass	Amorocho & Reina, 2007
	Sponges	Arthut & Balazs, 2008
	Scyphozoans	Carrión-Cortez et al., 2010
	Nematodes	Rguez-Baron et al., 2011, (unpublished data)
	Annelids	
	Anemones	
	Pennatuláceos	
	Hidrozoos	
	Jellyfish	
	Red crab	
	Tunicates	
Caretta caretta	Seaweed	Parker et al., 2005
	Polychaete	Peckham et al., 2011
	Jellyfish	
	Barnacles	
	Scyphozoans	
	Gastropods	
	Cephalopods	
	Crustaceans	
	Fish	
	Fish eggs	
Lepidochelys olivacea	Seaweed	Carr, 1961
	Groundfish	Mortimer, 1982
	Crabs	Márquez, 1990
	Oysters	
	Sea Urchins	
	Sessile tunicates	
	Shrimp	
	Jellyfish	
	Salps	
	Red crab	
	Barnacles	
	Mollusks	
Eretmochelys imbricata	Cnidarians	Carr & Stancyk, 1975
	Sponges	Mortimer, 1982
	Mangrove	Leon & Bjornadal, 2002
	Tunicates	
	Bryozoans	Parker et al., 2009
	Mollusks	Carrión-Cortéz (unpubl. data)
	Seaweed	

One important purpose of morphometric studies is to understand the variations in size and shape of an organism's body within a broad evolutionary context. This tool allows us to relate morphology with ecological attributes such as predation, competition, environmental performance in obtaining food and mating (Davis et al. 2010); enabling us to answer questions such as "how different are two organisms?" or "with which factors differences in shape are correlate?".

In this regard, Geometric Morphometrics allows the analysis of variation in shape based on the location of landmarks (Adams et al. 2004), which by taking into account the criterion of homology in which they develop are biologically definable and comparable between specimens, and they constitute specific natural points which are located on biological shape according to a scale (Slice et al. 1998). According to Bookstein (1991), there are three main types, which correspond to three ways of addressing epigenetic explanations that are in principle subject of these measurements:

Type I: Juxtaposition of tissues. Includes conventional points in which homology is based on a robust biological interpretation (anatomical: sutures and insertion of different structures, etc.). Although its location is limited to the edges of structural components of a shape or to regions defined by where different tissues are juxtaposed, it is not determined by general contour characteristics (MacLeod 2001).

Type II: Maximum curvature or other local morphogenetic processes. These include the protuberances apices and invagination valleys. The skeletal processes where muscle adhesion occurs are also included.

Type III: Extreme points. Include points that characterize a region of body design that may include information of a finite number of separate regions (extreme points of a diameter, intersections in segments found between type I landmarks). Although they are considered "deficient" because they do not have all the coordinates and it is not common to include them in the analysis, its displacement is significant mainly when are located in segments or areas of the structure, whose boundaries are defined by type I landmarks. Due to the variable nature of this type of landmarks and its dependence on a wide variety of conditions, they have been redefined as semi-landmarks (Bookstein 1997; MacLeod 2001). Polly (2012), defines these as marks that are positioned arbitrarily using an algorithm, often by defining extreme (or limit) landmarks, within which a specific number of semi-landmarks are placed.

To define landmarks that will represent shape(s) structure(s) or specimens to be evaluated, it is important to consider three main factors:

- The landmarks should proportion information that allows us to test the proposed hypotheses.
- They should adequately represent the shape and.
- They must be presented in all specimens.

In such a case, if what is sought is to relate morphology with ecological aspects such as changes in feeding habits on a inter or intraspecific level, landmarks must be located on the individuals craniomandibular system and focus on those structures involved in the feeding process (prey capture).

For each individual, landmarks are quantified on a bidimensional plane, at coordinates (x, y); once all landmarks on all of the specimens coincide as closely as possible, the differences between the coordinates for each landmark can be comparable.

To achieve this, landmark configuration from one specimen is overlapped on another as to coincide as closely as possible (Rohlf & Marcus 1993); the variation that is not due to shape and is generated by changes in position, rotation and scale of specimens is then removed (Slice et al., 1998). The most common method of superimposition is Procrustes analysis, which estimates the position and orientation parameters minimizing the sum of the distances squares between the configurations of two corresponding landmarks (Bookstein 1991).

This is achieved in three steps (Slice & Rohlf 1990; Polly 2012):

- The centroid of each individual is moved to the origin (0, 0); the centroid of the shape for each pair of landmarks is subtracted (Figure 7a).
- Generally all shapes are scaled to the same size (which usually corresponds to the centroid size; this is calculated from the mean shape and each of the "new" shapes) (Figure 7b).
- The shape is rotated around the origin, until the sum of the distances squares between corresponding landmarks is minimized (Figure 7c).

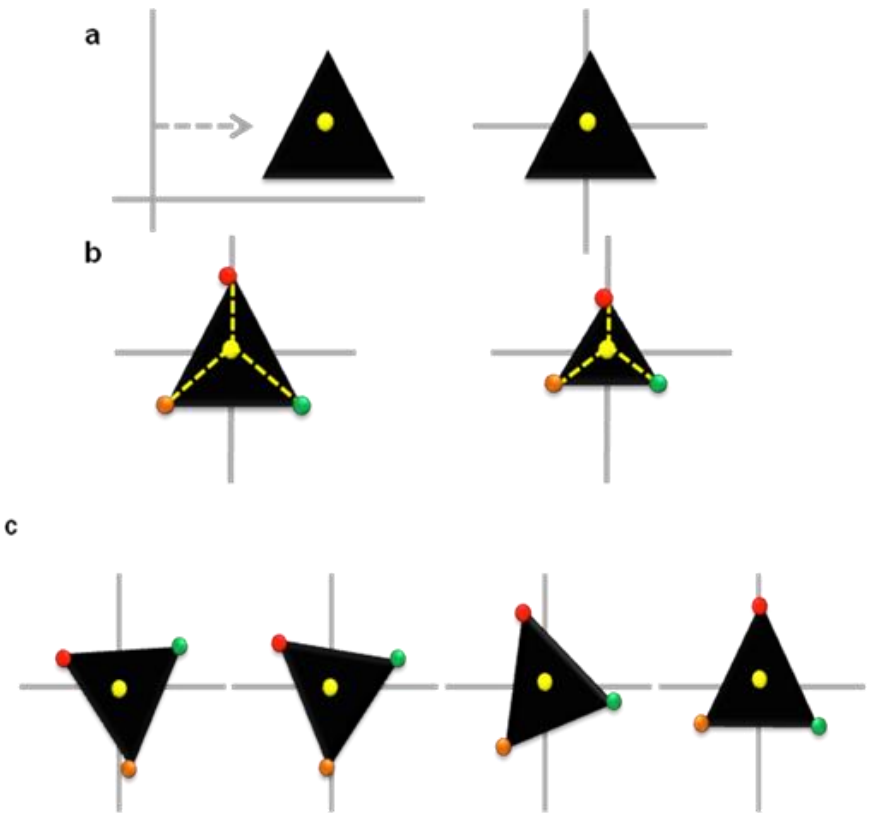

Figure 7. Processes of (a) position, (b) scale and (c) rotation that are removed by Procrustes analysis.

The result of this process is the elimination of size effects, thus the first principal component is shape. Additionally, this allows the configurations of landmarks to be directly comparable when putting in the same coordinate system and facilitates the formulation of hypotheses regarding organism shape, by minimizing the distances between landmarks, ensuring that any difference between shapes is "true" (Polly 2012).

For each specimen, iterations of the process are performed, obtaining the average shape (consensus configuration), after this, the landmark configurations are placed in a common coordinate system, and these are used as shape variables (Adams et al. 2004).

The most common method to visualize the differences between a specimens shape is by thin-plate splines. In these representations it is assumed that landmarks are placed on an infinitely thin metal plate; if we have two specimens, one of the configurations of landmarks may be found on a straight plate while for the other, the plate must be deformed to adjust to the first configuration (Bookstein 1991). The difference in shape between the two specimens, can be expressed as the energy needed to adjust the straight plate to its new shape (bending energy). The deformation of the plate shows where the differences between specimens exist, considering the variation of each landmark separately (Figure 8).

This form of visualization allows the interpretation of the results directly within the context of cranial anatomy and has been used for a little over a decade (i.e., Klingenberg & Zaklan 2000; Klingenberg et al. 2001a; Bookstein et al. 2003).

Finally an ordination plane of the distribution of the shape in terms of the Procrustes distances is obtained. The visualization method by thin-plate splines from relative warps, was developed by Bookstein (1989, 1991) to analyze the morphometric variation within a population using landmarks; each warp is taken as a change in the direction of the shape in respect to the average. These are used to describe the trends in the variation of the shape between specimens and can be interpreted as the deformation in a physical space for each landmark, with respect to the mean. Furthermore, these warps can be shown as displacement vectors for each landmark with respect to reference specimen (Rohlf 1993), facilitating the visualization of the location, direction and magnitude of morphological change.

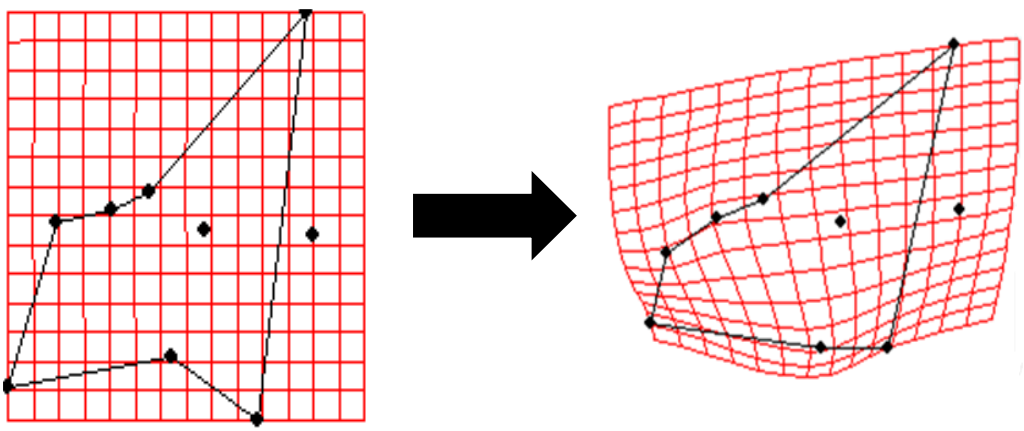

Figure 8. Deformation grid for landmarks with respect to a mean shape in a physical space (thin-plate spline).

Lastly, morphometric distances are considered as the main measure of differences between individuals, for all variables that were represented; for Geometric Morphometrics the primary measure of differences would be the Procrustes distances, i.e., distances between shapes after performing superimposition (Polly 2012).

Specifically with respect to morphometric analysis of the skull, we must take into account that the parts of this structure are integrated with each other as they develop, operate and evolve together. However integration is not absolute, but is divided into modules which are relatively independent within the entire unit (Klingenberg 2008, 2010). Thus the concepts of integration and modularity are closely linked and must be considered together. This is demonstrated in the example shown in the following sections.

Geometric morphometric methods are suitable for the investigation of morphological integration and modularity for several reasons. Firstly, it provides a wide range of powerful statistical tools to answer specific biological questions concerning modularity and integration. The combination of the geometry with multivariate statistics ensures that the shape of a structure is completely characterized without repetition. Additionally, all spatial relationships between landmarks or other geometric characteristics included are taken into account, without the need to define a set of features to include in the analysis *a priori*.

FUNCTIONAL ANALYSIS BY BIOMECHANICAL MODELS

Biomechanics in functional morphology covers the physics of biological materials and the environment that surrounds them, with the respective consequences for organism shape, function and evolution (Schwenk 2000). It is based on mechanics, engineering, anatomy and physiology among other disciplines; through which a precise description and analysis of the movement of the systems that composed living organisms and its causes are realized.

Measuring the performance associated with the mandibular system of vertebrates allows us to analyze how processes of feeding, prey capture, mating and defense from predators occur. The bite force is closely linked to the design of the craniomandibular system and therefore can be used as an indicator of the overall performance of the whole organism in relation to its morphology (Anderson 2009).

The study of biomechanics allows us to design and explore models that explain how mechanical forces act within organic systems and structures of living beings. The increased force provides an ecological advantage in the resources distribution and niche differentiation, diminishing competition and increasing the spectrum of availability prey allowing for the coexistence of several species within the same habitat (Kiltie 1982; Herrel et al. 2001; Herrel et al. 2005; Dumont et al. 2005; Wroe et al. 2005).

Biomechanical models outline the physical and physiological principles that determine the functions in organisms, allowing ecological and functional inferences based on morphology (Westneat 2006). In general, the jaws of vertebrates is a simple lever which closure is permitted by the adductor mandibulae muscles, which pull the jaw around a rotation point (formed by the jaw joint) creating a third order mechanism (Figure 9) (Westneat 2010). In this the effort lies between the resistance and fulcrum, and it is characterized because the input force being higher than the output force; it is employed when increased velocity transmitted to an object is required or the distance travelled by this.

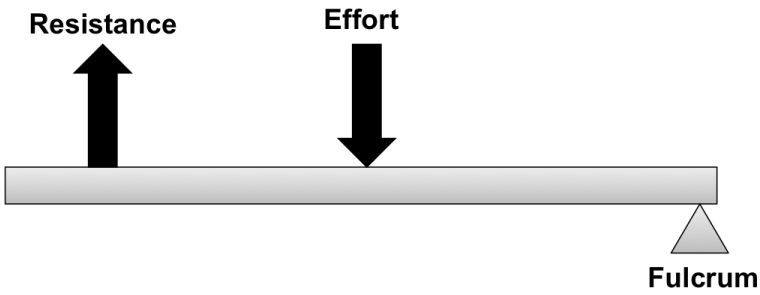

Figure 9. Third order simple lever.

The generation of force and velocity through this system, depends on the input force (I_f) and the distance between the point at which the effort is generated, regarding the input (I_f; I: in-lever arm) and output forces (O_f; O: (out-lever arm) and the fulcrum. The above generates a corresponding (d) displacement (Figure 10).

For sea turtles the rotation point (fulcrum) of the lever would consist by the quadratojugal-mandibular joint (towards the articular bone), around which would turn the other bone elements by the action of adductor mandibulae complex that presents several parts: these originate in the parietal, quadratal and supraoccipital bones (Figure 11) and converge in a tendon that is mainly inserted into the lower jaw (dentary, with small insertions in the squamosal bone after the jaw joint) (Wyneken 2004).

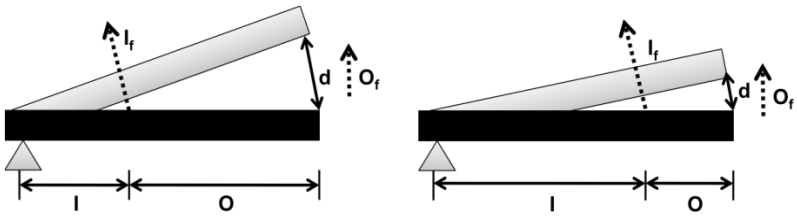

Figure 10. Generation of force and velocity in a third lever order. Input force (I_f), output force (O_f), in-lever arm (I), out-lever arm (O) and displacement (d). Taken and modified: Herrel & Aerts 2003.

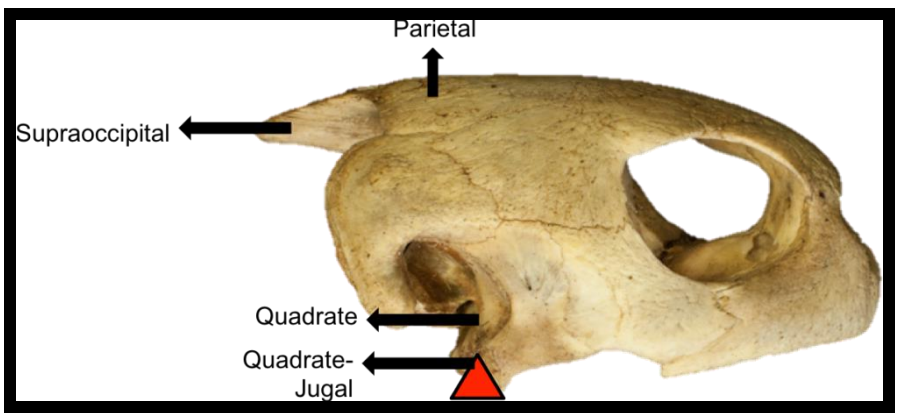

Figure 11. Cranial structure of *C. mydas* highlighting the bones in which adductor mandibulae complex is origin and the quadratojugal-mandibular joint (fulcrum of lever: red triangle).

EXAMPLE

To show the scope of Geometric Morphometrics, we developed a preliminary exercise in order to describe the main differences in the morphological features of the mandibular system of the four species of sea turtles listed above (*C. mydas*, *C. caretta*, *L. olivacea* and *E. imbricata*), distributed along the west coast of the Baja California Peninsula and Gulf of California.

We digitized 129 images of the lower jaw of the four species (*C. mydas*: 35, *C. caretta*: 78, *L. olivacea:* 11 and *E. imbricata:* 5), retaining the same conditions when photographing each specimen. Subsequently landmarks (Table 2, Figure 12) were located to describe the mandibular design and to carry out the comparisons. In this process we employed type I, II and III landmarks (mentioned above), because using only the first type is not enough, particularly when the design of the structure to be analyzed is composed by surface, contours or edges (as applicable) where it would be difficult to locate them. Given these considerations located landmarks corresponded to (Figure 12, Table 2):

Subsequently relative warps for each landmark were obtained, creating a matrix in which the shape variables are grouped. We obtained thin-plate splines, which as explained above help us to visualize the changes in the disposition of landmarks with respect to the mean shape, as deformations generated in the grids when they try to adjust to the average shape.

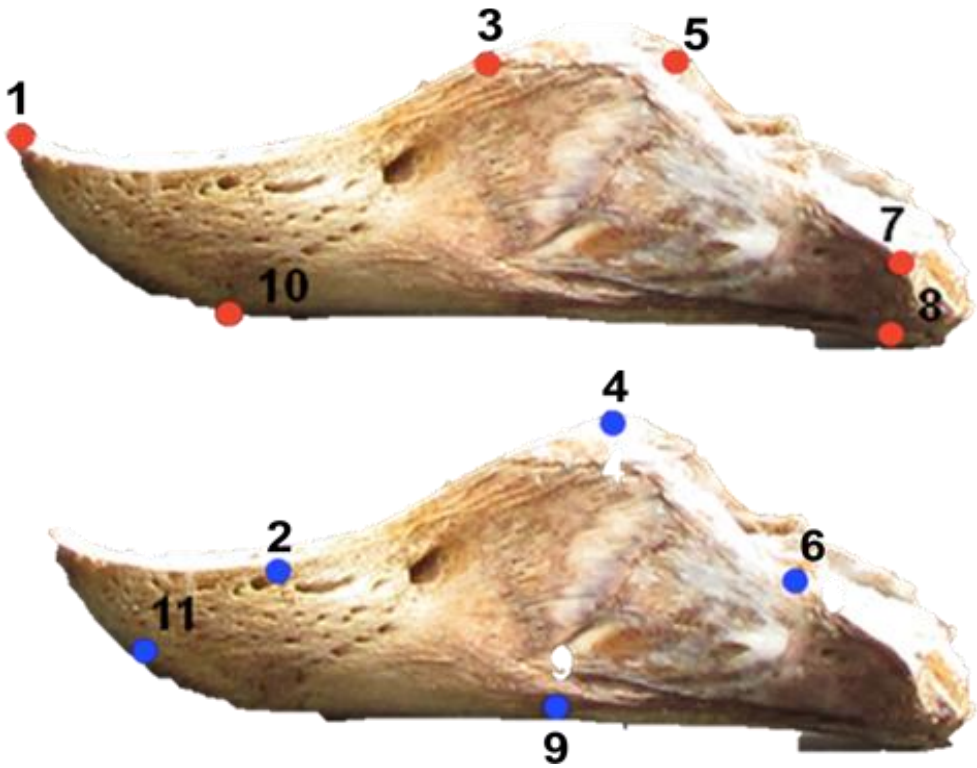

Figure 12. Landmarks (top) and semi-landmarks (below), placed to describe mandible shape.

Table 2. Landmarks and semi-landmarks placed to describe the mandibular shape of the four sea turtles species

Landmarks	Location	Type
1	Anterior tip of the dentary	I
3	Anterior tip of the ascending process of articular bone	II
5	Posterior tip of the ascending process of articular bone	II
7	Posterodorsal margin of articular bone	I
8	Posteroventral margin of articular bone	I
10	Posteroventral margin of the dentary	II
Semi-landmarks		
2	Dorsal edge of the dentary bone	III
4		III
6		III
9	Ventral edge of the dentary bone	III
11		III

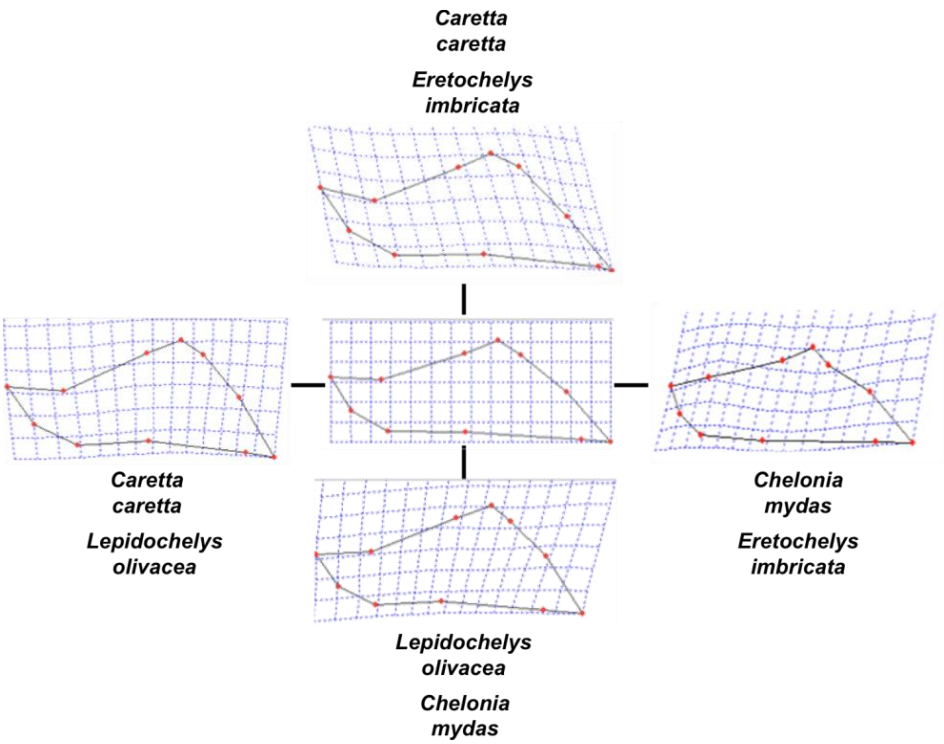

Figure 13. Thin-plate splines obtained from landmarks placed on the lower jaw of the four sea turtles species.

From the thin-plate splines (Figure 13) it is possible to identify that the main differences in the lower jaw of the four species occur at the anterior region of the dentary. It corresponds to the left end of the first axis of variation, where we would find *C. caretta* and *L. olivacea* characterized by a dentary that differs markedly from the ascending process of the articular

bone, which is wide and whose apex is blunted (Figure 14). Towards the right end of the ordination plane we would find *C. mydas* and *E. imbricata*, characterized by the absence of a marked difference between the dentary and the articular bone, whose ascending process has a pointed apex (Figures 13 and 14). These differences could be generating variations in feeding performance for these species, as it is in the lower jaw that the movement to carry out this process is generated, and the anterior region of the dentary would constitute the point of grip or press prey.

The second axis of variation was characterized by differences presented towards the posteroventral area of articular bone; in this case, the positive values are associated with the species *C. caretta* and *E. imbricata* characterized by a sharp termination of the articular bone (posterior region of the lower jaw) compared with negative values that relate to *L. olivacea* and *C. mydas* in which a blunt termination was observed (Figures 13 and 14). As for the variations due to the first axis, the differences could be affecting mandibular performance: the posterior region of the mandible is the area of junction with the skull and therefore, the rotation point that allows the movement of this structure. However, in this example we do not quantify the performance variables of the four species by using biomechanical models.

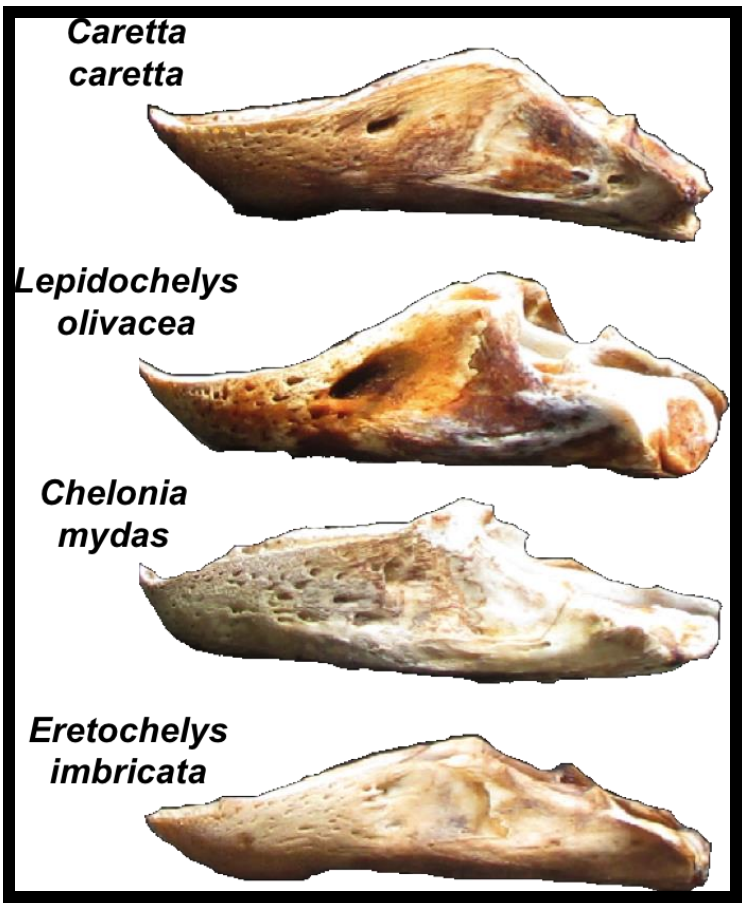

Figure 14. Dentary structure for the four sea turtles species.

We can then determine the relevance of the anatomic features whose morphological variation (measured from the Geometric Morphometrics technique) has functional consequences in the feeding performance of sea turtles. The success in the feeding event and more specifically during the prey capture can be developed using different strategies, which are facilitated by the characteristic shape of each species. This ultimately allows us to define the ecological role of each species in the ecosystem and if processes of interspecific competition are present or not.

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Chapter 2

ECOTOXICOLOGY OF SEA TURTLES

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ABSTRACT

This chapter is a review of studies in the toxicology of sea turtles and describes some ecotoxicological analytical procedures. A variety of chemical pollutants (heavy metals, persistent organic pollutants and polycyclic aromatic hydrocarbons) are found in the tissues and bones of sea turtles, including: kidney, muscle, gonads, lung, heart, pancreas, liver, adipose tissue, eggs, blood, faeces, carapace, spleen, bone, spinal cord, brain and urinary bladder. These pollutants have been reported in six species of sea turtle: *Caretta caretta, Chelonia mydas, Dermochelys coriacea, Eretmochelys imbricata, Lepidochelys kempii* and *Lepidochelys olivacea*. Moreover, this chapter will describe studies of *Chelonia mydas* ecotoxicology in Brazil and highlight ecotoxicological prospects in these species as tools for the conservation of sea turtles.

Keywords: oxidative stress biomarkers, metals, POPs, PAHs

INTRODUCTION

Coastal areas are subjected to constant pressure by human activities, due to their socio-economic importance and provision of various resources upon which local populations depend. Currently, coastal regions cover less than 20% of the planet's surface. However, these areas contain more than 45% of the human population; host 75% of megacities (with populations greater than 10 million people) and produce approximately 90% of global fishery

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stocks. Coastal regions constitute an important area of food production through farming, fishing and aquaculture. These regions are a focal point of industrial development and transport due to: significant mineral resources (including oil and natural gas) and heavy tourism demands. Beyond this, coastal areas are an abundant reservoir of biodiversity and ecosystems, upon which significant global functions depend (GESAMP 2010).

Pollution of coastal regions is associated with direct or indirect anthropogenically driven introduction of substances or energy into the marine environment, resulting in deleterious effects. Harm to living resources, hazards to human health and the hindrance of marine activities including fishing, reduction in quality of sea water or amenities likely result. Stressors resulting from pollution are associated with features of toxicity, persistence and bioaccumulation of substances in living organisms. Groups of potentially harmful substances that exist in marine environments, and for which critical issues have been identified include: oil derivatives, domestic sewage, heavy metals, radioactive materials and organochlorines (GESAMP 2009). In this scenario, estuaries and coastal regions generally act as the final receiving body of these substances. The sensitivity of estuarine and coastal regions to environmental impacts will depend on the ecological and biogeochemical characteristics of each particular region, including the presence or absence of human activities in that area (Lana et al. 2006). It is necessary, therefore, to design indicators of estuarine and coastal areas, able to provide reliable scenarios to diagnose the quality of the environment and the impacts of compromised systems on living organisms.

Ecotoxicology can be defined as the science of assessing effects of toxic substances on ecosystems. The primary goal of this field is the protection of entire ecosystems, and not merely isolated components (Hoffman et al. 2003). Previously, reptiles have been used as indicators or sentinels of environmental pollution. These species play a significant role in the transport of pollutants between trophic networks, which is underestimated by a lack of studies (Gardner & Oberdörster 2005); however further emphasizes their potential for environmental pollution modelling. The direct and indirect effects of environmental pollution and climate change can hardly be quantified, further inhibiting our understanding of potential factors that influence populations and their habitats. Long term monitoring of populations of reptiles should be standardized in order to identify possible causes of the decline in reptiles worldwide (Gibbons et al. 2000).

ECOTOXICOLOGY AS A TOOL FOR CONSERVATION OF SEA TURTLES

Sea turtles represent an interesting species to be used as biological indicators of environmental pollution, as they are subject to various threats linked to the dramatic ontogenetic changes in their life cycle. Development of sea turtles begins in the terrestrial environment, before moving to a pelagic phase, and finally returning to coastal areas to feed. As such, these individuals occupy a wide variety of ecosystems such as rocky shores, mangroves, coral reefs, seagrasses and offshore environments (Plotkin 2003; Bolten 2003). Developing within a wide range of environments increases the possibility of interacting with anthropogenic impacts including pollution, predation or changing environments (Marcovaldi et al. 2006; Gilman et al. 2007).

Adding to their high mobility throughout different ecosystems, the long life history of sea turtles enables an extended exposure period to polluting substances, both spatially and temporally. These attributes make these species an excellent model of pollution indicators or sentinels for monitoring the degree of costal contamination. A variety of chemical pollutants have previously been reported for sea turtles (heavy metals, persistent organic pollutants and polycyclic aromatic hydrocarbons) (Keller et al. 2004; Hamann et al. 2010; Lazar et al. 2011; Camacho et al. 2013).

Review studies regarding the toxicology of sea turtles have assessed six species (*Caretta caretta*, *Chelonia mydas*, *Dermochelys coriacea*, *Eretmochelys imbricata*, *Lepidochelys kempii* and *Lepidochelys olivacea*), however, *C. mydas* and *C. caretta* are more highlighted in this field. Studies with heavy metals and persistent organic pollutants (POPs) have identified a range of contaminants in various sea turtle tissues (kidney, muscle, gonads, lung, heart, pancreas, liver, adipose tissue, eggs, blood, faeces, carapace, spleen, bone, spinal cord, brain, urinary bladder). Differences in the accumulation rate depends on species, sizes and tissue type, along with environmental differences between species including: pelagic or sedentary life strategy, trophic levels, food items, and growth rates (Caurant et al. 1999; Pugh & Becker 2001; Gardner et al. 2006; Talavera-Saenz et al. 2007; Keller et al. 2006; Innis et al. 2008; Andreani et al. 2008; D'Ilio et al. 2011).

Some studies have been conducted to identify concentrations of heavy metals in sea turtles, reporting a higher accumulation of copper (Cu) and lead (Pb) in the kidney (Lam et al. 2004; Anan et al. 2001; Caurant et al. 1999) and copper (Pugh & Becker 2001; Fitzgerald 2004; Lam et al. 2004; Anan et al. 2001; Caurant et al. 1999; Innis et al. 2008; Andreani et al. 2008) and iron (Fe) (Aguirre 2004) in the liver. Silva et al. (2014) reported that zinc (Zn) concentrations for *C. mydas* in the muscle was the highest of all metals analysed, and the highest concentrations of lead, cadmium (Cd) and zinc were found in the kidney. The liver, however, was found to contain the highest values of silver (Ag) and copper.

Others studies that investigated metal profiles as environmental markers in green turtles demonstrated excellent correlations between the state of pollution in these foraging environments and the metal accumulation in the turtle (Talavera-Saenz et al. 2007; Franzellitti et al. 2004). Storelli et al. (2008) in the Mediterranean region, showed that the distribution of metals at the subcellular level and in the cytosol of liver and kidney cells plays a crucial role in the accumulation of metals such as Cd, Cu and Zn for *C. mydas*. However, many controversies related to the physiological and metabolic differences possible within the same species and exposure levels associated with the migratory routes remain (Fitzgerald 2004). Previous studies determined the concentrations of heavy metals in green turtles from the Caribbean (*C. mydas*) and loggerheads from the Italian Mediterranean (*C. caretta*), and evaluated hepatic and renal concentrations of methalothioneins (MT) as biomarkers of metal exposure, thus providing a useful tool in the long-term conservation of sea turtles (Andreani et al. 2008).

Assessments of persistent organic pollutants (POPs) have increased in recent years, as these pollutants possess characteristics to pose a significant environmental risk. POPs are persistent in the environment at a decadal scale; capable of becoming highly concentrated in fatty tissues. These can then become subsequently transferred great distances along trophic levels; a process linked with serious health effects in humans and other living organisms (WHO 1993).

Additionally, these substances undergo processes of bioaccumulation greater at each increasing level along the food chain (Peruguini et al. 2007). POPs reported within sea turtles include polychlorinated biphenyls (PCBs), organochlorine compounds (OCs) and perfluorinated compounds (PFCs) as the most studied. Among OCs, organochlorinated pesticides and hexachlorobenzene (HCB) are the two most important classes investigated (Cobb & Wood 1997; Alam & Brim 2000; Corsolini et al. 2000, Storelli & Marcotrigiano 2000; Keller et al. 2004, 2005; Storelli et al. 2007; Monagas et al. 2008; Oros et al. 2009; Richardson et al. 2010; Lazar et al. 2011; D'Ilio et al. 2011; Camacho et al. 2012, 2013).

Despite significant oil spills at sea, the impact of oil on sea turtles remains relatively unknown. However, studies have assessed polycyclic aromatic hydrocarbons (PAHs) due to the potential for metabolizing and subsequent biomagnification of these substances within the marine food chain (Camacho et al. 2013). Oil exposure can affect turtles via increased egg mortality and developmental defects; direct mortality due to oiling in hatchlings, juveniles, and adults; negative impacts to the skin, blood, digestive and immune systems, and salt glands of individuals (Milton et al. 2003). Stressor conditions for exposure to PAHs can inflict long-term impacts on sea turtle populations, due to decreased fitness of sea turtle individuals, and subsequent increases in population susceptibility to environmental pressures. Previously, Hutchinson and Simmonds (1992) proposed a relationship between low-level chronic exposure to contaminants and the occurrence of cutaneous fibropapillomatosis in sea turtles. Camacho et al. (2012) reported baseline concentrations of PAHs in loggerhead sea turtles from two populations of the West Africa coast; however, they suggested that further studies are necessary to evaluate the long term deleterious effects of PAH exposure.

The use of non-invasive biomarkers in apex species that are in danger of extinction such as seals, dolphins, hawks, cormorants, herons and whales has been reported in previous studies (Casini et al. 2003; Fossi et al. 1999). The application of non-invasive biomarkers for species threatened with extinction allows the use of animals found in the wild as indicators of the state of conservation of ecosystems and long-term monitoring. Techniques investigating the detection of vitellogenin in green turtles, as non-invasive biomarkers, found associations between the expression of this precursor molecule for egg yolk proteins, lipovitellins and phosphovitins with xenoestrogens as environmental stressors (Sifuentes-Romero et al. 2006).

The first study that implemented non-invasive biomarkers was developed with green turtles in Uruguay through the use of porphyrins for the detection of heavy metals and PAHs (Berrondo 2007). Porphyrins are byproducts of oxidative metabolites, intermediate metabolites of heme biosynthesis and are synthesized by organisms via cellular respiration. Porphyrins include Coproporphyrins and Uroporphyrins.

These metabolic products are stored in heritopoyetic tissues (liver, kidney) and excreted in urine and faeces (Berrondo 2007; Fossi et al. 1999). The presence of pollutants such as PAHs, PCBs and heavy metals alter hemosynthesis by interfering with biosynthesizing enzymes and subsequently altering the profile of excreted porphyrins. Through accumulation of uroporphyrins it is possible to identify the presence and concentration of PAHs, and through accumulation of coproporphyrins and uroporphyrins it is possible to detect PCBs (Berrondo 2007).

Sea turtles applied as bioindicators of pollution in marine ecosystems are of increasing interest because they are long-lived species that may bioaccumulate organic and inorganic contaminants from food, sediment and water (Andreani et al. 2008). These characteristics enable ecotoxicological studies to assess the response of sea turtle populations to pollutants in

ambient environment. This is enabled by the recovery of valuable information from dead animals of endangered populations, promoting the development of monitoring protocols over long-term periods that link mortalities with the harmful effects of pollutants.

Analytical Procedures Used in Sea Turtles Ecotoxicology Studies

The differences in metabolized forms of chemical substances necessitates the detection and evaluation of the impact of pollutants in exposed organisms. This leads to the study and development of morphological, molecular, biochemical or physiological biomarkers that detect biological effects on organisms (Bianchini et al. 2006).

This chapter will use the biomarker terms defined by Livingstone (1993): body fluids, cells or tissues, physiological, behavioural or energetic responses of organisms that may indicate the presence or exposure to contaminants. We then describe any biomarkers used in the ecotoxicological assessment of sea turtle studies.

Chemical Determination of Metals: Use of Tissue Residues in Sea Turtle Ecotoxicology

Although metals are considered contaminants, it is important to remember that they are natural substances (Walker et al. 2004). Through geological and biological cycles, they are redistributed naturally in the environment; in rocks, minerals, soil, water and air. These levels are generally low and widely dispersed. Rainwater dissolves rocks and physically transports the material to rivers and streams, and eventually into the ocean (Goyer 1991; Novotny 1995). The atmosphere represents a major entry pathway of certain metals into the ocean.

In terms of human exposure and toxicological significance, anthropogenic activities present greater importance in relation to the concentration of metals in the environment, as metal concentrations likely increase with human activities (Silva et al. 2014). Metals have been used throughout human history to make utensils, machinery, and in mining and smelting, while more recently such elements have been used in industry, agriculture and medicine. These activities generate an increase in environmental levels of metals.

With the increased levels of these contaminants in the environment their toxic effects can be felt in many sectors of the biota. This is due to metals being highly reactive and bioaccumulative, i.e., organisms are not able to completely eliminate the metals absorbed. Living things require small amounts of metals to perform vital functions in the body; however, these elements in excessive levels can be toxic (Silva et al. 2014). Other metals, such as mercury, lead and cadmium, have no function in organisms and their accumulation can cause serious illness, especially in vertebrates. When released as industrial waste in water, soil or air, these elements can be absorbed by plants and animals nearby, causing poisoning along the food chain, since they tend to accumulate in aquatic biota (Mazzuco 2008).

For a metal to exert its toxicity, it must cross the membrane and enter the cell. If the metal is of a lipid soluble form, such as methylmercury, it easily penetrates the membrane. However, when attached to proteins such as cadmium bound to metallothionein, the metal

enters the cell by endocytosis, while other metals such as lead, can be absorbed by passive diffusion. The toxic effects of metals typically involve interaction between the free metal and cellular target, such as specific biochemical processes and/or cellular and subcellular membranes (Cope et al. 2004).

Based on the above, it denotes that all forms of life are affected by the presence of metals, depending on the concentration and chemical form of these. As previously mentioned, many metals are essential for the growth of all types of organisms, from bacteria to humans, but they are only required at low concentrations and can damage biological systems when present in high concentrations (Silva et al. 2014). The manifestation of toxic effects of metals is associated with their concentration, which can be distributed throughout the body, affecting various organs and altering biochemical processes and cellular structures (Salgado 1996).

In the marine environment, bioaccumulation of metals occurs in many ways, but mainly through the ingestion of food and suspended particulate matter containing metals, and directly from the acquisition of metals from bottom sediments pore waters and removal of metals in solution (Kennish 1997).

The concentration of metals in the tissue of vertebrates has been used as a parameter to evaluate the quality of diverse environments. In the marine environment, mammals, turtles and birds are often affected by pollution due to the longevity of these groups. Furthermore, the position of these species in the trophic web creates susceptibility associated with the cumulative power of cadmium, chromium, lead, mercury and others. This can result in serious damage to the health of diverse populations (Furness & Rainbow 1990).

Assessing hazards and risks to life through metal concentrations in biological tissues is very important, because these residues generally provide a better understanding of the exposure conditions experienced by an organism compared to the concentrations of contaminants in the surrounding environment. In order to use these data for wildlife preservation, we should exercise caution with a number of factors regarding contaminant accumulation; results represent the bioavailability according to the physical-chemical characteristics of the area and chemical speciation of the contaminant as well as ecological and physiological characteristics of organism, which may influence uptake and accumulation (Hopkins 2005).

Reptiles are important members of ecosystems and generally have life history characteristics that make them vulnerable to the accumulation of metals (long life, high trophic level, aquatic habitat). Sea turtles are of increasing interest as potential bioindicators of metal pollution in marine ecosystems, due to the long life of metals in vertebrates, and because these species can accumulate inorganic contaminants through food, water and sediments (Andreani et al. 2008). Since sea turtles inhabit coastal waters, human activities can endanger these animals.

Due to all of these factors, several studies have measured the levels of metals in the tissues of juvenile and adult sea turtles worldwide (Anan et al. 2002; Barbieri 2009; Bezerra et al. 2012; Day et al. 2005; D'Ilio et al. 2011; Godley et al. 1999; Maffucci et al. 2005; Sakai et al. 2000a, 2000b; Silva et al. 2014; Storelli et al. 2005).

The literature shows that muscle, kidney and liver tissues are the most analyzed for quantification of metals, while organic compounds are usually investigated in the tissues of liver and adipose tissue.

Thus, such tissue samples are collected (in addition to samples from other tissues such as heart, pancreas, gonad, skin, brain and lung) in dead individuals. Already in living individuals

are collected blood, skin biopsy fragments carapace. The determination of contaminants in the blood is very important because this is the first means of transport of these elements throughout the body and before targeting organs, which may indicate a recent contamination. The elements most frequently analyzed are cadmium, Cu, Hg, Ni, Pb, Se and Zn are the most investigated metals in sea turtles. Cu, Ni, Se and Zn have an essential role in metabolism and animal growth. Cu in particular plays a role in oxygen transport, energy production and enzyme activity (D'Ilio et al. 2011). Zn is essential for the structure and function of many proteins and enzymes for proper functioning of the immune system. Cadmium is generally accumulated in the kidneys over the long-term; this is mainly due to its connection to metallothioneins, as Cu and Zn tend to accumulate in the liver (Andreani et al. 2008) and Pb in the bones. In the aquatic environment, inorganic mercury is microbially transformed into methylmercury, a more bioavailable and toxic organic form (MeHg), with a strong tendency to biomagnify in aquatic food webs (D'Ilio et al. 2011).

For quantification of elements in tissues of sea turtles, the protocol is generally the same, or very similar in all studies, except for some differences in relation to the volumes of reagents employed. The samples are thawed, weighed and usually 1 g of wet weight is placed in an oven to dry (60°C). After this process, the samples are then digested in concentrated nitric acid (HNO3 65%) and then diluted with MilliQ water®. The metal concentrations are determined by atomic absorption spectrophotometry (AAS). The two types of atomizers used in AAS are flame and graphite furnace; and there is a third method, by hydride generation. In the case of Hg, samples are usually analyzed through the technique of atomic absorption spectrophotometry with cold vapor (CV-AAS) using a hydride generator coupled to an atomic absorption spectrophotometer.

As more of the form used to quantify metals in tissues of sea turtles is through atomic absorption spectrophotometry with flame. Briefly, the method involves determining the presence and amount of a particular metal in a solution based on the principle that the steady state free atoms can absorb light at a certain wavelength. The absorption is specific to each element. The source most commonly used for atomic absorption measurements is a hollow cathode lamp consisting of a tungsten anode and a cylindrical or zirconium cathode, constructed with the metallic element of interest itself, and supported by a glass tube containing inert gas, such as argon. At one end the electrodes are positioned while the other end is sealed with a transparent window (usually quartz) calibrated for the wavelength of interest (Krug et al. 2004). To assess the concentration of a sample by atomic absorption spectrophotometry, the method involves creating a calibration curve with standardized element solutions and then inserting the sample points on the curve. This yields a concentration to be subtracted against the calibrated points to white.

Data are generally expressed in µg metal/g dry weight tissue; however, some studies express results in wet weight, so such samples do not pass through the drying process. There are no baseline data on the concentration of metals in tissues of sea turtles. Baseline data are typically used to perform a comparison with levels in other environments. However, caution is needed when comparing individuals of a given environment with another geographical area as there are a variety of factors that influence the accumulation of contaminants in wildlife. For example, sex: there may be differences due to changes in behavior, feeding ecology, or reproduction, and in addition, adult females maternally transfer contaminants to their eggs resulting in a disposal route for such individuals (Guirlet et al. 2008).

Age is another factor that may influence the accumulation of contaminants, e.g., metals are accumulated continuously throughout the life of an animal, which would be expected to result in higher residues in the tissues of older animals. This may be true for most species of sea turtles but not for *C. mydas*. A number of studies have reported negative correlations between concentrations of metals in the tissues (As, Cd, Cu, Mn, and Zn) and the turtle size (curved carapace length, CCL) (Gordon et al. 1998; Saeki et al. 2000; Sakai et al. 2000a, b; Silva et al. 2014). An explanation for this trend is the variation in eating habits between youth and adults of *C. mydas*.

In general, green turtle juveniles feed on zooplankton whereas adults graze on grass and seaweed after returning to their feeding grounds in coastal waters (Sakai et al. 2000b). Vegetable items have comparatively lower concentrations of metals than zooplankton, such that the amount of metal available through diet for adults is lower than that of juveniles. A maximum exposure would then be expected to occur early in the life cycle, when the dietary intake of green turtles is based on higher trophic level prey in the food chain (Silva et al. 2014). McKenzie et al. (1999) suggested that the charge of the metal body in green turtles could decrease as the animal grows, due to a dilution effect associated with the reduced intake of contaminants as individuals grow and alter carnivorous feeding habits to herbivorous.

The global importance of marine turtles in the transport of contaminants throughout food webs and ecological systems represents another very interesting situation. For example, turtles are known to be major contributors of nutrients and energy in ecosystems of coastal dunes during the nesting season. Indeed, in some systems where spawning is intense, as in Tortuguero in Costa Rica, approximately 80,000 green turtles lay an average of over 100 eggs per spawning. Turtles can be one of the most important biological transporters of energy and nutrients.

Since maternal transference in sea turtles typically involves high concentrations of contaminants passed on to eggs, the role of turtles in contaminant transport from the ocean to the food chains of coastal dunes should be evaluated (Hopkins 2005). So, working with concentration of contaminants in tissues is very important to increase awareness about the wildlife being affected by human manipulation of the environment, and how this ultimately affects other environments.

To reach our main goal in the conservation of these endangered species, future studies should focus on how a contaminant is accumulated, which influences its accumulation and division among the tissues, and that the accumulation is important for the body. Thus, the tissue concentrations of metals will be more useful to understand the concentration of the surrounding environment and the possible effects resulting from this exposure.

OXIDATIVE STRESS BIOMARKERS

All cells utilize oxygen in respiration for generating energy, a fundamental process without which much of life could not exist. The oxidation process creates free radicals as a byproduct, which in large proportions can cause damage to cells. However, oxidative stress is caused by an imbalance between the production of such reactive oxygen species (ROS) and the ability of a system to rapidly detoxify these biological intermediate reagents or repair the resulting damage.

Oxidative stress represents a large increase in cellular reduction potential or a large decrease in the reducing capacity of redox molecules. This reducing environment is preserved by enzymes that maintain this state through a constant input of metabolic energy. Imbalances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all cellular components, including proteins, lipids and DNA (Gardner and Oberdorster 2005). Thereby, enzymatic and molecular concentrations related with redox processes can be used as biomarkers for ecotoxicology assessments.

Oxidative stress can cause severe damage and cellular death – moderate oxidation can trigger apoptosis, whereas severe oxidation may cause necrosis. Assessing the oxidative stress of cells can determine the toxic effect of pollutants in exposed individuals, elucidating the degree of injury to which they are subjected and to identify detoxification pathways in organisms.

Reduce Glutathione (GSH)

Reduced glutathione (GSH) is a tri-peptide molecule found intracellularly in high concentrations, essential to all aerobic organisms (Huber et al. 2008). GSH provides a protective function to the cell by reducing oxidative species (*ex*. ROS) giving rise to their importance in the evaluation of oxidative stress (Manaham 2000). Many reactions of GSH involve a highly polarizable sulfhydryl group. This group is a strong nucleophile for reactions with electrophilic chemical compounds, making it an electron donor to other compounds and highly reductive. Thus, GSH is a protective biochemical cell against ROS and electrophilic compounds generated by oxidative processes, both in body and in the environment (Huber et al. 2008).

Concentration of GSH can be determined by the method of Sedlak and Lindsay (1968) for GSH and nonprotein thiols. For each 250 µl of sample (ex. supernatant of liver, homogenized with 0.3 mg for tissue in 0,5 mL of potassium phosphate buffer 0.1 M) 50 µl of trichloroacetic acid (TCA) is added to 48% concentration for protein precipitation. Subsequently, samples are centrifuged at 1,000 x g for 15 min at 4°C. 50 µl microplate sample of supernatant (soluble protein fraction) and 230 µl Tris- base buffer (400 mM, pH 8.9) are added to readings. Blank sample are used with 230 µl of Tris base and 400 mM and 50 µl of TCA 8% in PBS. Finally, 20 µl DTNB (5,5 '-dithio- bis -2- nitrobenzoic acid) is added to 2.5 mM (in 25% methanol buffer, 300 mM Tris- base, pH 8.9) and immediately measure for absorbance (λ = 415 nm). Determinations of GSH content are made by comparison with a standardized curve for GSH (0, 1, 2, 4, 8, 16, 24 and 32 mM GSH). Unit measurements are expressed in micromoles of GSH and nonprotein thiols per milligram of protein.

Glutathione S-Transferases (GST)

Glutathione S-transferases (GST) are a group of isoenzymes and can be of cytosolic, mitochondrial or microsomal origin. GSTs perform a number of functions within cells: removal of reactive oxygen species (ROS) and the conjugation of reduced glutathione (GSH) with hydrogen sulfide group, essential in the process of cellular detoxification (Sheehan et al.

2001). GSH also act as transport proteins and are related to the transfer of lipid hydroperoxide (LOOH) between lipid structures such as vacuoles and microsomes (Leaver & George 1998).

The activity of GST is measured using the method of Keen et al. (1976), however with certain modifications, through indirect determination for thioether substrates GSH (endobiotic molecule) formed from 1-chloro-2,4 -dinitrobenzene (CDNB, synthetic substrate to determine activity of most GST isoforms) measuring absorbance (λ = 340 nm). Samples are thawed on ice, subjected to ultrasound (6 cycles of 4s each) and centrifuged at 9,000 x g for 30 min at 4°C. A volume of 50 µl of supernatant (soluble protein fraction) of the sample is added to wells of a 96-well microplate, and 100 mL of assay medium containing 1.5 mM GSH, CDNB 2 mM potassium phosphate buffer 0.1 M (pH 6.5) is added immediately before readings are conducted. Gradual increases in absorbance are monitored and record by an absorbance microplate reader at 15 second intervals for a total period of 3 minutes. Total GST activity is expressed in micromoles of thioether formed per minute per milligram of protein.

Glutathione Peroxidase (GPx)

Glutathione peroxidase is an antioxidant enzyme that converts H_2O_2 into H_2O. This reaction with glutathione is then oxidized to glutathione disulfide, which can be converted back to glutathione by glutathione reductase in an NADPH-consuming process (Dröge 2002).

GPx activity may be measured by the method of Flohé and Günzler (1984) by monitoring the decrease of NADPH concentration during reaction with H_2O_2 as a substrate (at λ = 340 nm). One unit of GPx activity is defined as the amount of enzyme that oxidizes 1 µmol of NADPH min^{-1}.

Superoxide Dismutases (SODs)

The superoxide dismutases (SODs) are antioxidant enzymes that constitute the first line of defence against ROS. Cu, Zn-SOD is principally a cytosolic enzyme and Mn-SOD is mainly found in the mitochondria. Their function is to dismutate cellular concentration of O2•− to hydrogen peroxide (H_2O_2) and oxygen (Lesser 2006). SODs are the most important of theose defences, when coupled with the necessary mechanism for full detoxification of ROS.

Determination of total SODs isoforms enzymes can be assayed following the method of Suzuki (2000). This occurs via through inhibition of the reduction of nitrotetrazolium blue chloride (NBT) by the superoxide radical (O2•−), which is generated as a by-product of xanthine/xanthine oxidase system. Absorbance is measured per minute at λ =560 nm order to calculate the rate of change over time. One unit of SOD activity is defined as the amount of enzyme needed to inhibit the reaction by 50% of the superoxide radical NBT reaction. Activity is expressed in units of SOD per mg of protein.

Catalase (CAT)

Catalase is an important enzyme involved in the detoxification of H_2O_2 from cells, which may be converted into water in a similar manner to glutathione peroxidase (GPx).

Furthermore, decreased catalase and increased SODs activities can accelerate H_2O_2 generation by increasing the conversion of O_2^- into H_2O_2·. This may cause cellular oxidative damage such as lipid peroxidation and/or cell death (Dröge 2002). Furthermore, prolonged hypoxia in green turtles has been associated with elevated CAT and SOD activity (Valdivia et al. 2007).

The assay for CAT may be performed following the method of Pippenger et al. (1998), for the disappearance of H_2O_2 (10 mM) in KPi (50 mM, pH 7.0) at λ = 240 nm. One unit of enzyme activity is defined as the amount of catalase needed to reduce 1 μmol of H_2O_2 per min. Results are expressed in units per mg of protein (Valdivia et al. 2007).

Protein Carboxylation (PCO)

Oxidative stress results in the formation of carbonyl groups (e.g., aldehydes and ketones group) with a biochemical response termed as protein carbonylation (PCO) (Cattaruzza & Hecker 2008). Protein carboxylation tends to be hydrophobic and resistant to proteolysis, which can cause cellular damage via chemical changes in these macromolecules (Wong et al. 2008; Suzuki et al. 2010).

Determination of protein carbonyls can be performed indirectly by reactions with 2,4-dinitrophenylhydrazine (DNPH) to form dinitrophenyl hydrazones, which are detectable at 358-370 nm (Levine et al. 1994; Quinlan & Gutteridge 2000). 200 μl of supernatant samples (soluble protein fraction) are added to 800 μl of DNPH to 10 mM (prepared in 2 M of HCl) following centrifugation. Blank samples contain an equal volume of DNPH. Samples are vortexed for 5 minutes, and kept in water bath at 30°C for 1.5 h. After incubation, the proteins are precipitated by addition of 1 ml TCA (trichloroacetic acid) to 28%. It is subsequently centrifuged at 9,000 x g for 10 min. The protein pellet is washed by suspension (3x in ethanol/ethyl acetate 1:1) and then homogenized by vortexing and centrifuged. Proteins are resuspended in 6 M guanidine chloride, centrifuged at 9,000 x g for 5 min to remove insoluble material.

Carbonyl content is determined through spectrophotometry (λ = 360 nm) in microliter plates using the molar absorption coefficient for the hydrazone of 2.1 x 10^4 M^{-1} cm^{-1}. Before and after the reaction occurred proteins were quantified (in a reserved rate of soluble protein fraction, and proteins in guanidine chloride respectively) to avoid errors due to losses during the procedure. Results are expressed as nanomoles of carbonyl per milligram of protein (quantified after the test and corrected by the initial concentration of proteins).

Lipid Peroxidation (LPO)

Lipid peroxidation represents a cellular process which can be measured as a proxy for oxidative stress (Dröge 2002). Toxic effects resulting from oxidation of cellular components such as thiols, enzyme cofactors, proteins, nucleotides and lipids, mediated by reactive oxygen species (ROS) and reactive nitrogen species (ERN), generally known as free radicals (FR), trigger this process by removing hydrogen atoms to methylene group of polyunsaturated fatty acids chains in cell membranes, altering the fluidity and functionality of these (Lima et al. 2001).

Production of lipid hydroperoxides is verified by the FOX method (ferrous iron oxidation ammonium sulphate) and quantified by complex formation of Fe^{+3} - xylenol orange (source of light absorption) in the presence of butylated hydroxytoluene as a stabilizer (Jiang et al. 1991, 1992).

Samples are sonicated for 2 min on ice, after addition of methanol (500 µl) and centrifuged at 1,000 x g for 10 min at 4°C. For reading, 30 µl of sample (soluble protein fraction) is added to 270 µl of reaction medium and pipetted into the microplate [(xylenol orange at 100 mM, 25 mM H_2SO_4, BHT (butylated hydroxytoluene) at 4 mM, $FeSO_4.NH_4$ (ferrous ammonium sulfate) at 250 mM (in 90% methanol)]. The reaction is completed after 30 minutes at room temperature and capped microplates, to reduce evaporation of methanol. Absorbance of samples are measured (λ = 70 nm; reading range = 550-570 nm), with blank sample and positive control (30 µl of 50% methanol and 1 µl of hydrogen peroxide 30%, respectively).

Antioxidant Capacity (ACAP)

Animals use molecular oxygen (O_2) for the oxidation of food and power generation. During this process, reactive oxygen species (ROS) are continuously produced in aerobic organisms as byproducts of cellular respiration, causing numerous deleterious effects to the cells (Halliwell & Gutteridge 2007). ROS are continuously produced as unwanted toxic byproducts of normal metabolism of various endogenous processes.

It is estimated that approximately 1 to 3% of O_2 consumed in animal systems is converted to ROS, which mainly include the superoxide anion (O2•-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH•) (Storey 1996). In a normal healthy cell, antioxidant defenses act to reduce ROS. These defenses include the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferases (GST) and glutathione reductase (GR). Beyond these there are non-enzymatic antioxidant defenses including: ascorbic acid, flavonoids, α tocopherol, lipoic acid and glutathione. As such, there is an important balance between the production of pro-oxidant and antioxidant defenses. However, increases in ROS production may overcome the antioxidant defenses, resulting in a state known as oxidative stress. Such stress can lead to damage of macromolecules and changes in critical cellular processes such as lipid peroxidation, inactivation of enzymes, oxidation of DNA bases and protein degradation.

Oxidative stress can occur when cells are subjected to stressful environmental factors, such as exposure to intense light, ultraviolet (UV), contaminants (metals and organic compounds), heat shock, hypoxia, and hyperoxia (Borges 2013). Organic metal compounds can stimulate ROS production by a variety of biochemical pathways, including redox cycle bipiridílicos herbicides; redox transition metal (e.g., Co, Cr, Ni, Va) with O2 and other ROS, self -oxidation of specific oxygenases (e.g., cytochrome P450 (CYP); induction of enzymes (e.g., CYP reductase flavoprotein); and disruption of electron transport through the membrane by depletion of lipophilic contaminants and antioxidant defenses (Livingstone 2003).

When the production of free radicals and/or reactive species exceeds the capacity of action of antioxidants, it promotes the oxidation of biomolecules, generating specific metabolites and oxidative stress markers, which can be identified and quantified. Such markers are derived mainly from the oxidation of lipids (malondialdehyde - MDA), proteins

(protein carbonyls) and DNA (quantification of oxidized bases). Another approach to the evaluation of oxidative stress is by employing indirect methods, based on the total antioxidant capacity against peroxyl radicals (ACAP).

There is limited published data regarding sea turtles, however previous studies have examined possible correlations between chemical contaminants (Labrada-Martagón et al. 2011) and ingestion of garbage (Yoshida 2012) with respect to indicators of oxidative stress in the blood of green turtle (*C. mydas*), without assessing total antioxidant capacity.

ACAP is determined by detection of ROS via fluorimetry (excitation/ emission: 485/520 nm) using 2',7'- dichlorofluorescein-diacetate (H_2DCF-DA) as a substrate. Each sample is homogenized (1:5, w: v) in a buffer solution and exposed to peroxyl radicals generated by thermal decomposition (37°C) of 2,2'- azobis (2 methylpropionamidine) dihydrocloride (ABAP, 1 mM). Fluorescence is then measured for 30 min. in a fluorometer. ACAP is determined based on the difference between the area of fluorescence generated in the presence and absence of ABAP, and the normalized fluorescence value obtained in the absence of ABAP.

MICRONUCLEUS ASSAY

The Comet assay (test of individual cells in agarose gel) is a technique for the evaluation of damage and DNA repair in proliferating and non-proliferating cells individually. This technique employs extremely small cell samples. Cells with increased DNA damage show an increase in the migration of chromosomal DNA in the nucleus towards the anode, which resembles the shape of a comet (Speit & Hartmann 1999). The Comet assay has broad applications in genetic toxicology, genotoxicity in vitro, in vivo tests, and biomonitoring. Comet assay slides are analyzed by first adding 20 µl of ethidium bromide for staining, after which are immediately analyzed under an epifluorescence microscope with 400x magnification in a blind test, with 1000 cores on each blade. Cores are classified according to the damage, as the length of the tail formed after electrophoresis. Classified core is as follows: 0 (no apparent damage), 1 (little damage), 2 (medium damage), 3 (maximum damage) and 4 (core destroyed or apoptosis) (Ferraro et al. 2004).

Monitoring was performed *C. mydas* by evaluating the genotoxic potential of mixtures of pesticides. This was achieved by micronucleus tests in peripheral blood erythrocytes (biomarker of exposure and effect on DNA). A clear reduction in micronucleated cell number was observed as sampling sites moved away from the Canal. This aids in developing a protocol for analysis and repair of DNA damage by comet assay on individuals in the rehabilitation center Sea Turtle Karumbé (Montevideo) (Borrat et al. 2011).

ORGANIC POLLUTANTS INTO SEA TURTLES

Regarding the presence of organochlorine pollutants in tissues of sea turtles, previous studies (Aguirre et al. 1994; Rybitski 1995; Corsolini et al. 2000; Gardner et al. 2003; Mckenzie et al. 1999; Juarez 2004, Innis et al. 2008; Camacho et al. 2012, 2013) have reported the presence of PCBs in tissues and eggs of different species (*C. caretta*, *C. mydas*,

*D. coriacea, L. olivace*a). However, it remains difficult to relate cause of death with presence or concentration of pollutants due to lack of basic data for comparison (Pugh & Becker 2001; Guillete 2000). It is necessary to generate baseline information of pollution levels in wildlife populations in environments with low anthropogenic impact, as this enables comparisons with populations in polluted regions.

The first studies on this topic have reported the presence of Aroclors 1254, 1242, 1248, 1260 (McKim & Jonson 1983; Thompson et al. 1974) but recent studies report enroll as coplanar congeners 77, 126 and 169 as well as mono- ortho 28, 56, 60, 66, 108, 118, 105, 156, 189 and 153 by highlighting the occurrence in most studies conducted both in the Atlantic, Mediterranean and Pacific (Pugh & Becker 2001; Corsolini et al. 2000; Alam & Brim 2000). There are records of the presence of DDT in tissues of loggerhead turtles (*C. carreta*) (Pugh and Becker 2001) and lindane (Mckenzie et al. 1999). In *D. coriacea* the presence of the pollutant aldrin and its derivatives has been reported (Mckenzie et al. 1999). Among the most common pollutants found in sea turtles are chlordanes cis-chlordane, trans-chlordane, cis-nona chlordane, trans-nona chlordane, ocichlordane, heptoclor epoxodico) recorded for *C. mydas*, *C. caretta* and *D. coriacea* (Pugh & Becker 2001). The presence of endosulfan and hexachlorobenzene in samples of *C. mydas* and *L. olivacea* have also been reported (Gardner et al. 2003).

CASE STUDY: *CHELONIA MYDAS* ECOTOXICOLOGY IN BRAZIL

There are few studies on the ecotoxicology of marine turtles in Brazil, and most have been conducted with green turtles (*C. mydas*). These report concentrations of metals in the tissues of animals found stranded dead. Herein, we talk about 5 papers, 3 of which are already published and the other 2 from undergraduate degree completion projects.

Barbieri (2009) analyzed the concentrations of some metals (Cd, Cu, Ni, Mn and Pb) in tissues (liver and kidney) of 30 green turtles (*C. mydas*), 15 adults and 15 juveniles, which were found stranded dead along the Estuary of Cananéia, along the south coast of the state of São Paulo, between January 2005 and September 2006. In this study organotropism was found, which had already been reported for sea turtles, with Cd levels being higher in the kidney. This study found no significant differences between adults and juveniles. For concentrations of Cu, there were no significant differences in the liver between adults and juveniles. On the other hand, concentrations of nickel in adult livers were significantly higher than those of juveniles and liver tissues were significantly different than that of kidneys. Levels of lead were higher in adults, and the means were not significantly different in both organs.

Comparing these results with other studies, Barbieri (2009) found levels of Cd and Pb lower than those reported for the North Atlantic and both China and Japan. However, this has not enabled conclusions regarding whether this is due to the lower concentrations of these metals in the South Atlantic, or dietary differences between populations of different feeding areas.

Bezerra et al. (2012) determined the concentration of mercury in fragments of shell in 25 green turtles (*C. mydas*), of which 20 were living individuals that were trapped in fishing gear

and 5 found dead at a beach on the coast of Ceará (Northeast Brazil). Of these 25 animals, 22 were considered juveniles and 3 subadult/adults.

The highest levels of mercury were observed in juvenile green turtles, with a significant negative correlation between the size of the animal (CCL) and Hg concentration. And, as mentioned earlier in this chapter, the authors related this correlation factor to the change in eating habits between juvenile and subadult/adults of this species. When green turtles are recruiting to coastal habitats their diet changes from an omnivorous diet of a pelagic turtle to a herbivorous diet of an immature coastal turtle. This change results in feeding exclusively on benthic algae and sea grass when they reach sexual maturity (Arthur & Balazs 2008; Bolten 2003; Cardona et al. 2010). When the animal feeds on a more omnivorous diet, it is exposed to higher levels of organic mercury than when feeding on benthic plants such as the case with mature animals. The author points out that, in this way, juvenile turtles preference towards coastal habitats makes them more susceptible to contamination by mercury that is most abundant in these environments, as has been observed on the Ceará coast.

Regarding comparisons of levels found on the Ceará coast and elsewhere, few studies have used fragments of shells of *C. mydas* as a tool for assessing contamination. With these results, Bezerra et al. (2012) concluded that concentrations of Hg found in the carapace of green turtles of the Ceará coast are lower than those observed in the shells of other marine species, and much lower than those reported for internal organs of sea turtles.

Moreover, levels are generally lower than Hg content found in top of carnivorous animals in the marine chain, such as birds and marine mammals.

Silva et al. (2014) determined the levels and distribution of metals in tissues of green turtles (*C. mydas*) stranded at Cassino Beach, Rio Grande, Rio Grande do Sul State (Southern Brazil). Tissue samples (liver, kidney, muscle, and gonads) were collected of 29 juvenile green turtles, where gonads were histologically analyzed for sex determination. Based on results, the sex ratio was calculated as 1.25:1 (female: male).

Relationships between sea turtle size (measured as curve carapace length, CCL) and metal concentrations were not significantly different between males and females; therefore, data were pooled for analysis. In relation to non-significant differences between males and females, with regard to contamination by metals, it is in accordance with data reported for *C. caretta* from the Italian coast in the Mediterranean Sea (Franzellitti et al. 2004; Maffucci et al. 2005; Andreani et al. 2008). In addition, gender differences were not observed for *E. imbricata* and *C. mydas* of Japan (Anan et al. 2001).

Silva et al. (2014) found organotropism between metals analyzed, as mentioned in literature, with highest levels of Cu (Lam et al. 2004; Gardner et al. 2006; Talavera-Saenz et al. 2007; Andreani et al. 2008; Barbieri 2009) and Ag found in the liver; Pb, Cd and Zn in the kidney. In general, Cu levels in the liver were higher than those reported for green turtles in other regions, with the exception of Costa Rica (Andreani et al. 2008) and China (Lam et al. 2004), which were similar, indicating that Cu is present at relatively elevated concentrations in juvenile green turtles from southern Brazil. This trend could be associated with high levels of Cu available in coastal waters and associated biota from this region. Continuous and accelerated urban growth, as well as intense industrial and agricultural development occurring along the southern coast of Brazil over the last decades have been considered as major sources of metals released in estuarine and coastal waters in these regions (Seeliger & Knak 1982; Baumgarten & Niencheski 1998; Niencheski et al. 2005, 2006; Barbosa 2007). Pb concentrations found in the tissues of green turtles in the present study were higher than those

reported for *C. mydas* in other regions such as Costa Rica (Andreani et al. 2008), Mexico (Gardner et al. 2006; Talavera-Saenz et al. 2007), China (Lam et al. 2004), and Cyprus (Godley et al. 1999). Thus, juvenile green turtles that use regions of the southern Atlantic coast in Brazil for feeding and development have had greater concentrations of Cu and Pb compared to other regions. It is difficult, from data reported by Silva et al. (2014), to provide an explanation for variations in levels of metal contamination in tissues of green turtles from different regions.

Differences in quality of marine environments between locations may be a possibility. This would lead to an irregular intake of metals through the food chain. Discrepancies found in green turtles from other regions could be explained by several factors, including differences in eating habits, as well as geographic and temporal differences in environmental exposure to metals.

It is worth emphasizing the importance of analyzing possible correlations among metal concentrations in tissues of sea turtles, and few studies have analyzed these. Silva et al. (2014) found positive correlations between non-essential metals (Ag, Cd and Pb) and essential metals (Cu and Zn) in the liver and kidney of green turtles. These results suggest that correlations observed between non-essential metals and essential metals in tissues of the green turtle *C. mydas* is likely associated with metal-induced metallothionein synthesis induced by Zn and/or Cu. In this case, the essential metals could induce the synthesis of these proteins to protect against the potential toxic effects of non-essential metals (for review: Miles et al. 2000; Coyle et al. 2002).

Medeiros (2011) evaluated the concentrations of Cd, Pb and Hg present in the liver, kidney and muscle of 11 green turtles stranded in northern and middle coast of Rio Grande do Sul State, southern Brazil, from October 2009 to April 2010. Organotropism was previously reported in literature, and Medeiros (2011) concluded that the most striking feature of his study was the presence of high levels of Cd in the kidney and liver of green turtles-analyzed. The presence of high levels of Hg were also highlighted, which makes clear that populations of *C. mydas* are under heavy anthropogenic pressures in coastal regions of Rio Grande do Sul State (Brazil) and that these concentrations of Cd and Hg may be directly or indirectly influencing the population decline of this species. Lorente (2010), unlike the aforementioned studies, examined the occurrence of chlorinated compounds in green turtles collected along the Rio Grande do Sul coast. Therefore, 11 livers were collected, 11 muscles, 2 kidneys and 2 hearts; a total of 17 juveniles. The identification and quantification of PCBs and pesticides were performed on a gas chromatograph with electron capture detector. Low levels of organochlorine compounds detected in *C. mydas* collected on the southern coast of Rio Grande do Sul are possibly related to food habits of this species (organisms of low trophic level), once a major route of organic contaminants accumulation is the diet. Of the two major groups analyzed (PCBs and pesticides), the predominance of PCBs in almost all groups and environments suggests these compounds are highly stable and have been widely used, resulting in a higher bioavailability for turtles unlike chlorinated pesticides. For all analyses reported certified standards were used.

PROSPECTS FOR ECOTOXICOLOGY OF SEA TURTLES

A problem that is of concern to many researchers is the recent increase in fibropapillomatosis (FP) around the world. Focusing research on identifying the cause and understanding pathogenesis of the disease is of primary importance, so that management strategies can be developed to minimize its impact on populations of endangered green turtles (Herbst et al. 1999). FP is a disease that has been characterized as growth of skin tumors, which develop mainly in soft tissues, although can also grow on the carapace and plastron.

Most tumors are benign, however can disrupt basic functions of the individual (Foley et al. 2005).

Some studies have reported physiological changes in animals affected by the disease, including anemia, immunosuppression, hypoproteinemia, electrolyte imbalance, elevated liver enzymes, and propensity to acquire systemic bacterial infections (Work & Balazs 1999; Aguirre & Balazs 2000; Foley et al. 2005).

Regarding of sea turtle ecotoxicology, environmental contaminants have been identified as one of the possible factors contributing to the development of FP on sea turtles by reducing immune function (Balazs 1991). The increased prevalence of FP and other clinical diseases in sea turtles gives merit for further investigation of potential role of contaminants in their etiology.

Some efforts have been made in several geographic areas to elucidate possible correlations between environmental pollution and FP. In some places this correlation is indeed found, which justifies the belief that there may be a relationship, but in other places a lack of correlation confounds understanding of this disease.

In Brazil, several research groups are working with this theme. Recently in the VI and VII Meeting of Research and Conservation of Sea Turtles in the Western South Atlantic (ASO), two studies regarding this topic were presented in poster form.

Silva and Bianchini (2013) above reported some data on this, demonstrating that turtles deemed clinically healthy had considered size (CCC) lower (36.76 ± 1.18 cm) than those with FP (43.2 ± 2.52 cm). Furthermore, turtles with the disease had a lower mean value of cholesterol, bilirubin direct, sodium and Cd.

Rossi et al. (2013), collected blood samples of green turtles from different areas of the Brazilian coast and determined the concentration of PCBs a priority by European legislation: congeners 28 (triclorado), 101 and 118 (pentaclorados), 138 and 153 (hexaclorados) and 180 (heptaclorado).

These authors observed no differences between turtles with and without FP or between the sites studied, although catches have occurred in different Brazilian States.

It is important to note that observations of sample populations with reduced biological variability (sex and size) provide the most reliable results in terms of disease response. Biochemical parameters may vary between genders and stage of life; it is therefore important to minimize these effects to ensure representative observations. Due to the magnitude of the Brazilian coast, researchers continue to focus on studies of this nature so that we may understand a little more about fibropapilomatosis.

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Chapter 3

SEA TURTLE PHOTO-IDENTIFICATION

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ABSTRACT

Individual recognition of marine turtles is necessary to advance a variety of research initiatives. Unique identification of individuals can help researchers analyze population size, stability and density, site fidelity, movement patterns, and seasonal habitat use for example. Identification of individuals can detect frequency of rescued and rehabilitated turtles as well as frequency of nesting females to certain nesting beaches. Datasets collected from these individuals overtime can aid international conservation measures in terms of decision making. Turtle identification is currently based on four semi-successful methods: Passive Integrated Transponders, (PIT tags), flipper tags, living-tags, and satellite tracking. Since each method has significant drawbacks and only limited benefits, photo-identification marking provides a viable solution. This technique presented here involves photo imagery of the scales around the face of each individual turtle. Researchers identify the quantity of scales around the face, their shape and spatial arrangement. Scale arrangement and characteristics are presumed to be unique to each turtle, like a fingerprint. PITMAR is a photo-identification computer algorithm and a facial picture database created by Fundacion Neotropico whose main goals are the control and monitoring of individual sea turtles.

INTRODUCTION

Individual recognition of specimens is needed in research and can be used for a wide range of important analyzes. For example, as researchers identify and collect data on individual turtles, they can better understand population size, density, and stability.

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Researchers can identify the degree to which individual associate with one another, site fidelity, movement patterns, and seasonality. At rescue and rehabilitation centers, identification of individual sea turtles can help veterinarians understand frequency in admissions. At nesting beaches, biologists can identify spawning aggregation sites and frequency of nesting by individual females.

The accuracy of these studies is necessary in decision making and implementation of international conservation measures. Different marking systems are currently used to provide researchers the ability to differentiate each individual within a natural population. To be reliable, any wildlife identification method must have a marking technique unique of each individual and constant throughout time to allow subsequent re-identification for long-term studies. The system should also not be invasive, nor influence individual behavior or how other animals behave with the marked specimens. Sub-lethal effects affecting the survivorship of the individual or modifying its recapturing feasibility should also be avoided.

Little work has been undertaken on non-invasive or non-traumatic identification of reptiles. Toe-clipping technique in lizards consists in the removal of no more than two toes per limb in a coded numbering sequence. Applied to lizards it could have potentially negative consequences on their behavior and survivorship, more in arboreal species, reducing their clinging performance (Bloch & Irschick 2004), than in fossorial species, but both are affected. Even though natural foot or toe loss is observed in wild lizards and they can survive with that damage, the welfare of the individual and the natural population of origin must be deeply analyzed before using this method. Looking for non-invasive, non-stressful, and economically sustainable marking procedures, the use of different kinds of physical patterns to identify individuals by pictures is widespread and applied to a growing list of wildlife species.

Photo imagery is a non-invasive and potentially effective means of individual recognition for wildlife. Depending on the image capturing method, the results and quality can be highly variable. For example, aerial photos are collected by aerial means as sea turtles float on the surface of the ocean, from land, or underwater. If pictures are to be taken underwater, light refraction and artifacts from particulate matter in the water column, swimming speed of the individual, and picture angle must be taken into account. Obviously the picture quality affects the level of certainty of the identification and match success.

Since the 1960's researchers have used photo-recognition of individual whales through identification of patterns on the underside of each fluke (Aarabi *et al*. 2000, Beekmans *et al*. 2005, and Kniest *et al*. 2010). Photo-identification has also been used in several species with characteristic marks like body color patterns in octopi, *Wunderpus photogenicus*, (Huffard *et al*. 2008), and dorsal head pattern in salamanders, *Eurycea tonkawae*, (Bendic *et al*. 2013). Photo identification on the flanks of grey nurse sharks, *Carcharias taurus*, both in captive and wild individuals has shown success (Bansemer & Bennett 2008) allowing researchers to re-identify an individual 14 years after the first time it was photographed.

Other studies include: photo-identification of Baltic grey seals *Halychoerus grypus*, (Hiby *et al*. 2007) through identification of head and neck fur markings, blue whales, *Balaenoptera musculus*, (Olson 2009), through identification of mottling on the back and flanks of individuals, African penguins, *Spheniscus demersus*, (Sherley *et al*. 2010) through identification of chest plumage patterns, and whale sharks *Rhincodon typus*, (Meekan *et al*. 2008, Rowat *et al*. 2009) through identification using spot patterns in the area behind the 5th gill.

The use of photographic identification is a promising alternative marking technique to the above-mentioned invasive method of toe-clipping in lizards. For example, photos have been successful in detecting unique black spot patterns on the ventral scales of wall lizards, *Podarcis muralis* (Sacchi *et al*. 2007). Changes in color, but not pattern, were observed in this species allowing re-identification up to 4 years after the first pictures were taken. For species with less clear ornamentation patterns, the study of individual pholidosis has shown that shape, distribution and size of scales from specific areas of the body can be effectively used to tell apart specimens.

In terms of conservation applicability for endangered species, the survival rates of Florida Manatees, *Trichechus manatus latirostris*, (Langtimm 2004) have been successfully estimated using photo- identification of individual body marks and scars.

Even some species of crustaceans are likely to benefit from photo-identification techniques like the painted crayfish, *Panulirus versicolor*, as photos aid in identification of variable pigmentation patterns on the first abdominal sclerite, (Frisch & Hobbs 2007). This case is particularly interesting because it is very difficult to recognize individuals with classical methods in long-term studies due to the tag loss after molting.

In the case of European lizards, *Podarcis muralis* and *Lacerta bilineata* (Sacchi *et al*. 2010), the use of Interactive Individual Identification System (I^3S) software is used to identify unique spot patterns. Unique patterns form at the intersections among pectoral scales, in these two species. , This technique proved accuracy and validity as a recognition system of this software. When a large number of individuals are registered in the database, computer algorithms are used in analysis and reduce time used in specimen query time.

This kind of software has been successfully used for individual recognition in the following cases: Rosalia longhorn beetle, *Rosalia alpine,* based on the contour digitization of the spots present on the beetle's elytra, (Caci *et al*. 2013) whale sharks, *Rhincodon typus,* using an astronomical pattern-matching algorithm to identify unique individual spot patterns (Arzoumanian *et al*. 2005Collaboration among researchers even has led to the development of initiatives such as The Pacific Islands Photo-Identification Network, (PIPIN), a growing network of researchers studying cetaceans in the Pacific Islands Region through the use of photo-identification techniques.

METHODS

Sea Turtle Non-Photographic Identification Methods

In the case of sea turtles, which are air-breathing reptiles, four methods are currently used by researchers on a global scale. Each of these methods has limited benefits and significant drawbacks.

1. *PIT Tags:* (Passive Integrated Transponders) or microchips are harmless small implantable devices injected subcutaneously or intramuscularly into the sea turtle, usually near the shoulder region, posterior to the neck. This method involves stressful handling of each turtle and momentary pain. Similar in size to a grain of rice, PIT tags consist of a chip, a capacitor and inert wire encased in glass. This technique

requires expensive hand scanners to read the unique identifying number implanted in the individual sea turtle. The drawback is that these scanners are not globally calibrated or readily available in every site. Scanners and tags operate in frequencies from 125 kHz to 134.2 kHz (older used 400 kHz frequency with poor range and for that reason it is recommended to avoid those), so not all scanners can read all PIT tags. The radio frequency of the scanner excites the PIT tag which reflects the radio waves in form of a unique alphanumeric code detected by the scanner. PITs can migrate, or could be ejected before the entrance wound heals (producing the tag loss), or be broken by aggressive behavior (Van Dam & Diez 1999; Runyan & Meylan 2005; Smyth *et al*. 2013). While this may be a good marking system at a local level, it does not provide a universal solution.

2. *Metal Flipper Rings, Flipper tags:* Flipper tags are small metal clips, like earrings, attached to the front or hind flipper. This individual tagging method has been used for decades. This methods has demonstrated efficacy, however, tags deteriorate over time or become detached. Some researchers are concerned that tags can become entangled in marine debris, thus posing a serious threat to the turtle (Nichols *et al*. 1997, Reisser *et al*.2008). Additional drawbacks of flipper tags are the increased hazard of infections or the impaired swimming performance (Balasz 1999, Reisser *et al*. 2008). Sometimes to compensate the risk of tag loss, researchers mark the individuals in each of the four flippers thus multiplying the jeopardy associated to this marking method. Other materials have been tested. Brightly colored plastic tags could increase the risk of predation because they may affect the animal´s concealment capability. Metal tag insertion procedures are also painful and stressing. During egg deposition, they are attached to females and should only be achieved by trained and experienced researchers as the inaccurate implantation site of metal tags could be the origin of tissue tears or lead to necrosis (Wright 2001). Position and distance from the flipper edge, animal size, tag material, biofouling and time since the tag was applied also affects the probability of its loss (Balazs 1982; Limpus 1992; Bjorndal *et al*. 1996; van Dam & Diez 1999; Reisser *et al*. 2008). Many countries only allow the use of this marking method for registered and authorized researchers. Our recommendation is that it should be removed worldwide from the suggested tagging protocols list for marine turtles.
3. *Living tags*: This method was developed in the 1980s. This method involves an invasive surgical technique by removing two bone fragments of separate carapace scutes, one dorsal (dark colored) and one ventral (lighter), and implanting them in a new location on the sea turtle. Coding of selected scutes used in this procedure is consistent for the release year, so researchers can identify the time lapse of the tag (Hendrickson & Hendrickson 1981). This is a traumatic process, generally performed at the hatchling stage and with a limited number of possible combinations. As it is an autograft, no immune response is observed, however, this tagging method requires the turtle to remain in captivity and not to be released until each fragment is healed and fused with plastron and carapace respectively (Mrosovsky *et al*. 2003).
4. *Satellite Tracking*: This method started in the 1980s, which involves attaching or harnessing an electronic global positing transmitting device to the carapace of a sea turtle. The device sends signals to a satellite and records the coordinates. The accuracy of the location depends directly on the number of signals received in each overpass by different satellites, from an individual emitter. Depending on the

accuracy and model, additional information such as diving depth, water temperature, swim speed or submergence time, can be received from the transmitter. While this system provides very important information, each device has a very high cost, can be damaged, and is shed with the carapace scales. The use is temporary and due to the high cost, provides only a very small sample within a population thus offering limited efficiency. In addition, all methods to attach satellite transmitters, may affect negatively hydrodynamic efficiency or long distance swimming abilities on marine turtles, or even they could increase their likelihood of entanglement in marine debris. All satellite tags that are commercially available, currently work via the ARGOS system. This system requires an initial set-up with the ARGOS program to purchase satellite use over a specified amount of time. There are different transmitters with a wide range of options and prices. Cost depends on the battery life and amount of data collected. To conserve battery power, these devices are prepared to transmit only when the turtle is at the water surface and can be programmed to work in on/off time cycles. The attachment of the satellite tag to the carapace requires stressful turtle restraint, especially when adult individuals are handled. It is done by one of the following methods: gluing (two components epoxy resins, fiberglass resin or both simultaneously) for hard shelled species. For leatherbacks a harness or tether are used. (Yasuda & Nobuaki 2005; Blumenthal *et al*. 2006; Godley *et al*. 2007).

Sea Turtle Photo-Identification

Sea turtle photo-identification is a non-invasive and harmless marking method, not requiring direct manipulation of individuals and thus reducing stressful handling. Photo imagery does not require tags, thereby avoiding problems of tag loss, hydrodynamic drag and entanglement with floating debris. This method is cost-effective and can be applied to global populations. No special camera equipment is required, even a Smartphone or simple camera can be used to capture the photos. Frames can be extracted from video sequences to obtain useful images for the system. With high definition cameras, pictures can even be taken from a long distance without disturbing the individual turtle. As swimming turtle surface to breathe, their neck exposes the head to facilitate good quality images for individual identification.

While there are drawbacks, they are minimal and easily prevented. As with other species, the main problem with photo-identification based systems is the large amount of time needed to process manually each picture and that it has to be achieved by highly trained personnel to reduce error. Inexperienced or poorly trained workers can cause misidentification or data loss. With a growing amount of available pictures, the total time involved in processing them becomes unmanageable so the use of computer aided individual recognition and the development of more accurate software is helping in this titanic task.

Soft Shelled Sea Turtles

Leatherbacks, *Dermochelys coriacea*, lack scales on their carapace or skin. This increases challenges of attaching satellite tags. The high cost of scanners needed to read PITs has also reduced the efficacy of this identification technique for this critically endangered species

In 1996, McDonald *et al*. published a study illustrating that the ‘pineal spot’ of leatherbacks, a de-pigmented cloudy looking pink macula in the dorsal surface of the head (directly over the

pineal gland, thus its name), is distinct enough to be used as a unique identifier (McDonald *et al*. 1996).

Software Usage: Later, the use of identification software has validated this method, for a determined moment in time, showing exclusive individual differences in the spot for a sample of more than 400 leatherback turtles (with 100% accuracy), while eliminating the false negatives (Buonnantoni 2008). Other authors have used this system too with high levels of success (Pauwels *et al*. 2008, Zeeuw 2010). Although the results are promising, stability or the possible deviation within the pineal spot pattern over time (Figure 1), still have to be confirmed in long-term studies.

Figure 1. A stranded Leatherback, *Dermochelys coriacea*, found in Tenerife, being transported to the Turtle Hospital. Notice the pineal pink spot.

The software used to compare and match or reject the identification of an individual in different pictures relies in the Scale Invariant Feature Transform or SIFT (Lowe 2004) that is capable to identify changes in illumination, occlusion, noise and viewpoint. SIFT selects, in a given image, local points of interest or key points giving information in descriptor vectors not affected by image scaling, rotation, illumination or 3D camera viewpoint, with reduced probability of distortions caused by noise, clutter or occlusion by pollution or scars.

Before starting the algorithm matching procedure, the picture has to be manually cropped to a smaller image with the background removed. The red, green and blue components have to be merged into a grayscale image. Finally the contrast needs to be controlled and balanced with a correction factor to highlight the pink spot on the leatherback, thus enhancing contrast and removing flashlight. SIFT finds those regions detached from the background, determining their centers and the best scale and orientation relative to the dominant gradients, giving key points and key point descriptors with data epitomizing local gradient information.

Comparing images: To compare two different images, SIFT quantifies their similarity by matching a group of key points based on their corresponding descriptors within a given tolerance level. This step is improved by adding more sets of compared key points in the

images. A large number of consistent key points will show a match between the two individuals compared. A low number of key point results in a false paired match, indicating two different individuals. An intermediate number of matches need new additional verification achieved by a later test. In this case the results are presented to the human operator for a final confirmation or rejection of the matching (Pauwels *et al*. 2008; Zeeuw 2010).

Other Sea Turtles

Except for leatherback turtles, the remaining species of marine turtles have scales with individual shape patterns that facilitate their identification. Throughout the life cycle of a turtle, changes in color of *tomium* (scales forming the upper and lower parts of the beak), face, carapace and plastron, make color markings inappropriate as tools for photo-identification (Feliz *et al*. 2010). In the case of hawksbill turtles, *Eretmochelys imbricata*, tympanic scales present more utility for photo-identification than other cephalic scales due to a high individual variation in number, shape, and arrangement as well as stability over time and visibility in pictures (Feliz *et al*. 2010).

In some areas and programs, manual processing of pictures is still used with the help of volunteers. To reduce the amount of data to be processed, simple keys, similar to those used to identify species, have been developed with the inclusion of discriminatory features to increase accuracy and working speed. With the help of statistical techniques and a classification criteria matrix the number of steps needed to identify an individual are reduced (Lloyd *et al*. 2012).

Using a ranking system for the characteristics of the scales and assigning them a priority value according to the utility to separate individuals, different factors are used in the developed keys. These factors are (Lloyd *et al*. 2012):

- Number of parietal scales
- Number of parietal scales with tick
- Fronto-Parietal scale with tick
- Frontal scale with tick
- Presence of extra Fronto-Parietal scale
- 2 temporal scales
- 4 temporal scales
- 5 temporal scales
- Prefrontal scale with tick
- Supraocular scale with tick
- Interparietal scale meets Frontoparietal scale
- 3 temporal scales
- Number of spots at parietal scale base

Scale characteristics used in the key by Lloyd *et al.*:

- Tick: lighter color border of the scale extends lineally into a scale.
- Spot: small dark patch of skin, located at the base of Parietal scale or on neck.
- Dot: lighter small dot inside a scale.

- Frontoparietal extra scale: subscale inside Frontoparietal scale.
- Interparietal meets Frontoparietal scale.

Photo-identification of scales for hard-shelled sea turtles requires knowledge of the scale anatomy on the head; dorsal side. Here we present a numerical classification scheme to classify each scale using the following identifying numbers: 1 Prefrontal, 2 Interprefrontal, 3 Frontal, 4 Frontoparietal, 5 Supraocular, 6 Temporal, 7 Parietals, 8 Interparietal, 9 Occipitals. (Figure 2 and Figure 3)

Figure 2. Identification scheme of dorsal scales; shown here a loggerhead turtle, *Caretta caretta*.

Figure 3. Identification scheme of dorsal scales; shown here green Turtle, *Chelonia mydas*.

Photo identification when used to positively identify illness in sea turtles, such as fibropapilloma, a disease shown in turtles with tumors, can be a useful tool. For example, Studying the regression of fibropapilloma afflicting green turtles in the Hawaiian Islands, Peter Bennett and Ursula Keuper-Bennett (with pictures taken since 1988) started to identify each individual using facial scales patterns as shown in their work published together with George Balazs (Bennett *et al*. 2000).

Figure 4. *Chelonia mydas* scale face pattern.

Flipper scale pattern can also be used in a similar way for photo-identification (Bennett *et al*. 2000). However, the high frequency of flipper loss or scale alteration in terms of shape and arrangement makes this method not as widely efficient as head scales (Figure 5).

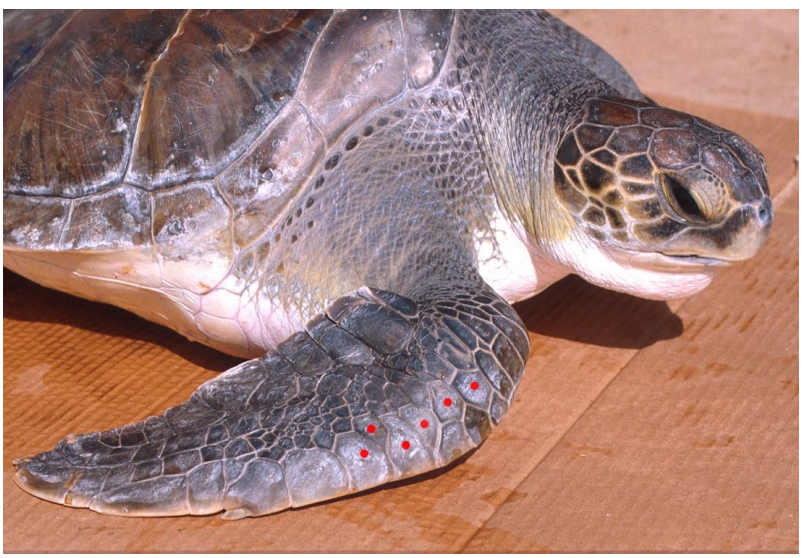

Figure 5. Singular shaped scales (highlighted with red dots) useful for the photo-identification of this individual of Green Turtle, *Chelonia mydas*.

The use of facial scales for the identification of sea turtles has clear advantages over dorsal head scales because of the large number of facial scales available for analysis. Facial scales have higher variability in number and polygonal shape, thus providing a wider range of possibilities (Figure 5). The following identification scheme of each lateral facial scale is presented here: 1 Tomium, 2 Supraocular, 3 Temporal, 4 Post ocular, 5 Sub temporal, 6 Tympanic, 7 Central (Figure 6). In each individual, both sides of the head present differences. When possible, the storage and use of the two profile photos increase the accuracy and reliability of this method. This makes the patterns exclusive to each individual on a global scale, making it serve as a virtual "fingerprint". Again, some research teams use manual identification of facial scales analyzing their number, shape and position when large groups of photos are available. Grouping images helps to reduce the amount of files and increase accuracy and working speed (Reisser *et al*. 2008, Schofield *et al*. 2008).

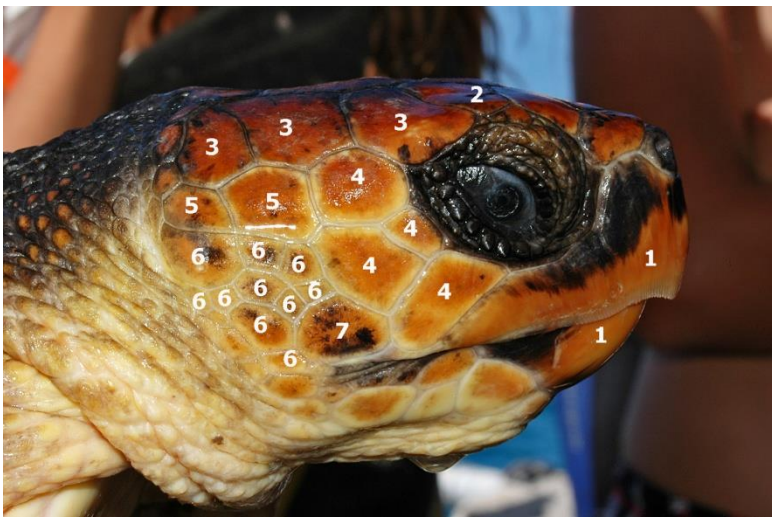

Figure 6. Naming Facial Scales in Turtles. 1 Tomium, 2 Supraocular, 3 Temporals, 4 Postoculars, 5 Subtemporals, 6 Timpanics, 7 Central.

One research trend uses the encoding of each scale according to its position and its shape (Jean *et al.* 2010). By dividing the arrangement of facial scales into rows starting from the eye extending to the neck, the first digit of the code indicates in which row the scale is located. The second digit of the code indicates the position of the scale in that row, counting from the upper jaw to the top of the head. The third digit is the number of sides of the scale. Finally, all the codes from one side of the head are arranged to compose a single profile formed by one digit followed by a series of 3-digit codes (every scale codes). The profiles of both sides define the identity of one individual (Jean *et al*. 2010, Chassagneux *et al*. 2013). Through manual analysis of the pictures, one by one, researchers use an identification tree to split the sample into sequential fields based on the shapes of post-ocular scales. The characteristics analyzed are: the identification scheme and pattern of tympanic and central scales, the shape of sub temporal scales and the shapes of temporal scales (Schofield *et al*. 2008).

Several software algorithms have been used or developed to help in the processing of pictures of facial scales of sea turtles. The design of identification software for turtles with scales has the advantage that a single algorithm can effectively work for all the species. For

these computer aided systems, pictures have to be preprocessed and standardized to allow its use. Below we present PITMAR, a software designed specifically for the photo-identification of sea turtles.

P.I.T.MAR. (Programa de Identificación de Tortugas Marinas = Marine Turtles Identification Program).

Neotropico Foundation is a private non for profit organization, declared for public interest by Spanish Government. We have been working in the Canary Islands since 2000. In collaboration with the Island Council (Cabildo de Tenerife), Neotropico Foundation rescues and rehabilitates all the stranded marine turtles in the island of Tenerife. With a number of entries ranging between 50 and 100 turtles per year, more than 1000 ill turtles have recovered healthy conditions to be released back to the sea in the past 15 years.

In terms of photo-identification of previously treated individuals, we have accumulated photographic images of individual turtles since 2005. Neotropico Foundation started storing images of both sides of the head of every released sea turtle in Tenerife (Figure 7) and we began developing the first steps to our successful photo-identification program; PITMAR (Program to Identify Marine Turtles). In year 2013, Neotropico Foundation was awarded a grant by Fundacion Biodiversidad (a public Foundation associated with the Environmental Ministry of Spain) to develop the PITMAR individual recognition algorithm and its associated database.

Using both facial scale patterns of the turtle in combination with other data, PITMAR allows the identification of every individual of a potential population up to tens of thousands of individuals. As stated before, the data set formed by the number of scales of this region, their shape and disposition is unique to each turtle as if it were a “fingerprint”. This disposition does not vary in shape or number throughout the life of the animal, being naturally modified exclusively by size.

Figure 7. Recapture of Green Turtle, *Chelonia mydas*. First picture was taken by Neotropico Foundation on 16 December 2008. The recapture picture took place 19 August 2011. When the number of stored pictures increases, an automated recognition system has to be applied.

In the wild, traumatic alterations can occur and then they could alter this disposition of scales. To account for alterations, the opposite side of the face region of the same individual can be used for comparison along with other characteristics used in identification and

comparison (size, weight, scars, flipper loss, etc.). For instance, an individual registered the first time with a missing front left flipper will not be included in the file search of those registered later with front left flipper present. The program allows queries of such characteristics to help narrow the search field once the database expands.

Each picture has to be first edited by a human operator to optimize contrast, sharpness and size. In the first phase of development of PITMAR, the operator will also need to mark the intersection points of each face scale, producing a cloud of spots. This procedure is calculated to take 3 to 5 minutes of work. In the near future and with the development of the algorithm, the software should be able to search the intersection point itself, presenting them to the operator for correction or acceptance, thus facilitating the human workload.

PITMAR uses the cloud of spots produced from each picture to analyze similarities with other spot patterns stored in the database looking for positive matches (Figure 8). A total or high level of coincidence would indicate a recapture of the animal, very low level of pattern matching will be considered as a new turtle. If PITMAR finds a stored registry with an intermediate level of matching to the pattern analyzed it will present the results to the operator for final decision on marking it as a recapture or a new registry.

For a greater accuracy of the system, if the opposite side of the turtle's face is available, it will also be used in the matching process thus limiting occurrence of a false positive or false negative.

PITMAR is freely accessible through internet in a web based platform (www.pitmar.net). In any country, with a simple digital camera and a connection to Internet it will be possible to know if the photographed turtle is registered and all the available data stored.

Search of individuals filtered by species, or geographic location, or by user, are also available on PITMAR.

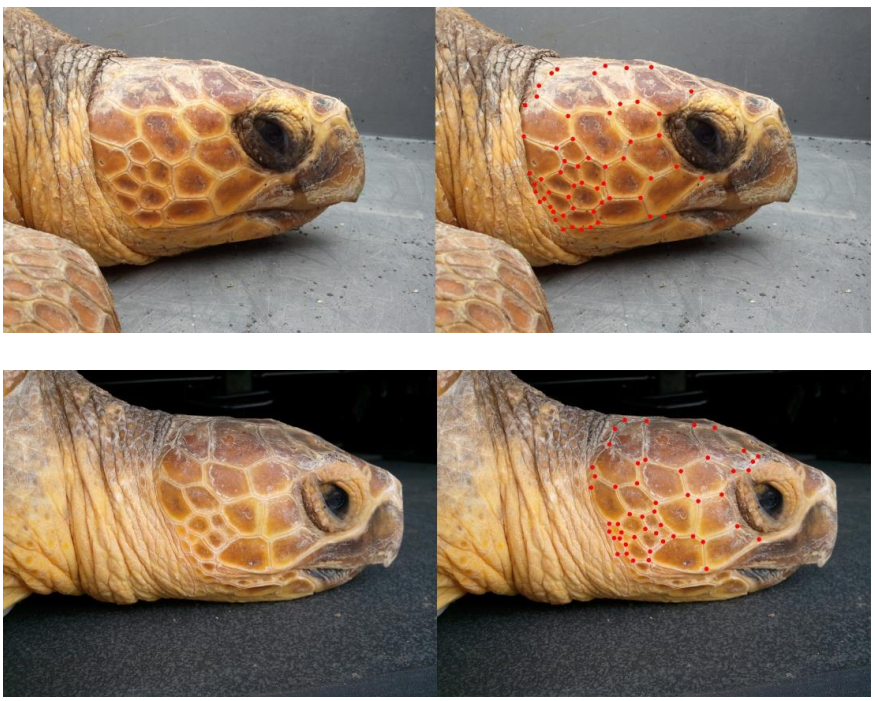

Figure 8. Two Loggerhead, *Caretta caretta*, individuals showing the cloud of spots used by PITMAR.

For injured animals that need to be handled or are going to stay in Rescue Centers for a while, the use of transponder tags are recommended as a collateral marking method and for research purposes.

The potential application of PITMAR to thousands of sea turtle researchers promotes this software as a highly viable conservation tool. It not only provides a beneficial scientific utility program but also encourages a bond for international scientific collaboration. In the associated webpage for PITMAR, environmental education materials will be readily available for use or download making PITMAR also a highly useful device system for educators, naturalists and the general public.

PITMAR is developed in three levels that work jointly.

Level 1. Database

Types of Users

External user: Once registered anyone can use the search tools, view information of the individuals searched, and submit potential new registries to an Operator or Administrator to be introduced in the database. Operator user: Needs to login to use the application. Operator can modify his/her own user and password details, use the search tools, add new capture data to an existing registry and add new turtle specimens to the database. Administrator user: Needs to login to use the application. Administrators can access the general configuration panel, access user profiles or specimens and capture data to modify, delete, insert or search in the database.

User Details

- Id: Internal, autonumeric identifier. Invisible to users.
- User: Nickname (alphanumeric).
- Password: Log in password (alphanumeric). Stored coded and can't be recovered, just modified.
- E-mail: e-mail address.
- Name: Name of the user (alphanumeric).
- Organization: Name of the organization (if applicable).
- User Level: External, Operator, Administrator.

Specimen Details

- Id: Internal, autonumeric identifier. Invisible to users.
- IdPITMAR: Identifier code for each individual formed by year (4 digits)-species code (9 digits)-Autonumeric. Autogenerated. Non modifiable.
- IdTransponder: PIT code.
- IdRingtag: Ring tag code.
- User Id: Identification of the user responsible for the first entry.
- Gender: Male / Female / Unsexed.
- Species: Latin name / common name

- Notes: Observations and singularities of the individual.
- General picture.
- Right side head picture.
- Right side data: geometrical data for picture recognition algorithm.
- Left side head picture.
- Left side data: geometrical data for picture recognition algorithm.

Capture Details

- Id: Internal, autonumeric identifier. Invisible to users.
- IdPITMAR.
- User Id: Identification of the user responsible for the recapture.
- Date.
- Site: nominally explicit capture site.
- Coordinates: in several formats. Optional place selection over maps.
- LRC: straight carapace length
- LCC: curved carapace length
- ARC: straight carapace width
- ACC: curved carapace length
- RFL: right forelimb present
- LFL: left forelimb present
- RRL: right rear limb present
- LRL: left rear limb present
- Notes: Observations and singularities of the capture circumstances (nesting, feeding, basking, wounded) as well as possible pathologies or traumatic wounds observed in first inspection.

Level 2. Monitoring System of Cephalic Scale Pattern

This module will execute the analysis of the given images from the facial area of the turtles head to recognize the different patterns that serve to detect if that individual has been previously registered in the system (and therefore it is a recapture) or if it appears for the first time (and then a new identifier has to be generated).

Level 3. Interface of Use

The inter-operatively with the system will be done via Web, so that any user of the system will be able to access simply using a navigator. Although the use of a Web platform implies small restrictions for the interface, these are totally compensated by the ease of use, the low resources consumption for the administrators, the possibility to use the application from any place of the planet that has an Internet connection and, mainly, the multiplicity of systems on which this tool can be used.

This last point is of enormous importance since, independent of the operating system (Microsoft Windows, Linux, Macintosh, etc.) or the device (computer, notebook, smartphones, tablets, etc.) visitors or operators, will have access to the tool whenever an internet connection is available. The development of the interface was achieved with standards of prevailing Web building software in mind to make the application operate properly via any browser (Internet Explorer, Mozilla Firefox, Operate, Konqueror, etc.). Also the recommendations of WAI for the accomplishment of accessible Webs will be considered.

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Chapter 4

TOPOLOGICAL TOOLS FOR EVALUATING THE STRUCTURAL IMPORTANCE OF SEA TURTLES IN TROPHIC NETWORKS

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ABSTRACT

The use of topological analysis tools in marine trophic networks offers a wide range of possibilities to investigate how the different species are involved in the structuring of a network, as well as to evaluate how important they can be in their organization. This approach allows us to identify the importance of the species from its connectivity and its contribution to the spread of direct and indirect effects in the network and to determine their participation in the mechanisms of ecosystem control. This, to determine if they can be considered as keystone species. Topological analysis will be conducted using a data base from the trophic network of Bahia Magdalena (Mexican Pacific) to determine if green turtles (*Chelonia mydas*) can be considered a highly important structural species based on their connectivity, betwenness centrality and closeness centrality, key species or even species with low topological redundancy, which would give them an important role in the network. Furthermore, the results will deliver evidence on how sea turtles are involved in the different mechanisms of ecosystem control, and whether the absence of these organisms could create major structural gaps in the organization of the network, such as to affect the identified large-scale patterns.

INTRODUCTION

A central theme in the study of trophic networks is how interspecific relationships affect ecosystem dynamics and stability (Pimm 2002; De Ruiter et al. 2005). The importance of these interactions has given rise to the development of concepts such as multispecific management (May et al. 1979; Yodzis 2000) or ecosystem approach (Grant et al. 1997), which recognize the need to know the role of not only one species, but of most or all species to model more accurate responses to the dynamics of each system under study (Jordán et al. 2006). Some authors have suggested that, although changes in species composition are an important indicator to identify perturbed ecosystems, a holistic knowledge allowing identification of structural and functional effects could emerge from the study of communities as networks interconnected by trophic interactions (Dunne et al. 2002; Bascompte et al. 2005). Owing to the relatively stable characteristics of trophic networks, these interactions can provide information on species relationships within a community and how human activities could be degrading ecosystems (Dell et al. 2005).

Network analysis provides a number of tools that can support quantitative community ecology. In particular, there exist techniques for quantifying the positional importance of species (ecosystem components) in food webs. Species that are of high importance in a trophic network can be in either central (like hubs) or unique positions. The latter can be interpreted as species having non-redundant neighborhoods. As a result, their extinction (or overfishing) has profound effects on the ecosystem. This is quite sound in ecology, as a suite of field and theoretical results support the importance of indirect interactions.

Several mesoscale indices have already been suggested in network science, most of them, considering distance between nodes (e.g., closeness and betweenness centrality; Wasserman & Faust 1994). Some of these indices have been applied to ecological problems (Estrada 2007; Jordán et al. 2007). Others have been slightly modified and adapted to ecology (see net status (Harary 1961) and keystone index (Jordán et al. 1999)) or simply developed by ecologists (measuring apparent competition (Godfray et al. 1999; Müller et al. 1999)).

The knowledge about the ability of sea turtles to affect their ecosystem structure and function has been considered one of the most important objectives to be met in the study of the ecology of these species (Bjorndal 2000), in that way we may establish critical habitats for their conservation. Seagrasses are considered the main dietary component in several life stages of green turtles (Bjorndal 1980). In fact, the green turtle is the most abundant large vertebrate consumer of seagrasses in the world (Ogden 1980). In northwest of Mexico, eelgrass (*Zostera marina*) was found in diet samples at canal del infiernillo (Felger & Moser 1973), Bahía Magdalena (Lopez-Mendilaharsu et al. 2005), Laguna de San Ignacio, Punta Abreojos and Laguna Ojo de Liebre (Rguez-Baron unpublished data).

Like other sea turtle species *Chelonia mydas* shows a slow grow and delayed sexual maturity which, along with the intrinsic characteristics of its life history, allows it to spend several decades in different habitats where it uses and modifies the environment (Chaloupka & Musick 1997). Among the main challenges for the sea turtles conservation, is the determination of their ecological role (Bjorndal 1997), because the diet composition strongly influences in their life history, growth and reproduction rates (Wallace et al. 2009). Particularly in immature animals, the selection of diet items has implications on the duration of future life stages and consequently in the required time to reach reproductive maturity,

which is an important attribute in their population dynamic (Seminoff et al. 2003; Jones et al. 2009).

Green turtles from the Eastern Pacific are considered the most carnivorous population of the *Chelonia mydas* complex (Bjorndal 1997). Although generally they feed on algae and seagrasses (Mortimer 1982; Seminoff et al. 2002; Lopez-Mendilaharsu et al. 2005), it has been reported that green turtles are able to ingest pelagic red crabs (*Pleuroncodes planipes*) (Lopez-Mendilaharsu et al. 2005), sea pens (*Ptilosarcus undulates*) (Seminoff et al. 2002; Lemons et al. 2011), tunicates (Amorocho & Reina 2007), hydrozoans, scyphozoans, nematodes, annelids (Carrion-Cortez et al. 2010), sponges (Seminoff et al. 2002) and anemones (Rguez-Baron et al. 2011). A mixed diet has apparently the evolutionary advantage for some species to experience and learn about the optimization of ingested nutrients. The consumption of animal tissues has been associated to protein requirements of immature turtles to enhance their body growth and earlier sexual maturity (Bjorndal 1985).

Associated to these ecological features, Bahia Magdalena probably is one of the more studied green turtle feeding grounds at the Eastern Pacific. Some of the studies include turtle movements inside the channels (Brooks et al. 2009), population dynamics (Koch et al. 2007; López-Castro et al. 2010), diet selectivity and differences in dietary intake associated with turtle size (López-Mendilaharsu et al. 2005, 2008) and health indices across blood biochemistry values and body condition index (Labrada-Martagon et al. 2010a, 2010b). One relevant result is that reported by Koch et al. (2007) suggesting that juvenile of *C. mydas* could live over 20 years inside the lagoon complex until reaching sexual maturity.

Owing to the variety of approaches used to study trophic networks and the large number of topics studied in these systems, we present in this chapter a brief review of a topological analysis of trophic network and its application to assess the importance of green turtles in the coastal lagoon ecosystems.

METHODS

Topological Approach

Building the Trophic Network

Base on the diet matrix of the biomass-balance model (Ecopath) for Bahía Magdalena (Cruz-Escalona et al. 2013) a binary matrix of trophic interactions was building, where 1 represents the presence of prey *i* in the diet of predator *j* and 0 the absence of it. Thus, the number of interactions was determined for all species (predators and prey) in the network. Hence, this analysis focused on trophic interactions between species, not on strength or direction of such interactions, therefore it shows specific information about the topological properties of the studied network area.

To assess the positional importance of the different nodes in the trophic network, and thus infer the positional importance of the species or trophic components under study, we used local and mesoscale indices, whose application in topological analysis has been extensively discussed in multiple publications (Jordán 2001; Jordán & Scheuring 2002; Jordán et al. 1999, 2006; Abarca-Arenas et al. 2007; Gaichas & Francis 2008). These indices are:

Node Degree (D)

This is the most easily applied but least informative index because it only takes into account the number of other nodes connected directly to node *i*. Thus, the degree of node *i* (D_i) is the sum of its prey (in-degree, $D_{in,i}$) and predators (out-degree, $D_{out,i}$), and was computed using NetDraw as $D_i = D_{in,i} + D_{out,i}$.

Centrality Indices

The first index is betweenness centrality (*BC*), which is based on quantifying how often node *i* is on the shortest path between each pair of nodes *j* and *k*. This index was computed using the UCINET IV software package (Borgatti et al., 1996). The standardized index for node *i* (BC_i) is:

$$BC_i = \frac{2 \times \sum_{j \leq k} g_{jk}(i) / g_{jk}}{(N-1)(N-2)}$$

Where $i \neq j$ and k, g_{jk} is the number of equally shortest paths between nodes *j* and *k*, and $g_{jk}(i)$ is the number of these shortest paths to which node *i* is incident (g_{jk} could be equal to 1). The denominator is twice the number of pairs of nodes without node *i*. This index thus measures how central a given node is in terms of being incident to many shortest paths in the network. If BC_i is large for trophic group *i*, it indicates that the loss of this node will have many rapidly spreading effects in the web.

The second centrality index used was *CC* (closeness centrality), which is based on the proximity principle and quantifies how short the minimal paths from a given node to all other nodes are (Wassermann & Faust 1994). This index was also computed using UCINET IV (Borgatti et al. 1996), and its standardized form (*CCi*) is expressed as:

$$CC_i = \frac{N-1}{\sum_{j=1}^{N} d_{ij}}$$

Where $i \neq j$, and d_{ij} is the length of the shortest path between nodes *i* and *j* in the network. This index thus measures how close a node is to the rest of nodes. The smallest value of CC_i will be for that trophic group that upon being removed will affect the majority of other groups.

Keystone Index (KI) (Jordán et al. 2006)

It is used to characterize the importance of species in ecosystems according to their position in the trophic network; it is also known as index of topological importance. This index considers information additional to the nodes directly connected to one another and was defined in detail by Jordán (2001) and Jordán et al. (2006). It is expressed as:

$$K_j = \sum_{c=1}^{n} \frac{1}{d_c}(1 + K_{bc}) + \sum_{e=1}^{m} \frac{1}{f_e}(1 + K_{te})$$

Where n is the number of predators eating species i, d_c is the number of prey of the c_{th} predator and K_{bc} is the bottom-up keystone index of the c_{th} predator. Symmetrically, m is the number of prey eaten by species i, f_e is the number of predators of the e_{th} prey and K_{te} is the top-down keystone index of the e_{th} prey. For node i, the first summation of the equation quantifies bottom-up effects (K_{bu}), whereas the second summation quantifies top-down effects (K_{td}). After rearranging the above equation, the terms that contain the values of K ($\sum K_{bc}/d_c + \sum K_{te}/f_e$) refer to indirect effects (K_{indir}), whereas those that do not contain K ($\sum 1/d_c + \sum 1/f_e$) refer to direct effects (K_{dir}). The sums of these values of effects ($K_{bu} + K_{td}$ and $K_{indir} + K_{dir}$) equal $K_i = K_{bu,i} + K_{td,i} = K_{dir,i} + K_{indir,i}$. In addition to informing about the number of direct connections among nodes, the keystone index informs on how these neighbors are connected to one another (Jordán et al. 2006), emphasizing vertical interactions over horizontal ones (e.g., trophic cascades vs. apparent competition). It also characterizes positional importance, separating direct from indirect effects, as well as bottom-up from top-down effects in the trophic network (Jordán 2001). The keystone index was computed using the FLKS 1.1 software package (provided by F. Jordán), which is designed for characterization of vertical positional importance of species in food webs.

These mesoscale indices have been favored over other more local statistics, such as the distribution of trophic connections (Dunne et al. 2002; Montoya & Solé, 2002), or more global ones, such as food web connectance (Martinez 1992) because the latter for example reflect the global topology of the network but do not provide information on the specific position of individual nodes or their more distant interactions, thus preventing the analysis of important indirect effects, such as apparent competition and trophic cascades (Holt & Lawton, 1994; Menge 1995). Furthermore, mesoscale indices are recommended when the purpose of the study is to understand relationships within a community (Jordán & Scheuring 2002) and especially when one wants to quantify the relative importance of a given species with respect to the rest of species in a system (Jordán et al. 2006).

The Topological Importance Index (TI) (Jordán et al. 2009)

It is used to analyze indirect interactions of various lengths separately (up to a 3-step-length). It assumes a network with undirected links where interspecific effects may spread in any direction without bias (we are interested in interaction webs, in the broadest sense, but considering only indirect chain effects [Wootton 1994]). The effect of species j on species i, when i may be reached from j in n steps, is defined as $a_{n,ij}$, when n=1 (i.e., the effect of j on i is direct): $a_{1,ij} = 1/Di$, where Di is the degree of node i (i.e., the number of its direct neighbors including both prey or predatory species). We assume that indirect chain effects are multiplicative and additive. When the effect of step n is considered, we define the effect received by species i from all **N** species in the same network.

$$\psi_{n,i} = \sum_{j=1}^{N} a_{n,ij}$$

Which is equal to 1 (i.e., each species is affected by the same unit effect.). Furthermore, we define the *n*-step effect originated from a species *i* as:

$$\sigma_{n,i} = \sum_{j=1}^{N} a_{n,ij}$$

Which may vary among different species (i.e., effects originated from different species may be different). Here, we define the topological importance of species *i*, when effects up to *n* steps are considered

$$TI_i^n = \frac{\sum_{m=1}^{n} \sigma_{m,1}}{n} = \frac{\sum_{m=1}^{n}\sum_{j=1}^{N} a_{m,ij}}{n}$$

Which is simply the sum of effects originated from species *i* up to *n* steps (one plus two plus three…up to *n*) averaged over by the maximum number of steps considered (*n*). With this index, it is possible to quantify the origins of effects influencing a particular species, i.e., the internal interaction structure of the network.

The $a_{n,ij}$-values for species *j* had been defined as its "trophic field" (Jordán 2001). For long indirect effects, every species is connected to every other. It is reasonable to define a *t* threshold of $a_{n,ij}$-values separating strong interactive partners from weak interactors. Given a maximum length of indirect effects (*n*) and a threshold for interaction strength (*t*), every node may be characterized by its effective trophic range (Jordán et al. 2009). Since the sets of strong interactors of two, or more, nodes may overlap, it is important to quantify the positional uniqueness of graph nodes. The "trophic field overlap" (TO^n_{ij}) between nodes *i* and *j* is the number of strong interactors appearing in both *i*'s and *j*'s effective range. The sum of all *TO*-values between species *i* and others ($\Sigma TO^{n,t}_{ij}$ summed over all j with $i \neq j$) provides the summed trophic field overlap of species *i* ($TO^{n,t}_i$), and this may be normalized by dividing it with the maximum value ($TO^{n,t}_{max}$) for a given network ($relTO^{n,t}_i = TO^{n,t}_i / TO^{n,t}_{max}$). Note that all this is determined by *t, n* and the topology of the network. We define the "topological uniqueness" of species *i* as $TU^{n,t}_i = 1 - relTO^{n,t}_i$. Here, we used $n = 3$ and $t = 0.001$. This index may contribute to the problem of how to quantify species and role and redundancy in ecosystems (Bond 1994; Luczkovich et al. 2003; Shannon & Cury 2003). This index was computed using CosbiLabGraph (Valentini & Jordán 2010).

Key Player Problem (KPP)

To determine whether sea turtles species belong to the key element set of the ecosystem under study (defined as "topological keystone species complexes" by Jordán et al. (1999, 2006) and Libralato et al. (2006)) we used the "key player" problem approach of ecological network analysis (Borgatti 2003a). This approach is used to determine the importance of different species combinations in maintaining network integrity. More specifically, we used Key Player Problem 2 (*KPP-2*), which works under the assumption that "if information is spread from node n, which nodes must be selected to reach the other nodes in the fastest way

in an intact network?" (Benedek et al. 2007). The analysis was run with the Key Player 1.44 software package (Borgatti 2003b).

Application of Topological Analysis to Evaluate Ecological Role of Sea Turtles

We built a trophic network using stomach content data of representative functional groups found in Magdalena-Almejas Bay, located in the south-west coast of the Baja California Sur peninsula (24º 16 'N and 25º 45' N and 111º 20 'W and 112º 18' W). This system has three different geomorphological zones: Canals Zone (137 km^2) located northwest; Magdalena Bay (883 km^2) located in the center of the complex, and Almejas Bay (370 km^2) located in the southeast (Figure 1) (Álvarez-Borrego et al. 1975). Information about diet matrix was taken from Cruz-Escalona et al. (2013) and includes a 24 trophic components.

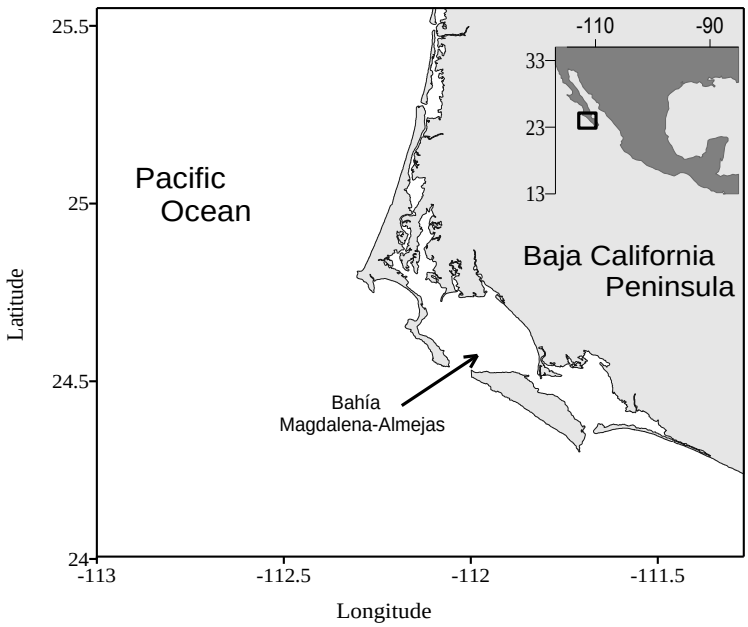

Figure 1. Geographic location of Bahia Magdalena in Baja California Sur, Mexico.

Results of Topological Analysis

Based on the degree of node (*D*), suprabenthic invertebrates were the highest value node (18), followed by sea lions (14), other crustaceans and penaeid shrimps (12). *Chelonia mydas* obtained a degree of node of 9 and was ranked tenth within the 24 nodes (Figure 2).

Centrality indexes showed that suprabenthic invertebrates have the highest betweenness value (*BC* = 43.2) followed by the green turtle (*BC* = 37.1) and sea lions (*BC* = 14.2). Meanwhile green algae (Chlorophyta), brown algae (Phaeophyta), red algae (Rhodophyta), and gray whale showed the lowest intermediate values (Figure 3a). As for species closeness (*CC*), suprabenthic invertebrates (*CC* = 52), sea lions (*CC* = 56) and penaeid shrimps (*CC* = 58) had the most representative values (Figure 3b). As with the degree of node, turtles showed a middle point value (10 position) in terms of closeness index within the network.

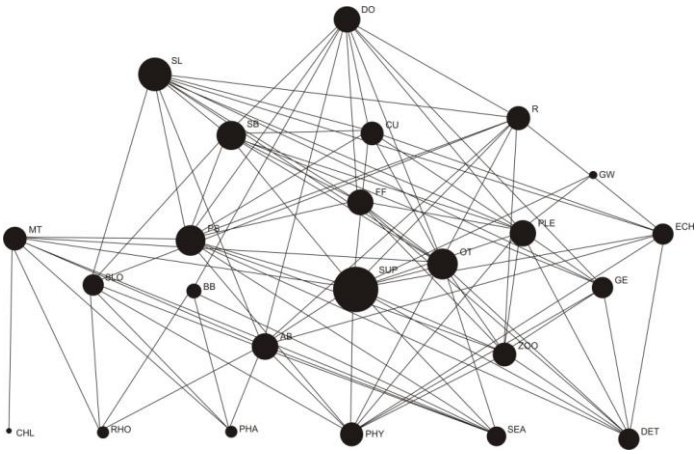

Figure 2. Degree of node index of the studied area's trophic network. The size of the node is proportional to the importance value. Explanation of the initials in the Table 1.

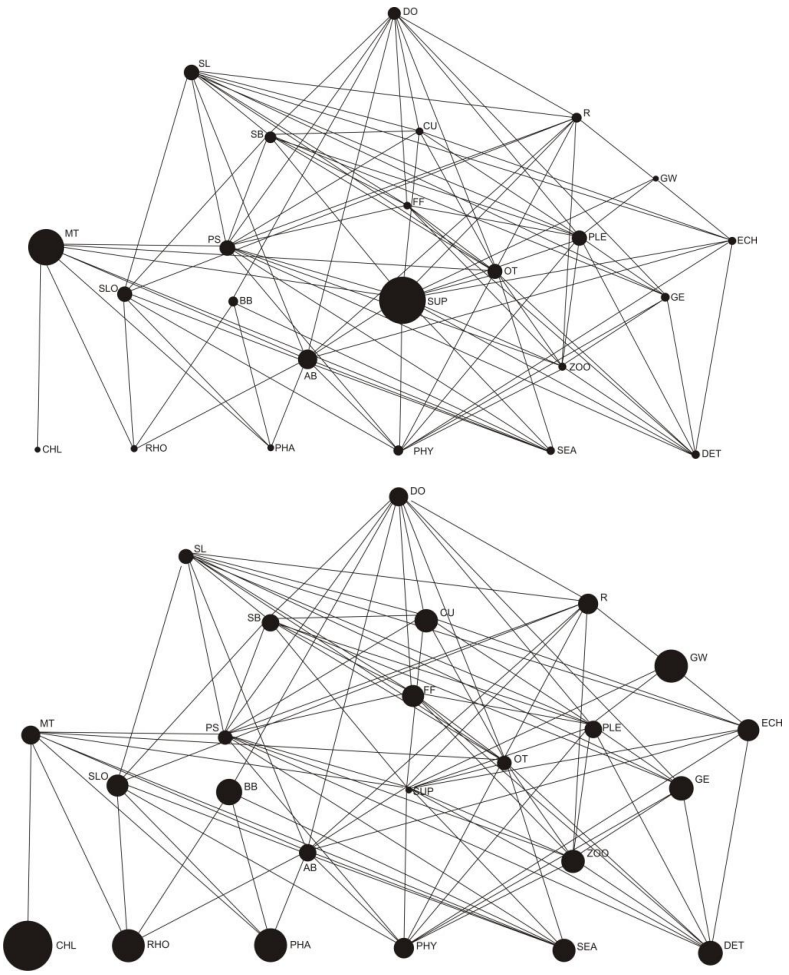

Figure 3. Centrality indexes of the studied area's trophic network. A) Betweenness Index (BC), the size of node is proportional to the importance of the node. B) Closeness Index (CC), the size of node is inverse to the importance of the node.

The keystone index (*K*) showed the highest values for dolphins and sea lions, suggesting that their removal could result in significant structural changes in the trophic network. On the other hand, green turtle is in the eighth position, play the main role in this network as a predator (K_{td}), directly affecting its prey, algae, sea grass and some invertebrates (K_{dir}) and with few predators in the network (Table 1).

Table 1. Results of keystone index of Magdalena Bay

Rank	Species or trophic component	K_{bu}	K_{td}	K_{dir}	K_{indir}	K
1	Dolphins (DO)	0,00	10,68	4,02	6,66	10,68
2	Sea lions (SL)	0,00	10,22	3,81	6,41	10,22
3	Phytobenthos (PHY)	7,08	0,00	2,38	4,70	7,08
4	Detritus (DET)	6,74	0,00	2,18	4,56	6,74
5	Suprabenthic invertebrates (SUP)	4,97	0,44	4,01	1,41	5,42
6	Zooplankton (ZOO)	3,81	0,24	1,61	2,43	4,04
7	Sea bass (SB)	0,16	3,78	2,13	1,81	3,94
8	Green turtles (MT)	0,16	2,90	2,56	0,50	3,06
9	Skates (R)	0,16	2,19	1,46	0,89	2,36
10	Corvinas (CU)	0,29	1,89	1,38	0,80	2,18
11	Seagrass (SEA)	1,96	0,00	1,23	0,73	1,96
12	Other crustaceans (OT)	1,10	0,68	1,51	0,28	1,78
13	Penaeid shrimps (PS)	1,10	0,68	1,51	0,28	1,78
14	Abalone (AB)	0,85	0,85	1,49	0,22	1,70
15	Flatfishes (FF)	0,51	1,01	1,12	0,40	1,52
16	Echidoderms (ECH)	0,58	0,70	0,99	0,29	1,28
17	*Pleuroncodes planipes* (PLE)	0,72	0,54	1,05	0,21	1,26
18	Spiny lobster (SLO)	0,37	0,85	1,15	0,07	1,22
19	Rhodophyta (RHO)	1,05	0,00	0,76	0,28	1,05
20	Phaeophyta (PHA)	1,05	0,00	0,76	0,28	1,05
21	Gerreidae (GE)	0,51	0,54	0,91	0,14	1,05
22	Black brant (BB)	0,09	0,74	0,80	0,03	0,83
23	Chlorophyta (CHL)	0,13	0,00	0,11	0,02	0,13
24	Grey whales (GW)	0,00	0,10	0,07	0,03	0,10

K_{bu}= Importance index from bottom to top, K_{td}= Importance index from top to bottom, K_{dir}= Importance index of direct effect, K_{ind}= Importance index of indirect effects.

Regarding the topological importance index (*TI*), which shows those species that have the smallest topological redundancy into the network, revealed that the Dolphins and Sea Lions were the more difficult replacement nodes (Figure 4). The green turtle was at an intermediate level of topological redundancy suggesting that in this ecosystem this species has a trophic function with other repetitive mesopredators. This can be observed in the trophic overlap established between the turtle and stingrays by invertebrates (e.g., crustaceans, suprabenthic invertebrates, shrimp) and with the lobsters and Black Branch by primary producers. On the other hand, the absence of natural predators for this species in the study area influences the outcome of topological significance found.

The graphical representation of the values of *TI* showed that invertebrates and bony fishes have high redundancy in the network and therefore the values (node size) of this index are low in most of the same (Figure 4).

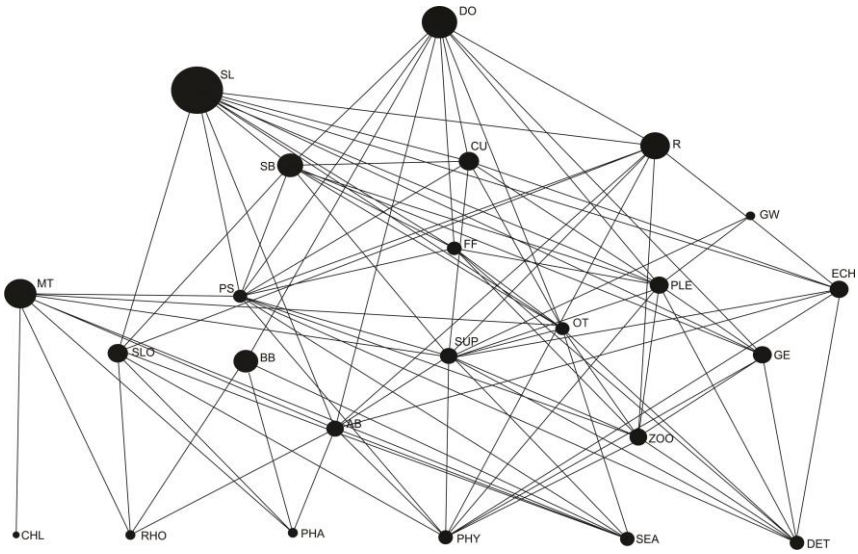

Figure 4. Topological importance index of the trophic network of the study area. The size of the node is equal to the index value.

Finally, analysis of key groups "*KPP-2*" showed that it is composed of a single species, the pelagic red crab (*Pleuroncondes planipes*), being this the most influential in the trophic network interactions, as the dispersion of any effect from this species reaches 100% of the nodes in this network. In this case, being such a short trophic network it is feasible for this to happen, but as more complex trophic networks are analyzed it will be more likely to observed in greater detail the participation of species of interest in the group of keystone species.

CONCLUSION

The topological indices represent a novel and useful tool to evaluate in an easy way, the structure of a trophic network and how the different elements that compose it take part in that structure. In the present study we have implemented these analyses in order to assess the structural importance of the green turtle in a marine trophic network, and make comparisons among our results with other previous approaches which have focused on studying the function of these species in ecosystem through the mass and energy flows and their effects over the population structure of their prey.

The outcomes of this study show how green turtle (*Chelonia mydas*) is a sort of structural value intermediate in the structure of the trophic network in the Bahia Magdalena which is due to an intermediate connectivity and proximity to other network nodes. This means that the species of interest does not provide a high number of trophic interactions with other network components; thus, the proximity of the same for all these nodes is moderate. From this, it follows that green turtle has a mild participation in the velocity dispersion of indirect trophic effects that may occur in this network (trophic cascade, apparent competition, competition for exploitation among others, see Menge 1995). Thereby, the absence of this species would have little current velocity dispersions of these effects, which are considered fundamental for the stability of trophic networks.

Nevertheless, another scenario is presented when the intermediation index (*BC*) outcomes are analyzed. *Chelonia mydas* turned out to be identified as the second most valuable species. This result suggests that if the green turtle does not have a high number of connections within the network, it does play an important role in the dispersion of indirect effects within the same network. This occurs due to the fact that the species of interest becomes an important connection in the flow of trophic interaction among primary producers and top predators. Thus, the loss of this species would force many of the pathways through which the indirect effects are spreading within the network to change and therefore could significantly impact the current network structure.

These results are in line with what some studies have suggested with functional perspective. For example, grazer species may have significant positive effects on grazed areas via their role in enhancing nutrient recycling or availability (McNaughton et al. 1997; Augustine et al. 2003). Repeated cropping by turtles has been found to result in increased nitrogen concentrations in *Thallasia testudinum* in the Caribbean, which was interpreted as improving the nutritional quality of those areas (Bjorndal 1980; Zieman et al. 1984). Moreover, it has been suggested that, historically, grazing by sea turtles played an important role in the maintenance of healthy seagrass beds by removing the seagrass canopy and preventing the build-up of organic matter in seagrass beds (Jackson et al. 2001, Bjorndal & Jackson 2003).

Aragones et al. (2006) through experiments simulated cropping by green turtles by manual removal of plants or plant parts to resemble that made by the animals. The changes to plant chemical composition following grazing were long-lasting, with both *Halophila ovalis* and *H. uninervis* showing significantly elevated whole-plant N concentrations in response to all treatments after 11 and 13 months, respectively. On the other hand, Fourqurean et al. (2010), made protecting experimental plots from grazing by sea turtles caused an increase in the biomass of seagrasses and an increase in the structural complexity of the seagrass canopy, as the length and width of the seagrass blades increased. This shows that green turtles may modify significantly the seagrass beds. Also, through observational evidence, Goatley et al. (2012), showed that green turtles and hawksbill (*Eretmochelys imbricata*) may function as both grazing and browsing herbivores on coral reefs. The green turtles appear to be an important component in tropical seagrass ecosystems in structuring communities through cropping.

From the point of view of ecosystem control, Keystone Index analysis (*K*) identified the top depredators as those who have the top down network control almost exclusively. (See K_{td} in Table 1). In this index, the green turtle showed that its structural importance in the network regarding the mechanisms of ecosystemic control is mainly given by the direct trophic effects (K_{dir}) or predation being greater the contribution as predator than prey (K_{td} vd K_{bu}) and therefore dispersing more strongly its direct effects than indirect for the rest components of the network. This latter result corresponds with the findings in our analyses of topological importance (*TI*) which indicated that *Chelonia mydas* is a species with average levels of topological redundancy and that it shares its predatory function with primary producers with lobsters and Black Brant.

This exercise represents the first study, which uses topological analyses to assess the structural importance of green turtle within a trophic network. The outcomes of this study show this is an important tool for the target mentioned and provides useful information to implement management measures for this species. It is recommended that such studies in

focal areas and hotspots of marine turtles at different life stages, and with this, give a new perspective to the assessment of ecosystem importance of these species.

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Chapter 5

RESEARCH TECHNIQUES IN THE STUDY OF MARINE TURTLE STRANDINGS

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ABSTRACT

Although subject to several caveats, stranding data may provide information on geographic ranges, seasonal distribution and life history of marine turtle aggregations in nesting, foraging/development, and migratory areas. Strandings are the main source of biological samples for ecological studies on marine turtles. Samples are collected either from live and recovered turtles or from dead ones through necropsy. Moreover, strandings provide data on sex ratios of local aggregations, diseases, and feeding ecology, among other topics. Two methods can be used to record stranded marine turtles on beaches: (1) creating a marine stranding network or (2) organizing beach surveys over the area of interest. In order to provide valuable information, stranding networks must use replicable protocols and be able to record the majority of stranding events. However, the discovery and reporting of washed carcasses depends on observation effort and public awareness, and economic resources are critical for the effectiveness of the network. Beach surveys for stranding records need constant effort over areas of interest. Stranding data can be recorded across wide spatial and temporal ranges at high resolution due to the low cost per unit effort compared to in-water or aerial-based studies. Stranding data is often considered as non-representative of populations at sea, since the probability of stranding varies widely in space and time, as predators, scavengers, winds and sea currents may prevent carcasses from reaching the shore. However, strandings yield reasonable data on the occurrence and distribution of marine turtle species in the adjacent marine area. Thus, stranding data, while an imperfect measure of marine turtle abundance, distribution and activity, provides additional information that can complement research studies at sea. Analyses of stranding data can also contribute to a better implementation of conservation measures and management on endangered marine

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turtles in coastal waters through the identification of (1) the areas probably used by marine turtles, and (2) anthropogenic mortality sources in the region of interest. Strandings can also be used for public awareness on the conservation of these threatened species.

INTRODUCTION

This chapter tries to summarize the most relevant techniques used in the study of marine turtle strandings and all the potential data that can be obtained from them. An approach to studying marine turtle aggregations in feeding and development areas can be represented by stranding data, because stranding records do not require high costs as surveys at sea do. Many turtles are found dead, injured or ill on the beach or floating at sea, in coastal waters, especially near important foraging areas.

In recent years, there has been a rising awareness on the global threats affecting marine turtles, such as incidental captures by different fishing gears (Lewison et al., 2004a, b; Lewison & Crowder, 2007; Peckham et al., 2008; Tomás et al., 2008a; Wallace et al., 2008, 2010; Casale, 2011), climate change (Hawkes et al., 2009; Poloczanska et al., 2009; Fuentes et al., 2011), marine pollution (Bugoni et al., 2001; Tomás et al., 2002; Storelli & Marcotrigiano, 2003; Tourinho et al., 2010; Ivar do Sul et al., 2011) and habitat loss in nesting and in marine feeding areas (Musick & Limpus, 1997; Bolten, 2003; Fish et al., 2008). However, it is difficult to determine the relative importance of different threats, particularly those occurring at sea, because knowledge on population demography is still scarce in many areas, and it is essential to understand the impact of each threat on every population for its conservation (Casale et al., 2010). One approach to the study of marine turtle aggregations in foraging areas at sea is the use of stranding data. Carcasses of marine turtles, marine mammals and other marine vertebrates encountered on shorelines can provide valuable information for estimations of mortality rates at sea, and also on causes of death if the animals arrive fresh and can be necropsied (Geraci & Loundsbury, 1993). However, probability of stranding varies widely in space and time, and usually does not exceed 10–20% of total mortality at sea, even in near-shore waters, since predators, scavengers, wind, and currents prevent carcasses from reaching the shore (Epperly et al., 1996; Hart et al., 2006; Mancini et al., 2012). At greater distances from the shore, stranding probability diminishes even more and animals that die offshore may never strand (Koch et al., 2013). Hence, stranding data are often considered as non-representative of population sizes and mortality at sea (Epperly et al., 1996; Siebert et al., 2006; Peltier et al., 2012). However, strandings yield reasonable data on the occurrence and distribution of marine vertebrate species in the adjacent marine area (Maldini et al., 2005; Pyenson, 2010; Peltier et al., 2012). Inferences on stranding data are subject to a number of caveats, i.e.: seasonal and spatial variation in recording, inter annual variation in surveying, influence of coastal currents in stranding distribution, etc. (Lewison et al., 2003). Yet, when integrated over wide spatio-temporal extents, they can provide information about geographic ranges, seasonal distribution and life history of marine turtles, particularly for life stages underrepresented in the literature such as juvenile individuals (Witt et al., 2007; Tomás et al., 2008a).

Most of the literature on strandings focuses on reports of isolated cases. Although valuable because of their detail (they can sometimes provide new biogeographic records and

extensions of the reported range of a species), isolated reports are not integrated into any overall scheme that provides an accurate reflection of stranding patterns and spatio-temporal distribution. To be valuable, data has to be collected in a consistent way, using standard protocols, on the greatest possible number of specimens and over a long period of time. Only then can the information collected contribute to an understanding of the size, shifts or variations, and causes of mortality in a marine vertebrate population.

Defining a Stranding

There have been different proposals for defining a marine turtle stranding: e.g., dead or alive turtles washed or found floating coastal waters (Tomás et al., 2008a); turtles founded dead, injured or ill on the beach or floating at sea (Casale et al., 2010); any dead or alive marine turtle, or remains, washed ashore (Vélez-Rubio et al., 2013); or carcasses of marine turtles encountered on shorelines (Koch et al., 2013). Researchers should decide and describe the different categories in detail within each study (strict stranding, floating in coastal waters, etc.), to allow for comparisons among studies on the same species in different areas. Furthermore, it would be recommended to reach a consensus for this same reason. Nonetheless, in many cases turtles are found near the shore or in coastal waters, and they can be analysed as a stranding; although notes on findings must be taken for filtering data according to the aims of the study and for further comparisons with data taken elsewhere.

On the Interest of a Marine Turtle Stranding

Data on stranded turtles provide essential information about at-site life stages, seasonal distribution, and geographic ranges of marine turtles (Hart et al., 2006; Chaloupka et al., 2008; Casale et al., 2010). Stranded animals present an evident scientific potential but strandings also offer a source of biological material for population studies such as age determination from skeletochronology (e.g., Zug et al., 2002), parasite quantification and general health condition (Deem et al., 2009), and tissue samples for stable isotope analyses (e.g., Cardona et al., 2010), toxicity (e.g., Storelli et al., 2005) and genetic studies (e.g., Naro-Maciel et al., 2012).

However, stranding based studies present a series of caveats linked to random sampling. Most stranded turtles have suffered health problems or were ill before dying, thus having a non-normal behaviour. Therefore, information on healthy individuals of the populations is scarce in this type of studies. On the other hand, stranding causes are not always identifiable, which limits the accuracy with which different impact threats can be assessed.

Nonetheless, stranding studies based on long-term data series can constitute the baseline for conservation planning and management, because they are an easy way to obtain information on local marine turtle populations. Such studies are also very important in raising local conservation awareness, particularly among fishermen, authorities and other stakeholders, and in engaging communities and the general public interested in marine turtle conservation.

What to Do When Finding a Marine Turtle Stranded on the Beach? Organizing the Response

In each country or region, marine turtle strandings can be recorded by different actors (Environmental authorities, universities/research centres, civil organizations, NGOs, etc.). These groups normally receive the information through a 24 h hotline specifically set for this purpose. In many cases the police, park rangers or coast guards directly contact the organization in charge. In many regions, several of these entities are part of a coordinated stranding network (see below).

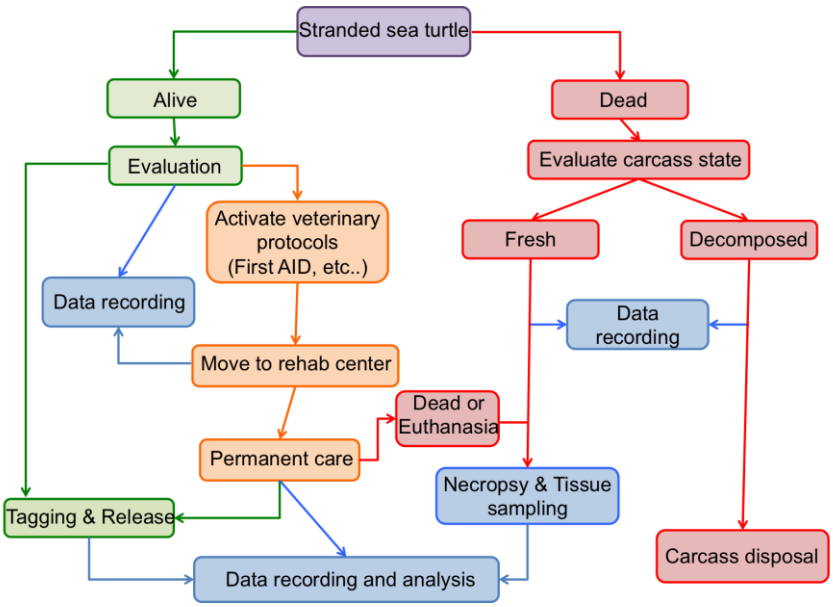

Figure 1. Response flowchart for when a marine turtle is found stranded on the beach. Two paths can be followed when encountering a stranded turtle. If alive, we need to evaluate the health condition of the turtle; if the evaluation is successful we can gather information and take samples, tag the turtle according objectives and means, and release it (in green). If the animal is not in good health, we take the turtle to a rehabilitation centre and activate veterinary protocols (in orange). If the turtle is dead (in red) we should first evaluate the carcass state, if decomposed we just gather as much information as possible and proceed to the disposal of the carcass; if the carcass is fresh we can conduct a necropsy to obtain more information and samples. Blue boxes indicate the most important points for collecting data during the procedure.

The quality and the quantity of information obtained from stranded turtles depends on a number of factors, including: the specimen´s location and state of decomposition, the workteam's motivation and organization, equipment availability, supplies, and shipping and storing of samples. For that reason, it is crucial to organize the response to strandings. An ideal diagram is presented in order to organize the response when finding a stranded marine turtle (Figure 1). There are two main flow lines in the diagram that may activate different protocols: in green and orange we represent when stranded turtles are founded alive (activating veterinary or tagging/sampling protocols); and red represents when they are found dead (activating necropsy or carcasses disposal protocols). In different parts of the chart (blue boxes) we indicate when data recording can be carried out. It is crucial to log as much information as possible along the process. The diagram can be adapted in any particular case

or location, depending on the organization responsible, the interest and motivations of the studies in the area, the extension of the study area, etc.

METHODS

Stranded Marine Turtle Sampling Techniques

How should strandings be monitored? Normally the stranding of a marine turtle is a sporadic event that can occur at any point of the coast distant from our place of work or residence. It is necessary to use simple and clear protocols, easy to implement, and covering large coastal areas. We present three non-exclusive sampling procedures to optimize the recording of marine turtle strandings. Depending on the stretch of coast to be covered and the region, stakeholders and institutions in charge may choose which of these procedures are most adequate for optimizing data recording and aims.

Stranding Network

The main objectives of a stranding network are (1) to provide rapid and effective actions that serve to recover live stranded animals, (2) to protect the public from possible diseases transmitted by the stranded animals, and (3) to gain the maximum scientific information. A stranding network's general aims should mainly focus on:

- The efficiency and speed of action after the stranding report.
- Recording as many of the strandings as possible (in addition to reporting strandings, systematic surveys should be planned, see section 3.2)
- Increasing knowledge on stranded species conservation biology (in case they are in some way threatened).
- Providing as much information and as many samples as possible.
- Setting up a tissue bank and sample collection.
- Finding partners for the managing of strandings
- Regularly publishing reports on strandings and associated information.
- Providing expertise and training to local and national authorities, local communities, park rangers, beach rescue staff, etc.
- Developing public awareness on conservation of threatened marine species (marine turtles in our case) and the threats affecting them.

To reach these objectives, collecting the appropriate information from a stranded live or dead turtle requires team organization for a quick and effective response. For this reason, a stranding network should host: (1) an emergency response team with well trained staff of both veterinaries and biologists to perform different tasks adequately (such as species identification, recording biometric data, performing a necropsy, taking samples, rehabilitation tasks, etc.), and/or to collaborate with specialist institutions; (2) the necessary equipment to examine the animals *in situ* and to transport them when necessary; (3) a facility for medical treatment and rehabilitation; (4) equipped facilities for carrying out necropsies on carcasses; and (5) an appropriate protocol for collecting, analyzing, and storing samples and data. As we

have said, networks must be coordinated via 24-h telephone hotline or via email, and/or social networks. Depending on the reach of the network, stranding events are reported by the general public, fishermen, lifeguards or civil organizations. Other organizations that could benefit the stranding network (police, coast guard, municipal authorities, park rangers, education and research centers, wildlife and conservation groups) should be informed of the network's existence. It is therefore necessary to develop awareness campaigns to involve all these actors. Useful actions are, for example, increasing information availability before holidays and in touristic areas; or providing training courses and workshops to park rangers, lifeguards and other workers aiming to increase reporting effort. The main aim of the network is being able to record 100% of strandings in a determined coastal area during a certain time period.

The network must function within the legal framework established by regional and country authorities, and cooperate with them to ensure effective action and reach long-term goals.

In an optimal scenario, a stranding network would span a country's entire coast. However, according to the length and features of the coast, the general context of the country, and the conservation status of the marine turtle aggregations, several work teams could be expected. A leader should coordinate each team and a national coordinator ought to manage the activities of all teams in order to acquire an overall picture of the situation and to obtain data and conclusions on strandings at a national scale (Bradai, 2009). Wider geographical scale networks (e.g. covering strandings over coasts around basins or enclosed seas) are also desirable, although coordination among working teams and countries is not always easy (but see section 3.3).

Beach Monitoring

Beach survey efforts can be performed over areas of special interest and as a complement to stranding networks. These surveys have to be constant and should be boosted in specific areas where high numbers of strandings occur, and in areas with on-going in-water studies. For systematic and regular monitoring, the area covered by the surveys must be of a length that can be surveyed at least monthly, and accessible to monitoring staff. Depending on the geomorphology and the length of the beaches and coasts, and also on the resources available, monitoring can be conducted by motor vehicle, bike or by foot. The use of motor vehicles must be carefully considered during surveys over beaches where marine turtle nesting may also occur. Since limited resources may not allow surveying the entire coastline of a country/region, beaches designed as important in previous studies can be chosen for monitoring. Long term monitoring of these beaches will also provide much needed information on population status and threats. When other on-going studies are absent, and resources are limited, pilot beach surveys and interviews to fishermen and coastal inhabitants must be carried out to choose the most important beaches to monitor. One advantage of beach monitoring for strandings, and also of stranding networks, is that in addition to carcasses or injured animals, they can report sporadic or exceptional marine turtle nesting events, providing new locations for the nesting range of different species (e.g., Tomás et al., 2008b).

Integrated Databases

An interesting way to coordinate and organize data on strandings over large areas, or around basins, and for long periods of time is the use of databases. These databases may be

hosted in committed research institutions or by environmental authorities. The creation of such databases can emerge from international conventions and agreements. Costs of hosting the database can proceed from local, national or international projects, or through funding provided by institutions and countries involved in the agreement. These databases should offer basic data reports, with online access, and may serve as contact means among professionals or working teams. The following are some examples of existing databases:

- TURTLE database operated by Marine Environmental Monitoring (UK): TURTLE is multi-agency project that in 2001 started acting as a repository for records of marine turtle sightings, strandings and incidental captures. Reports of such events are received from members of the public, government agencies or marine environmental organizations. These groups usually collect morphometric and pertinent additional data (e.g. geographic location) from stranded individuals and provide for either rehabilitation or necropsy. Appropriate data is then passed to the TURTLE coordinator who validates and subsequently adds it to the project database, which also contains historical records of marine turtle sightings, strandings and captures since ca. 1758. http://www.strandings.com.
- Sea Turtle Stranding and Salvage Network, STSSN (USA): this network was formally established in 1980 to document and collect information on strandings of marine turtles along the U.S. Gulf of Mexico and Atlantic coasts. The network, which includes federal, state and private partners, encompasses the coastal areas of the eighteen-state region from Maine to Texas, and includes portions of the U.S. Caribbean. Data is compiled through the efforts of network participants who document marine turtle strandings in their respective areas and contribute that data to the centralized STSSN database. By accessing the website, a citizen can report a stranded marine turtle to the state coordinator. http://www.sefsc.noaa.gov/species/turtles/strandings.htm.
- The United Kingdom Turtle Code: (UK): a code for reporting marine turtle sightings in the UK. All users can help in the effort to protect endangered marine turtles by providing information about their encounters with these spectacular creatures in UK waters. The website also provides information on marine turtles and what to do when finding a sick or entangled turtle on the beach. http://www.euroturtle.org.
- Turtle Research and Monitoring Database System, TREDS (Pacific island countries): this system provides highly valuable information for Pacific island countries and territories to manage their turtle resources. TREDS can be used to collate data from strandings, tagging, nesting, emergence, and beach surveys as well as other biological data on turtles. http://www.sprep.org/marine-turtles/turtle-research-and-monitoring-database-system-treds.
- Stranding Network Locator (STRAND) (worldwide): The Sea Turtle Rehabilitation and Necropsy Database (STRAND) is an effort to streamline and improve the accuracy of the reporting process. Here you can find all the organizations around the world with stranding network or that coordinate the strandings at beaches. Information on sea turtle strandings is collected by hundreds of individuals in USA and around the world. This data is used by local, regional and national resource managers to make decisions regarding fisheries, dredging operations and other

activities that have the potential to impact sea turtle aggregations. Accurate and timely data delivery is crucial for making effective management decisions. http://www.seaturtle.org/strand/contact.shtml.

- The State of the World´s sea turtles, SWOT (worldwide): SWOT works directly with field-based sea turtle researchers across the globe, regularly compiling the most current data available in order to provide an up-to-date global picture of sea turtle status. http://seaturtlestatus.org.

Studies Based on Stranded Marine Turtles

In this section we explain how to prepare and organize a study based on turtle strandings, what the potential goals and restrictions should be, and what data and samples it is best to collect. Finally, we expose some examples of current studies on marine turtle strandings.

Scopes and Limitations

Stranding records, once analysed with due caution for intrinsic biases, represent a valuable information source on both mortality factors and the spatio-temporal distribution of marine turtles (Casale et al., 2010). Yet stranding records cannot constitute an accurate scientific source of data because they are random phenomena, dependent on parameters such as cause-related mortality, interaction with human activities, environmental features (currents, tides, or prevailing winds); where in many cases several of these factors are convergent (Epperly et al., 1996; Hart et al., 2006).

Most marine turtle strandings involve individuals that perished at sea due to natural or anthropogenic causes such as encounters with fishing gear (NRC, 1990). However, most carcasses show no clear evidence of the cause of death (Sis & Landry, 1992; Turtle Expert Working Group, 1998; Hart et al., 2006). As the carcasses of animals which died in open waters may decompose while trapped in currents and eddies, the number of recorded marine turtle strandings likely represents a minimum estimate of mortality (Murphy & Hopkins-Murphy, 1989; Epperly et al., 1996). Moreover, the relationship between the number of turtles that died offshore and the numbers of stranded turtles remains unknown (Peckham et al., 2008).

Hence, stranding data are often considered as non-representative of populations at sea (Epperly et al., 1996; Siebert et al., 2006; Peltier et al., 2012). However, strandings yield reasonable data on the frequency of occurrence and distribution of marine vertebrate species in the adjacent marine area (Maldini et al., 2005; Pyenson, 2010; Peltier et al., 2012), and the threats that affect them in coastal waters close to study areas (Coles & Musick 2000; Mooreside, 2000). Live turtles incidentally captured in coastal waters and dead carcasses found on shore certainly provide important information on age, size, abundance, growth rates, main sources of threat, geographical patterns, and feeding of local marine turtle aggregations (MacLeod et al., 2004; Chaloupka et al., 2008).

Data Collection

Marine turtle stranding data collection relies on the people that report the stranding event, and is highly dependent on their training and on the resources available for the task. Type and amount of recorded data should be adjusted to the aims of the program, and to research and conservation purposes in a particular area during a particular period; although there are many constraints (mainly economical, but also social, political, etc.) that may limit data recording.

There are different data form models for data recording on stranded turtles, some of which are presented in Appendix I of the present chapter. In the process of design of data forms we have to consider the person filling out the forms. The information recorded in the data sheet must be easy to understand and adequate for the recorder in each case, i.e.: researchers, fishermen, volunteers, tourists, etc.

Regardless of the type of recorder and constraints, basic data that needs to be recorded from a marine turtle stranding should include:

- Name, address and phone number of the observer/recorder.
- Date and hour of the stranding or detection of the carcass.
- Exact location (latitude/longitude). If coordinates are no available, include local name of the place/beach and/or physical description of the stranding site.
- Identification (confirmed by a trained person) and description of the animal (size, weight, sex if possible, etc.). Asking for photographs is highly desirable.
- Condition of the turtle (alive, dead, fresh, decomposed, bones).
- Final disposition of the turtle (left on beach, buried, salvaged, taking to facility, etc.).

Stranding Location

Stranding date and time refers to the moment the stranded turtle was first reported or encountered. For the stranding location recorders can provide a detailed description of the stranding site by using reference points (inlets, fishing piers, lighthouses, water tanks, etc.). If a GPS device is available, whoever reports the stranding must provide latitude and longitude of where it took place. This information is important for mapping the strandings and displaying their spatial distribution along the coast, from where areas of high stranding concentration can be identified.

Species Identification

In order to identify turtle species, it is recommended to keep manuals, books or identification guides handy (e.g., Eckert et al., 1999). If identification is not clear (advanced state of decomposition, possible hybrid, etc.) it is best to write down the species as unknown or unidentified. Depending on the studied area several different species can be detected, since some of the marine turtle species have a restricted distribution (e.g., the Flatback turtle *Natator depressus* restricted to North Australia and adjacent waters) while other are globally distributed, and some of them can share feeding or nesting habitats. Here we provide a brief description of the seven extant marine turtle species for their identification (modified from Pritchard & Mortimer 1999):

Family Dermochelyidae

Dermochelys coriacea: Leatherback (E); Tortue luth (F); Tortuga laúd, baula o siete quillas (S).

Family Cheloniidae

Chelonia mydas: Green turtle (E); Tortue verte (F); Tortuga verde o blanca (S)
Chelonia mydas agassizii 1: Black turtle (E); Tortue noire (F) Tortuga negra (S)
Natator depressus: Flatback turtle (E); Chelonée à dos plat (F); Tortuga aplanada (S)
Eretmochelys imbricata: Hawksbill (E); Tortue imbriquée (F); Tortuga carey (S)
Caretta caretta: Loggerhead (E); Caouanne (F); Tortuga cabezona, boba o caguama (S)
Lepidochelys kempii: Kemps ridley (E); Chelonée de Kemp (F); Tortuga lora (S)
Lepidochelys olivacea: Olive ridley (E); Chelonée olivâtre (F); Tortuga olivácea (S)
(E) English, (F) French, (S) Spanish

Leatherback Turtle (*Dermochelys coriacea*)

This species differs from other the marine turtles in its anatomic and physiologic characteristics. Adults lack scales, scutes and claws. It´s carapace is reduced and formed by a mosaic of small bones. The carapace surface resembles leather, with the skin being several centimetres thick in this part of the body. Dorsal coloration is predominantly black, with variable degrees of white or paler spotting; spots can be bluish or pink on the neck and the base of the flippers. Light pigment predominates ventrally. Forelimbs are long in relation to the body, if compared to the other marine turtle species. Males and females have a characteristic pink spot on the dorsal side of the head (Pritchard & Mortimer, 1999). This species is the largest marine turtle and the heaviest reptile. Adults attain a curved carapace length (CCL) of 130-210 cm, and a weight of 250-900 kg (Goodman & Belskis, 2012).

Green Turtle (*Chelonia mydas*)

This species has a broad oval carapace, with 4 pairs of lateral scutes. It has a relatively small head, with one pair of prefrontal scales, and a slightly serrated, non-prominent beak. Individuals have a single claw on each flipper. Carapace coloration in adults is very variable, normally brown. Head and limbs are dark grey, while the underside is yellowish. This species is the largest of the hard-shelled turtles. Adults attain a straight carapace length (SCL) of 90-120 cm, and a weight of 120-230 kg (Goodman & Belskis, 2012). In the East Pacific we can find the black turtle (*Chelonia mydas agassizii*), currently considered as subspecies of the green turtle. The black turtle has a slightly smaller size, darker carapace and skin colour, and the posterior part of the carapace narrower than the green turtle (*Chelonia mydas*).

Flatback Turtle (*Natator depressus*)

This specie has a very broad and rounded carapace, with upturned lateral margins. The carapace presents 4 pairs of costal scutes; the scutes are very thin and have a softer texture than in other cheloniid turtles, with seams often disappearing in old adults. Dorsal coloration

is uniformly olive-green in hatchlings and adults, and yellowish ventrally (Pritchard & Mortimer, 1999). The species may reach approximately 100 cm of CCL and about 90 kg in weight. It is confined to waters of tropical Australia and southern New Guinea and Indonesia.

Hawksbill Turtle (*Eretmochelys imbricata*)

This is the only species to have imbricated scutes on the carapace. It has a narrow, oval-shaped carapace with a strongly serrated posterior margin and overlapping scutes. Dorsal coloration of the carapace is dark to light brown, and the ventral side is light yellow to white (Pritchard & Mortimer, 1999). Adults attain a SCL of 65-90 cm and a weight of 60-80 kg (Goodman & Belskis, 2012). This turtle is highly sought after in some countries where their colourful scutes are exploited to use in jewellery, even though this practice is illegal worldwide.

Loggerhead Turtle (*Caretta caretta*)

It´s carapace is moderately broad, being approximately 80% of the turtle's length. Flippers are relatively short, with 2 claws on each forelimb. Dorsal coloration is primarily brown with occasional individuals retaining some tan or even black, ventral color ranges from yellow to orange (Pritchard & Mortimer, 1999). The head is large in relation to the rest of the body and triangular in shape. Adults attain a SCL of 90-115 cm, and a weight of 100-180 kg (Goodman & Belskis, 2012).

Kemp's Ridley (*Lepidochelys kempii*)

This species has a relatively short and wide, almost circular carapace which presents a modest marginal serration or scalloping. Juveniles of Kemp's ridley turtle present high vertebral projections, but the carapace is smooth and low in adults. Juveniles are dorsally grey, while a light olive-green colour can be seen in adults. They are ventrally are white or light yellow. The individuals of this species have small pores (four per side) near the posterior margin of the inframarginal scutes in the plastron. Adults attain a SCL of 60-70 cm, and a weight of 35-50 kg (Goodman & Belskis, 2012). The species is distributed almost exclusively in the Gulf of Mexico and Eastern USA, being also reported occasionally in European coasts and even in the Western Mediterranean (Tomás et al., 2003 and references therein).

Olive Ridley Turtle (*Lepidochelys olivacea*)

It is a small turtle with a triangular head. This species presents a short and wide carapace very similar in shape to its congeneric species *L. kempii*, but less wide in adults. The dorsal coloration is dark olive-green in adults, and it is ventrally creamy yellow (Pritchard & Mortimer, 1999). As with *L. kempii*, olive ridleys habe small pores (four per side) near the

posterior margin of the inframarginal scutes of the plastron. They can be distinguished from *L. kempii* because *L. olivacea* individuals have a variable number of coastal scutes in the carapaces (six or more per side). Adults attain a SCL of 70-80 cm, and a weight of 35-60 kg (Goodman & Belskis, 2012).

Sex Determination

Marine turtles are difficult to be sexed externally until they reach maturity. Depending on their nesting aggregation and population, marine turtles reach sexual maturity at different sizes. This size is generally around 90 cm curve length for green turtles, 130 cm curve length for leatherbacks, 83 cm curve length for loggerhead turtles, 85 cm curve length for hawksbill turtles, 65 cm curve length for kemp's ridleys, 70 cm curve length for olive ridleys and 90 cm curve length for flatback turtles. If the turtle found stranded is of a smaller size than the minimum adult-size described either globally or, if known, locally, then the turtle must be recorded as 'immature' or 'sex undetermined'. However, sex can be determined if a necropsy (if the turtle is dead), laparoscopy, hormonal blood analysis or other test (if the turtle strands alive) can be undertaken. In the case of adults, the length of tail can be used for sex determination, with males having a long tail (exceeding the posterior edge of the carapace) while females possess shorter ones (rarely reaching the posterior edge of the supracaudal scutes). Premature diagnostics based only on external characteristics must be carefully considered, as there have been a few recent cases where turtles showing premature masculine secondary sexual characters were later identified as females after a veterinary diagnostic test (see Crespo et al., 2013). If means are available, laparoscopy examination of the gonads is the most effective way to determine sex and the sexual maturity of the specimen, although this technique must be supervised and carried out with veterinary care and training by experts.

Photography Documentation

It is important to photograph the carcasses at the stranding site, using a reference scale (e.g., a graduated scale with an identification number to code the animal and other relevant information, Figure 2). Photographs of the animal on the stranding site may provide important information (e.g., on stranding cause, epibionts, etc.) that can be lost or cannot be collected later after moving the carcass or during the necropsy.

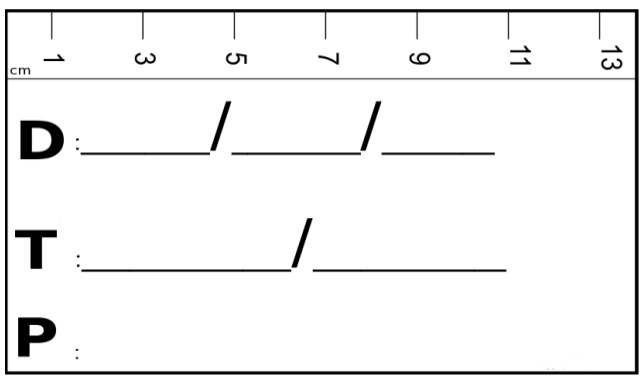

Figure 2. An example of an identification white board with a centimetre scale to be placed near or on the carcass when taking photographs. Capital letters indicate D: date, T: time and P: place of the stranding.

Figure 3. Detailed images of interesting features to be recorded in stranded turtles. The figure includes pictures taken for individual identification such as scars from tag removals (A), deformities caused by predators or during development (B), external abnormalities (ectoparasites and epibionts (C) or tumours (D)), or wounds resulting from stranding causes (e.g., missing limbs because of entanglement (E) or dredge strikes (F)). Photos: Karumbe.

Standard photographs to be taken include the following planes: ventral (plastron) and dorsal (carapace), head (dorsoventral and rostrocaudal), forelimbs and hindlimbs, and pictures of any mark (including tag scars or carapace traumas, Figure 3). All photographs should allow for species identification via carapace shape, scale pattern and, if present, notches. In addition to these photographs, it is necessary to take detailed images of any external abnormalities (e.g., epibiont concentrations, tumours, wounds, missing limbs or eyes, deformities, skin lesions, etc.).

Some of these marks and scale patterns (for instance in the dorsoventral portion of the head) can be used for individual turtle identification in live stranded and released turtles (Reisser et al., 2008) in case resources limit the use of artificial tagging.

Tagging

The primary purpose of tagging is to identify a marine turtle as an individual (Bjorndal, 1999). Physical means of identifying marine turtles include uniquely painted or coloured marks, tattoos, carapace tags or drilled holes, flipper (metal or plastic) tags, coded wire tags, "living tags", and PIT (Passive Integrated Transponder) tags. Hence, a stranded turtle coming from other nesting or foraging areas distant from the studied coast, where other researchers could be tagging, may have one or several of these tag types. Thus, staff recording strandings must check stranded live turtles or carcasses for tag presence.

- Flipper Tags: All four flippers have to be checked, and when finding a tag the information on it has to be recorded. These tags normally have an alphanumeric code for identification and a contact address. Presence of scars produced by previous tag removal should be noted. Most flipper tags used are made of metal (monel or inconel style) or plastic materials (Figure 4).

- PIT Tags: The detection of this type of tags requires electronic equipment (scanner) to read the tag code. They may be either numeric or alphanumeric. All areas on the turtle´s body should be scanned, even if one PIT tag has already been found, because some turtles may carry more than one PIT tag (e.g., a turtle captured and tagged by two different teams), or because the tag can migrate inside the turtle´s body. There is no consensus on the placement of PIT tags, therefore project personnel should examine all possible body parts (i.e., left and right shoulder muscles, left and right fore flippers, left and right rear flippers, and both sides of the neck) for existing tags.

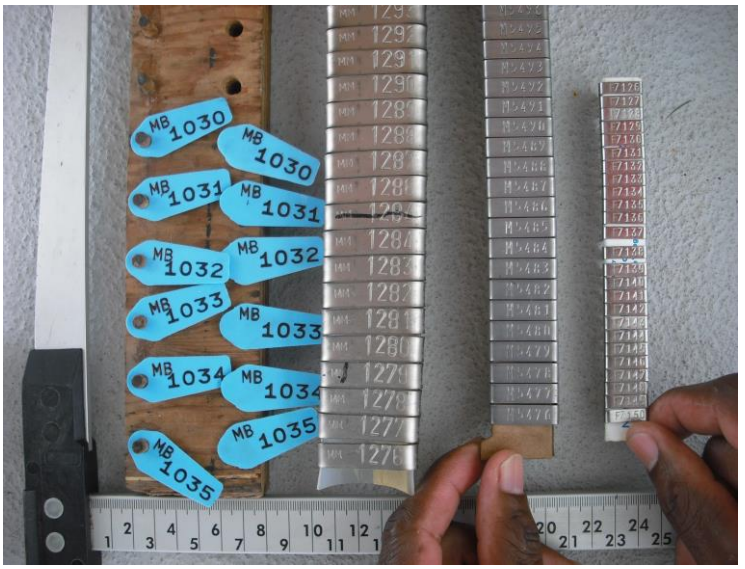

Figure 4. Example of flipper tags (left: plastic Jumbo model; right: different sized metal tags) used to tag marine turtles. Photo: G. Martinez Souza.

- "Living" tags have occasionally been used to identify cohorts of hatchlings or yearlings released in a given year. Contrasting pigmented marks are created by the surgical exchange (referred to as "autografting") of small pieces of tissue between the carapace and the plastron. These marks are retained and increase in size as the animal grows (Balazs, 1999; Eckert & Beggs, 2006). Hence, careful observation of carapace and plastron is needed.

State of Decomposition of the Carcasses

It is necessary to classify the state of decomposition of the carcasses found. Such state will determine how the stranding will be managed. It is very important to try to estimate the time elapsed since the turtle stranded on the beach until it was encountered.

Depending on a turtle´s condition or a carcass´s degree of decomposition, the turtle can be used for different kinds of studies, and sampling collection must be planned accordingly. The degree of decomposition can be classified in many ways following different objectives or situations in different areas. Here we propose a classification with 5 categories, from 0 to 4, being: 0 Live turtle, 1 Freshly dead, 2 Starting to lose scales/losing eyes, 3 Lost scales/Very rotten, 4 Dried carcasses, Bones only (Figure 5).

Figure 5. Strandings classified regarding condition of the animal. decomposition sate 0: alive; decomposition sate 0 1: fresh; decomposition sate 0 2: without eyes, losing scales; decomposition sate 0 3: rotten; decomposition sate 0 4: dried carcasses, only bones. Photos: Karumbe.

Morphometrics

There are several ways to measure a turtle, but using a method appropriate for a particular study consistently is most important. Linear measurements can be taken with either callipers (straight-line measurements) or a flexible measuring tape (curved measurements). The decision on what to use depends on the accuracy, precision, cost, and convenience required by the study (Bolten, 1999). For instance, straight measurements of the carapace are more accurate than curved ones, since the latter may vary depending on the state of decomposition of a carcass, which can be inflated due to gas accumulation. However, it´s very important to define measurements clearly on data forms. When describing turtle size, five standard linear measurements are commonly presented: carapace length, carapace width, tail length, head width, and plastron length; although other measurements can be taken if convenient.

For hard-shelled turtles, a few different carapace length measurements have been used according to some manuals (see Pritchard et al., 1983; Bolten, 1999):

1) Minimum straight carapace length (SCLmin) or Minimum curved carapace length (CCLmin) is measured from the nuchal notch of the carapace (nuchal scale) to the posterior notch at the midline between the two supracaudal scales, using a calliper or a flexible measuring tape respectively.
2) Straight carapace length notch to tip (SCLn-t) or Curved carapace length notch to tip (CCLn-t) is measured from the nuchal notch to the posterior tip of the supracaudals, using a calliper or a flexible measuring tape measure. For consistency, in the curved measurement, the longest supracaudal should be used because the tips of the supracaudals are often not symmetrical.
3) Straight carapace width (SCW) or curved carapace width (CCW) is measured at the widest point, using a calliper or a flexible measuring tape, respectively. There are no anatomical reference points for these last measurements, and they are just taken at the point of maximum carapace width.

4) Tail lengths: Total tail length (TTL) is the distance from the midline of the posterior margin of the plastron to the end of the tail following the curvature of the tail. Post-cloacal tail length (PTL) is the distance from the mid-cloacal opening to the end of the tail following the curvature of the tail.
5) Head width (HW), plastron length (PL) and plastron width (PW) are less frequently measured in marine turtles than are carapace length and width; although these measurements can also be of interest in certain studies. Plastron length (PL) is measured along the midline of the plastron. Some variation in measurement is introduced because frequently the anterior and/or posterior edges of the plastron scutes do not completely overlay the anterior and/or posterior edges of the underlying bone. PL should be measured along the midline from the anterior edge to the posterior edge of the underlying bone when it extends beyond the scutes (Bolten, 1999). Plastron width (PW) is measured at the widest point of the plastron, and there are no anatomical reference points for this measurement. HW is measured at the widest point with a calliper. PL can be measured with a calliper, or with a measuring tape (curved), along the midline line. Straight measurements of the plastron are recommended for dead turtles, because decomposition can result in the inflammation of the body, thus altering the curved measure. HW is measured at the widest point with a calliper.

For leatherback turtles there are some variations in biometric measurements that need to be considered (see Biasatti, 2004):

1) Both straight carapace length (SCL) and curved carapace length (CCL) are measured from the nuchal notch (anterior edge of the carapace at the midline) to the posterior tip of the caudal peduncle. Curved measurements are made along the side of the midline (vertebral) ridge using a flexible measuring tape. The end of the tape should be securely positioned at the junction between skin and carapace, and the tape pulled taut to the caudal peduncle, allowing the tape to continue the natural position alongside the ridge.
2) Carapace width is measured at the widest point; again there are no anatomical reference points. Straight carapace width (SCW) is measured with a calliper. Curved carapace width (CCW) is measured with a flexible measuring tape; the tape measurement does not follow the curvature of the ridges, but rather spans from ridge crest to ridge crest.

Head width can be measured at the widest point with a calliper. Tail length should be measured as described for hard-shelled turtles, and plastron length should be measured with a calliper along the midline from the anterior edge to the posterior edge.

Sample Collection

In this section we include a descriptive list of samples that can be collected from stranded turtles for different studies on biology, conservation, diseases, and health status of individuals of the different species.

Live Turtles

In addition to activating the veterinary protocols to evaluate the condition of the turtle and allow its recovery, obtaining important information from the live turtles can help increase knowledge on the biology and conservation of these species.

At the moment of stranding, oesophagus contents can be collected for dietary studies through the oesophageal lavage technique (see Forbes & Limpus, 1993; López-Mendilaharsu et al., 2008, and Carrión-Cortez et al., 2010 for details on the technique). Such contents provide information on immediate feeding behaviour and associated threats, such as debris ingestion.

It is also very important to gather information by routinely classifying and counting epibionts (barnacles, leeches, algae, etc.) on the carapace and skin of turtles. Total number, location and percentage of the turtle´s skin and carapace covered must be recorded. In some species and locations, like the green turtles in Uruguay (Vélez-Rubio, 2011), high epibiont loads may be indicative of health problems. Massive colonization leads to the inability to dive and flotation problems (Bellido et al., 2010; Flint et al., 2009; Badillo, 2007). Epibionts can help us know the turtles movements and migrations (e.g., Báez et al., 2002), and help to identify some behaviours such as brumation (Castro et al., 2007).

Several techniques can be used for sex determination, invasive approaches include (1) laparoscopy (a miniature telescope to view directly inside the peritoneal cavity through minor surgery), (2) gonadal biopsy sampling (small pieces of gonadal tissue can be evaluated histologically to determine the sex of the animal), (3) ultrasonography (ultrasound images, although perhaps less effective on smaller turtles where gonads are less developed), and (4) hormonal levels (testosterone radioimmunoassay, RIA (Owens et al., 1978) If the means and equipment are available, non invasive techniques such as ultrasonography are preferable for sex determination in live turtles (Limpus et al., 1994; Braun-McNeill et al., 2007; Wyneken et al., 2007; Blanvillain et al., 2008; Crespo et al., 2013).

Biopsy samples may be collected from various tissues to provide information on the life history and population biology of local aggregations. There are many manuals and protocols for taking different biopsy samples (e.g., Jacobson, 1999; NMFS, 2008; Flint et al., 2009). Skin biopsies have been collected for toxicological assessment (including toxin levels and presence of heavy metals, polychlorinated biphenyls and organochlorine compounds), microbiological assessment (including isolation and identification of bacteria, viruses, fungal elements and pathogenic protozoa), genetic studies, or foraging behaviour and potentially distributional patterns (stable isotope studies). Blood samples may be also taken for toxicological assessment or genetic studies, and for sex maturity (hormonal levels) or dietary studies (stable isotopes studies). Carapace biopsies are also taken for foraging behaviour and potential distribution patterns (stable isotopes studies). Muscle biopsies can be collected to determine aerobic and anaerobic metabolic capacity, thermal tolerance, and also for stable isotope analyses or genetic studies. Biopsies can also be obtained from visceral structures from live turtles, but in most situations this will be performed in a veterinary hospital under general anaesthesia (see Jacobson, 1999).

Dead Turtles

Initially, when a dead turtle is found onshore, our first interest is the cause of stranding. Normally, due to problems such as difficult access to many carcasses, degree of decomposition, or lack of resources, the real cause of stranding is difficult to determine, and

therefore the impact of many threats is probably underestimated. For example: interaction with fisheries are not easy to assess; only some fishing gears leave evidence on the carcasses (e.g. longline hooks and lines, net fragments and the corresponding injuries). Moreover, some injuries are not always easy to assign with certainty to an interaction with fishing gear (Casale et al., 2010). Ingestion of solid debris is also difficult to assign as the main cause of stranding unless there is a clear physical evidence of obstruction or perforation of the digestive tract. These and other threats (e.g. hypothermia, diseases, etc.) affecting marine turtles are difficult to identify.

Necropsies are one of the basic tools for determining the underlying cause/s of death when a turtle succumbs to illness or injury or is euthanized, if an *antemortem* diagnosis was not obtained. Necropsies also yield general information useful for management, including diet and reproductive condition of a turtle (Annex IV). A good necropsy involves a thorough external and internal examination of a carcass, including careful observations of lesions or abnormalities, and the procurement, labelling, and storage of organ and tissue samples. Laboratory tests on properly preserved tissue allow wildlife disease specialists to systematically evaluate potential causes of mortality (Work, 2000; Bluvias & Eckert, 2010). Even if an animal is in a state of fairly advanced putrefaction (lacerated skin, viscera distended by putrefying gases, rotten smell, etc.), it may be interesting to examining a carcass in order to collect as many samples as possible, including digestive contents, fishing gear remains (hooks, lines, etc.), evidence of other possible threats, bone samples, carapace scales, etc.

Once morphometric measurements and external examinations have been completed, an internal examination may be conducted. A list of tools and equipment required for performing a *post mortem* necropsy is provided in Annex II and III.

The recommended necropsy procedure for an internal *post mortem* examination of a marine turtle starts with the removal of the plastron. It includes the examination of forelimbs, coelomic mesentery, heart, thyroid, liver, trachea, tongue, oesophagus, coelomic cavity, lungs, urogenital system, kidneys, adrenal glands, distal aortas, central nervous system, brain, salt glands, and gastrointestinal tract. To avoid faecal contamination, it is best to remove the whole gut; closing the extremes with strings and to proceed with the internal examination of gut contents after all other tissues and organs have been examined (Flint et al., 2009).

In addition to a standard *post mortem* examination, samples of the eyes, muscle, skeleton, blood and faeces may be collected for studies on histology, toxicology, microbiology, ageing, serology, and parasitology. Parasites found in gut contents must also be collected. It is critical that all organs be systematically examined in the same order on every turtle during the study. For more details on necropsy and sample collection the following three necropsy manuals can be consulted (the first two are also available in Spanish):

- http://www.sefsc.noaa.gov/turtles/TM_470_Wyneken.pdf.
- http://www.nwhc.usgs.gov/hfs/Globals/Products/Turtle%20manual%20english.pdf.
- http://www.uq.edu.au/vetschool/content/vet-marti/PM.Guide.MSF.pdf.

The quality and the quantity of the samples taken depend on the sort of necropsy intended. It is frequent to carry out the necropsy *in situ* (at the same place of stranding at the beach). necropsies and sample collection are limited by many factors and working conditions.

A necropsy can be improved considerably if we can move the carcass to a veterinary or University facility, with a fully equipped necropsy room. Nonetheless, necropsy procedures can be modified and adapted to the possibilities and needs of each research group, research and conservation interests, available resources, or the biological questions and aims per suited.

Actions on Live Animals, First Aid In Situ

When a marine turtle is found alive on the beach, in addition to engaging in transport the protocol to take it from the beach to a rehab centre (see Walsh 1999), there are first aid technics to give the damaged turtle assistance in order to increase survival probabilities:

- Taking the turtle carefully away from the shore prevents it from getting hit by breaking waves.
- A turtle should be put under the shade.
- Inclining a turtle 45º with the head down helps it eject any water it may have swallowed; although the turtle may not seem to have drowned, water may have entered during or before the stranding.
- A turtle should be taken and held carefully by its shoulders and rested on the person´s knees, the front flippers should be moved backward-forward for the water to come out.
- As a last step, the turtle should be left resting in the shade until it recovers or it is time to transport it to a rehab centre. Pouring some water over the carapace and flippers keeps the animal wet. Care should be taken not to pour water into the marines.

External Physical Examination

When the turtle is stabilized, it can be examined to detect signs of illness, scars, wounds or internal injuries. Animals should be examined externally from head to tail for any abnormalities or damage. Photos of any abnormality or for species identification should always be taken. When examining the carcass, the team should do the following:

External damage:

- Plastron, carapace, and skin: check for presence of fresh or old wounds (Ulcerations of the skin of the plastron are more common in chronic debilitated animals), presence and number of epibionts, abnormal growths on the skin, etc. Check if epibionts are fixed to critical parts such as narines, mouth or eyes.
- Cloaca: check if there is something protruding out of the cloaca
- Narines: look for presence of mucus or leaking blood (possible internal wound) or liquid (possible presence of water in the lungs cause by drowning).
- Mouth: Note any ulcers, cuts, plaques, growths, presence of hooks, fishing lines, blood, spots or lumps in the oral cavity.
- Eyes: check if eyes are collapsed, cloudy, or weepy, or if there are abnormal warty growths around them.

- Flippers: check for abnormal growths, presence of fishing line wounds, strangulations causing ischemia and/or necrosis, or hooks embedded in the flipper.
- Finally, check for any other abnormality: lumps, bumps or exudates in unusual places.

General body condition: Turtles in good condition will usually have a nice rounded plastron. In severely emaciated turtles, the plastron is dished in and concave, and in the neck area the back of the skull shows a prominent occipital process that becomes very conspicuous in starved turtles.

Response to stimuli: Evaluate behavioural responses to stimuli. There are three response levels: (1) alert (aware, responsive to environmental stimuli), (2) weakly responsive (responsive only after a lot of stimulation), and (3) non-responsive (not responding to touch). If the turtle is not responding to skin stimuli, try touching one of its eyes to observe a reaction.

Hydration condition: Dehydration normally causes weepy eyes and dry skin.

Breathing rate and type: a continuous wheeze and suffocated breath may indicate a pulmonary disease.

If the turtle seems to be in very good condition after all this examination process, evaluate the buoyancy and locomotion of the turtle before releasing it back to the sea.

Diagnostic Techniques

If facilities and resources are available, the condition of sea turtles can be further evaluated through complementary diagnostic techniques, which include:

- Biological sampling: blood sample (for a complete blood count and serum chemistry tests), microbiology (2 cloaca swabs, 1 conjunctive swab and 1 nasal swab), and faeces examination (bacterial cultures, presence of parasites, etc.).
- X-ray images for internal damage in bones and viscera.
- Echography study for a general examination of celomic cavity.
- Computerized Axial Tomography (CAT scan), Magnetic-Resonance Imaging (MRI), etc.

Examples of Studies Based on Stranded Marine Turtles

In the last decades, several studies on marine turtle strandings have been published at different geographical scales in many parts of the world (Southern Spain, Bellido et al., 2010; Northeast Spain, Tomás et al., 2008a; Turkey, Yalcin-Ozdilek & Auregii 2006; Argentina, Gónzalez Carman et al., 2011; Uruguay, Vélez-Rubio et al., 2013; Peru, Rosales et al., 2010).

Here we present some recent studies based on strandings and a brief description of the applications of the stranding data collected in them:

- "The Impact of Turtle Excluder Devices (TEDs) and Fisheries Closures on Loggerhead and Kemp's Ridley Strandings in the Western Gulf of Mexico" Lewison RL, Crowder LB and Shaver DJ (2002): these authors evaluated the efficacy of TEDs and other management actions (e.g., fisheries closures) on loggerhead (*C. caretta*) and Kemp's ridley (*L. kempii*) turtle populations by analysing a long-term stranding data set from the western Gulf of Mexico. Analyses suggest that both marine turtle population growth and shrimping activity have contributed to the observed increase in strandings. Their data set included 1795 and 1279 strandings of loggerhead and Kemp's ridley turtles, respectively. Their analyses suggest that TEDs can be effective in reducing strandings, depending on the level of TED compliance. Because stranding data cannot reflect the proportion of turtles that escape unharmed from TEDs, they were unable to directly measure the reduction in mortality resulting from TED regulations and to verify mortality-reduction estimates from previous research.
- "Back-calculating length from skeletal growth marks in loggerhead sea turtles *Caretta caretta*" Snover ML, Avens L and Hohn AA (2007): these authors used a Skeletochronology technique for the assessment of individual growth rates in marine turtles. They obtained both humeri and carapace length measurements from 243 free-ranging loggerhead marine turtles found stranded and dead on beaches along the Atlantic and Gulf of Mexico coasts of the US from Maryland to Texas. As the loggerheads collected along the coast of the US are primarily neritic, their sample was comprised of juvenile and adult turtles between 44.7 and 106.1 cm SCL. They also suggest that, with proper application, back-calculation in combination with skeletochronology can be a powerful tool for studying the growth dynamics of individual marine turtles.
- "Spatio-temporal patterns of juvenile marine turtle occurrence in waters of the European continental shelf" Witt MJ, Penrose R and Godley BJ (2007): authors present data spanning approximately 100 years regarding the spatial and temporal occurrence of marine turtle sightings and strandings in the northeast Atlantic. Records of loggerhead (n = 317) and Kemp's ridley (n = 44) turtles occurring on the European continental shelf were most prevalent during the autumn and winter, when waters were coolest. In contrast, endothermic leatherback turtles (n = 1,668) were most common during the summer.
- "Cause-specific temporal and spatial trends in green sea turtle strandings in the Hawaiian Archipelago (1982–2003)" Chaloupka M, Work TM, Balazs GH, Murakawa SKK and Morris R (2008): These researchers recorded five species of marine turtle in 3,861 strandings over a 22-year period (1982–2003) in the Hawaiian Archipelago. Green turtles comprised 97% of these strandings with size and gender composition reflecting the demographic structure of the resident green turtle population in Hawaiian waters. The most common known cause of the green turtle strandings was the tumour-forming disease, fibropapillomatosis (28%) followed by hook-and-line fishing gear-induced trauma (7%), gillnet induced trauma (5%), boat strike (2.5%), and shark attack (2.7%). The specific mortality rate (conditional probability) for fibropapillomatosis was 88%, 69% for gillnet gear and 52% for hook-and-line gear.

- "High mortality of loggerhead turtles due to bycatch, human consumption and strandings at Baja California Sur, Mexico, 2003 to 2007" Peckham SH, Maldonado-Diaz D, Koch V, Mancini A, Gaos A, Tinker MT and Nichols WJ (2008): here investigators assessed anthropogenic mortality of endangered North Pacific loggerhead turtles in the coastal waters of Baja California Sur, Mexico, through the synthesis of 3 information sources: (1) intensive surveys of an index shoreline from 2003 to 2007; (2) bimonthly surveys of additional shorelines and towns for stranded and consumed carcasses from 2006 to 2007; and (3) observations of bycatch by 2 small-scale fishing fleets.
- "Sea turtle strandings reveal high anthropogenic mortality in Italian waters" Casale P, Affronte M, Insacco G, Freggi D, Vallini C, D' Astore PP, Basso R, Paolillo G, Abbate G and Argano R (2010): authors recorded a total of 5938 stranded loggerhead turtles measuring from 3.8 to 97cm curved carapace length (mean: 48.3 cm) for the period of 1980–2008. Their results suggest that anthropogenic factors are the cause of at least 52% of turtle strandings, plus an uncertain but considerable proportion of turtles drowned as a consequence of incidental capture by bottom trawlers.
- "Marine Sponges, Other Animal Food, and Nonfood Items Found in Digestive Tracts of the Herbivorous Marine Turtle *Chelonia mydas* in Hawaii" Russell DJ, Hargrove S and Balazs GH (2011): A total of 2,471 digestive tract samples taken from *Chelonia mydas* along the six main Hawaiian Islands (Kaua'i, O'ahu, Moloka'i, Maui, Läna'i, Hawai'i) were examined between 1975 and 2010. Authors analysed 127 mouth, 43 oesophagus lavage, 2,201 forestomach, 61 stomach, and 39 intestines samples. Although the usual diet of *C. mydas* comes from algae and sea grasses (plant material), animal material has been found in samples taken over the past 35 yr. They reported thirty different kinds of other animals were found in the samples. And among animal food items known to have nutritional value, the protein sponge *C. chucalla* could be contributing as an important nutritive factor.
- "Estimating At-Sea Mortality of Marine Turtles from Stranding Frequencies and Drifter Experiments", Koch V, Peckham H, Mancini A and Eguchi T (2013): researchers evaluated the magnitude and distribution of at-sea mortality of marine turtles along the Pacific coast of Baja California Sur, México during 2010–11. They used a combination of counting stranded animals and drifter experiments. They found a total of 594 carcasses during the study period, with loggerhead (62%) and green turtles (31%) being the most common species. Their study showed that drifter trials combined with beach monitoring can provide estimates for deaths at sea in order to measure the impact of small-scale fisheries, which are notoriously difficult to monitor for by-catch.

Parallel Studies That Can be Conducted Associated to Marine Turtle Strandings (in-Water Studies, Aerial Surveys)

Although stranded turtles give us valuable data about local aggregations, the probability of stranding varies widely in space and time. Strandings do not usually exceed 10–20% of total mortality even in coastal waters, since predators, scavengers, winds and currents may

prevent carcasses from reaching the shore (Epperly et al., 1996, Hart et al., 2006, Mancini et al., 2012, Koch et al., 2013). Stranding probability diminishes for deaths at greater distances from shore even more as animals that die offshore may never strand. It is therefore extremely difficult to estimate total mortality when using stranding frequencies only, even in near-shore waters (Epperly et al., 1996, Hart et al., 2006, Mancini et al., 2012, Koch et al., 2013). For that reason, it is very important to complement with other techniques, normally orientated to in-water studies.

Drifter experiments allow us to understand the main currents that influence the study area and may help explain and predict the spatio-temporal patterns of the strandings (see Hart et al., 2006).

Bycatch surveys (either on-board or interview-based) for estimating catches and at-sea mortality are very useful to assess the impact of different fishing gear on marine turtle bycatch.

For population studies:

- Mark-recapture technique through intentional capture to study growth, movements and populations dynamics (Chaloupka & Musick, 1997). This technique consists in "marking" (any method of identifying individual turtles) and "recapturing" (any method of re-identifying a marked individual at a later time) (see Gerrodete & Taylor, 1999).
- Satellite tracking is used to characterize broad scale behavioural patterns, inter-seasonal variability, and general high-usage areas among different marine turtle populations. However, it does present some limitations due to a large instrument size, high cost, and low positional accuracy and dive resolution. Ultrasound and radio tracking studies movements and home range (e.g.: Van Dam & Diez, 1998; Seminoff et al., 2002); and time depth recorders (TDRs) enable the research of scaling in dive capacity and habitat use (Blumenthal et al., 2009). In addition, TDRs and ultrasonic tracking combined (Makowski et al., 2006) have successfully elucidated movements and diving behavior.

Aerial surveys are a proven method for examining parameters such as abundance and distribution, and are especially beneficial because they allow large areas to be surveyed in a relatively short amount of time. However, the identification of marine turtle species during aerial surveys can be challenging and inexperienced or untrained observers are likely to be limited in their ability to identify species. In addition, because of their tendency to dive and remain submerged when startled, marine turtles often present observers with the challenge of having to identify species from a single viewing (see Goodman & Belskis, 2012). These surveys can be a cost effective alternative for surveying large lengths of coastline. Such aerial surveys could also be used to obtain other management information such as human usage patterns.

CONCLUSION

The main interest in long term stranding studies is obtaining biological and ecological conclusions on marine turtle species, with implications on their conservation. Bearing in mind the caveats associated to these studies, the analyses of stranding data can contribute to a better implementation of conservation measures and management on endangered marine turtles of different populations in different areas of the world. This can be accomplished through the identification of the areas used by these species, and the anthropogenic sources of mortality in the region covered. The establishment, continuity and improvement of stranding networks over time is one of the most important achievements. Networks have a twofold benefit: (1) the development of long-term temporal series of data which, integrated with complementary studies on distribution, sexual maturity, habitat use and environmental preferences, will help to assess threats affecting endangered marine turtles over time; and (2) the raising of awareness at different social levels, increasing public interest in marine turtle conservation.

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ANNEXES

Annex I. Example of Stranding Data Forms (Modified from STSN)

SEA TURTLE STRANDING AND SALVAGE NETWORK - STRANDING REPORT

OBSERVER'S NAME / ADDRESS / PHONE:

First_____ M.I.____ Last _____

Affiliation _____

Address _____

_____

Area code/Phone number _____

STRANDING DATE:

Year 20☐☐ Month☐☐ Day☐☐

Turtle number by day ☐☐

State coordinator must be notified within 24 hrs; this was done by

Phone:

E-mail:

Species: (check one)

☐ CC = Loggerhead
☐ CM = Green
☐ DC = Leatherback
☐ EI = Hawksbill
☐ LO = Olive Ridley
☐ UN = Unidentified

Check Unidentified if not positive. Do not guess.

STRANDING LOCATION: ☐Offshore (Atlantic or Gulf beach) ☐Inshore (bay, river, sounds, inlet, etc.)

State _____ District _____

Descriptive location (be specific) _____

_____

Latitude _____ Longitude _____

Carcass necropsied? ☐ Yes ☐ No
Photos taken? ☐ Yes ☐ No
Species verified by state coordinator? ☐ Yes ☐ No

Condition: (check one)

☐0 = Alive
☐1 = Fresh dead
☐2 = Moderately decomposed
☐3 = Severely decomposed
☐4 = Dried carcass
☐5 = Skeleton, bones only

FINAL DISPOSITION: (Check)

☐ 1 = Left on beach where found: painted? ☐ Yes* ☐ No (5)
☐ 2 = Buried: ☐ on beach / ☐ off beach; carcass painted before buried? ☐ Yes ☐ No
☐ 3 = Salvaged: ☐ all / ☐ part(s), what/why? _____
_____
_____
☐ 4 = Pulled up on beach/dune; painted? ☐ Yes* ☐ No
☐ 5 = Alive, released
☐ 6 = Alive, taken to rehab. facility, where? _____
_____
☐ 7 = Left floating, not recovered; painted? ☐ Yes* ☐ No
☐ 8 = Disposition unknown, explain _____
_____
*** If painted, what colour?** _____

Sex:

☐ Undetermined
☐ Female ☐ Male
Does tail extend beyond carapace
☐ Yes; how far? _____ cm / in
☐ No
How was sex determined?
☐ Necropsy
☐ Tail length (adult only)

TAGS: ***Contact state coordinator before disposing of any tagged animal!!***

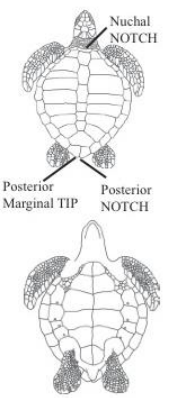
Checked for flipper tags? ☐ Yes ☐ No
Check all 4 flippers. If found, record tag number(s) / tag location / return address
_____
_____
_____

PIT tag scan? ☐ Yes ☐ No
If positive response, record location (flipper)
_____

Check for living tag? ☐ Yes ☐ No
If found, record location (scute number & side)

CARAPACE MEASUREMENTS: (see drawing)

Using calipers		**Circle Unit**
Straight length (NOTCH-TIP)	_____	cm/in
Minimum length (NOTCH-NOTCH)	_____	cm/in
Straight width (widest point)	_____	cm/in
Using non-metal measuring tape		**Circle unit**
Curved length (NOTCH-TIP)	_____	cm/in
Minimum length (NOTCH-NOTCH)	_____	cm/in
Curved width (Widest Point)	_____	cm/in
		Circle unit
Weight ☐ actual / ☐ est.	_____	kg / lb



Modified from NMFS - Sea Turtle Stranding and salvage network

Mark wounds / abnormalities on diagrams at left and describe below (note tar or oil, gear or debris entanglement, propeller damage, epibioata, papillomas, emaciation, etc.) **Please note if no wounds / abnormalities are found.**

_____
_____
_____
_____
_____
_____

Annex II. Basic Field Equipment for Stranding Studies

- Photo Camera
- Data taking forms with a specific design according objectives.
- Pencil and rubber (avoid ball pens since ink can be deleted by sea water, sea spray or rain)
- Measuring equipment (flexible measuring tape, calliper rule) and weighing equipment (net or harness and scales preferably)
- Geographical Position System (GPS)
- Discard gloves
- Knives, scissors, scalpel, plastic knives, string or other cutting material. Surgical material is preferably
- Appropriate bottles and containers for the various samples taken
- Unused plastic bags
- Portable coolers (Styrofoam or polyurethane boxes for sample preservation and transport).
- 'Waterproof' markers
- Chemical products to preserve samples (ethanol 70%, formalin 4% or 10%, etc.)

Annex III. Equipment for Necropsy in Facilities

- Coveralls or other appropriate clothing
- Rubber boots or shoe covers
- Rubber gloves
- Masks
- Photo camera
- Calipers and flexible tapes
- Necropsy forms and notebook
- String, labels, assorted bottles, water proof pen
- Forceps of several sizes
- Tissue cutting board
- Necropsy knives and sharpener
- Scalpel blades (#20 and #10) and handles
- Postmortem shears
- Alcohol lamp or butane burner
- Matches or lighter
- Ethanol 70% and formalin
- Containers of different sizes
- Fixative for electron microscopy such as Trumps solution (should be kept chilled)
- Sterile whirl-pack bags
- Cryotubes
- Microbial culturette swabs
- Microbial transport media

- Dry ice and ice chest or cooler
- Scale
- Stryker saw

Annex IV. Example of Necropsy Data Form (Modified from Work 2000)

NECROPSY DATA SHEET
(all measurements are metric)

Species_____ ID#_____ Date Collected_____Date Necropsied_____
mmddyy mmddyy

Collection site_____

History:_____SEX (M/F/U) AGE:_____

Weight_____(kg) SCL ____ CCL_____PL ____SCW____CCW_____

(Circle most appropriate term(s)). Add notes as you see fit.
BODY CONDITION: (Good, fair, poor)
POST-MORTEM CONDITION: (Fresh dead, ~1 day old, >2 days old)

EXTERNAL EXAM (Skin, carapace, eyes, nostrils, cloaca)

MUSCULOSKELETAL: (*Pectoral muscle atrophy*-None, moderate, severe*; Fat*: firm, soft, jelly-like; *body cavity*-Lots of fluid, small amounts of fluid, no fluid)

LIVER: (*Surface*: smooth, rough, granular, wrinkled*; Consistency*: firm, friable*; Color*: homogenous/mottled, red, black, brown, purple, tan, yellow.)

HEART: (*Surface*: smooth, rough, granular, wrinkled*; Consistency*: firm, friable*; Color*: homogenous/mottled, red, black, brown, purple, tan, yellow.)

LUNGS: (*Surface*: smooth, rough, granular, wrinkled*; Consistency*: firm, friable, spongy*; Color*: homogenous/mottled, pink, tan, yellow, grey, red, brown; **TRACHEA**-*Lumen*: smooth, rough*; Color*: homogenous/Mottled, tan, white, red, brown, green, pink.)

SPLEEN: (*Surface*: smooth, rough, granular, wrinkled*; Consistency*: firm, soft*; Color*: homogenous/mottled, brown, tan, red, black, brown, yellow.)

KIDNEY: (*Surface*: smooth, rough*; Consistency*: firm, soft*; Color*: homogenous/mottled, brown, tan, red, black, brown, yellow.)

GONAD: (*Surface*: smooth, rough*; Consistency*: firm, friable*; Color*: homogenous/mottled, red, black, brown, purple, tan, yellow.)

THYROID: (*Surface*: smooth, rough*; Consistency*: firm, friable*; Color*: Translucent/mottled, orange, red, tan, yellow.)

ORAL: (Mucosa: smooth, rough, granular, pitted*; Color*: homogenous/mottled, pink, tan, yellow, grey, red, brown); Contents?

ESOPHAGUS-*Mucosa*: smooth, rough*; Color*: homogenous/Mottled, tan, white, red, pink.) Contents?

CROP: (*Mucosa*: smooth, rough*; Color*: homogenous/mottled, tan, red, yellow, black, brown,) Contents?

STOMACH: (*Mucosa*: smooth, rough*; Color*: homogenous/mottled, tan, red, yellow, black, brown) Contents?

SMALL INTESTINES: (*Mucosa*: smooth, rough*; Color*: homogenous/mottled, tan, red, yellow, black, brown) Contents?

LARGE INTESTINES: (*Mucosa*: smooth, rough*; Color*: homogenous/mottled, tan, red, yellow, black, brown) Contents

BLADDER: (*Mucosa*: smooth, rough*; Color*: homogenous/mottled, tan, red, yellow, black, brown) Contents

BRAIN: (*Surface*: smooth, rough*; Consistency*: firm, friable*; Color*: Homogenous/mottled, tan, red)

SALT GLAND: (*Surface*: smooth, rough*; Consistency*: firm, friable*; Color*: Homogenous/mottled, brown, pink, tan, orange)

SAMPLES:
Formalin:_____

Frozen:_____

Other:_____

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Chapter 6

GENETICS AND CONSERVATION OF SEA TURTLES

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ABSTRACT

Knowledge on sea turtle life cycle has been considerably improved over the last decades due to the increased application of genetic techniques. The aim of this chapter is to describe some molecular markers and techniques (Microsatellite Analysis, Restriction Fragment Length Polymorphisms, Single Nucleotide Polymorphism, mitochondrial DNA haplotypes, DNA Barcodes, Next-generation sequencing) used in genetics studies to encourage researchers to contribute to the conservation process of sea turtles. Molecular techniques provide information to different levels of ecological and biological issues. All of them have limitations and their application will be largely determined by the information that is being sought with the use of a molecular marker system, and the availability of resources for the development of these techniques.

The chapter includes illustrated case studies of conservation genetics to *Dermochelys coriacea*, *Eretmochelys imbricata* and *Caretta caretta*.

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INTRODUCTION

The aims of conservation genetics are assess genetic status of populations and propose management measures for genetic diversity preservation and prevent genetic risks that could affect persistence of populations. To this end molecular markers have been relevant for studies in different species. Nevertheless, application of molecular markers is not limited solely to this, in conservation it can also contribute to understanding of evolution history, demography and ecology of species in danger of extinction.

A successful conservation strategies for sea turtles will require information related to distribution, biology and dynamic population. Based on development of genetic analytical procedures, researches have been generating data that are important for managing implications (Bowen & Witzell 1996; Jensen et al. 2013).

Research efforts in this respect, will make it possible to determine population structure of sea turtles and promote sharing of samples and data for global knowledge of life history of these endangered species.

There is a noticeable increase of studies that tend to contribute to understand sea turtles ecology. Among them we can cite natal homing (Meylan et al. 1990; Bowen et al. 2004; Bowen & Karl 2007a), male philopatry (FitzSimmons et al. 1997; Shamblin et al. 2012b), infer genetic structure (Bowen et al. 1994; Dutton et al. 1996, 1999; Encalada et al. 1996; Bass & Witzell 2000; Naro-Maciel et al. 2007; Reis et al. 2010a, Duchene et al. 2012), demonstrate multiple paternity (Kichler et al. 1999, Hoekert et al. 2002; Joseph & Shaw 2011), detect hybridization (Karl et al. 1995; Lara-Ruiz et al. 2006; Reis et al. 2010b; Garofalo et al. 2012), reveal mating behaviour (Jensen et al. 2006), deduce migration trends by inferring connections between rookeries and foraging areas (Bowen et al. 1995; Encalada et al. 1998; Lahanas et al. 1998; Bolten et al. 1998; Roberts et al. 2004; Dutton et al. 2008; Boyle et al. 2009; Blumenthal et al. 2009), resolve taxonomic questions to define priorities for conservation management (Karl & Bowen 1999; Bowen et al. 2005; Campbell & Godfrey 2010; Naro-Maciel et al. 2008, 2010; Wallace et al. 2010).

A complete review of scientific literature about molecular genetics was compiled by Lee (2008) concerning sea turtle research and explains in detail the use of nuclear (nDNA) and mitochondrial DNA (mtDNA) markers.

In another more recent publication, Jensen et al. (2013) described the advances and contribution of molecular genetic studies in aspects such as sea turtle phylogeography, gene flow, dispersal, feeding groups, migratory behavior, mating systems, sex ratios of breeding populations, reproduction biology, conservation and management.

The aim of this chapter is to describe molecular marker techniques used in conservation genetics and explain advantages and limitations of application of these to help researchers to contribute to the sea turtle conservation process, encouraging the development of research with molecular techniques.

Furthermore, in the chapter there are illustrated case studies of genetic conservation of three species of sea turtles (*Dermochelys coriacea*, *Eretmochelys imbricata* and *Caretta caretta*).

METHODS

Analytical Procedures Used in Sea Turtles Genetic Studies

With the advance of molecular biology techniques, different methods for detecting genetic polymorphism were developed at the DNA level. DNA molecular markers serve as a reference to detect transmission of a chromosome segment from one generation to another. Molecular markers of mitochondrial and nuclear DNA are available for analyses but none of them can be regarded as optimal for all research applications.

Molecular markers are DNA fragments with a defined position within a species genome. Within a marker, if differences in the DNA sequence are observed in a locus (different alleles), they will be called polymorphisms. Markers can also be highly polymorphic (with multiple alleles) or low polymorphic (with few alleles).

These markers reflect differences in the DNA sequences, and separate loci can provide independent tests, thus using many loci can yield extreme sensitivity because of a trade-off between precision and convenience (Sunnucks 2000). Processes such as recombination, selection, genetic drift in different genes and regions of the genome can generate different genealogical histories. Thus each marker (locus) can be considered a sample of the genome and combining results from many loci provides a more precise and statistically powerful way of comparing populations and individuals (Selkoe & Toonen, 2006).

The polymorphism detection can be based on the unique features of each marker type. They can be classified as i) variation on the number of repetitions in a sequence (e.g., microsatellites, minisatellites) and ii) markers that detect specific changes in the genome (e.g., Restriction Fragment Length Polymorphisms (RFLP), Single Nucleotide Polymorphism (SNP), DNA barcodes, etc.)

Mitochondrial DNA has often been used as a marker in investigations of molecular diversity, reconstruction of historical patterns of population demography and speciation. It is characterized as being highly variable due to its elevated mutation rate compared to nuclear DNA, which generates signals for population history over short periods of time. Mitochondrial DNA has highly variable regions (e.g., control region or D-loop) which are typically flanked by more conserved regions (e.g., ribosomal DNA) (Galtier et al. 2009). It has two genes of ribosomal RNA (rRNA), the 12S and 16S rDNA. For analysis of genetic diversity of levels in superior categories such as in the phyla is usually used 12s rDNA, while in analysis of average categorical levels such as in families or genera is used 16s rDNA (Gerber et al. 2001). Characteristic features of various mtDNA and nuclear DNA markers are summarized in Table 1. DNA barcoding is a method that uses COI in animals for rapid and accurate identification of species (Ratnasingham & Hebert 2007). A mitochondrial fragment of 648-bp (*Folmer region*) of the cytochrome c oxidase I gene (COI) has been standardized for molecular taxonomy and identification. However, one of biggest criticisms of DNA barcode is that it is based on information from a single marker. The use of genes (one or more) from a haploid genome can affect correct the identification of species.

In situations where incomplete lineage sorting (due to recent radiation), introgression or pseudogenes are present, the correct taxonomic identification can be wrongly inferred and will interfere in the correct identification of a species, since it is not possible to distinguish these situations from a correct taxonomic classification (Teletchea 2010).

Table 1. Characteristics of some molecular markers: Mitochondrial DNA and nuclear DNA

Type	Molecular marker	Advantage	Limitation
Mitochondrial DNA (mtDNA)	mtDNA Haplotypes Control region (d-loop)	• Hypervariate • Used for identification of species and sub–species Degrades slower than nuclear DNA • It can be used in degraded or old samples • Evolves about 10–fold faster than nuclear DNA • Useful to study evolutionary relationship and biodiversity	• The level of efficiency of mtDNA repair mechanisms has been implicated in influencing substitution rates. • To infer a concordant species tree should be used mtDNA sequences not less than two coding genes in size • Not used for assessed gene-specific, species-specific, and lineage specific evolution in mtDNA. • Maternal inheritance of mtDNA restricts it explore events at the maternal angle • mtDNA is not use for assessed recent loss of genetic variation and any individual-level events such as identity, individual dispersal, and mating systems
	DNA Barcodes - Mitochondrial cytochrome c oxidase subunit 1 gene (COI)	• Short stretch of DNA (‘barcode’) is commonly used to allocate an unidentified individual to a species • High levels of variation between specimen barcodes from previously accepted species are held to uncover cryptic species • Used for species identification, biodiversity analysis and conservation management	• No clear distinction between ranges of genetic distance values typical of intraspecific variation Vs. interspecific divergence • Error rates associated with the use of threshold values and distance measures for species detection in some taxa • DNA barcodes are not an adequate source to be used alone in taxonomy to discover a species
Nuclear DNA	Microsatellites (Synonyms: STR, SSLP, SSRP, SSR or STMS)	• Specific locus (in contrast to multi-locus markers, such as minisatellites or RAPDs) • PCR Based (small amounts of tissue are needed, allowing the use of highly degraded DNA) • Very high polymorphism (multiallelic) • Large number of loci • Codominant (mendelian inheritance) • Simple interpretation of results • High reproducibility • Automation	• Requirement prior knowledge of some part of genome for the specie in question • Initially slow • Initially expensive • High cost to isolate the loci • Unilocus (although they can be multiplexed) • Possible presence of null alleles • Information obtained requires extensive knowledge of statistics.
Nuclear DNA	Restriction Fragment Length Polymorphisms (RFLPs)	• Comprise a potentially unlimited number of loci (high genomic coverage) • Codominant Highly reproducible). • Inexpensive • Easy to design • Applicable to analysis of SNPs	• Some species exhibit low polymorphism (compared to other molecular markers) • Require high amount of DNA • Procedure is laborious and slow • Requires that a variation generates or abolishes a restriction enzyme recognition site • Some restriction enzymes are expensive • Exact genotyping cannot be achieved in the event that there is more than one nucleotide variation in a restriction enzyme recognition site • Long time from start to completion of the analysis • Not suitable for high-throughput analysis
	Single Nucleotide Polymorphisms (SNPs)	• Low rate of mutation (greater stability than SSR) • Very abundant • Continued development of new analytical methods for detection • Simplification of cross-comparative studies.	• Expensive to isolate and characterize (sequencing) • Low level of information to a single SNP

Genetic research in sea turtles is focused in the analysis of maternal lineages, population genetic structure, migratory trends and phylogeography. In the studies with sea turtles four types of nuclear markers have been used (Microsatellites, scnDNA, RFLP, nuclear sequences) and mitochondrial markers. Microsatellites are often used in sea turtle studies, 119 microsatellite loci have been recognized of which 25 are of *Caretta caretta*, 12 of *Chelonia mydas*, 18 of *Dermoclehys coriacea*, 10 of *Lepidochelys olivacea*, 4 of *Lepidochelys kempii*, 11 of *Natator depressus* and 39 of *Eretmochelys imbricata*. Sea turtle hybridization has been studied with molecular markers such as mtDNA, scnDNA and RFLPs (Karl et al. 1995; Seminoff et al. 2003; Witzell & Schmid 2003), research is also being done on the combined use of morphological analysis and mtDNA (Lara-Ruiz et al. 2006; Reis et al. 2010b; Garofalo et al. 2012), and nuclear markers (Vilaça et al. 2012).

In mitochondrial phylogenetic research of sea turtles markers such as cytochrome b (Cytb) (Bowen et al. 1993), D-Loop, ND4 (Dutton et al. 1996), 12S and 16S (Naro-Maciel et al. 2008) and recently the complete mitochondrial DNA genome has been used (Duchene et al. 2012). DNA Barcoding of sea turtles was reported first for 5 species from Brazil by Vargas et al. (2009).

Subsequently, Naro-Maciel et al. (2010) established barcode sequences of all sea turtles species in the Atlantic and Pacific and agreeing the utility of mitochondrial COI for the purposes of barcoding even though they are ancient taxa with slow molecular evolution.

Microsatellite Analysis (Simple Sequence Repeats - SSRs)

Microsatellites are polymorphic DNA loci that contain repetitions of 1-6 nucleotides, found at high frequency in nuclear genomes of most taxa. They represent the more polymorphic class of molecular markers currently known and used (Balloux & Lugon-Moulin 2002). Allelic variation, number of repeats and allelic frequencies are available for thousands of markers across numerous organisms. The ability of researchers to choose from such a large selection of highly informative markers has made microsatellite analysis a widely accepted tool for linkage studies, association studies, and identification of individual organisms (Selkoe & Toonen 2006).

Microsatellites are highly informative and based on the amplification by PCR of individual regions. Each amplified segment can have a different size and corresponds to a different allele from the same locus. The polymorphism is given by the number of repetitions in a loci. For example, in a loci with $(CA)_n$ repeats, where n is the repeat number, the different alleles will have a distance of two base pairs between them (e.g., $(CA)_{11}$ and $(CA)_{12}$). Then either the repeat number (e.g., 11, 12, 13, etc.) or the allele size (e.g., 100 bp, 102 bp, 104 bp) will be extracted and used in statistical analysis (Balloux & Lugon-Moulin 2002).

Development of microsatellite primers for a new species is difficult, laborious and expensive, although genomic era could facilitate this process. Although microsatellites are extremely useful for genetic analysis, there are difficulties concerning their use: (i) they are expensive to develop, as a large number of sequences must be cloned and only a small number of these will be useful for development of SSR markers; (ii) the primers may not amplify any PCR product; (iii) the primer may produce very complex, weak or nonspecific amplification patterns; (iv) the loci may not be polymorphic (Madesis et al. 2013).

Even though microsatellites mainly occur in noncoding sequences, development of expressed sequence tags (EST) in databases revealed that microsatellite repetitive sequences also occur inside coding sequences. Information obtained by EST libraries has been recently used for development of SSR markers (Scott et al. 2000).

Although technology progresses, new genomes and EST libraries become available with help of bioinformatics approaches, development of SSR markers based on EST's through data mining has become a fast, efficient and relatively inexpensive, compared to development of genomic SSRs.

PCR-RFLP Analysis (Restriction Fragment Length Polymorphisms)

Restriction Fragment Length Polymorphism (RFLP) is characterized as a single combination of restriction enzymes. The technique for detection of RFLPs involves fragmentation of a DNA sample to obtain the difference in specific sequences of nucleotides that are recognized and are cut with specific restriction endonucleases.

In the PCR-RFLP, the digestion is done in a fragment of interest which was amplified by PCR. The detection of polymorphisms in the DNA cutting (or amplified fragment) is performed in an electrophoresis and determining the number of fragments and relative sizes. RFLP analysis can be used in many different settings to accomplish different objectives determination of paternity, characterization of genetic diversity and characterization of breeding patterns in animal populations (Zhang & Hewitt 2003).

The use of PCR-RFLPs as markers to trace the transmission of genes associated with them is useful in that it has the genetic advantages as not depending on ontogenetic status of animal (Brito & Edwards 2009). Moreover, the RFLP technique allows an analysis of all the genome; include inexpensiveness and not requirement for advanced instruments. Furthermore, the design of PCR-RFLP analyses generally is easy and can be accomplished using public available programs.

Disadvantages of technique include the requirement for specific endonucleases and difficulties in identifying the exact variation in the event that several SNPs affect the same restriction enzyme recognition site. Moreover, PCR-RFLP consists of several steps, and it requires relatively time for analysis. This technique is not suitable for the simultaneous analysis of a large number of different SNPs due to the requirement for a specific primer pair and restriction enzyme for each SNP (Berg Rasmussen 2012).

SNP (Single Nucleotide Polymorphism)

SNP is believed to be the most recent generation of molecular markers, based on the identification of the substitution of one nucleotide for another, representing two alleles. Its frequency oscillates between 1 in 600 and 1 in 1000 base pairs, depending on the organism. They include the classic technique of RFLP but not exactly by detecting the appearance or abolition of a restriction site (Vignal et al. 2002). The fact that they can be detected on "arrays" (chips) from the solid phase without the use of electrophoresis gels is an advantage. The most direct method for detection is sequencing DNA segments, previously amplified by PCR, of several individuals representing the diversity of population.

Primers are designed to amplify DNA fragments and could be derived mainly from interest genes or sequences reported in databases corresponding to express sequences (expressed sequence tags, EST) (Brumfield et al. 2003). This marker has been shown to be mostly bi-allelic, determined by the low mutation rate of single substitutions that cause the SNP. The advantages of this marker are codominant inheritance and its abundance in the genome. Its disadvantages are associated with genotyping methods, which include high cost equipment such as microchips, spectrometry mass, quantitative PCR or sequencing (Morin et al. 2004).

Mitochondrial DNA (mtDNA) Haplotypes

The extensive use of mtDNA haplotypes also means that the limit of resolution of this marker for distinguishing population genetic structure has probably been reached (Lee 2008). Mitochondrial DNA haplotypes are based in gene sequencing process and four techniques are widely utilized in this process, but the Sanger method is more commonly used because it has been proven technically easier to apply and it has been automated by PCR, also easily applied to long strands of DNA including some entire genes.

The mitochondrial genome contains approximately 16,500 base pairs (bp) and encodes a small fraction of mitochondrial proteins. The mtDNA contains 38 genes: 2 rRNA (12S and 16S), 22 tRNA and 13 structural genes, which encode different subunits of the enzyme complexes of the oxidative phosphorylation system (Arif & Khan 2009). The largest non-coding region, known as the control region or D-loop, occupies 1122 base pairs (aprox.). This region is often used for its high mutation rate and high variability among different populations (Gerber et al. 2001).

The study of mitochondrial DNA is particularly recommended when working with highly degraded samples, as expected in ancient DNA. It is estimated that a cell can contain up to hundreds of mitochondria, and each mitochondrion coexist within 1000 to 10000 copies of mitochondrial DNA. This high number of mitochondrial DNA molecules within the cell makes its recovery in cases where the starting DNA is very low or very degraded much more efficient than, for example, the nuclear DNA or autosomal (Meiklejohn et al. 2007).

Evolutionary properties of mtDNA (metabolically active, highly oxidative environment, complex mutation process, highly variable in space and time) make that it is marker for resolving problems of conservation but not is the ideal marker of molecular diversity and taxonomic identification (Galtier et al. 2009). Nevertheless, one should be cautious with gene-specific, species-specific, and lineage specific evolution in mtDNA. Besides, mtDNA to be a maternal inheritance is a useful auxiliary marker to nuclear DNA (Duchene et al. 2011).

DNA Barcodes

DNA barcodes have been formed from the cytochrome c oxidase subunit 1 mitochondrial region (COI) and have been used to differentiate species (Hebert et al. 2003a, 2003b).

Closely related species can be differentiated because individuals within the same species have similar barcodes (Hebert et al. 2004). Despite the potential of DNA barcodes there are still many drawbacks associated with the use of these techniques.

One concern is that DNA barcodes are not an adequate source to be used alone in taxonomy to discover a species (Rubinoff et al. 2006; DeSalle 2006). It is still generally accepted that DNA barcodes are useful for identifying species after taxonomy has been established. DNA barcoding technique involves gene sequencing for the COI region to generate the DNA barcode for each species. Tissue from the specimen is used for DNA extraction; with the COI is amplified in a PCR and then sequenced. Once the COI sequence has been obtained, it is placed in the Barcode of Life Data Systems (BOLD) database. This is a searchable repository for barcode records, it provides an identification engine based on the current barcode library and monitors the number of barcode sequence records and species coverage (Rubbinof 2006).

DNA Barcode can be developed and used to determine identity of eggs sea turtles and meat in areas where these are consumed or trafficked. This method has used in conjunction with Library Barcode of Life (Barcode of Life Database - BOLD) discovering the illegal presence of products of these endangered species (Vargas et al. 2009).

Moreover DNA barcodes are of utility in conservation biology, being more accepted for identification of samples as of control region haplotypes because is widely utilized in genetic studies.

Next-Generation Sequencing (NGS)

In conservation genetics, new techniques as Next-generation sequencing (NGS) have allowed the development of genomic conservation field. The NGS is characterized by high-throughput DNA sequencing techniques that are opening novel fields and applications as: genomics with detailed analysis of individual genome stretches; precise analysis of RNA transcripts for gene expression, reliable and precise quantification of transcripts for identification and analysis of DNA regions interacting with regulatory proteins in functional regulation of gene expression (Ansorge 2009).

These technologies permit rapid genome-wide characterization and profiling of mRNAs, small RNAs, transcription factor regions, structure of chromatin and DNA methylation patterns, microbiology and metagenomics (Mardis 2008).

NGS techniques are based in randomly fragmenting DNA or RNA into smaller pieces and constructing DNA libraries. These are sequenced at a high coverage and the sequenced reads are then mapped into the reference genome of the species (De Magalhães et al. 2010). The choices of NGS technique depend on the objective of the study and experimental design.

NGS technologies more commons utilized are: i) Roche (454) GS FLX sequencer that works on the principle of pyrosequencing which uses the pyrophosphate molecule released on nucleotide incorporation by DNA polymerase that produces light from the cleavage of oxyluciferin by luciferase; ii) Illumina genome analyzer which is based on concept of sequencing by synthesis to produce sequence reads of ~32-40 bp from tens of millions of surface amplified DNA fragments simultaneously and iii) Applied Biosystems SOLiD sequencer uses a unique sequencing process catalyzed by DNA ligase that is based in couples oligo adaptor-linked DNA fragments with 1-mm magnetic beads that are decorated with complementary oligos and amplifies each bead–DNA complex by emulsion PCR (Mardis 2008).

Ouborg et al. (2010) identify different questions and possible conservation genomic approaches that can be resolved by NGS techniques:

1. To assess the impact of habitat fragmentation on selectively important variation
    a. Use of genome-wide SNPs to obtain a representative estimate of genetic variation
    b. Perform a genome scan to distinguish neutral from non-neutral markers
    c. Comparison of patterns of neutral and non-neutral variation
    d. Undertake an association-mapping approach to find correlations between markers and phenotypic traits important for adaptation
    e. Candidate-gene studies can be used to search for frequency changes of alleles in relation to environmental change
2. To identify genetic mechanisms underlying inbreeding depression
    a. Population transcriptomics for identify genes associated with inbreeding depression, in different life-history stages and many genotypes
    b. Quantitative trait locus mapping for identify genomic regions associated with inbreeding-depression phenotypes
    c. Selection experiments on gene-expression phenotypes
3. To characterize the role of gene-environment (G x E) interactions
    a. Population transcriptomics for performed in combination with full factorial experiments to identify genetic, environmental and GxE effects in transcript profiles
    b. Perform epigenetic screening, using methylation-sensitive AFLP or high-throughput bisulfite sequencing, of small and large populations in high and low quality habitats.
4. To identify the role of phenotypic plasticity in the response to environmental challenges
    a. Epigenetic manipulation experiments to manipulate methylation levels, and study phenotypic effects in relation to population size, inbreeding level and environmental variation
    b. Screening of methylation levels as a function of the level of phenotypic plasticity in relation to level of inbreeding
5. To characterize the effects of habitat fragmentation on gene expression and genomic pathways
    a. Use microarrays or RNA-seq to screen for changes in genome wide gene expression profiles in response to inbreeding and population size
    b. Screen gene-expression variation in high- and low-diversity populations and genotypes to disentangle direct gene effects from regulatory changes

Studies Case of Conservation Genetic for Sea Turtles

Dermochelys coriacea: Molecular Data and Its Use on Leatherback Turtle's Conservation

The leatherback sea turtle, also known as giant turtle in some countries of its range (e.g., Brazil), is the largest of the seven sea turtle species found in the world. They are found in all

ocean basins but the largest nesting populations are located in the Atlantic Ocean: Gabon (Fossette et al. 2008; Witt et al. 2011) and French Guiana/Suriname (Fossette et al. 2008).

Besides, population nesting in Brazil (Espírito Santo State), although very small (about six nests in 1993/1994) (http://tamar.org.br/interna.php?cod=76), shows an increasing trend in the annual number of nests (Antônio P. Almeida, personal communication).

Despite some differences in its biology compared with the other sea turtles species, such as size, long migration capabilities (Pritchard 1976; James et al. 2005), and longtime spending on foraging areas (James et al. 2005), the leatherback turtle is a target of the most important threats affecting all sea turtles species: fisheries bycatch (i.e., incidental capture by marine fisheries operations targeting other species), plunder eggs and meat for human consumption and coastal development (Mast et al. 2005). It is current classified as critically endangered by the International Union for Conservation of Nature and Natural Resources Red List of Threatened Species (IUCN 2015).

A new more accurate context named Regional Management Units (RMU) has been used since 2010 aiming to prioritize conservation and research of marine turtle's species (Wallace et al. 2010). In the light of this novel framework the leatherback populations worldwide are compounded by seven RMU´s distributed in the three oceans. Three of them from the Atlantic Ocean (North West, South East and South West Atlantic), two from the Indian Ocean (North East and South West Indian) and two from the Pacific Ocean (East and West Pacific). For these seven separated RMU´s different levels of threats and risks were evaluated (see Wallace et al. 2011 for details).

Three out of seven RMU´s are considered at High risk-Low threats (HR-LT), two at Low risk-Low threats (LR-LT), two at Low risk-High threats (LR-HT) (Wallace et al. 2011) meaning that the more than 70% (five out seven) of the leatherback´s populations in the world are facing, at least, one from the two major concern in marine turtle conservation.

Molecular data are used in a range of studies regarding conservation of endangered turtle species. The majority of the works regarding molecular techniques deals with the mitochondrial DNA (mtDNA) in a wide range of topics: phylogeography (Dutton et al. 1999, 2007, 2013; Vargas et al. 2008) and mixed stock analysis using mtDNA control sequences (Vargas et al. 2008, 2013; Prosdocimi et al. 2014); barcodes using mtDNA cytochrome oxidase c subunit I (COI) sequences (Vargas et al. 2009); investigation of illegal trade using PCR-RFLP of cytochrome b (Moore et al. 2003). Some recent studies are including microsatellites markers to improve the resolution of the phylogeographic analysis (Dutton et al. 2013), to better understand the demographic history, population dynamics and behavior (Rivalan et al. 2006; Molfetti et al. 2013) to estimate effective population size (Rivalan et al. 2006) and also to reveal multiple paternity in leatherbacks (Crim et al. 2002).

The delineation of the aforementioned RMU's (Wallace et al. 2010) and further conservation priorities actions (Wallace et al. 2011) are also improved by molecular data, showing that a large amount of contexts can be abetted by using molecular tools to improve the knowledge on endangered species and, as a final goal, to avoid the extinction levels as much as possible. In the present chapter, we intend to revisit information on, how and in which context, some studies using molecular data can be useful in the conservation of the critically endangered leatherback sea turtle.

The contexts explored are: (1) the mating systems and multiple paternity, (2) the phylogeography of this species and the mixed stocks at foraging grounds, (3) the population

dynamics, demographic histories and estimations of effective population size, (4) the delineations of RMU´s, and (5) barcodes and PCR-RFLP to identify species.

Phylogeography and Mixed Stocks at Foraging Grounds

Although the ability for extensive dispersal in the marine environment many marine species exhibit population genetic structure (Dutton et al. 2013). Phylogeography studies deals with structure levels and their distributions among geographic regions. Only five publications, concerning structure among an overall of about 20 different populations were found for leatherbacks (Dutton et al. 1999, 2007, 2013; Vargas et al. 2008; Molfetti et al. 2013). Despite only seven RMU´s were recognized for this species (Wallace et al., 2010), most of the results from genetic structure values based on mtDNA sequences studies showed high levels of structure among the overall studied nesting populations. Great variation is found if the populations represents different ocean basins, as revealed by Vargas et al. (2008) and whose found which 52.8% of the genetic variation occurring between the Atlantic and Indo-Pacific groups, 36.5% found within populations, and only 10.7% among populations within the Atlantic and Indo-Pacific groups.

The use of nuclear markers (microsatellites) is also revealing new opportunities to understand different gene flow patterns between different gender and among diverse populations (see Dutton et al. 2013 and Molfetti et al., 2013 for two examples of divergent results using mtDNA and nuDNA to infer genetic structure).

Molecular markers can also be used to learn the origin of individuals from feeding aggregations (mixed populations) based on the comparisons with a set of source populations by using Mixed Stock Analysis (MSA) (Bolker et al. 2007). Comparing haplotypes distributions among nesting and foraging grounds, beside the use of MSA, Vargas et al. (2008) revealed a major contribution to a Brazilian pelagic aggregate from Atlantic nesting populations (mean 96.1%) and the existence of six individuals bearing an orphaned haplotype (Dc_A2) for leatherbacks.

This haplotype, considered an orphaned haplotype because no match with nesting population were found at that moment, had its origin ascertain (Gabon and/or Western Africa) by genetic studies that reanalyzed samples from other study with longer sequences and number of rookeries surveyed (Dutton et al. 2013; Vargas et al. 2013).

Together, analysis of mtDNA control region sequences and tag return data have been used effectively to support the natal homing hypothesis (Dutton et al. 1999), to ascertain the origin of individuals from feeding aggregation in Mixed Stock analysis (MSA) approaches (Vargas et al. 2008, 2013; Prosdocimi et al. 2014) and also to recognize bias effort in samples sites collection by finding animals in feeding aggregation with haplotypes not yet recognized for any rookery sampled.

Population Dynamics, Demographic Histories and Estimations of Effective Population Size

The knowledge obtained by using genetic markers in order to understand demographic histories, population dynamics (recent and historical trends) and effective population sizes

(Ne) are essential to outline the real situation about populations regarding loss of genetic diversity and their prospects of long-term survival. For leatherback turtles, two studies used mtDNA and microsatellites data to infer demographic scenarios for population from Atlantic Northwest (NW).

The first one found that a leatherback turtles´s nesting population from French Guiana and Suriname, called Maroni, it can be considered as part of a large metapopulation, whose boundaries remain currently unknown (Rivalan et al. 2006). Despite the high population size known for leatherbacks from this region (2750-20,000 individuals) effective population sizes measured (from 60 to 220 depending on the mutation rate and the mutation model) (Rivalan et al. 2006) are below the lower level of target effective populations sizes for conservation programs for endangered species (500 to 1000) (Lynch & Lande 1998). Contrariwise, Molfetti et al. (2013) in their study revealed that leatherbacks population's dynamics from three different regions from Atlantic NW may be driven by an island model rather than a severe metapopulation model that would suggest successive cycles of extinction and recolonization. Despite the great importance of the aforementioned knowledge to better understand the species historical, recent and consequently future abundance, trends for populations from other regions of occurrence of this species remains unknown.

RMU´s and Conservation Priorities

A recent publication evaluated five criteria (population size, recent trend, long-term trend, rookery vulnerability and genetic diversity) to develop a risk matrix that is used together with a threats matrix as an attempt to plan the global conservation priorities for marine turtles (Wallace et al. 2011). As we can see, the last criterion, genetic diversity, is exclusively calculated by using analyses of the mtDNA and the number of known or inferred genetic stocks (Wallace et al. 2011). Despite this approach is based on biological and environmental data and does not use solely the information about genetic data, is clear the importance of its use in this new and more powerful strategy to guide conservation priorities (Wallace et al. 2010, 2011).

Matings Systems and Multiple Paternity

When a genetic study is aiming to identify aspects from the paternal lineage from marine turtles, the nuclear DNA has to be used rather than the mtDNA, because the last one is maternally inherited, failing to reveal aspects from the paternal ancestry. Once recognized for some species of sea turtles, the multiple paternity (FitzSimmons 1998, Jensen et al. 2006) was evaluated for leatherbacks (Crim et al. 2002; Stewart & Dutton, 2011). Using microsatellite data, Crim et al. (2002) concluded that leatherbacks from Playa Grande (Costa Rica) exhibit low levels of multiple paternity (10%), with a maximum of three males contributing per each family. They also found evidences of polygyny (two females mating with the same male) by analyzing and matching the genotypes of hatchlings and females from different families. Another study genotyped leatherback females and hatchlings from Sandy Point National Wildlife Refuge (St. Croix, US Virgin Islands) and found multiple paternity levels of 41.7%

(Stewart & Dutton 2011). Both studies showed that multiple fathers contribute unequally to the clutches.

Barcodes and PCR-RFLP to Identify Species

In this chapter we found that assays using COI sequences (DNA barcodes) and PCR-RFLP of cytochrome b can be used to differentiate, at least, five marine turtle species from each other (Moore et al. 2003, Vargas et al. 2009). The use of turtle meat and eggs as food in human populations is reported and "take" is considered one of the five threats studied to guide global conservation priorities (Wallace et al. 2011). Thus, barcode and/or PCR-RFLP methodologies can be applied wherever turtle meat and eggs are eaten or trafficked, as a way of identifying species-source, playing an important role to combat illegal trade. They can also be used during field work, when identifying lost nests, animals stranded on beaches or those killed as part of the bycatch in fishery nets (Vargas et al. 2009).

As we can see, besides the indirect use in management strategies (improving the knowledge on a large range of biological concepts: phylogeography, mating systems, population dynamic, etc.), the molecular data can likewise be useful in direct actions and policies for the conservation of marine turtle species.

Eretmochelys imbricata

The hawksbill turtle (*Eretmochelys imbricata*) is a species that inhabits the tropical waters of the Indo-Pacific and Atlantic oceans (IUCN 2015). It is considered as critically endangered by the IUCN and is listed in the Appendix I of the Convention on International Trade in Endangered Species (CITES).

The first studies using molecular markers in *E. imbricata* started in the late 1970's. The earliest studies aimed to uncover the taxonomic relations between diverse turtle species (Frair 1979; Chen et al. 1980, see also Mao and Chen 1982 for a review in immunoeletrophretic studies with turtles), and used the serum proteins to uncover the relationships between different species.

Among these techniques, electrophoresis, agglutination, and immunoprecipitation were used to uncover the affinities between different turtle species. Chen et al. (1980) for example, using immunological distances tried to estimate the divergence time between the different genera, which showed to be concordant with data from fossil record.

Since the 1990's, DNA data have been used to investigate the phylogenetic position of *E. imbricata*. The first study using DNA data, published by Bowen et al. (1993), using the mitochondrial gene cytochrome b, observed an evolutionary deceleration in the mtDNA rate among all turtle species, and that spongivory in *E. imbricata* evolved from a carnivorous ancestral. Soon after, another studies using other markers followed, dealing with population genetics and other aspects of the evolutionary history of *E. imbricata* (Broderick et al. 1994; Bass et al. 1996; Bowen et al. 1996a; FitzSimmons et al. 1995; Karl et al. 1995).

Among the new markers, we can list the use of the control region (also called D-loop) of the mitochondrial DNA (Bass et al. 1996; Bowen et al. 1996b), nuclear markers such as microsatellites (FitzSimmons et al. 1995), and anonymous single-copy loci (Karl et al. 1995). Even though the first studies using the mitochondrial DNA used restriction enzymes to estimate the nucleotide divergence (Bowen et al. 1993; Broderick et al. 1994), the first studies

using small sequences of the mtDNA were published in 1996 (Bass et al. 1996; Bowen et al. 1996b).

Currently, diverse markers have been used to study the genetic diversity in *E. imbricata*. These include: microsatellites, restriction fragment length polymorphisms, and mitochondrial DNA. Each marker has its pros and cons and has been used for a variety of genetic conservation studies. The control region (CR or D-loop) of the mtDNA is the most widely used marker when studying *E. imbricata*. It has shown to perform well in phylogenetic (Naro-Maciel et al. 2008) and in phylogeographic studies (Leroux et al. 2012; Vilaça et al. 2013; Bass et al. 1996; Velez-Zuazo et al. 2008), and for species identification (Vargas et al. 2009; Naro-Maciel et al. 2010). This region has been used until today to study diverse aspects of life history. Until recently, only a short stretch of the D-loop was used in *E. imbricata*, corresponding to a sequence of 384 bp (Bass et al. 1996). This fragment formed the baseline data (or reference data) for genetic diversity studies. This short fragment proved to be powerful to perform multiple-stock analysis (given a mixed stock population and a set of probable source population, this method aims to estimate the fraction in a mixed stock population that comes from each source population), and to distinguish between rookeries demonstrating that they are independent entities. Different rookeries within Caribbean and Atlantic Ocean could be characterized according to their haplotype frequencies.

In more recent years, this fragment of the D-loop has been extended to 832 bp (Abreu-Grobrois et al. 2006). This longer fragment has proven to be more efficient in distinguishing between rookeries, since it presents more polymorphic sites than the short fragment, and allowed the unfolding of several short fragments of 384 bp in different haplotypes. Using this fragment, even though most major rookeries could be distinguished by their haplotype frequency or by exclusive haplotypes, some nesting aggregates separated by a few hundred kilometers could not be differentiated in terms of haplotype frequencies. Studies published with this longer fragment could unfold haplotypes previously considered as a single one, and even differentiate geographically close nesting aggregates (Leroux et al. 2012). For example, the haplotype Q, present in Mexican rookeries and several Caribbean feeding grounds, was identified as three different haplotypes (EiA23, EiA41, and EiA43).

Also the rookery of Guadaloupe could be described as a distinct demographic unit, and the total haplotypes that compose the baseline were extended, allowing a better resolution between close nesting areas.

When comparing these longer fragments with the 384 bp in Caribbean samples, Leroux et al. (2012) showed that 75% of total variability was present within the 384 fragment. Also the last 170 bp of the 832 bp fragment showed no variation, and therefore, a 740 bp fragment is sufficient for describing the different *E. imbricata* populations. Using the 740 bp fragment, Vilaça et al. (2013) found new *E. imbricata* haplotypes (hybrids excluded) that distinguished Brazilian rookeries from Caribbean, and also unfolded new haplotypes from the previously 384 bp short sequences. The mtDNA gene Cytochrome oxidase c subunit I (COI) have also been successfully used to uniquely identifying *E. imbricata* samples (Vargas et al., 2009; Naro-Maciel et al. 2010).

These two studies showed that *E. imbricata* can be distinguished from other sea turtle species, with some diagnostic (autapomorphic) characters. Vargas et al. (2009) found six diagnostic characters for Brazilians *E. imbricata*, while Naro-Maciel et al. (2010) found only one for samples from Puerto Rico and Australia.

Mitochondrial haplotypes have been proved to be useful to distinguish *E. imbricata* from other sea turtle species, between rookeries and feeding areas, and to estimate populational parameters of diversity and female gene flow (Bass et al. 1996). But because mitochondria is only one locus, information on diversity across the genome is lost. Also because mitochondria is female inherited, any information from male dispersal (or any other information male-driven) is not recovered.

Microsatellites on the other hand, because are nuclear markers, are informative for the male-mediated gene flow. The first study that used microsatellites for *E. imbricata* showed that these markers are useful for gene flow analysis and paternity, because the loci showed different allele frequencies between rookeries (FitzSimmons et al., 1995). Since then, several loci were specifically isolated for *E. imbricata* (Lin et al. 2008; Miro-Herrans et al. 2008; Shamblin et al. 2013).

Currently, a total of 39 loci microsatellite are available, with the number of alleles varying from 2 to 24 (based on a maximum of 40 samples typed), therefore showing a wide range of polymorphism. Other loci developed for other sea turtle species have also been tested for *E. imbricata*, with several showing successful amplification, and even high levels of genetic diversity (see Phillips et al. 2013 for examples).

Despite the low resolution for detecting population structure, microsatellites have been successfully used to investigate multiple-paternity in *E. imbricata* (Joseph & Shaw 2011; Phillips et al. 2013). Phillips et al. (2013) not only managed to determine the rate of multiple paternity, but also estimated the frequency of polyandry and polygyny, and estimated the frequency, duration and viability of sperm storage. Using a set of 33 microsatellite loci (isolated for *E. imbricata* and other species), they were able to show that almost 91% the offspring was sired by a single male, and those male did not fertilize more than one female in the same season.

Anonymous single loci nuclear (scnDNA) loci were also used in *E. imbricata* samples. These scnDNA loci are initially amplified in a PCR reaction, and after digested with restriction enzymes (Karl et al. 1992, 1995; Karl & Avise 1993). Initially developed for *Chelonia mydas* (Karl et al. 1992; Karl & Avise 1993), scnDNA showed to be able to distinguish different sea turtle species, even though they did not any polymorphisms in *E. imbricata* samples from Brazil (Vilaça et al. 2012). Despite the lack of polymorphisms within *E. imbricata*, these loci are useful to differentiate *E. imbricata* from other sea turtle species, and especially useful when studying hybridization (Karl et al. 1995; Vilaça et al. 2012). Using a restriction fragment length polymorphism (RFLP) generated by the digestion of the PCR product of these scnDNA loci, diverse cases of hybridization involving *E. imbricata* and other three sea turtle species were investigated, and F1 hybrids could be distinguished from later generation (>F1) hybrids (Karl et al. 1995; Vilaça et al. 2012). One advantage of these markers in hybridization detection is their low cost (only a PCR and few low cost restriction enzymes are required) and quick results. When comparing microsatellites with PCR-RFLP, even though the microsatellites markers developed so far are not able to show high population structure, microsatellites have a higher mutation rate, which increases the ability to track coalescence events and detect population-specific alleles.

Nuclear sequences have been standardized for sea turtles only recently (Naro-Maciel et al. 2008). The first study used five different nuclear loci (four exons and one intron) to investigate the phylogeny of sea turtles. Subsequently, populational studies using the same loci, but shorter fragments, were published (Vilaça et al. 2012, 2013; Vilaça & Santos 2013).

Vilaça et al. (2012) investigated the population structure in the Brazilian coast and the patterns of hybridization with other three sea turtles species. Using these five nuclear loci, no population structure was observed in Brazilian rookeries and feeding aggregations, but one loci (Cmos) showed a small differentiation between two rookeries. Two loci (R35 and Cmos) showed private haplotypes for rookeries and feeding areas. In contrast, one loci (RAG2) was monomorphic across Brazilian populations. When comparing the data for these five nuclear loci with other species, most haplotypes found in *E. imbricata* were private, even though two were shared with other turtle species (two RAG1 haplotypes were shared with *L. olivacea*, and the monomorphic BDNF loci possessed the same haplotype as *C. caretta*). Despite the low polymorphism of the nuclear sequences, they were proven to be useful in hybridization, and investigating possible introgression (i.e., the backcrossing of hybrids with one or both parental species) between hybrids of four turtle species in Brazil.

Nuclear markers have the advantage to provide information from male gene flow, but when comparing different populations, they usually fail to show population structure. This inability of nuclear markers to recover population structure was hypothesized to be due to the high levels of male gene flow, but it might also be because of the low evolutionary rates observed in the karyotype, mitochondrial and nuclear genomes of sea turtles (Bowen et al. 1993; FitzSimmons et al. 1995; Matsuda et al. 2005). If this is the case, only a large amount of nuclear markers will permit the distinction between different populations.

When comparing the different markers available for *E. imbricata*, the most commonly used is the control region of the mtDNA. Considering the resolution given by these two types of markers, mtDNA is more efficient in distinguishing between rookeries and feeding areas, but nuclear markers showed to be extremely relevant for studies involving hybridization or paternity investigation.

The use of Next Generation Sequencing (NGS) for developing nuclear markers for *E. imbricata* is promising, since it allows the isolation of hundreds or thousands or markers with relatively low cost. Even though on sea turtle species have its genome sequenced (Wang et al. 2013) and have populational studies using mitogenomic data (Shamblin et al. 2012c), for *E. imbricata* the use of large-scale data was used only in phylogenic studies (Duchene et al. 2012). Duchene et al. (2012) used data from the entire mitochondrial (around 16,000 bp) from samples from Indo-Pacific (n = 3) and Atlantic (n = 1) Oceans to estimate the divergence time and phylogenetic position of all sea turtle species. The topology and confidence of the trees was similar to the ones obtained by Naro-Maciel et al. (2008) using few mtDNA genes and five nuclear loci, but the divergence dates obtained had a smaller confidence interval. Even though the mitochondrial DNA can be considered as one loci, the use of larger sequences (or the entire mtDNA) can increase the resolution of the analysis and provide more confidence in the results.

Studies with other non-model animal species, with conservation interest, have shown that is possible the development of hundreds of microsatellite markers and Single Nucleotide Polymorphisms (SNPs) by sequencing a small fraction of genome. Techniques like RAD-Seq (Baird et al. 2008; Miller et al. 2007) and Genotyping-By-Sequence (GBS, Elshire et al. 2011) seems promising in dealing with species without a reference genome.

These two methodologies randomly sequence a fraction of the genome, and can be useful for marker discovery, hybridization, phylogenomics, or selection studies.

Caretta caretta

The loggerhead sea turtle, *Caretta caretta*, is widely distributed in tropical and temperate waters around the world (Pritchard & Trebbau 1984). In Brazil, loggerheads are the most abundant sea turtle species nesting along the country's coastline (Marcovaldi & Marcovaldi 1999). The nesting beaches range from the north of Rio de Janeiro (southeast coast) to Sergipe states (northeast coast); and nesting density is greatest on beaches of Bahia state (Marcovaldi et al., 2005). In fact, the Brazilian nesting population of loggerheads is one of the largest in the world, after the super-aggregations at Masirah, Oman, and Eastern Florida, US (Marcovaldi & Chaloupka 2007; NOAA 2010).

Currently, *C. caretta* is internationally listed as "endangered" by the International Union for Conservation of Nature and Natural Resources Red List of Threatened Species (IUCN 2015). In Brazil, the loggerhead sea turtle was considered "vulnerable" by the Ministry of Environment - MMA (Martins & Molina 2008). However, this status was recently reevaluated to "endangered" by Santos et al. (2011).

Loggerheads have a long history of exploitation in Brazil. Prior to 1980 nearly all eggs laid along the coast were removed, and most nesting females were taken for meat (Marcovaldi et al. 2005). The establishment of Projeto TAMAR (The Brazilian Sea Turtle Conservation Program) by the Brazilian government in 1980, and the enactment of full legislative protection of all sea turtle species in 1986 have contributed significantly to the improving status of the Brazilian loggerhead stock (Marcovaldi & Marcovaldi 1999; Marcovaldi & Chaloupka 2007). However, in more recent years, loggerheads have become exposed to other hazards such as coastal development (Marcovaldi et al. 2006), marine debris (National Research Council 1990; Bugoni et al. 2001; Milton & Lutz 2010), and incidental capture in coastal gillnet and pelagic longline fisheries operating in southern Brazilian waters (Soto et al. 2003; Kotas et al. 2004; Sales et al. 2008). Protection of the Brazilian loggerhead stock is of great importance for the global conservation of this species.

Genetic analyses have been used worldwide to investigate genetic diversity and rookery structure, phylogeography, foraging ground composition, rookery contributions to foraging aggregations, migratory patterns, natal homing behavior, taxonomic relationships, paternity, and hybridization in sea turtles (Bowen & Karl 2007; Bowen et al. 2007; Bjorndal & Bolten 2008; Jensen et al. 2013).

Bowen et al. (1994), in a phylogeographic survey based on restriction-site analyses of mtDNA with 176 samples from rookeries in Greece, Brazil, South Africa, Oman, Japan, Australia, and US, demonstrated the existence of two primary lineages in loggerheads. Both lineages are found in both Atlantic-Mediterranean and Indian-Pacific basins, probably due to the ability of this temperate-adapted species to migrate around southern Africa (Bowen et al. 1994). On the basis of a molecular clock for marine turtles calibrated at 2%-4% per million years (m.y.) (Avise et al. 1992), the deepest bifurcation in the loggerhead mtDNA phylogeny would be around 2-4 m.y. old (Bowen et al. 1994). The absence of a clear matrilineal separation between these oceanic basins could be explained by gene flow around southern Africa that perhaps occurred through the last 20,000 years. These ocean basins were relatively isolated by geography and climate in the Pleistocene. During interglacial periods, the expansion to higher latitudes was possible because of warmer temperatures.

An alternative explanation is that major mtDNA lineages have been retained in both ocean basins for several million years and recent inter-oceanic exchange of mtDNA haplotypes has resulted in the similarity of haplotypes in separate oceans (Bowen et al. 1994).

Bayesian phylogenetic analysis of ~800 base pair (bp) mtDNA fragments has recently confirmed the presence of two major loggerhead lineages globally (Shamblin et al. 2014). The deepest bifurcation among loggerhead lineages was estimated at 4.3 m.y.: haplogroup II (containing CC-A2 haplotypes and derived variants) was characterized by shallow structure relative to haplogroup I (represented primarily by CC-A1, CC-A4, and CC-A11 variants). The deepest divergence among clade I lineages occurred between Western Pacific/

Southeastern Indian Ocean haplotypes (haplogroup IA) and the remaining Atlantic and Indian Ocean haplotypes (haplogroup IB). This coalescent was dated at 2.7 m.y. A clade containing Brazilian haplotypes, Caribbean CC-A14, and CC-A11.6 from Oman diverged from the remaining haplogroup IB lineages approximately 1.0 m.y. (see Shamblin et al. 2014 for details). Encalada et al. (1998), based on 380 bp mtDNA control region sequences of 249 Atlantic and Mediterranean loggerhead turtles from 10 major nesting areas, defined six demographically independent groups: (1) North and South Carolina, Georgia and Northeast Florida, US, (2) Southern Florida, US, (3) Northwest Florida, US, (4) Quintana Roo, Mexico, (5) Bahia, Brazil, and (6) Peloponnesus Island, Greece.

At that time, these authors suggested that climate, natal homing, and rare dispersal events defined the loggerhead biogeographic scenario. Other studies, also based on 380 bp fragment of the mtDNA, have demonstrated genetic partitioning within the Northwest Atlantic (Shamblin et al. 2011), Southwest Atlantic (Reis et al. 2010a), and Mediterranean (Carreras et al. 2007). Despite clear indication of genetic structure through significant frequency differences, widespread haplotype sharing across ocean basins has limited the utility of the 380 bp sequences as a population marker in mixed stock analysis.

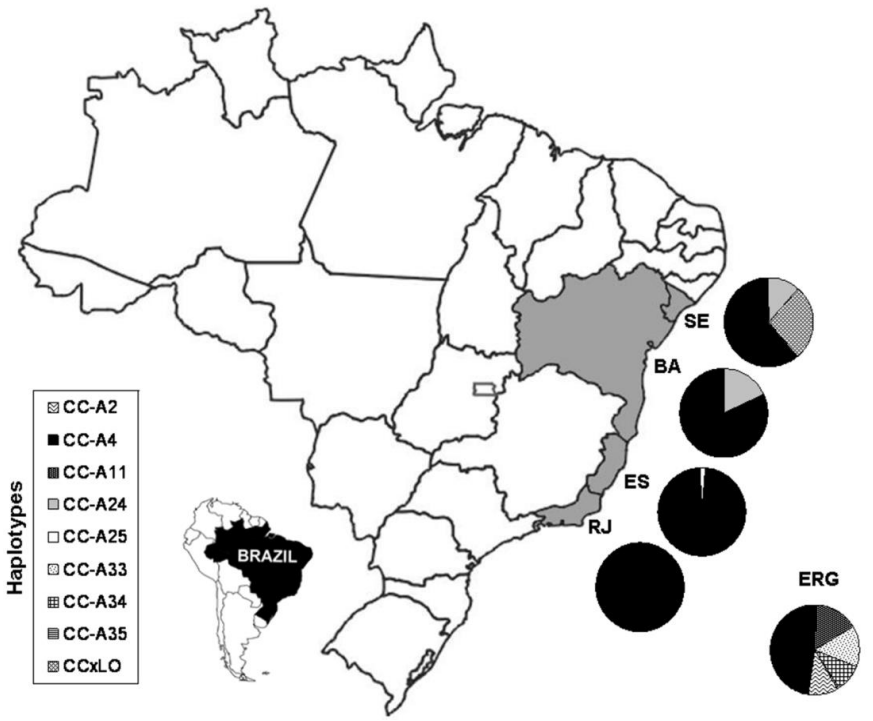
Recently, Wallace et al. (2010) have proposed the definition of nine Regional Management Units (RMUs) for loggerhead turtles, based on the integration of multiple tools and techniques, including genetic analyses (mtDNA and nDNA), site-based monitoring, mark-recapture studies and telemetry. Studies using longer sequences (~800 bp) have currently confirmed six RMUs and recognized at least 18 demographically independent management units (MUs), based on female natal homing (Shamblin et al. 2014). In the present chapter, we intend to review information on (1) the population genetic composition of Brazilian loggerhead rookeries, foraging aggregates and bycatch reports, (2) the phylogeography of this species and (3) hybridization cases. For this propose, we are revisiting the results described by Reis et al. (2009, 2010a, 2010b), Vilaça et al. (2012) and Shamblin et al. (2014). Reis et al. (2010a) analyzed 204 female loggerheads from rookeries in Rio de Janeiro (N = 64), Espírito Santo (N = 50), Bahia (N = 39), and Sergipe (N = 51) states, during the nesting seasons (September to March) of 1996/1997, 2003/2004, 2004/2005 and 2005/2006. These authors also sampled 125 loggerheads captured at Elevação do Rio Grande (ERG) as incidental take in the longline fishery. Complementarily, Reis et al. (2009) analyzed samples from one unusual loggerhead nesting specimen from Rio Grande do Norte and from five specimens that were incidentally captured in fisheries in Rio de Janeiro (N = 2), Sergipe (N = 1), Rio Grande do Norte (N = 1) and Ceará (N = 1) states. For both studies, a 627 bp consensus sequence was produced, but only a fragment length of 380 bp was used for comparisons with data from other Atlantic and Mediterranean rookeries and foraging aggregations.

Shamblin et al. (2014), through the establishment of an international working group, brought together data of loggerhead turtle rookeries in the Atlantic, Mediterranean and western Indian Ocean, combining information on 380 bp and ~800 bp control region

sequences from the literature or generated from novel samples (as from Brazil: Rio de Janeiro N = 49, Espírito Santo N = 23, Bahia N = 32 and Sergipe N = 27; Cape Verde; Oman; and South Africa). Finally, Reis et al. (2010b) presented the distribution and frequency of interspecific hybrids within loggerhead nesting beaches in Sergipe state (Abaís, Pirambu and Ponta dos Mangues; N = 51) based on mtDNA analyses, and Vilaça et al. (2012) discussed the high frequency of hybridization and introgression among sea turtle species on the Brazilian coast through mitochondrial and nuclear markers.

Brazilian Rookeries

Four distinct loggerhead control region haplotypes of 380 bp were observed among the 204 turtles sampled from Brazilian rookeries: CC-A4 (86.3%), CC-A24 (6.4%), CC-A25 (0.5%), and CCxLO (6.8%) (Figure 1, Table 2).



Data from Reis et al. (2010a).

Figure 1. Surveyed locations on the Brazilian coast and loggerhead turtle mtDNA haplotype frequencies for rookeries of Rio de Janeiro (RJ), Espírito Santo (ES), Bahia (BA) and Sergipe (SE), and for the foraging aggregation of Elevação do Rio Grande (ERG).

Endemic haplotypes (CC-A4, CC-A24, CC-A25), found only in Brazilian rookeries, create a unique Brazilian haplotype profile (Reis et al. 2010a). The CCxLO haplotype, only found in Sergipe, was attributed to specimens considered hybrids because they have the typical *Lepidochelys olivacea* mtDNA haplotype, but the external morphology of loggerheads (64.29%) or a mixture between loggerheads and *L. olivacea* (35.71%; Reis et al., 2010b). Brazilian rookeries have low values of genetic and nucleotide diversity (Table 5).

The genetic diversity of the Brazilian rookeries decreased from north to south. Sergipe had higher values with three different 380 bp haplotypes, Bahia had two haplotypes, Espírito Santo also had two different sequences, but one of them at a low frequency, and finally Rio de Janeiro had only one haplotype (Figure 1; Table 3; Reis et al. 2010a).

When considering the mtDNA fragments of ~800bp, CC-A4 haplotype was subdivided into three variants: CC-A4.1, CC-A4.2 and CC-A4.3 (Table 4; Shamblin et al., 2014). Rio de Janeiro rookery showed only two haplotypes and lower values of haplotype diversity, while Espírito Santo, Bahia and Sergipe, three haplotypes each and close values of haplotype diversity (Table 4; Shamblin et al. 2014).

A TCS network with all loggerhead 380 bp haplotypes from Brazil and Atlantic-wide rookeries revealed that the rookery haplotypes from Brazil and some from US were closely related (Figure 2). These US haplotypes included CC-A1 (the most frequently detected haplotype in western North Atlantic rookeries), CC-A11, and CC-A14 (Reis et al. 2010a).

Table 2. 380 bp mtDNA control region haplotypes from loggerhead sea turtles found at Brazilian rookeries (including an uncommon nesting site*), foraging grounds and bycatch

	Rookeries					Foraging aggregation	Bycatch			
Haplotypes	RJ	ES	BA	SE	RN*	ERG	RJ	SE	RN	CE
CC-A1					1					
CC-A2						13			1	
CC-A4	64	49	32	31		59	2			
CC-A11						19				
CC-A17										1
CC-A24			7	6						
CC-A25		1								
CC-A33						18				
CC-A34						15				
CC-A35						1				
CCxLO				14				1		
Total	64	50	39	51	1	125	2	1	1	1

Locations: Rio de Janeiro (RJ), Espírito Santo (ES), Bahia (BA), Sergipe (SE), Rio Grande do Norte (RN*), Ceará (CE) and Elevação do Rio Grande (ERG). Data from Reis et al. (2009, 2010[a], 2010b).

Table 3. Standard diversity indices (mean +/- SD) calculated for Brazilian rookeries and foraging grounds based on 380 bp of the mtDNA

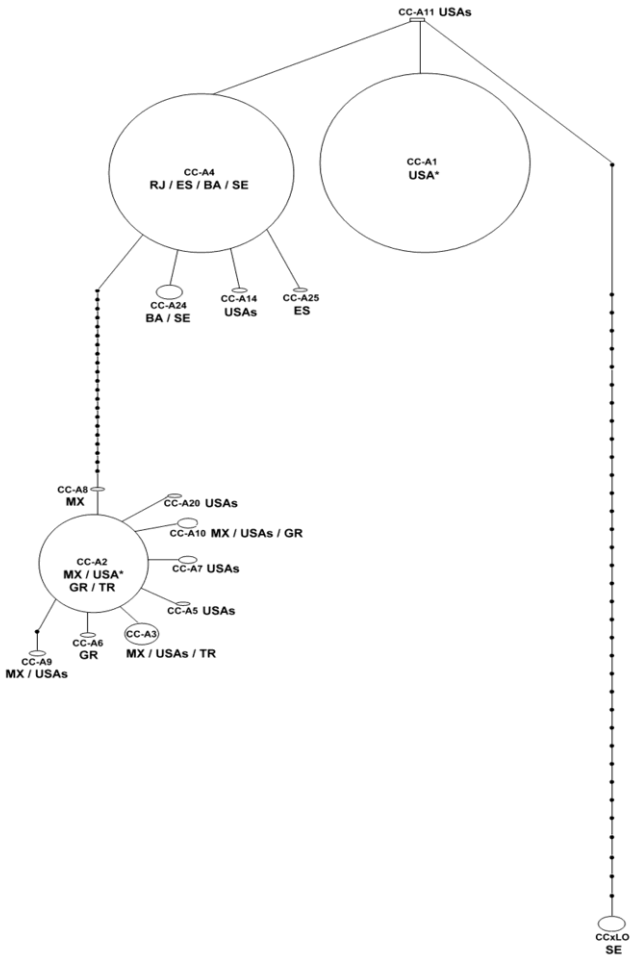
Rookeries	N	*h*	π
Brazil	190	0.1380 +/- 0.0327	0.000372 +/- 0.000606
Rio de Janeiro	64	0	0
Espírito Santo	50	0.0400 +/- 0.0380	0.000107 +/- 0.000320
Bahia	39	0.3023 +/- 0.0795	0.000810 +/- 0.000957
Sergipe	37	0.2793 +/- 0.0826	0.000749 +/- 0.000915
Foraging grounds	N	*h*	π
Elevação do Rio Grande	125	0.7140 +/- 0.0310	0.01744 +/- 0.001560

CCxLO hybrids from Sergipe (N = 14) were not included in the calculation. N = sample size, *h* = haplotype or genetic diversity, π = nucleotide diversity. Data from Reis et al. (2010a).

Table 4. 800 bp mtDNA control region haplotypes from loggerhead sea turtles found at Brazilian rookeries and corresponding standard diversity indices

Haplotype	RJ	ES	BA	SE
CC-A4.1	9	8	14	6
CC-A4.2	40	11	17	17
CC-A4.3		4		
CC-A24.1			1	4
N Total	49	23	32	27
Hap Div	0,306	0,648	0,542	0,553
Hap Div SD	0,071	0,055	0,039	0,084

Locations: Rio de Janeiro (RJ), Espírito Santo (ES), Bahia (BA) and Sergipe (SE). Data from Shamblin et al. (2014).



Circles/ovals are approximately proportional to haplotype frequencies. Brazilian rookeries: Rio de Janeiro (RJ), Espírito Santo (ES), Bahia (BA), and Sergipe (SE). Other rookeries: Mexico (MX), southern United States (USAs), southern and northern United States (US*), Greece (GR), and Turkey (TR). Data from Reis et al. (2010a).

Figure 2. TCS network of loggerhead turtle mtDNA haplotypes from Atlantic and Mediterranean rookeries. Lines between haplotypes represent one mutational step; small black circles are hypothetical haplotypes; the rectangle identifies the most probable ancestral haplotype according to the coalescent theory.

Pairwise F_{ST}, estimates of maternal gene flow (*Nm*) and the exact test of population differentiation (see Reis et al. 2010a) revealed that Rio de Janeiro and Espírito Santo rookeries were significantly different from those of Bahia and Sergipe, and no significant difference was found between Rio de Janeiro and Espírito Santo, or between Bahia and Sergipe. These results suggested that Brazil has two loggerhead genetic population units: one represented by Rio de Janeiro and Espírito Santo (Southern stock), and the other by Bahia and Sergipe (Northern stock). AMOVA global F_{ST} (see Reis et al., 2010a) indicated significant genetic structuring among the Brazilian sampled rookeries. However, AMOVA F-statistics did not confirm this two-stock structure. Due to the limited information provided by mitochondrial DNA markers (especially with shorter fragments), complementary studies based on larger sequences of mtDNA and biparentally inherited nuclear markers were then recommended to confirm and better understand the genetic structure of Brazilian loggerhead populations. In fact, Shamblin et al. (2014), analyzing ~800 bp mtDNA haplotypes, suggested that the strongest demographic partitioning within the Brazilian nesting aggregation may occur between the rookeries of Rio de Janeiro relative to all others in the country. Analyses based on the 380 bp haplotypes and using the combined sample sets from Reis et al. (2010a) and Shamblin et al. (2014) supported recognition of Northern and Southern Brazil MUs with a break between Bahia and Espírito Santo.

So, based on these results, Shamblin et al. (2014) proposed the recognition of three MUs within the Southwest Atlantic RMU: Northern coast (Sergipe and Bahia), Espírito Santo and Rio de Janeiro. In light of the subdivision of CC-A4 obtained with the expanded control region fragments, analysis with larger sample sizes from the Brazilian rookeries is warranted to better resolve the number of MUs and their boundaries.

Neutrality tests and mismatch distribution analyses (see Reis et al. 2010a) suggested that Brazil has experienced recent demographic and spatial expansions, resulting from bottlenecks or founder effects.

Brazilian Foraging Aggregation

Elevação do Rio Grande (ERG) is a seamount chain located ca. 800 km off the south coast of Brazil that rises to within 350 m of the sea surface. Similar to the Azores Archipelago in the North Atlantic (Bolten et al. 1998), ERG is an important foraging ground and oceanic developmental habitat for immature loggerheads in South Atlantic waters (Marcovaldi et al. 2006; Sales et al. 2008). Among the 125 loggerhead turtles sampled from the foraging aggregation atERG, six distinct haplotypes were found: CC-A2 (10.4%), CC-A4 (47.2%), CC-A11 (15.2%), CC-A33 (14.4%), CC-A34 (12%), and CC-A35 (0.8%) (Figure 1; Table 3; Reis et al. 2010a). For the first time, CC-A2, CC-A11, CC-A33, CC-A34 and CC-A35 haplotypes were reported for a Brazilian foraging aggregation (ERG). CC-A2 and CC-A11 have been reported from northwestern Atlantic rookeries (Bolten et al. 1998; Bowen et al. 2004; Reece et al. 2006; Shamblin et al. 2012a), and CC-A2, also from Mediterranean rookeries (Carreras et al. 2006; Yilmaz et al. 2011; Casale et al. 2013; Garofalo et al. 2013). CC-A33 is identical to CC-P5, already registered for rookeries of Western Australia, Queensland and New Caledonia (FitzSimmons et al. 1996; Boyle et al. 2009) and CC-A34 is identical to CC-P1, also registered for these same rookeries (Bowen et al. 1995; Boyle et al. 2009) and for Japan (Watanabe et al. 2011). CC-A35 represented an orphaned haplotype

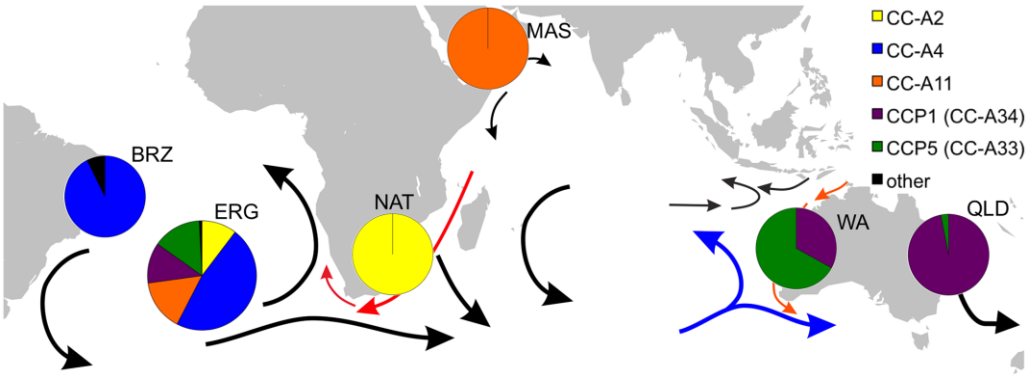
since its source rookeries are still unknown. The foraging aggregation (Elevação do Rio Grande) had high haplotype diversity and a moderate nucleotide diversity (Table 3) compared with other Atlantic-wide foraging aggregations. Generally, foraging aggregations also show higher diversity indices than rookeries because rookeries host philopatric females while foraging aggregations can receive individuals from many source rookeries.

Mixed Stock Analysis (MSA) indicated a major contribution to the ERG foraging aggregation from Brazil (mean 59.5%) and a non-significant contribution from the US, Mexico, and Turkey (see Reis et al. 2010a). This result is expected due to the presence of the Brazilian exclusive haplotype (CC-A4) in 59 out of 106 individuals from the foraging aggregation used for the MSA. The second highest contribution detected by MSA was from Australia (28.5%), because haplotype CC-A34 has only been reported from Australian rookeries. Another significant contribution was also detected from Greece, due to the presence of CC-A2 among the Brazilian foraging aggregation, which is present at high frequencies in Greece. Although CC-A2 is also frequently observed in US, the MSA takes into account that the contribution from a particular rookery should include all of the most frequent haplotypes (Reis et al. 2010a). The occurrence of CC-A4, an endemic Brazilian haplotype, in the ERG aggregation indicates that those turtles belong to the Brazilian loggerhead genetic stock, which was corroborated by the MSA results. However, the ERG aggregation is a mixed stock with haplotypes from worldwide rookeries. The haplotypes CC-A33, CC-A34 and CC-A35 form an independent cluster in phylogenetic reconstructions and probably have a common origin. The MSA results must be interpreted with care, since this analysis assumes that all source rookeries are known, but African and Indo-Pacific rookeries have been very poorly surveyed.

Thus, the proposed contributions from Australia and Greece may well derive from rookeries on the west coast of Africa or Indo-Pacific rookeries (Reis et al. 2010a). Dispersal patterns for oceanic juveniles modeled under the assumption of passive drift supported the likelihood of dispersal of South African juveniles into the South Atlantic but did not indicate connectivity between the South Atlantic and more distant rookeries in the Indian Ocean basin (Mansfield & Putman 2013).

Nonetheless, confirmation of CC-A2.1 from South Africa and CC-A11.6 from Oman (Shamblin et al. 2014) warrants reconsideration of the possibility of long distance migratory connectivity between the South Atlantic and Indian Ocean basins (Figure 3).

Furthermore, Caraccio et al. (2008), analyzing 14 loggerheads incidentally captured by coastal trawl and 29 by pelagic longline fisheries in Uruguayan waters, found the same haplotypes identified among the ERG samples: CC-A2 (N = 6, 14%), CC-A4 (N = 32, 74%), CC-A11 (N = 1, 2.3%), CC-A33 (N = 2, 4.6%) and CC-A34 (N = 2, 4.6%), with genetic diversity (*h*) equal to 0.4319 +/- 0.0872 and nucleotide diversity (π) equal to 0.014616 +/- 0.007978. All turtles analyzed from the Uruguayan continental shelf (N = 14), classified as adults or late maturing juveniles, showed CC-A4 haplotype, which is only found at Brazilian rookeries. On the other hand, turtles studied from the oceanic area (N = 29), classified as juveniles, were genetically diverse and presented haplotypes that pertained to distance nesting colonies: CC-A2 (present in the US, Mexico, Mediterranean and South African rookeries), CC-A11 (present in the US and Oman), CC-A33, CCA34 (found in the Indo-Pacific Ocean), and also CC-A4. These data suggested that some adult individuals move on the continental shelf carrying out feeding migrations from the nesting beaches in Brazil towards higher latitudes.



BRZ is combined Brazilian rookeries (Reis et al. 2010a); NAT is Natal, South Africa (Shamblin et al. 2014); MAS is Masirah Island, Oman (Shamblin et al. 2014); WA is Western Australia (FitzSimmons et al. 1996), QLD is Queensland, Australia rookeries (Boyle et al. 2009). The arrows indicate directionality of major surface currents: orange represents the Leeuwin Current; blue indicates the Western Australia Current, and red, the Agulhas Current. Data compiled by Shamblin et al. (2014).

Figure 3. Loggerhead turtle haplotype distribution for an oceanic foraging aggregation and major Indian Ocean rookeries. Control region haplotype (380 base pair) frequencies for the oceanic juvenile foraging aggregation from the Elevação do Rio Grande (ERG) and adjacent ridge and slope of the continental shelf in the South Atlantic Ocean (Reis et al. 2010a) and the RMUs in the South Atlantic, Indian, and South Pacific Ocean basins.

However, juvenile turtles that pertain to diverse nesting colonies carry out feeding migrations towards areas of high productivity in open waters of the southwestern Atlantic Ocean. Prosdocimi et al. (2015), analyzing 24 samples of bycatch and 37 samples of stranded loggerhead sea turtles (all of them classified as adults or sub-adults) on the coast of the province of Buenos Aires, Argentina, have found similar results. Both shorter (380 bp) and longer (760 bp) mtDNA sequence analysis showed that in the foraging grounds of the Argentinean coast only haplotypes from Brazilian nesting areas (CC-A4 = 98% and CC-A24 = 2% for shorter sequences, and CC A4.2 = 81%, CC A4.1 = 17% and CC A24.1 = 2% for longer sequences) were found. The homogeneous stock located relatively close to the rookery where individuals originated contradicts the paradigm of immature loggerhead sea turtles forming mixed stocks in foraging and developmental areas. The conservation of the stock in coastal areas of Argentina could benefit the nesting population in the nearby Brazilian rookeries, and could be achieved by conservation actions between these two countries, as well as Uruguay in between.

Additional Reports: Bycatch and Uncommon Nesting Site

Finally, among the samples from bycatch (N = 5) and an uncommon nesting site (N = 1), five distinct control region haplotypes were observed: CC-A1 (N = 1, unusual nesting report for Rio Grande do Norte), CC-A2 (N = 1, Rio Grande do Norte bycatch report), CC-A4 (N = 2, Rio de Janeiro bycatch reports), CC-A17 (Ceará bycatch report) and CCxLO (Sergipe bycatch report) (Table 4; Reis et al. 2009). For the first time, CC-A1 and CC-A17 haplotypes were reported for the Brazilian coast. The loggerhead sampled in Rio Grande do Norte

(6°13'39"S 35°03"01"W) is an unusual report because in Brazil the northern-most regular nesting region for this species is Sergipe. CC-A1, found for this sample, is a characteristic haplotype from north western Atlantic nesting and feeding grounds (Bowen et al. 2004; Reece et al. 2006; Shamblin et al. 2012a). But it has also been reported for Cape Verde rookery, as well as for feeding aggregations in the eastern Atlantic and Mediterranean Sea (Carreras et al. 2006; Monzón-Argüello et al. 2009, 2010; Garofalo et al. 2013). CC-A17, from a bycatch sample of Ceará (3°43'06"S 38°32'02"W), is typically found in juvenile aggregations from Andalusia, Madeira and Canary Islands and has recently been reported for Cape Verde rookery (Bolten et al. 1998; Monzón-Argüello et al. 2009, 2010). In fact, Ceará has been pointed through telemetry data as an important foraging ground for loggerheads in the northern coast of Brazil (Marcovaldi et al. 2010). The occurrence of these haplotypes (CC-A1, CC-A2 and CC-A17) suggests transoceanic migratory behavior of *C. caretta* and a possible origin for the Brazilian rookeries colonization. The natal homing behavior of *C. caretta* is also confirmed by the fact that there are some in-water loggerheads with haplotypes (CC-A2 and CC-A17) that are not found in Brazilian rookeries, suggesting these animals would return to their natal nesting areas for reproduction.

Brazilian Demography History

The CC-A4 haplotype is common to all Brazilian rookeries and foraging areas (Figure 1). This fact, associated with the Brazilian low nucleotide diversity (Table 3) and phylogenetic proximity among rookery haplotypes (Figure 2), suggested a common origin, with CC-A4 the probable ancestral haplotype of Brazilian populations (Reis et al. 2010a). These data indicate that Brazilian rookeries may have had the same origin. Because the Brazilian rookeries also show low genetic diversity values and low divergence between those haplotypes, the Brazilian populations were probably colonized recently, as also suggested by neutrality tests and mismatch distribution analyses (see Reis et al. 2010a). As genetic diversity decreases from northern to southern Brazil (Figure 1; Table 3), the authors suggested that colonization of the rookeries followed a north to south route along the coastline, probably influenced by the Brazilian warm current which flows from north to south. In fact, neutrality tests indicated that the Brazilian southern stock is more recent than the northern one. At times of glacial retreats, loggerhead nesting and foraging habitats expanded into higher latitudes. Encalada et al. (1998) suggested that, during these interglacial periods, an equatorial lineage may have colonized northern latitudes along the Florida peninsula, which explains the existence of different phylogenetic lineages in US.

Reis et al. (2010a) believe that colonizations in Brazil followed the same pattern. This hypothesis is supported by the fact that loggerheads show the propensity for occasional long-distance colonization, as indicated by the widespread distribution of some haplotypes. As maritime current dynamics have changed through the years; this colonization hypothesis should be re-evaluated considering paleogeography and paleocurrents.

Reis et al. (2009, 2010a) also hypothesized that, considering the phylogenetic proximity between CC-A1 and CC-A4 haplotypes (380 bp), the colonization of Brazilian rookeries could have been from the US stock, especially from the south. However, with expanded sequences (~800 bp) it is clear that the Brazilian haplotypes appear basal among haplogroup

IB lineages (see Shamblin et al., 2014 for details). Thus, the Brazilian rookery harbors lineages older than those nesting in the southeastern United States (Shamblin et al. 2014).

Global Population Structure and Phylogeography

Parwise F_{ST}, exact test of population differentiation, *Nm* estimation, and AMOVA (see Reis et al. 2010a) indicated strong genetic structuring among global rookeries. These data corroborate the natal homing behavior of loggerheads on a global scale. If female loggerheads return to natal sites for nesting, then rookeries would show pronounced differences with respect to female-transmitted genetic markers such as mtDNA (Bowen et al. 1994). However, natal homing in loggerhead turtles cannot be absolute, because new rookeries must be colonized by turtles hatched elsewhere.

Results of the Mantel test (see Reis et al. 2010a) supported the isolation by distance model between western Atlantic (northern and southern US, and Brazil) and Mediterranean (Greece and Turkey) rookeries. This model results from spatially limited gene flow with gene dispersal only between adjacent areas. However, long distance colonization is essential to explain the current global distribution of loggerhead rookeries. Despite the great migratory capacity of loggerheads, isolation by distance is a result of their philopatric behavior. As females tend to return to their natal region to nest, it is expected that new colonizations would occur in adjacent areas. Data also suggested that the most recently colonized rookeries are Greece and Brazil. Colonization into the Mediterranean Sea was most likely accomplished within the last 10,000 years, after the Wisconsin glaciation (Encalada et al. 1998).

Recently, Clusa et al. (2013), based on the new data available to the Mediterranean Sea (by Carreras et al. 2007; Garofalo et al. 2009; Yilmaz et al. 2011), suggested that loggerhead rookeries in the Mediterranean would be the result of at least two colonization events from the Atlantic, the oldest one in Libya and a most recent in Calabria, combined with local extinctions during Pleistocenic glaciations and re-colonizations from glacial refugia in Libya, eastern Turkey and western Greece.

Based on ~800bp mtDNA sequences, Shamblin et al. (2014) suggested the invasion of the Atlantic by Indo-Pacific loggerhead lineages via southern Africa, as demonstrated in the case of the haplogroup I. These authors also suggested that inter-oceanic exchange of loggerhead lineages likely occurred multiple times and in both directions.

Hybridization

Although hybridization events have previously been reported for marine turtle populations (Conceição et al. 1990; Bowen et al. 1992, 1994; Karl et al. 1995; Bass et al. 1996; Bowen & Karl 1996; Lara-Ruiz et al. 2006), hybrids between *C. caretta* and *L. olivacea* were reported for the first time on the Brazilian coast by Reis et al. (2009, 2010a, 2010b). Fossil evidence suggests that time of separation among these two species is around 10-20 million years ago (Zangerl 1980; Dodd & Morgan 1992). Sea turtles are likely the most ancient vertebrates hybridizing under natural conditions, since hybrids exist between *Caretta* and *Chelonia* genera, which were separated about 50 million years ago (Bowen et al. 1992,

1994; Karl et al. 1995). The capacity of sea turtles to generate fertile hybrids seems to be related to the slow chromosomal and anatomic evolution.

The maintenance of chromosomal number and structure may allow genomic compatibility among species (Bickham 1981). The fitness success of the carapace and accompanying morphological adaptations should favor interspecific mating. Moreover, a dearth of behavioral barriers to hybridization may be an issue, as well as the fact that male turtles are notably indiscriminate in mating preferences (Bowen 2003).

C. caretta x L. olivacea (CCxLO) hybrids were identified into the loggerhead nesting population (N = 14, 27%) and bycatch (N = 1) of Sergipe (Table 3). From the 14 individuals genotyped as CCxLO hybrids, nine (64%) presented *C. caretta* external morphology and five (36%), mixed morphological characteristics from both *C. caretta* and *L. olivacea*. In this last case, specimens show a combination of morphological characteristics from both species, i.e., number of lateral scutes on the carapace and on the head, the format of the carapace and the animal's biometry. These two types of hybrids led the authors to suppose that at least two hybridization events may have occurred between these two species, but both of them involving the same *L. olivacea* haplotype. Thus, even if more than one hybridization event had taken place, they involved the same *L. olivacea* mtDNA haplotype. An ancient event resulted in individuals with *L. olivacea* mtDNA haplotypes without morphological vestiges of this species. In this case, hybrids will most likely be F2 or >F2 and introgression probably occurred by backcrossing of female hybrids with *C. caretta* males. A second more recent event resulted in individuals with mixed morphological characteristics from both *C. caretta* and *L. olivacea*, possibly from direct interspecific coupling, therefore, F1. This scenario, however, cannot be distinguished from an ongoing process of hybridization over the last few millennia without nuclear DNA data.

An additional hypothesis considers the CCxLO hybrids as a result of a long and antique process of introgressive hybridization, although it predicts a morphological gradient of characteristics which was not observed in the present scenario. However, Vilaça et al. (2012), through the complementary analyses of 12 nuclear markers, indicated that most of these individuals in the crossings *C. caretta* x *L. olivacea* were F1 hybrids. Thus, some sort of reproductive barrier might be present affecting the fertility of the progeny.

Reis et al. (2010b) only observed hybrids between females *L. olivacea* and males *C. caretta* (see also Hahn et al. 2007). This suggests that only unidirectional hybridization and subsequent backcrossing occurred between these two species. Karl et al. (1995) suggested that a numerical predominance of hybrids with mothers from the more common species is due to a constant "error" rate in the choice of mate per female. Nonetheless, Vilaça et al. (2012) reported a unique hybrid between a female *C. caretta* and a male *L. olivacea*, from a bycatch sample of São Paulo state previously classified by morphology as a loggerhead, but identified by mtDNA as an olive ridley. It suggested that mating occurred in either direction, as hybrids with mitochondria of either parental species were detected.

The incidence of hybridization between loggerheads and olive ridleys in Sergipe, that hosts the largest Brazilian nesting population of *L. olivacea* (Silva et al. 2007), may be related to an overlap in the reproduction period and area of both species. Recalling that the *L. olivacea* nesting population is larger than the *C. caretta*, we assumed that the same proportion is maintained at the reproduction colony. Naturally, the availability of *L. olivacea* females was superior to *C. caretta*, favoring the interspecific mating of loggerhead males with olive ridley females. The scenario is even more severe since the sex ratio is female biased

(Marcovaldi et al. 1997). This may also be associated to global warming since it might alter the sex ratio of sea turtles, thus facilitating interspecific mating. Because of that, it is important to evaluate natural sex proportion in those species. Moreover, the monitoring of natural temperature changes is crucial to better understand this ongoing hybridization process.

The unusually high proportion of hybrids between *C. caretta* and *L. olivacea* in Sergipe (almost 30%), as well as in related hybridization events between *E. imbricata* and *C. caretta* or *E. imbricata* and *L. olivacea* in Bahia (Lara-Ruiz et al. 2006), and between *C. caretta* and *C. mydas* also in Bahia (Bowen et al. 1992, 1994; Karl et al. 1995), represents a serious concern for the conservation of these species in Brazil, which raises a controversial issue about conservation efforts focusing on hybrid populations (Allendorf et al. 2001).

Although hybrids are rare in populations, a few hybrids may form a bridge which allows a trickle of alleles to be transferred between species; thus, if species that hybridize are common, even low rates of hybridization per individual can have important evolutionary consequences (Mallet 2005).

Vilaça et al. (2012) suggested that hybridization on the Brazilian coast is a recent phenomenon, spanning at least two generations or approximately 40 years. These authors also suppose that sea turtle hybridization occurring in Brazil may be linked to overhunting and local warming of beaches due to coastal deforestation (Matsuzawa et al. 2002). These could be the direct causes of the recent decline of sea turtle populations, reaching its climax in the 1970s in Brazil, which could have triggered an increase in interspecies hybridization.

In fact, frequent examples of hybridization in nature are often attributed to environmental degradation (Mallet 2005). Therefore, the occurrence of hybrids among sea turtle species may point to the existence of anthropogenic pressure that needs to be investigated.

Equally, if we consider that the process of hybridization has important evolutionary consequences, it becomes essential to evaluate its impact on the genetic diversity and identity of those species.

Conservation Perspectives for *Caretta caretta*

Reis et al. (2009, 2010a, 2010b) have not distinguished new mtDNA haplotypes with the longer sequences of 627 bp. However, Shamblin et al. (2014) have subdivided CC-A4 into three variants based on ~800 bp fragments. Indeed, over the past few years, expanded mitochondrial control region sequences of about 760 to 817 bp has been used to increase resolution of population structure and mixed stock analysis (e.g., Monzón-Argüelo et al. 2010; Shamblin et al. 2012a, 2014; Garofalo et al. 2013). Up to now, results have been promising and novel polymorphisms have been detected. Nevertheless, additional data are needed from major Atlantic rookeries (including Brazilian ones) to evaluate the utility of the expanded sequences to improve resolution of those analyses.

The variation uncovered in CC-A4 from Brazil should be further explored in two directions: i) analysis with larger sample sizes from the Brazilian rookeries is warranted to better resolve the number of MUs and their boundaries, and ii) reanalyzing Brazilian oceanic juvenile samples for the longer control region fragment should be also investigated to let testing juvenile natal homing along the Brazilian coast.

Therefore, in a global scale, phylogeographycal and mixed stock analyses should be redone considering the recent data available for western Atlantic (Ruiz et al. 2008; Monzón-

Argüello et al. 2010; Shamblin et al. 2011, 2012a, 2014), eastern Atlantic (Monzón-Argüello et al. 2009, 2010), Mediterranean (Carreras et al. 2007, 2011; Garofalo et al. 2009, 2013; Chaieb et al. 2010; Yilmaz et al. 2011; Saied et al. 2012; Casale et al. 2013), and Indo-Pacific (Boyle et al. 2009; Watanabe et al. 2011; Shamblin et al. 2014). Additionally, due to the limited information provided by mitochondrial DNA markers (especially shorter fragments), complementary analyses based on larger sequences of the mtDNA, and biparentally inherited nuclear markers are also recommended to confirm and better understand the genetic structure and demographic history of loggerhead populations.

The existence of three loggerhead genetic population units in Brazil, as well as evidence of extensive hybridization between *C. caretta* and *L. olivacea*, will influence the development and implementation of appropriate management strategies in the country. The extent of hybridization between different species of the Family Cheloniidae must be investigated to understand the implications and causes of such events, and its impact on the genetic diversity and identity of those species. Natal homing in loggerhead females, which was corroborated by these data, means that each regional nesting population comprises an independent stock or an Evolutionary Significant Unit (ESU). Thus, an extirpated rookery will not be reestablished over a timescale compatible with human interests. Although new nesting beaches must be occasionally colonized, the frequency of such events is low. It reinforces the need of an adequate management plan for each ESU, designed to meet its specific needs, in order to guarantee the maintenance and conservation of loggerhead populations. Recently, Wallace et al. (2010) have proposed the definition of Regional Management Units (RMUs), based on genetic, demographic, geographic, and oceanographic considerations, to organize marine turtles into global units of protection.

For loggerheads, nine significant nesting aggregations have been globally recognized as RMUs: (1) Northwest Atlantic Ocean, (2) Southwest Atlantic Ocean, (3) Northeast Atlantic Ocean, (4) Mediterranean Sea, (5) Southwest Indian Ocean, (6) Northwest Indian Ocean, (7) Southeast Indian Ocean, (8) North Pacific Ocean, and (9) South Pacific Ocean. A tenth putative RMU has been also proposed for the Northeast Indian Ocean, but genetic and biological data are still lacking.

Additionally, Wallace et al. (2011) developed a new assessment framework that allows evaluating, comparing and organizing these RMUs according to categories of paired risk and threats scores, also highlighting important gaps in available information. The definition of RMUs and this risk and threats framework provide valuable guidance to the research, management and conservation of sea turtle species, including loggerheads.

Perspectives for Genetic and Conservation of Sea Turtles

The importance of a profound knowledge on endangered species biology is unquestionable to create management strategies that fit the excepted species behaviors. This genetic data chapter, made for three sea turtles species found within widespread geographic range, shows that studies on leatherbacks are less frequent. For example, only three studies on MSA of feeding aggregations were published (Vargas et al. 2008, 2013; Prosdocimi et al. 2014) compared with many (more than ten) for the other species (Jensen 2010; Table 5).

Scarce studies on phylogeography (Dutton et al. 1999, 2007, 2013; Molfetti et al. 2013; Vargas et al. 2008) and multiple paternity (Crim et al. 2002; Stewart & Dutton 2011) were

found. We did not find studies comparing data among different life cycles stages for leatherbacks, despite its great importance to better understand the combinations of forces that can affect the construction of genetic diversity, the spreading, the recruitment and the migratory behavior on all life-history stages of marine species (see Velez-Zuazo et al. 2008 for an example in *Eretmochelys imbricata*).

Table 5. Studies using Mixed Stock Analysis of marine turtle foraging aggregations, categorized by species and region

Loggerhead turtle (*C. caretta*) Overall = 17	Green turtle (*C. mydas*) Overall = 19	Hawksbill turtle (*E. imbricata*) Overall = 14	Leatherback turtle (*D. coriacea*) Overall = 3
	Caribbean		
Atlantic-US	Bass et al. 1998		
Sears et al. 1995	Lahanas et al. 1998		
Bass et al. 2004	Luke et al. 2004		
Bowen et al. 2004	Bolker et al. 2007	Caribbean	
Roberts et al. 2005	Bjorndal and Bolten 2008	Bowen et al. 1996b	
Reece et al. 2006	Richardson et al. 2009	Bass 1999	
Rankin-Baransky et al. 2001	Atlantic-US	Diaz-Fernandez et al. 1999	
Atlantic-East	Bass et al. 2006	Bowen et al. 2007	
Bolten et al. 1998	Bass and Witzell 2000	Velez-Zuazo et al. 2008	
Monzón-Argüello et al. 2009	Atlantic-Brazil	Blumenthal et al. 2009	
Atlantic-Brazil	Naro-Maciel et al. 2007	Browne et al. 2010	Atlantic-Brazil
Reis et al. 2010a	Proietti et al. 2009	Troëng et al. 2005	Vargas et al. 2008
Mediterranean	Bjorndal et al. 2006	Richardson et al. 2009	Vargas et al. 2013
Laurent et al. 1998	Prosdocimi et al. 2011	Australia	Prosdocimi et al. 2014
Maffucci et al. 2006	Naro-Maciel et al. 2012	Broderick et al. 1994 Bell et al. Unpub.	
Carreras et al. 2006	Proietti et al. 2012	Jensen et al. in prep	
Casale et al. 2008	Pacific-Hawaii	Indian Ocean	
Monzón-Argüello et al. 2009	Dutton et al. 2008	Jensen et al. Unpub.	
Pacific	Australia	Atlantic-Brazil	
Bowen et al. 1995	Dethmers unpub.	Vilaça et al. 2013	
Boyle et al. 2009	Jensen et al. 2013 - Chapter 5		
Caribbean	Jensen et al. 2013 - Chapter 3		
Engstrom et al. 2002	SE Asia		
	Jensen et al. 2013 - Chapter 4		

Adapted from Jensen 2010.

Despite most of the paper reviewed in this chapter had the mtDNA control region and/or the microsatellites as the molecular marker of choice, new tools and methods such as single nucleotide polymorphisms (SNPs) and mitogenomics can disclose important accompaniments to the molecular database that promise to overcome some of the constraints of previous studies (Jensen et al. 2013) for all marine turtle species.

CONCLUSION

To choose a molecular marker for conservation genetics analysis is very important, because a selection of inappropriate markers will result in incorrect conservation actions, so the choice of technique depends on the objective and preliminary information. Molecular techniques provide information to different taxonomic levels. All have their limitations and

application will be determined largely by the information been looked in the study and the availability of resources to the development of these techniques. Genetic data have provided important information on the distribution and migratory patterns of sea turtles in the southwestern Atlantic, reaffirming the importance of international cooperation in the management of this species.

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Chapter 7

PRACTICAL MANUAL ON CLINICAL CYTOLOGY AND HEMATOLOGY FOR SEA TURTLE CONSERVATION

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ABSTRACT

Wild animals, such as sea turtles, are becoming more common at clinics and veterinary practices. Diagnostic methods for these species are under development and more work is needed, including hematologic diagnosis and blood cytology procedures. The work presented here explores variable studies describing blood cells of sea turtles. Within red blood cell series, we present the following red cell types (erythrocytes), hemoglobin (Hb), Hematocrit (HCT), erythrocytes levels, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Within white blood cell series, we present the following: heterophils, eosinophils, basophils, monocytes, azurophils and thrombocytes.

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Hematologic values such as hematocrit, total counts, and hemoglobin and erythrocytes levels are also presented. Hematologic and blood cytology interpretation provide an excellent diagnostic tool for sea turtle health given cytological parameters of main blood cells. At the same time techniques for cell count and evaluation are detailed, with an interpretation of pathologies and physiological alterations. This work is aimed at biologists, veterinarians, cytologist and pathologist to assist examination of blood frotis between different sea turtle species.

INTRODUCTION

Mexico is one of the few places in the world where six out of the seven species of sea turtles nest (Márquez 2002), of which all are considered endangered. Sea turtles serve as good indicators of ecosystem contamination, mainly due to their long lifespan and broad distribution. Therefore, the understanding of their health could also be used as a proxy to determine ecosystem health.

Due to the level of research conducted on the green sea turtle (*Chelonia mydas*) in terms of conservation, this species is probably the most understood due to its historical value. This species is listed as endangered by national Mexican and international laws and treaties.

Hematological and cytological reference values can aid wildlife conservation efforts, especially to threatened and endangered species, such as sea turtles as in the case of this study. Health determination and possible afflictions of individuals from blood metabolite levels aid in the selection of more precise treatments, prevention and control of various pathologens. Results prevent speculation about the reasons behind depletion of health, such as when clinical observations are the only available information.

The best available hematological and biochemical reference levels are currently only available from countries other than Mexico. *Chelonia mydas*, is one species of sea turtle that nests along the Yucatan, Mexico coastline. The present study was performed using a practical manual sampling design, whose objective was to obtain blood samples to serve as reference values, and to find differences between apparently healthy and sick turtles. We compared our study to relative studies performed on a global scale to better understand the application of our results on this vastly migratory species. The present work recommends research techniques to establish reference levels to improve treatment efficacy by using levels closer to the reality of sea turtle populations in Mexican waters. The aim of this paper is to provide a relevant tool using manual blood parameter evaluations for people interested and involved not only on sea turtle conservation but also on wildlife in general.

Hematology

Every single cell of the analyzed blood components has a particular relevance because they are involved in different metabolic paths for carbohydrates, proteins and lipids. These metabolic routes are active on different levels in accordance to the associated organ. Therefore a modification on the metabolic route can be detected as a change on blood levels of various metabolites and consequently their levels within the related organ.

Enzymes are proteins that catalyze chemical reactions performed at the cell level and their level can be modified when cells decrease in activity, since intracellular components, included enzymes, are liberated. An increase in plasma concentration of certain types of enzyme can indicate variable levels of tissue damage which can alter reproductive capabilities. Therefore, the laboratory work determination of levels of metabolites can be used for the diagnosis of different pathologens.

Implementation of these diagnostic techniques can define concentration levels (reference levels) for each blood metabolite under normal conditions. References to the state of health can be determined as acceptable for each species in accordance to their gender, age, habitat, time of the year and even time of the day when samples were taken. This level of detail sets a solid baseline to measure changes in the levels of metabolites observed at any time.

It is extremely complicated to establish reference levels on wildlife fauna. Therefore, it is necessary to select a sample of various populations considered as "healthy" for each one of the sea turtle species to determine blood metabolites concentrations. This process needs to be supported by the level of experience of biologist, marine biologists, veterinarians, ecologist and chemist professionals involved on the study of wildlife health.

Limited information of sea turtle hematologic values and cytology techniques inhibits effective health diagnosis. These two parameters serve as excellent indicators for many aspects of sea turtle well being, including health and reproductive potential. Reference values among published research is not well documented in respect of providing a comparison between lower and higher limits of each chemical and hematological threshold. In this study we compare blood parameters ranges of *Chelonia mydas* to related studies in other areas with other species of sea turtles to present a hands-on approach. Blood samples can help scientists identify the ratio of healthy to sick turtles of a given population.

Blood samples can be counted by different methods, including manual and automatized counts, but problems arise due to the challenges in in differentiating erythrocytes from nucleated thrombocytes. Some studies have determined that the most reliable leucocytes count can be obtained by the use of automatized counters such as Cell Dyn 3500 and the associated computer program for veterinarians produced by Abbott Diagnostics, Abbott Park, IL. Nevertheless, it is still necessary for the researcher to modify the parameters to validate the count for each of the studied species and eliminate the variations between cellular lines. The manual analysis of blood samples must take into account various factors that are not necessary in automatic analysis.

Hematological studies of sea turtles started in the 1970´s intended on identifying correlations between diet and anemia conditions potentially present in *Chelonia mydas* (Frair 1977). From these early studies, a large number of blood value variations were documented, related to different factors such as: age, gender, stress, diet, temperature, hydration, etc., This has spurred additional research needs on this particular topic in order to understand how blood value variations might be related to certain health conditions of this endangered sea turtle species (Aguirre & Balazs 2000).

Despite the fact that hematological evaluation is a simple method where blood values are used as indicators of the health status of an individual, such as parasitic infestations (Watson 1999), no blood value research on *Chelonia mydas* have been done for nesting female turtles in Mexico.

The objective of the present study was to obtain baseline information of hematological values and compare the findings between apparently healthy and sick (parasitized) nesting *Chelonia mydas* sea turtles in the northeast coast of Yucatan, Mexico.

METHODOLOGY

Blood Collection in the Field

Methods vary on how to capture turtles to collect blood samples. Most turtles are caught manually by the use of snorkeling gear or scuba diving that result on less stress for the turtles. Yet, depending on the region, most sea turtles are captured by gillnets set in place overnight and checked every 2 to 12 hours. Individuals are then retained until a blood sample can be collected safely and precisely.

An important factor to take into consideration during blood sample collection is the injection site. Final counts and hematocrit value can differ depending on the area and method used for collection. For example, lymphatic vessels usually run in close proximity to blood vessels on reptiles, which can result in contamination of the sample from lymph fluid during extraction (Frye 1991). Therefore, the best places to collect blood samples from sea turtles are the carotid artery and the brachial plexus.

In the case for *C. mydas* population, researchers extracted blood samples by dorsal cervical sinuses puncture (Owens and Ruiz 1980); sampled was collected by a 6 or 10 ml syringe with a 22 by 1.5" needle that was introduced at a perpendicular angle at the neck. Samples were place on 5 ml Vacutainer® tubes that contained lithium heparin. Pressure was applied to the neck area after blood extraction to prevent hematoma formation and help with cauterization (Sykes and Klaphake, 2008).

Manual Preparation of Blood Samples

Samples should be diluted to a suitable concentration for counting cells. It is important to consider various aspects of using anticoagulants when analyzing blood samples of reptiles. Some anticoagulants can lead to the degradation of erythrocytes which can result in false positives of a healthy sea turtle. The three most common anticoagulants used for blood screening include: ethylenediaminetetraacetic acid (EDTA), heparin and lithium heparin. For example EDTA has been observed to cause degradation of erythrocytes in sea turtle blood samples (Muro et al. 1998) also been observed in samples from other reptiles, however it is appropriate to use for cell tinction. In contrast, lithium heparin, has been successfully used diagnostic analysis (Sykes and Klaphake 2008, Lawrence 1985) as a preservative of the blood samples collected. However, overall, EDTA is the better option for cell identification when compared to heparin. The present study was carried out during the nesting season, April to October 2008 and 2009, at northeast beaches, identified as "El Cuyo" and "Las Coloradas" in Yucatán, México. A total of 100 blood samples were collected from the dorsal cervical sinus of *Chelonia mydas* turtles using a Vacutainer system during egg laying. Physical examination and measurement of individuals were also performed during sampling.

Blood samples were placed in crystal tubes containing lithium heparin (Sykes and Klaphake 2008, Lawrence 1985). Erytrocytes (RBC) and leucocytes (WBC) were counted using the Natt and Herricks solution (Campbell 1995).

Hemoglobin (Hb), Hematocrit (Ht), Mean Corpuscular Volume (MVC), and Mean Corpuscular Hemoglobin Concentration (MCHC), were obtained using Beckman Coulter equipment. WBC differential count was carried out with stain of blood smears with Dip Quick Stain.

Individual turtles were classified as apparently healthy (AH) during clinical examination in the absence of parasites or sick (S) when parasites were present on their bodies.

The minimum/maximum values (Range) for each red and white series components was determined.

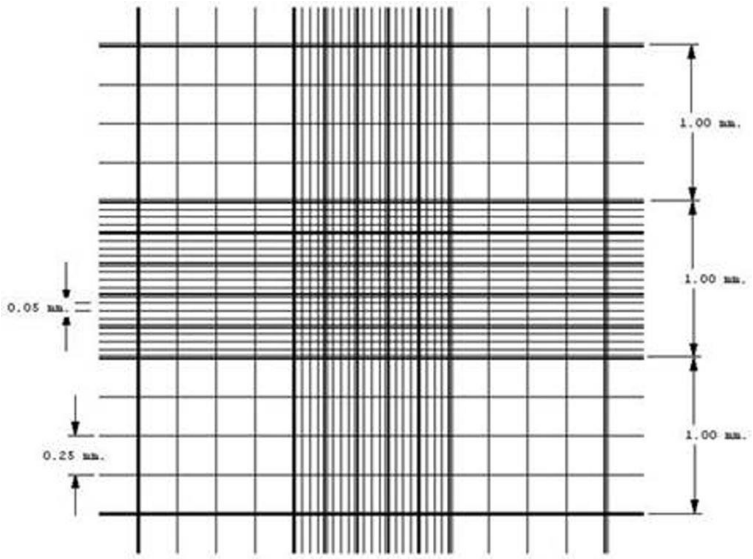
Counting Cells Manually

The Neubauer hemocytometer chamber for cell counting method is most commonly used (counting grid 4 x 4 lines); the Neubauer-improved chamber (counting grid 5 x 5 lines) with the application of the Natt and Herrick solution (Frye 1991); or the Unopette system (Becton-Dickinson, Rutherford, NJ) (Figure1).

1. Approximately 10 µl of dilution should be placed on the Neubauer chamber central area and left to rest for 5 minutes for cells to deposit.
2. Counts are performed on the four square sections at the corner and the central one. Square areas at the four corners are counted on addition to the central area of the chamber. The total number of erythrocytes per ml is calculated by multiplying the total count by 10,000.
3. Thrombocytes are counted at a Neubauer chamber with the application of Natt and Herrick solution to blood on 1:200 rate. In this case, counts include both sides of the chamber and the totality of the grid. Total number is multiplied by 1,000 to obtain thrombocytes/µl in blood.
4. Leukocytes are counted using same chamber and dilutant (Natt & Herrick 1952). One of the major disadvantages of the present technique is the complications to differentiate lymphocytes from thrombocytes and immature erythrocytes. Some authors suggest including the two latest cells in the final leukocytes account on the blood sample, this way the total count on the chamber can be corrected (Wilkinson 2001). Leukocytes by µl should be obtained to estimate the total leukocytes present on the 9 major fields of the Neubauer chamber. This number should be increased by 10% and the result multiplied by 200.

Unopette's technique provides better results than the Natt and Herrick system, however variation can result between species depending on the percentage of eosinophils present in relation to heterophils (Strik et al. 2007).

This method counts the number of leucocytes with 40x magnification in 10 fields; then the middle value of counts is multiplied by 1,000 and that represents the total number of leukocytes/µl, allowing to confirm the manual counts of these cells.



http://bdigital.eafit.edu.co/bdigital/PROYECTO/P660.62CDD259/anexos.pdf.

Figure 1. Neubauer-improved chamber counting grid detail (central area has a 5 x 5 lines grid).

Hematocrit Determination

Blood samples were analyzed to determine the hematocrit following the microhematocrit technique. Samples were placed in test tubes using a centrifuge machine to maintain homogenization (Nutator shaker by Clay Adams, Germany).

Each capillary tube was filled ¾ths by introducing the end into the test tube with blood samples and inverting the position. The capillary tube was sealed by maintaining the tube horizontal and melting the empty side using a Bunsen burner.

The tubes were centrifuged for 5 min at 11.000 RPM (Centrifuge LW Scientific, DSC-024, 14.490 xg, China), then the cellular volume was recorded a reader for microhematocrit.

Determination of Total Leukocytes and Erythrocytes

Blood samples were homogenized (APSA, SM, Mexico) and a Thoma pipette (precision ± 3%, Germany) was filled up with the resulting solution to be diluted at 1:200 ratio. This was achieved by filling up the pipette with blood solution to the 0.5 mark and completing with Natt and Herrick's solution to the 101 mark (Natt & Herrick 1952), followed by a 20 min settling period. Pipettes were shaken for 2-3 minutes (mechanical shaker Clay Adams, Nutator, Germany).

The first four drops were released to eliminate the liquid from the capillary proportion that was not mixed with blood, followed by the normal filling of the hemocytometer (EC Brad®, Germany) and including a 20-min settling period before count.

Erythrocytes were counted within 5 small squares along the grid in the central part of a slide at 40x magnification (Primostar microscope, Carl Zeiss®, Germany).

Counts were totaled using the following equations:

Number of Red blood cell x mm^3 = height x dilution x area.
Number Red blood cell x mm^3 = 1/10 x 1/200 x 1/5 = 1/10 000,

Number Red blood cell x mm^3 = Red blood cell counted x 10 000 to determine the total number of erythrocytes per liter.

Leukocytes were counted at 9 squares, also under a 40x magnification, then multiplied by 220 and divided by 1000. This final number was multiplied by 109 to determine the total number of leukocytes per liter.

To determine the section with the lowest superposition of cell counts, a differential leukocyte count was performed using the direct observation of a frotis sample stained with Wright solution at 10x magnification. Differential count implies the classification of 100 leukocytes that results on a percentage of the types of leukocytes present in the blood. Prepared slides were then observed at a 100x magnification (oil objective). Absolute value was obtained by multiplying the calculated differential count using the hemocitometer.

Thrombocytes were determined based on frotis stain of every 100 leukocytes using the technique by Sypek and Borysenko (Campbell and Ellis 2007).

Recording Cell Measurements

Microphotography was used to measure cells (camera Lieca DC and software 1M 1000, Germany) (Lieca Company 2005). The number of cells to be measured was determined by the Fuentes-Mascorro method which considers the percentage of the cell type present at the hemogram (Fuentes-Mascorro 2007). Frotis samples were used to perform the differential count during this process. Measurements of round cells were performed at the x and y axes juntion in accordance to the Cartesian organization, which defines length as the longest measurement. These measurements established maximum and minimum lengths (Fei-Yan et al. 2001). The morphological index (MI), was calculated by dividing the minimum by the maximum length where the length closest to 1 determine rounded shapes. Values assessed father away from 1 determine elliptical shapes[1].

Evaluation of Cell Morphology

To assess the morphology of cells, blood smears should be conducted without anticoagulant and immediately after extraction, air dried and stained using the Romanowsky techniques, such as May-Grünwald Giemsa (Campbell 1998) regardless of the technique used for tinction. This technique requires some time, but provides better results for the differentiation of leukocytes, thrombocytes and immature erythrocytes. Following these recommendations we can prevent “false positives” or “false negatives,” alterations on leukocyte and thrombocyte morphology problems on monocyte visualization or increase on

[1] Note - This measurement was not obtained for monocytes and basophils due to the presence of lobulated nuclei.

lymphocyte size. Another technique used for the same purpose is the Diff-Quick[2], although it may not provide the best differentiation between the types of leukocytes, and heterophils can be glued together making evaluation difficult (Muro et al. 1998). Reagent Formulae. “Diff Quick” solution I/II or kits can be purchased from IHC Store. Store Diff-Quik at 5°C to retard growth of organisms. "Diff-Quick" includes specific technical instructions: Exposure to alcohol should be as brief as possible to prevent excessive discoloration. Paraffin sections can be rehydrated using distilled water.

1. Using the Diff Quick” solution II stain for 30s
2. Counterstain (optional) with “Diff Quick” solution I for additional 30s
3. Rinse rapidly in tap water,
4. Rapidly dehydrate in absolute alcohol,
5. Clear and mount.

For smears/imprints using an air dry method absent of alcohol, follow these instructions:

1. Air-dry the smear,
2. Fix in “Diff Quick” Fixative (or methanol) for 30 s/drain.
3. Stain with “Diff Quick” solution II for 30 s/drain.
4. Counterstain (optional) with “Diff Quick” solution I for 30 s/drain.
5. Rinse in tap water to remove excess stain,
6. Rapidly dehydrate in absolute alcohol,
7. Clear and mount

Color Reference of Blood Cell Types Using Diff Quick

Platelets	violet to purple
heterophyls nucleus	dark blue
cytoplasm	pale
eosinophils nucleus	blue
cytoplasm	blue
granules	red to red/orange
basophils nucleus	purple or dark blue
granules	dark purple/black
monocytes nucleus	violet
cytoplasm	sky blue

[2] “Diff-Quick” is a proprietary brand of a Romanowski stain. The Romanowski group of stains are defined as being the black precipitate formed from the addition of aqueous solutions of methylene blue and eosin, dissolved in methanol. The variants of the Romanowski group differ in the degree of oxidation (polychroming) of the methylene blue stain prior to the precipitation. The stain class was originally designed to incorporate cytoplasmic (pink) staining with nuclear (blue) staining and fixation as a single step for smears and thin films of tissue (spread preparations of omentum). Minor modifications of working stain concentration and staining time have been made over the years for fixed tissue sections.

RESULTS

Blood samples were taken from 100 individual sea turtles (*C. mydas*), during egg deposition on Yucatan beaches. Individuals were categorized as apparently healthy (N = 84), and sick turtles (N = 16). Sick turtles were categorized when ectoparasites were present on their bodies.

The range of blood values between apparently healthy turtles and sick turtles are presented below (Table 1). Differences between the minimum range blood values, except monocytes, from the two animal groups were noted. It is important to mention that these blood values represent the first baseline information from this species in the area.

Table 1. Comparison of blood value ranges between healthy and sick sea turtles

Blood Category	Healthy Range	Sick Range
RBC	0.500 to 0.61410^6/µL	0.215 to 0.689 x 10^6/µl
Ht	31.5 to 41.4%	26.5 to 47.6 %
Hb	14.9 to 18.9 g/L	13.2 to 20.0 g/L
MCV	118.1 to 148.7 fl	87 to 152 fl
MCHC	30.2 to 39.1 g/L	25.0 to 45 g/L
WBC	4.7 to 6.9 x 10^3/µl	3.7 to 6.9 x 10^3/µl
Heterophils	3.0 to 82.56 x 10^3/µl	2.22 to 6.831 x 10^3/µl
Eosinophils	.094 to 7.74 x 10^3/µl	.037 to 0.897 x 10^3/µl
Lymphocytes	0.679 to 39.56 x 10^3/µl	0.333 to 3.588 x 10^3/µl
Monocytes	0 to 2.35 x 10^3/µl	0 to 0.555 x 10^3/µl

Table 2. Comparison of blood vale ranges between sick and apparently healthy *Chelonia mydas* sea turtles nesting on the northeast beaches of Yucatan, Mexico, with reference to other related studies

Blood Variable	ST[a] (n = 16)	AHT[b] (n = 84)	Study 1[c] (n = 30)	Study 2[d] (n = 25)[e]
RBC[f] x 10^6/µl	0.251 – 0.689	0.5 – 0.614	0.24 – 0.57	0.28 – 0.64
Hb[g] g/dl	13.2 – 20	14.9 – 18.9	No data	5.3 – 12.4
Ht[h] %	26.5 – 47.6	31.5 – 41.4	21 - 38	22 - 52
MCV[i] fl	87 - 152	118.1 – 148.7	509.4 - 1310	601.6 – 1446.4
MCHC[j] g/dl	25 - 45	30.2 – 39.1	No data	20 – 32.5
WBC[k] x 10^3/µl	3.7 – 6.9	4.7 – 6.9	2.6 – 12.1	0.2 – 4.3
Heterophils[l] x 10^3/µl	2.22 – 6.83	3.0 – 82.56	1.98 – 10.76	0.11 – 2.84
Eosinophils[l] x 10^3/µl	0.037 – 0.588	0.094 – 7.74	0 – 0.191	0 – 1.26
Lymphocytes[l] x10^3/µl	0.333 – 3.588	0.679 – 39.56	0.154 – 3.102	0.02 – 0.87
Monocytes[l] x 10^3/µl	0 – 0.552	0 – 2.35	0 – 0.360	0.0 – 0.13

Note: [a]Sick turtles, [b]apparently healthy turtles, [c]Montilla et al., 2006 [d]Samour et al., 1998.[e]Adult females, [f]Red blood cells, [g]Hemoglobin, [h]Hematocrit, [i]Mean corpuscular volume,. [j]Mean corpuscular hemoglobin concentration, [k]White blood cells, [l]Absolute values.

Based on 100 total samples, 84 were classified as apparently healthy turtles (AHT), and 16 classified as sick (ST) due to presence of parasites The blood values compared between the two turtle groups (AHT and ST) showed differences, especially the minimum range value, suggesting that the presence of parasites have an impact over these hematological variables (Table 2). The observed cells on blood smears were the following: different cell types of the red series and white series as eosinophilic leukocyte, 2) erythrocyte, 3) polychromatic erythrocyte, and 4) thrombocyte (Figures 2-5) are observed. These cells are observed in the green turtle of our work were seen eosinophilic leukocyte broken.

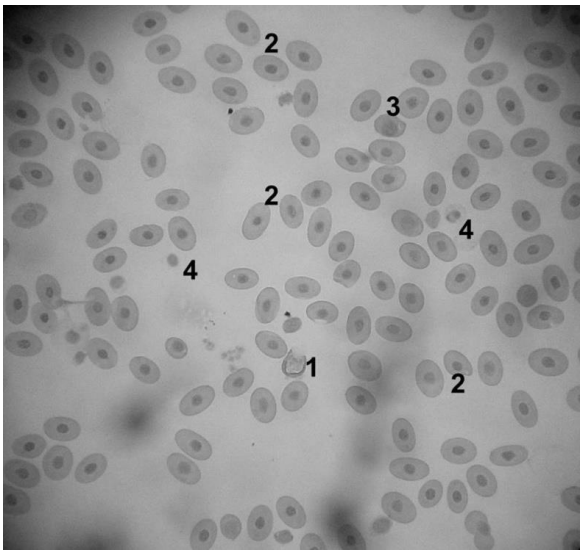


Figure 2. Blood smear of four types of blood cells identified: 1) eosinophilic leukocyte, 2) erythrocyte, 3) polychromatic erythrocyte, and 4) thrombocyte.

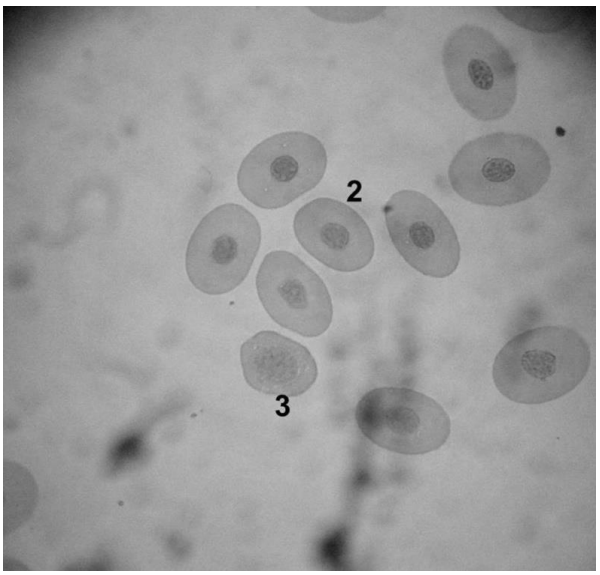


Figure 3. Red blood series cells: 2) erythrocyte, 3) polychromatic erythrocyte.

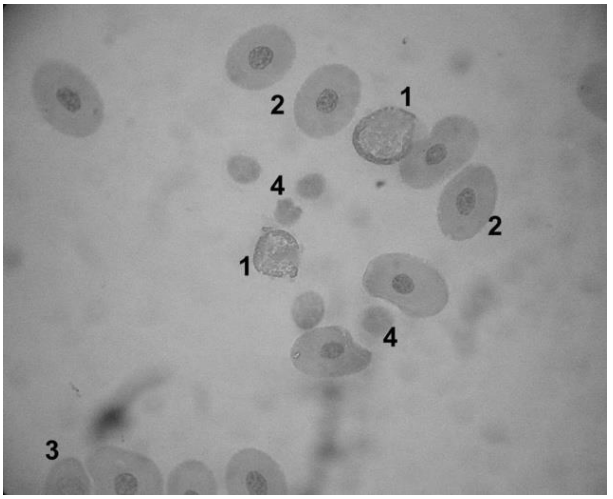


Figure 4. Cells on the red and white series: 1) eosinophilic leukocyte, 2) erythrocyte, and 4) thrombocytes.

Blood Samples as Reference Values

In order to determine reference values for nesting females in Yucatan, Mexico from the blood samples collected above, we compared our values to previous studies in other regions yet of the same species, *C. mydas*. Below we present a summary of the findings from comparable studies in Venezuela, just south of Mexico and presumably within the same population range as the sea turtles studied from Yucatan as presented above.

Gulf of Venezuela, Alta Guajira (Montilla et al. 2006)

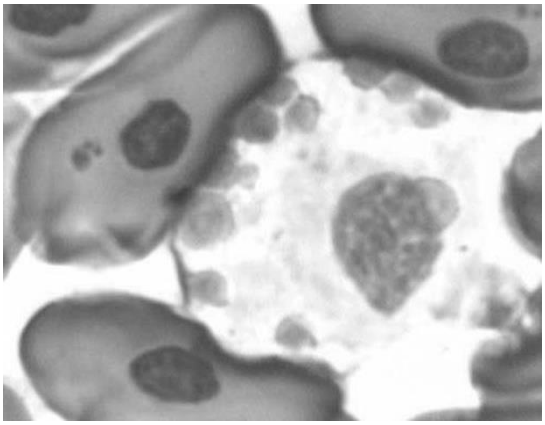
In this study, blood samples from, thirty individual turtles (N = 30) were taken from the dorsal cervical sinuses of *C. mydas.* Researchers recorded the following blood parameters: Red Blood Cell (RBC), White Blood Cell (WBC), Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), and differential count of leukocytes. Curved carapace length (CCL) and curved carapace width (CCW) was collected from each turtle in order to estimate the age.

Results from this study included average values of the hematological parameters as follows: RBC (0.42 × 106 µL), WBC (6.16 × 10^3µL), PCV (29.40%), MCV (730.17 fL), Heterophils (82.9% - 5.1023 × 10^3/µL), Lymphocytes (14.7% - 0.9081 × 10^3/µL), Eosinophils (0.47% - 0.0236 × 10^3/µL), Monocytes (1.97% - 0.1259 × 10^3/µL).

These results coincide with the reference intervals documented for the species. The variability in some of the parameters evaluated could be attributed to factors as age, sex, reproductive state, stress, temperature, and capture technique and analysis methods (Figure 5 and 6). A Mann-Whitney test concluded significant differences (P = 0.038) between male and female based on the MCV values.

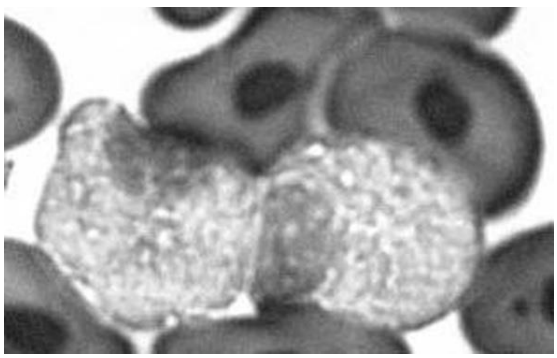
The Wildlife Refuge Aves Island, Venezuela

This area provides the main nesting area for *Chelonia mydas* in the country and the second largest breeding colony of relevant in the Caribbean (Prieto-Torres et al. 2012).



Magnification: 7000x.

Figure 5. Eosinophil cell of individual green sea turtle (*Chelonia mydas*).



Magnification: 7000x.

Figure 6. Heterophil cells of green sea turtle (*Chelonia mydas*).

Thus this study provides a good comparison to our study in Yucatan, Mexico to provide reference values. In this study, researchers determined hematological values from July and September 2010 based on blood samples of 64 turtles (52 females and 12 males) collected from the dorsal cervical sinus. A complete hematological study was performed using the following parameters: Red Blood Cell (RBC), White Blood Cell (WBC), Hematocrit (Hct), Hemoglobin (Hb), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) and Leukocytes differential count Researchers measured curved carapace length (CCL), curved carapace width (CCW) of all 64 turtles and tail length (LT), each male turtle, to estimate the minimum sexual maturity. Due to working conditions at the study area, samples were placed on ice and refrigerated for 30-60 min ice then processed at the scientific laboratory at the Simón Bolivar navy base RFSIA. The differential leukocyte count was performed on two frotis samples that were stained with a Dip-Quick solution, resulting in absolute values as percentages (Montilla et al. 2006). Results from this study included average values of the hematological parameters as follows, noting below turtles identified in the "sick" category: RBC (457.187 × 103^3µL), WBC (3.66 × 10^3µL), MCV (727.83 fL), Heterophils (52.3% - (1.990 × 10^3/µL), Lymphocytes (36% - 1.297 × 10^3/µL), Eosinophils (7.4% - 0.234 × 10^3/µL), Monocytes (3.8% - 0.123 × 10^3/µL), basophils 0.4% (0.0097×10^3/µL), Ht 30.77%, Hb 9.60 g/dL, MCHC

31.21 g/dL. Figure 7 below shows the differences of various cell types reported from this study that were used as baseline comparison model for the current study in Yucatan, Mexico. In this study, researchers noted emerging fibropapilloma disease present on front flippers and the cervical region of two of the female turtles. Mean CCL and CCW for nesting females was 112.1 cm and 101.4 cm, respectively. Recorded average measurements for males was 105.6 cm CCL, 96.2 cm CCW and 34.3 cm LT.

CONCLUSION AND DISCUSSION

It is imperative to create reference values for studies involving blood samples to determine the health status of wildlife populations. In the case presented here using sea turtles, reference values are applied when analyzing condition of nesting females and foraging populations. Based on results, researchers can then adapt conservation strategies in accordance to environmental conditions present in various regions.

Correlations between sea turtle morphology and hematological variables are important, in as well as differences between nesting females, hatchlings and migrating individuals. It is a general agreement that differential counts vary from previously reported literature, mainly due to the lack of standardized criteria to measure and differentiate types of leukocytes (Aguirre et al. 1995). Various publications describe the presence of erythrocytes, eosinophils, basophils, azurophils, neutrophils, lymphocytes, monocytes and thrombocytes on frotis samples from green sea turtles (*Chelonia mydas*), with the help of light microscopes (Work et al. 1998). For example, Aguirre et al. (1995) used the same methodology but classified leukocytes as heterophils, neutrophils, lymphocytes, eosinophils and basophils. Differences in cell classification are a result of the lack of identification using cytochemistry and/or the analysis of ultrastructure by electronic microscopy to corroborate results.

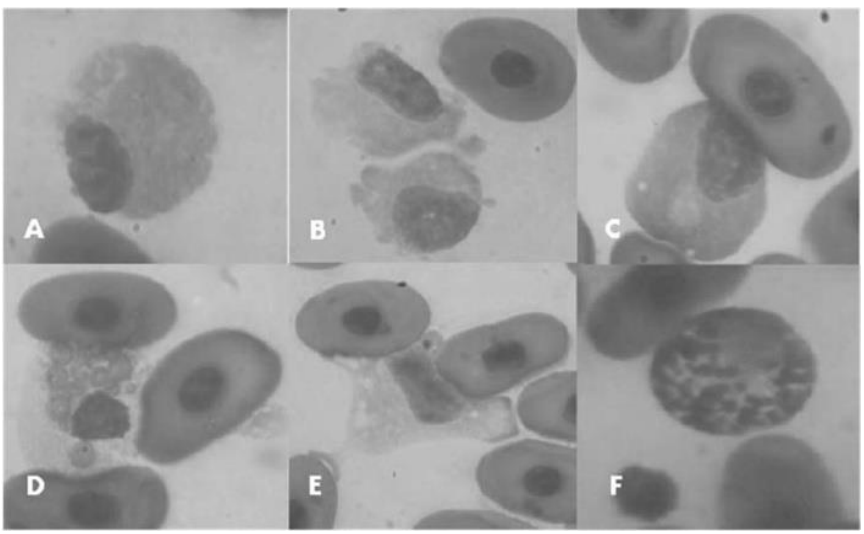


Figure 7. Blood cells observed from green nesting females (*Chelonia mydas)* at the Wildlife Refuge from Isla de Aves, during the 2010 nesting season. A) heterophil (7000x); B) reactive lymphocyte (6000x); C) reactive lymphocyte (7000x); D) eosinophil (6000 x); E) monocyte (5000x) and F) basophile (7000x).

Some authors describe the presence of neutrophils on reptiles, same that are reported as heterophils by others and that play the same role as neutrophils in mammals (Aguirre et al. 1995; Montilla et al. 2006; Work et al. 1998). Heterophils are defined as polymorphic-nuclear cells that are most frequently present on bird and reptile blood samples (Myers et al. 2004; Montilla et al. 2006; Flint et al. 2010).

The influence of climatic or environmental variations over the hematological values on reptiles is still controversial (Campbell 1995; Fuentes-Mascorro 2007; Work et al. 1995).

Studies to resolve this question occur in different times and are run for short periods, factors that can temper the observed results.

Some authors have pointed out that no significant differences exist between hematological values between males and females from some species (Rossini 2002). Nevertheless it has also been mentioned that difference on sizes, sex, temperature, corporal hydration and reproductive state may be responsible of the observed variations on hematological parameters (Bolten & Bjorndak 1992; Campbell 1995; Christopher et al. 1999; Rosskopt 2000). It is hard to compare the results from this study to those mentioned above, because most samples were obtained from juveniles where sex differentiation has not been achieved. This chapter presents findings from Yucatan, Mexico of 100 sampled nesting females in comparison to related studies from Venezuela in order to provide future studies a range reference values.

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Chapter 8

ARACHIDONIC ACID IS A MAJOR FATTY ACID IN GONADS OF CORAL REEF FISHES AND IMPROVES LARVAL SURVIVAL OF RABBITFISH *SIGUNUS GUTATTUS*

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ABSTRACT

The supply of wild fry of coral reef fishes for aquaculture has resulted in the deterioration of their natural stock status, causing public concern. Through a series of studies on the establishment of artificial-fry production technologies for coral reef fishes, we found that ovary, testis, eggs and fry of coral reef fishes have high or intermediate levels of arachidonic acid (ArA), which is a relatively minor component in temperate and cold-water species. In gonadal polar lipids of selected coral reef, in particular demersal fishes (19 species), ArA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) levels ranged from 6.0% to 19.4%, from 0.9% to 6.2%, and from 7.9% to 27.8%, respectively. It is notable that the major highly unsaturated fatty acids (HUFA) of polar lipids in all coral reef fish gonads are DHA and ArA (not EPA) in a ratio of about 2:1. This result allowed us to speculate that not only DHA but also ArA may be nutritionally much important for egg development and larval growth in coral reef fishes.

Thus, feeding trials were conducted to investigate the effects of dietary ArA supplementation on reproductive performance of coral reef rabbitfish (*Siganus guttatus*) broodstock. The number of spawning and the number of hatched larvae tended to be better in broodstock fed diets with ArA than in those fed a diet without ArA. Next, larval rearing tests were conducted to investigate survival and growth in rabbitfish fry fed live rotifers which had been enriched with or without ArA. Fry fed the rotifers enriched with a combination of DHA Protein Selco (Inve Aquaculture, Baasrode, Belgium) + 5% ArA (VEVODAR CRUDE ARACHIDONIC OIL, DSM Food Specialties, Delft, the Netherlands) showed significantly the best survival (44.4±4.5% for Day17 fry), although growth was not different among treatments. The present study indicates that ArA is not a minor component in coral reef fishes, and that dietary ArA is very promising for the improvement of fry production technologies of the coral reef fishes.

Keywords: arachidonic acid (ArA), DHA, EPA, coral reef, fry production, aquaculture

INTRODUCTION

Coral reefs are not only biologically but also economically productive waters, and in fact coral reef fishes such as grouper, snapper and rabbitfish are high marketable fish (Johnson, 2007). An increasing demand for such high value fish has intensively developed aquaculture of these species during these few decades, which has provided excellent food fish as well as the means to rise revenue for coastal communities around the waters. Aquaculture appears a semi-sustainable activity which allows for avoiding overfishing and destructive fishing practices such as dynamite fishing. However, aquaculture of coral reef fishes is based on wild-caught fry, that is, the industry still relies largely on the natural capture of seed fish in the wild, because of the shortage of artificial seed fish produced in hatcheries. The heavy supply of wild fry of coral reef fishes for aquaculture may lead to the deterioration of the natural stock status, causing public concern as a new threat.

Fry supply is the most important component in aquaculture, but the fry needed for aquaculture still come mostly from the wild as mentioned above, and thus due to poor production of the artificial fry, their availability is a major constraint in the development and extension of aquaculture not dependent on wild fry (Marte, 2003). Although hatcheries should have the responsibility to stably produce and supply healthy fry to famers, fry production of coral reef species remains variable due to poor fecundity of broodstock and low survival of hatched larvae. The nutritional condition of broodstock is one of the first determinants of egg and larval quality, and diet quality and nutrients have been shown to have a profound effect on egg nutritional composition and subsequent gonadal development, fecundity (total number of eggs produced), fertilization rate, normal embryo development, hatching rate and survival rate of hatched larvae (Izquierdo et al., 2001). However, nutritional information on broodstock is limited to a few species, and little information is available in coral reef fishes. In order to improve egg production/quality and larvae/fry quality through dietary manipulation, we have conducted a series of studies that was aimed at developing advanced artificial diets for coral reef fishes.

Through a study to investigate a difference in nutritional components between mangrove red snapper (a coral reef species) (*Lutjanus argentimaculatus*) and cold/cool-water species, we found that the EPA: ArA ratios were interestingly around 1 based on analyses of eggs and newly hatched-larvae of mangrove red snapper. The EPA:ArA ratios are 7.5 in Atlantic

salmon *Salmo salar* (cold water species) eggs (Cowey et al., 1985), and 11.5 in Japanese flounder *Paralichthys olivaceus* (cool water species) eggs (Furuita et al., 2000), respectively (Figure 1). In the "Research & Development" of fry production technology, the most innovative finding is that marine fish require essential fatty acids, or dietary docosahexaenoic acid (DHA) and EPA for the developments of gonads in broodstock and the normal growth and development in embryo/larvae. Practically, dietary EPA and DHA have successfully improved reproductive performance and egg/larvae quality such as fecundity, embryo development, hatchability and survival in several species (Watanabe et al., 1984; Izquierdo et al., 2001). In contrast, although ArA also is an essential fatty acid in higher vertebrates, the acid is a minor component in cold/cool-water species, and thus little attention was paid to the function of ArA in marine fish. Considering together the practical importance of essential fatty acids in hatcheries and the existence of ArA in an amount almost equal to EPA in the snapper's eggs, it has been highly speculated that ArA may be nutritionally much more essential for reproduction and larvae growth in coral reef fishes than in cold/cool water fishes. Thus, our efforts have been directed toward demonstrating the efficacy of ArA in the advancement of artificial diets for fry production of coral reef fishes. The present paper describes I: the wide distribution of ArA as a major fatty acid component in coral reef associated, in particular demersal fishes; II: the effects of dietary ArA supplementation on reproductive performance of rabbitfish broodstock; and III: the effects of DHA/ArA-enriched rotifers on survival and growth of rabbitfish fry.

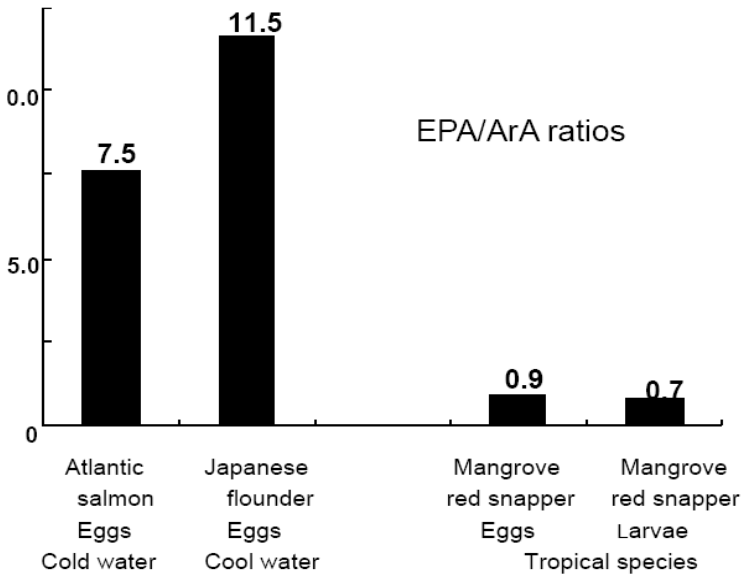

Figure 1. Comparison of EPA/ArA ratios of eggs in cold water, cool water and tropical species.

1. Wide Distribution of Arachidonic Acid as Major Fatty Acid Component in Coral Reef Associated Fishes

The details of sample species analyzed and fatty acid analysis are described in our previous papers (Ogata et al., 2004; Suloma and Ogata, 2011).

1.1. Coral Reef Associated, Demersal Fishes

Tables 1-17 list fatty acid composition of coral reef associated, demersal fishes in Philippines and Ishigaki Island, Japan. Grouper's data are shown in Tables 1-3, *Lutganidae* in Tables 4-10, *Siganedae* in Tables 11-15, and *Lethridae* in Tables 16-17. The most striking result is that ArA level is higher than EPA level in polar lipids of most wild fishes (gonads and muscle). In ovarian polar lipids, ArA levels ranged from 6.0% (*Epinephelus areolatus*) (Table 2) to 19.4% (*Lethrinus. atkinsoni*) (Table 16), while EPA levels ranged from 0.9% (*Sigunus virgatus*) (Table 15) to 6.2% (*Pristipomoides argyrogrammicus*) (Table 13). ArA in ovarian polar lipids was one of the top three fatty acid components in *Cephalopholis argus* (12.6%, Table 2), *E. quoyanus* (15.2%, Table 3), *Epinephelus. sp.*-1 (11.4%, Table 3), *Epinephelus sp.*-2 (11.7%, Table 3), *Lutjanus decussatus* (16.5%, Table 5), *L. erythropterus* (17.5%, Table 5), *Plectropomus leopardus* (Ishigaki, Japan) (19.2%, Table 10), *L. gibbus* (12.3%, Table 4), *L. miniatus* (17.3%, Table 16), *S. canaliculatus* (Ishigaki, Japan) (9.9%, Table 13), *L. atkinsoni* (19.4%, Table 16), *S. guttatus* (12.7%, Table 11) and *S. virgatus* (8.9%, Table 15). Irrespective of the maturity (see Suloma and Ogata, 2011), ovarian polar lipids had higher levels of ArA than EPA levels. DHA was also one of the top three fatty acid components in ovarian polar lipids. Ovarian DHA level in polar lipids was always higher than EPA in almost all of the species analyzed, ranging from 7.9% (*S. virgatus*, Table 15) to 27.8% (*S. canaliculatus*, Table 14).

Surprisingly, ArA was the top fatty acid component in testis polar lipids of *L. ornatus* (22.9%, Table 17), *L. nebulosus* (22.5%, Table 17) and *L. atokinsoni* (21.4%, Table 16). ArA levels in testis polar lipids of two *Siganus* species were intermediate but higher than EPA levels (Tables 11 and 15). Testes of the five species of coral reef fishes had average ratios of: ArA/EPA of 9.8, DHA/EPA of 6.5 and DHA/ArA of 0.8 in polar lipids and ArA/EPA of 5.5, DHA/EPA of 3.4 and DHA/ArA of 0.6 in neutral lipids, respectively. This result suggest that since the average DHA/ArA ratio was smaller in the testis than in the ovary, the optimum dietary ratio of HUFA during gonadgenesis might be different between males and females, and moreover that the degree of the physiological essentiality of ArA might be greater in testis than in ovary.

The characteristics of ArA, EPA and DHA distribution of the gonads in the coral reef demersal fishes are shown in Figures 2 and 3. In the present study, ovaries of 19 species of coral reef fishes had average ratios of: ArA/EPA of 4.8, DHA/EPA of 6.2 and DHA/ArA of 1.7 in polar lipids and ArA/EPA of 2.5, DHA/EPA of 3.8 and DHA/ArA of 2.5 in neutral lipids, respectively. This result indicates that unlike cold water species in the northern hemisphere, ovarian DHA/ArA ratio, not DHA/EPA ratio, is about 2 in tropical and coral reef fishes, suggesting that the DHA/ArA (not EPA) ratio of around or more than 2 may be optimum for broodstock diets, especially for ovary development in coral reef fishes. It should be again mentioned that the major HUFA are DHA and ArA for the coral reef group, and DHA and EPA for the cold/temperate group in about 2:1 of DHA-to-ArA or -to-EPA ratio in each group. We recommend a dietary ratio of DHA/ArA (not EPA) of about 2 or greater as an ideal for broodstock diets of coral reef fishes. In this connection, it is quite interesting that ArA, EPA and DHA levels and ArA/EPA ration of wild goose eggs was respectively 9.3%, 1.8%, 4.8% and 3.9 (Speake et al., 1999), namely the pattern of gonadal essential fatty acids in the coral reef fishes is similar to that in the goose rather than that in the cold/cool-water fishes.

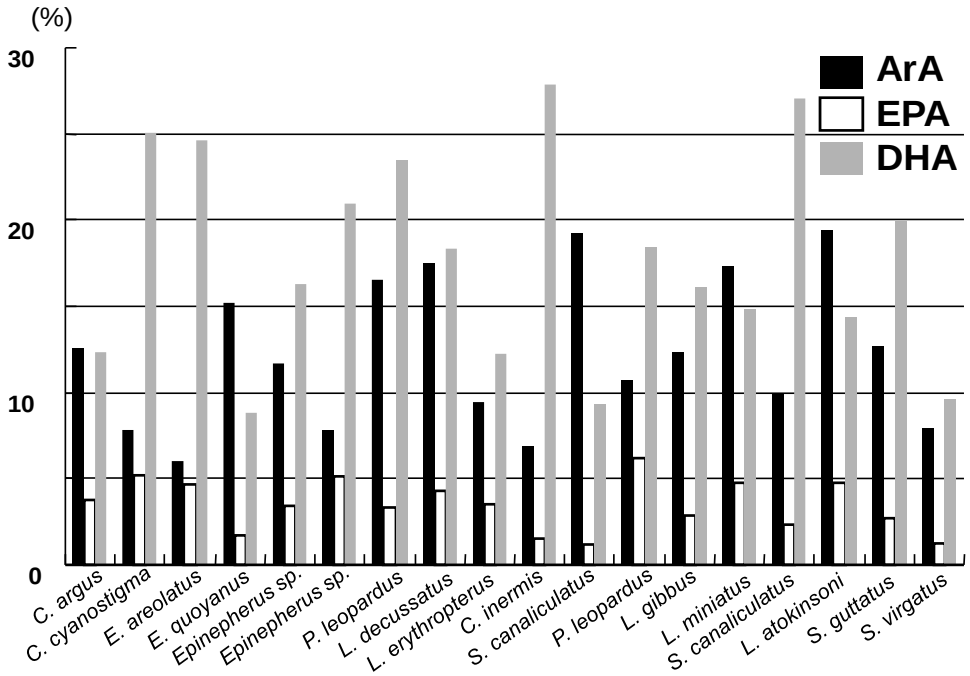

Figure 2. Levels (%) of ArA, EPA and DHA of ovaries in coral reef demersal fishes.

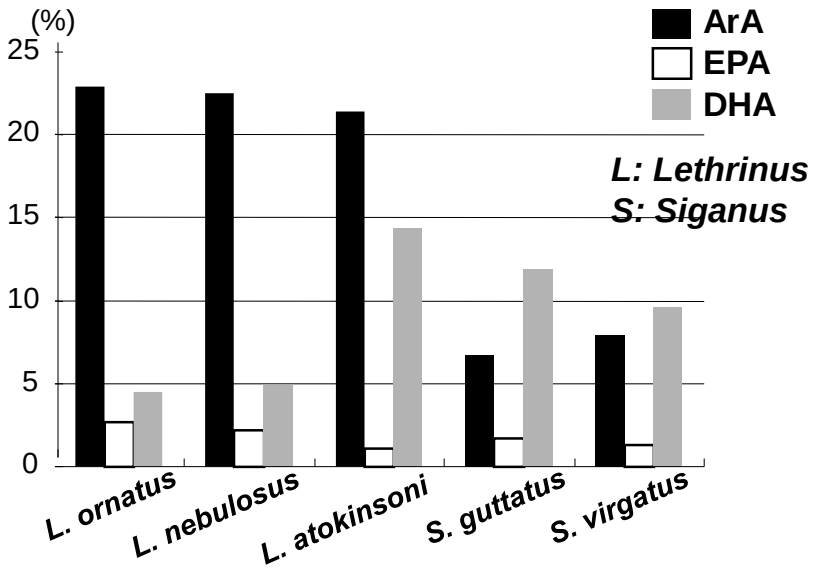

Figure 3. Levels (%) of ArA, EPA and DHA of testes in coral reef demersal fishes.

As an overall trait, ArA, EPA and DHA levels in neutral lipids were lower than those in polar lipids due to relatively high levels of 16:0, 18:0 and 18:1n-9 fatty acids in neutral lipids in gonad tissues. Yet, in neutral lipids, ArA levels were entirely higher than EPA levels throughout all the species.

Table 1. Fatty acid composition (%) of groupers

Source	Ishigaki Japan		Ishigaki Japan		Puerto Princesa	
Sample name	*Plectropomus leopardus* ovary		*Plectropomus leopardus* muscle		*Plectropomus. Leopardus* ovary	
Sample #	2		2		3	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	1.1	4.5	0.5	6.1	1.42	3.87
14:1	0.5	0.2	0.2	0.3		
15:0	0.3	0.8	0.2	0.9	0.58	1.00
16:0	19.0	31.4	20.7	34.5	25.58	28.46
16:1n-7	1.5	6.7	1.8	7.3	2.59	7.22
17:0	0.8	0.3	0.3	0.2	0.92	1.29
16:3n-6	0.6	0.9	0.5	0.9		
16:3n-3	0.2	0.3	0.2	0.3		
18:0	12.6	8.2	9.6	8.7	10.18	8.36
18:1n-9	7.3	9.4	8.3	9.5	7.63	16.78
18:1n-7	2.0	2.6	1.4	2.6	2.28	3.97
18:2n-6	0.9	1.6	1.3	1.3	0.78	0.90
18:3n-6	0.4	1.2	0.4	0.9	0.20	0.15
18:3n-3	0.1	0.5	0.2	0.4		0.34
18:4n-3	0.0	0.7	0.2	0.3	0.58	0.19
20:0	0.2	0.3	0.1	0.5		0.34
20:1	0.8	1.2	0.5	1.3		
20:2n-6	0.3	0.4	0.2	0.3	0.26	0.15
20:3n-6	0.8	0.8	0.9	0.7		
20:4n-6	**19.2**	**7.0**	**11.1**	**3.9**	**8.03**	**2.25**
20:3n-3	0.1	0.1	0.1	0.1		0.29
20:4n-3	0.2	0.5	0.3	0.3		0.23
20:5n-3	**1.2**	**2.1**	**2.2**	**1.2**	**5.07**	**2.39**
22:0	0.2	0.3	0.1	0.5		
22:1	0.4	0.5	0.3	0.6		
22:4n-6	4.6	1.9	3.1	1.6	1.91	1.66
22:5n-6	2.5	1.2	3.8	0.9	2.20	0.67
22:5n-3	2.1	2.1	2.8	1.7	1.51	1.24
22:6n-3	**9.3**	**5.1**	**22.6**	**4.4**	**20.88**	**12.03**
24:0	0.3	0.3	0.1	0.5		
24:1	2.4	0.6	0.4	0.8		
ΣSaturates	34.5	46.1	31.6	52.0	38.68	43.15
ΣMonoenes	14.8	21.3	12.7	22.3	12.49	27.96
Σn-6	29.3	15.0	21.3	10.6	13.15	5.29
Σn-3	13.3	11.4	28.6	8.8	27.74	15.70
Σn-3HUFA	12.8	9.8	28.0	7.7	27.45	15.44

Table 2. Fatty acid composition (%) of groupers

Source	Puerto Princesa		Puerto Princesa		Puerto Princesa	
Sample name	*Cephalopholis cyanostigma* ovary		*Cephalopholis argus* ovary		*Epinephelus areolatus* ovary	
Sample #	3		3		3	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	1.37	5.35	1.79	4.10	1.16	4.36
14:1	0.06	0.09	0.05	0.14	0.04	0.13
15:0	0.20	0.64	0.34	1.07	0.39	0.98
16:0	19.99	38.75	23.93	41.13	22.58	31.02
16:1n-7	2.74	8.74	2.81	7.94	2.94	10.52
17:0	0.25	0.30	0.20	0.28	0.89	0.98
16:3n-6	0.43	1.02	0.72	1.30	0.37	1.09
16:3n-3	0.26	0.52	0.35	0.55	0.23	0.59
18:0	12.39	8.69	11.26	16.68	11.93	7.68
18:1n-9	8.38	16.25	10.93	10.30	8.27	14.79
18:1n-7	1.30	3.49	1.84	3.03	1.91	3.79
18:2n-6	0.75	0.78	1.00	1.13	0.71	0.97
18:3n-6	0.27	0.33	0.57	0.43	0.28	0.20
18:3n-3	0.14	0.17	0.12	0.14	0.11	0.32
18:4n-3	0.16	0.12	0.22	0.10	0.25	0.03
20:0	0.17	0.14	0.14	0.29	0.13	0.19
20:1	0.63	1.11	0.57	0.99	0.46	0.74
20:2n-6	0.29	0.10	0.27	0.19	0.15	0.15
20:3n-6	0.37	0.10	0.67	0.20	0.24	0.11
20:4n-6	**7.78**	**1.34**	**12.55**	**1.77**	**5.53**	**1.30**
20:3n-3	0.06	0.06	0.07	0.08	0.03	0.18
20:4n-3	0.42	0.13	0.18	0.14	0.32	0.52
20:5n-3	**5.24**	**1.02**	**3.82**	**0.51**	**4.59**	**2.02**
22:0	0.14	0.10	0.12	0.07	0.13	0.09
22:1	0.17	0.09	0.18	0.10	0.24	0.11
22:4n-6	2.04	0.41	1.29	0.68	1.37	0.87
22:5n-6	2.57	0.41	1.91	0.13	2.73	0.73
22:5n-3	2.45	0.63	3.58	0.47	3.05	1.43
22:6n-3	**25.05**	**4.90**	**12.26**	**0.72**	**24.27**	**11.42**
24:0	0.15	0.11	0.11	0.07	0.22	0.06
24:1	0.11	0.04	0.33	0.11	0.09	0.00
ΣSaturates	34.56	53.98	37.79	63.63	37.11	45.27
ΣMonoenes	13.34	29.77	16.38	22.49	13.36	29.43
Σn-6	14.49	4.49	18.98	5.83	10.88	4.21
Σn-3	33.62	7.44	20.40	2.31	32.58	15.85
Σn-3HUFA	33.17	6.69	19.85	1.53	32.22	15.32

Table 3. Fatty acid composition (%) of groupers (gonads)

Source	Puerto Princesa		Puerto Princesa		Puerto Princesa	
Sample name	*Epinephelus quoyanus* ovary		*Epinephelus* sp. -1 ovary		*Epinephelus* sp. -2 ovary	
Sample #	2		2		1	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	1.60	1.96	0.84	2.11	1.82	4.45
14:1	0.07	0.19	0.15	0.14	0.03	0.15
15:0	0.35	0.94	0.26	0.76	0.24	0.70
16:0	18.06	29.28	23.37	32.07	22.54	36.08
16:1n-7	2.99	4.12	2.35	5.95	2.83	12.26
17:0	0.58	0.28	0.19	0.27	0.29	0.10
16:3n-6	0.87	1.30	0.50	1.15	0.57	0.88
16:3n-3	0.40	0.63	0.35	0.41	0.23	0.54
18:0	12.22	11.45	11.19	11.54	11.22	6.62
18:1n-9	14.03	18.23	11.17	16.23	9.99	16.98
18:1n-7	2.65	3.18	1.86	3.74	1.38	3.62
18:2n-6	0.97	0.63	0.83	0.48	0.71	0.94
18:3n-6	0.30	0.55	0.35	0.39	0.24	0.41
18:3n-3	0.14	0.65	0.17	0.60	0.17	0.35
18:4n-3	0.12	0.15	0.16	0.16	0.14	0.28
20:0	0.19	0.52	0.24	0.49	0.13	0.14
20:1	0.94	0.98	0.68	0.77	0.48	0.94
20:2n-6	0.24	0.24	0.23	0.14	0.24	0.19
20:3n-6	0.46	0.55	0.45	0.25	0.96	0.38
20:4n-6	**15.21**	**2.60**	**11.38**	**1.83**	**11.67**	**2.40**
20:3n-3	0.14	0.23	0.10	0.11	0.07	0.05
20:4n-3	0.12	0.15	0.51	0.20	0.45	0.35
20:5n-3	**1.69**	**0.96**	**2.16**	**1.13**	**3.40**	**0.86**
22:0	0.27	0.91	0.41	0.31	0.06	0.10
22:1	0.69	0.36	0.24	0.16	0.26	0.13
22:4n-6	4.28	4.18	4.65	2.84	3.26	0.77
22:5n-6	1.53	0.37	2.19	0.72	2.79	0.45
22:5n-3	2.26	0.50	1.63	0.59	2.73	0.77
22:6n-3	**8.78**	**7.35**	**9.59**	**5.97**	**16.28**	**2.39**
24:0	0.27	0.23	0.41	0.38	0.06	0.03
24:1	1.42	0.00	1.75	0.66	0.20	0.01
ΣSaturates	33.10	45.58	36.48	47.57	36.29	48.19
ΣMonoenes	22.03	27.05	16.46	26.99	14.97	34.07
Σn-6	23.85	10.42	20.58	7.81	20.43	5.96
Σn-3	13.52	10.46	14.50	2.72	23.32	4.54
Σn-3HUFA	12.85	8.96	13.89	1.60	22.85	3.60

Table 4. Fatty acid composition (%) of *Lutjanidae*

Source	Ishigaki Japan		Ishigaki Japan		Ishigaki Japan	
Sample name	*Pristipomoides Argyrogrammicus* ovary		*Pristipomoides Argyrogrammicus* muscle		*Lutjanus gibbus* ovary	
Sample #	2		2		2	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	0.6	0.9	0.2	1.9	0.9	7.2
14:1	0.1	0.1	0.1	0.2	0.1	2.9
15:0	0.4	0.4	0.4	1.2	0.5	0.9
16:0	17.6	16.2	20.7	29.5	22.4	24.3
16:1n-7	0.9	1.7	0.7	1.8	1.2	6.2
17:0	0.1	0.3	0.1	0.2	0.2	1.0
16:3n-6	1.4	1.4	0.8	0.8	1.0	1.5
16:3n-3	0.6	0.7	0.3	0.5	0.5	0.5
18:0	8.8	11.2	7.6	10.3	9.8	10.5
18:1n-9	13.6	12.4	5.3	7.2	9.5	9.7
18:1n-7	2.3	2.9	1.2	2.0	1.8	3.4
18:2n-6	1.1	1.2	0.9	0.9	1.6	1.3
18:3n-6	0.5	0.3	0.5	0.6	0.3	0.3
18:3n-3	0.2	0.3	0.1	0.3	0.2	0.4
18:4n-3	0.1	0.2	0.3	0.2	0.0	0.1
20:0	0.4	0.3	0.3	0.6	0.1	0.6
20:1	1.0	1.4	0.6	1.4	0.3	2.1
20:2n-6	0.4	0.3	0.2	0.3	0.4	0.4
20:3n-6	0.2	0.2	0.1	0.1	0.6	0.4
20:4n-6	**10.7**	**9.1**	**5.2**	**3.6**	**12.3**	**4.3**
20:3n-3	0.2	0.2	0.1	0.1	0.1	0.1
20:4n-3	0.3	0.3	0.2	0.2	0.6	0.4
20:5n-3	**6.2**	**4.9**	**2.7**	**2.5**	**2.9**	**1.5**
22:0	0.1	0.2	0.1	0.7	0.1	0.4
22:1	0.2	0.3	0.1	0.7	0.3	1.1
22:4n-6	1.6	1.5	0.9	0.5	5.5	1.9
22:5n-6	3.4	2.8	4.3	1.6	2.9	0.5
22:5n-3	2.4	2.2	1.9	1.2	3.1	1.4
22:6n-3	**18.4**	**16.7**	**39.7**	**18.6**	**16.1**	**3.7**
24:0		0.0	0.1	0.4	0.0	0.3
24:1	1.0	0.9	0.0	0.7	0.2	0.6
ΣSaturates	27.9	29.5	29.4	44.7	34.1	45.2
ΣMonoenes	19.1	19.6	7.9	14.0	13.4	26.1
Σn-6	19.3	16.8	13.0	8.5	24.6	10.7
Σn-3	28.4	25.4	45.4	23.8	23.5	8.2
Σn-3HUFA	27.4	24.1	44.5	22.6	22.7	7.0

Table 5. Fatty acid composition (%) of *Lutjanidae*

Source	Ishigaki Japan		Puerto Princesa		Puerto Princesa	
Sample name	*Lutjanus gibbus* muscle		*Lutjanus decussatus* ovary		*Lutjanus erythropterus* ovary	
Sample #	2		2		3	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	0.6	7.7	0.59	6.07	0.60	4.75
14:1	0.2	2.8	0.40	0.20	0.32	0.43
15:0	0.3	1.1	0.21	1.43	0.27	1.84
16:0	19.3	25.9	8.61	31.93	14.42	26.38
16:1n-7	1.5	5.3	1.17	6.32	0.96	7.74
17:0	0.3	0.3	0.16	0.56	0.23	0.43
16:3n-6	0.6	0.8	0.64	0.44	1.24	2.09
16:3n-3	0.3	0.5	0.24	0.52	0.26	0.87
18:0	9.3	13.7	12.44	14.26	15.72	12.83
18:1n-9	7.9	9.0	7.10	10.28	8.23	12.19
18:1n-7	1.7	2.8	1.93	3.40	1.83	3.81
18:2n-6	1.4	1.0	0.57	0.78	0.96	1.89
18:3n-6	0.3	0.5	0.37	0.20	0.41	0.69
18:3n-3	0.2	0.3	0.34	0.65	0.08	0.22
18:4n-3	0.0	0.1	0.09	0.13	0.09	0.11
20:0	0.1	0.7	0.07	0.36	0.10	0.34
20:1	0.5	1.9	0.90	1.91	0.27	1.57
20:2n-6	0.4	0.3	0.34	0.37	0.34	0.55
20:3n-6	0.6	0.3	0.51	0.37	0.67	0.59
20:4n-6	**10.8**	**4.1**	**16.48**	**1.64**	**17.47**	**4.80**
20:3n-3	0.1	0.1	0.10	0.03	0.06	0.10
20:4n-3	0.3	0.2	0.19	0.23	0.13	0.26
20:5n-3	**2.2**	**1.0**	**3.30**	**1.49**	**4.35**	**2.00**
22:0	0.1	0.6	1.01	0.40	0.08	0.11
22:1	0.4	1.1	0.38	0.51	0.31	0.32
22:4n-6	3.9	0.9	4.58	0.76	2.48	1.22
22:5n-6	3.6	0.5	3.99	0.53	1.78	0.45
22:5n-3	2.8	0.9	3.19	1.06	3.76	1.43
22:6n-3	**24.4**	**4.5**	**23.34**	**3.97**	**18.28**	**3.22**
24:0	0.2	0.5	1.14	0.38	0.05	0.04
24:1	0.1	0.7	0.36	0.34	0.05	0.07
ΣSaturates	30.2	50.5	22.80	55.39	31.38	46.69
ΣMonoenes	12.2	23.7	11.74	22.62	11.78	26.06
Σn-6	21.7	8.5	27.47	5.10	25.33	12.29
Σn-3	30.3	7.6	30.70	7.96	26.92	7.17
Σn-3HUFA	29.6	6.6	30.02	6.75	26.51	5.98

Table 6. Fatty acid composition (%) of *Lutjanidae*

Source	Tigbauan		Tigbauan		Tigbauan	
Sample name	*Lutjanus ehrenbergii* muscle		*Lutjanus malabaricus* muscle		*Lutjanus* sp.-3 muscle	
Sample #	3		3		2	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	0.17	2.44	0.16	2.85	0.13	2.64
14:1	0.02	0.10	0.02	0.09	0.02	0.46
15:0	0.18	0.78	0.21	0.85	0.15	0.75
16:0	20.71	30.40	24.58	33.60	21.07	32.84
16:1n-7	0.83	4.46	1.22	5.98	1.14	5.91
17:0	0.16	0.39	0.14	0.37	0.15	0.39
16:3n-6	0.51	1.18	0.55	1.38	0.49	1.18
16:3n-3	0.42	0.49	0.42	0.71	0.47	0.53
18:0	8.49	12.12	6.64	10.70	8.35	11.83
18:1n-9	7.52	14.73	9.56	18.48	8.87	16.41
18:1n-7	2.24	3.90	1.85	3.79	2.37	4.21
18:2n-6	0.75	1.05	0.88	0.63	0.69	0.66
18:3n-6	0.07	0.13	0.05	0.11	0.07	0.10
18:3n-3	0.16	0.34	0.18	0.40	0.13	0.29
18:4n-3	0.08	0.16	0.09	0.11	0.06	0.12
20:0	0.08	0.64	0.09	0.57	0.08	0.53
20:1	0.22	1.51	0.38	1.67	0.26	1.46
20:2n-6	0.24	0.33	0.27	0.40	0.19	0.21
20:3n-6	0.26	0.22	0.39	0.17	0.40	0.14
20:4n-6	**9.21**	**3.31**	**14.01**	**2.69**	**10.40**	**2.74**
20:3n-3	0.06	0.07	0.03	0.07	0.05	0.06
20:4n-3	0.15	0.23	0.18	0.12	0.23	0.18
20:5n-3	**4.46**	**2.29**	**5.49**	**2.54**	**4.24**	**1.77**
22:0	0.14	0.61	0.14	0.52	0.13	0.52
22:1	0.21	0.49	0.44	0.51	0.40	0.46
22:4n-6	1.37	1.11	2.62	0.78	2.22	1.17
22:5n-6	2.63	0.68	2.85	0.42	2.93	0.58
22:5n-3	2.48	2.04	2.40	1.37	3.02	1.64
22:6n-3	**30.92**	**7.72**	**18.50**	**3.77**	**26.16**	**4.45**
24:0	0.28	0.27	0.18	0.20	0.23	0.33
24:1	0.13	0.24	0.21	0.27	0.10	0.45
ΣSaturates	30.22	47.64	32.13	49.61	30.28	49.84
ΣMonoenes	11.10	25.42	13.67	30.81	13.09	29.35
Σn-6	15.04	8.01	21.62	6.58	17.40	6.79
Σn-3	38.72	13.34	27.30	9.08	34.36	9.01
Σn-3HUFA	38.01	12.28	26.57	7.81	33.66	8.04

Table 7. Fatty acid composition (%) of *Lutjanidae*

Source	Tigbauan		Tigbauan		Tanghalan	
Sample name	*Lutjanus* sp.4 muscle		*Lutjanus* sp.5 muscle		*Lutjanus argentimaculatus* wild fry	
Sample #	2		2		2 pooled	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	0.27	3.97	0.17	3.26	1.2	4.0
14:1	0.06	0.42	0.08	0.27		
15:0	0.31	1.40	0.19	1.13	1.0	2.2
16:0	20.53	26.78	19.13	30.80	22.8	24.9
16:1n-7	1.01	5.66	0.96	6.65	2.7	7.0
17:0	0.45	1.18	0.23	1.59		
16:3n-6	0.78	1.81	0.49	0.83		
16:3n-3	0.47	0.76	0.30	0.75		
18:0	7.96	11.09	8.59	14.54	11.8	10.1
18:1n-9	8.96	18.51	8.45	16.65	9.2	9.6
18:1n-7	2.59	4.45	2.39	3.75	2.9	4.4
18:2n-6	0.87	0.79	1.27	0.81	1.1	1.5
18:3n-6	0.30	0.24	0.24	0.46		
18:3n-3	0.12	0.62	0.24	0.30	0.3	0.8
18:4n-3	0.07	0.19	0.07	0.11	0.1	0.4
20:0	0.05	0.14	0.09	0.16		
20:1	0.40	4.94	0.22	4.02	0.4	0.6
20:2n-6	0.31	0.46	0.40	0.40		
20:3n-6	0.39	0.27	0.37	0.18	0.2	0.2
20:4n-6	**14.65**	**2.38**	**14.86**	**1.05**	**4.0**	**2.1**
20:3n-3	0.01	0.06	0.10	0.00		
20:4n-3	0.16	0.09	0.22	0.16	0.4	0.5
20:5n-3	**6.58**	**1.46**	**4.32**	**0.42**	**3.2**	**3.0**
22:0	0.36	0.54	0.04	0.46	0.3	0.4
22:1	0.19	0.88	0.14	0.85	0.1	0.3
22:4n-6	2.09	0.77	2.55	0.26	0.9	0.2
22:5n-6	3.00	0.29	2.85	0.19	trace	trace
22:5n-3	2.89	0.59	3.52	0.18	1.7	2.3
22:6n-3	**17.95**	**1.19**	**21.13**	**0.76**	**23.5**	**11.6**
24:0	0.17	0.06	0.14	0.06		
24:1						
ΣSaturates	30.11	45.17	28.58	52.00	38.1	43.5
ΣMonoenes	13.20	34.86	12.24	32.19	15.3	22.4
Σn-6	22.39	7.00	23.04	4.18	6.0	4.3
Σn-3	28.26	4.96	29.91	2.68	29.4	19.3
Σn-3HUFA	27.58	3.34	29.19	1.53	28.9	17.8

Table 8. Fatty acid composition (%) of *Lutjanidae*

Source	Igang-AQD		Igang		Palawan	
Sample name	*L. argentimaculatus* hatchery fry		*L. argentimaculatus* wild ovary		*L. argentimaculatus* hatchery ovary	
Sample #	2 pooled		3		3	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	1.7	4.5	0.84	3.24	1.29	4.86
14:1			0.16	0.12	0.10	0.33
15:0	0.4	0.6	0.30	0.57	0.52	1.14
16:0	21.8	21.2	18.97	28.61	19.50	29.86
16:1n-7	3.5	7.0	2.53	5.96	2.59	5.64
17:0			0.34	0.42	0.51	0.60
16:3n-6			0.63	0.46	0.62	0.51
16:3n-3			0.31	0.19	0.81	0.53
18:0	8.7	5.8	12.28	9.95	13.37	12.52
18:1n-9	12.9	16.6	13.07	26.16	9.96	16.27
18:1n-7	3.5	3.9	2.86	4.59	2.62	4.29
18:2n-6	4.3	7.1	1.42	1.92	0.81	1.02
18:3n-6			0.10	0.13	0.13	0.18
18:3n-3	0.6	1.6	0.24	0.31	0.33	0.44
18:4n-3	0.2	0.8	0.10	0.08	0.15	0.15
20:0			0.18	0.40	0.26	0.58
20:1	1.2	3.1	0.51	1.60	0.77	2.56
20:2n-6			0.22	0.30	0.24	0.39
20:3n-6	0.2	0.1	0.36	0.20	0.23	0.23
20:4n-6	**2.3**	**1.1**	**10.73**	**1.31**	**9.46**	**1.25**
20:3n-3			0.04	0.06	0.08	0.10
20:4n-3	0.6	0.7	0.10	0.16	0.21	0.34
20:5n-3	**4.3**	**3.5**	**1.93**	**1.11**	**1.72**	**0.92**
22:0	0.3	0.3	0.38	0.30	0.37	0.48
22:1	0.4	2.0	0.28	0.42	0.21	0.58
22:4n-6	0.6	0.1	2.34	0.58	1.73	0.61
22:5n-6	0.9	0.3	1.88	0.34	2.22	0.61
22:5n-3	2.5	1.8	1.92	0.92	1.80	1.16
22:6n-3	**18.0**	**6.4**	**14.35**	**2.99**	**15.16**	**4.52**
24:0			0.16	0.13	0.30	0.43
24:1			1.23	0.39	1.47	0.48
ΣSaturates	34.4	33.2	33.30	43.48	36.00	50.26
ΣMonoenes	21.7	32.9	19.73	39.06	17.19	29.59
Σn-6	7.5	9.9	13.45	4.33	13.99	4.34
Σn-3	26.6	15.3	18.96	5.82	20.27	8.16
Σn-3HUFA	25.4	12.4	18.31	5.24	18.92	6.96

Table 9. Fatty acid composition (%) of *Lutjanidae*

Source	Igang		Igang-AQD		Palawan	
Sample name	*L. argentimaculatus* Wild liver		*L. argentimaculatus* Hatchery liver		*L. argentimaculatus* Hatchery liver	
Sample #	3		3		1	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	0.89	1.73	1.82	2.17	0.67	1.12
14:1	0.07	0.07	0.23	0.19	0.08	0.04
15:0	0.42	0.40	2.31	2.21	0.39	0.43
16:0	24.03	29.00	32.47	31.67	24.39	28.88
16:1n-7	3.35	5.79	5.46	7.26	3.31	4.26
17:0	0.39	0.41	1.06	1.11	0.59	0.56
16:3n-6	0.55	0.49	1.77	1.65	0.68	0.36
16:3n-3	0.27	0.38	1.71	2.53	0.64	0.81
18:0	13.31	7.52	11.07	6.27	13	7.99
18:1n-9	15.03	33.49	15.78	25.24	14.64	31.23
18:1n-7	3.40	5.19	6.26	7.82	3.47	5.34
18:2n-6	1.48	1.30	1.17	0.94	0.89	0.83
18:3n-6	0.12	0.17	0.18	0.17	0.13	0.12
18:3n-3	0.18	0.14	0.33	0.39	0.37	0.24
18:4n-3	0.07	0.06	0.27	0.10	0.18	0.2
20:0	0.17	0.25	0.24	0.18	0.2	0.28
20:1	1.11	2.03	1.54	1.29	1.46	2.39
20:2n-6	0.24	0.30	0.17	0.14	0.32	0.17
20:3n-6	0.32	0.35	0.23	0.11	0.24	0.1
20:4n-6	**7.11**	**0.87**	**1.93**	**0.47**	**4.93**	**0.96**
20:3n-3	0.06	0.06	0.08	0.04	0.15	0.07
20:4n-3	0.12	0.37	0.10	0.07	0.21	0.2
20:5n-3	**1.21**	**0.57**	**0.50**	**0.15**	**0.97**	**0.47**
22:0	0.29	0.26	0.30	0.30	0.28	0.44
22:1	0.23	0.49	0.27	0.46	0.3	0.77
22:4n-6	1.18	0.30	0.32	0.18	0.88	0.33
22:5n-6	1.25	0.26	0.21	0.06	1.55	0.37
22:5n-3	1.42	0.54	0.88	0.35	1.12	0.63
22:6n-3	**14.60**	**2.76**	**2.54**	**0.41**	**16.2**	**2.99**
24:0	0.26	0.24	0.22	0.27	0.18	0.29
24:1	1.10	0.56	0.35	0.27	1.44	0.97
ΣSaturates	39.43	39.45	49.49	44.18	39.52	39.69
ΣMonoenes	23.90	47.37	29.89	42.52	23.25	44.04
Σn-6	9.84	3.48	5.97	3.70	7.19	2.55
Σn-3	17.91	4.88	6.42	4.05	19.84	5.62
Σn-3HUFA	17.39	4.30	4.02	0.98	18.66	4.36

Table 10. Fatty acid composition (%) of *Lutjanidae*

Source	Igang		Igang-AQD		Puerto Princesa	
Sample name	*L. argentimaculatus* Wild muscle		*L. argentimaculatus* Hatchery muscle		*L. argentimaculatus* Hatchery muscle	
Sample #	3		3		1	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	0.34	2.62	0.35	4.66	0.34	2.81
14:1	0.05	0.21	0.05	0.43	0.04	0.15
15:0	0.15	0.51	0.79	3.45	0.20	0.57
16:0	19.89	28.28	19.05	30.41	18.57	25.54
16:1n-7	0.97	4.81	2.02	8.24	0.90	3.79
17:0	0.18	0.40	0.38	1.05	0.27	0.41
16:3n-6	0.33	0.66	0.87	2.03	0.37	0.85
16:3n-3	0.18	0.52	1.30	2.02	0.28	0.66
18:0	9.53	11.15	9.03	10.07	10.00	10.63
18:1n-9	13.36	23.63	11.52	16.77	11.00	22.71
18:1n-7	1.67	3.82	3.21	6.34	1.45	3.67
18:2n-6	2.09	2.11	1.61	0.93	0.92	3.23
18:3n-6	0.12	0.11	0.15	0.19	0.09	0.14
18:3n-3	0.12	0.21	0.46	0.34	0.22	0.28
18:4n-3	0.07	0.06	0.13	0.11	0.08	0.04
20:0	0.08	0.33	0.28	0.42	0.11	0.36
20:1	0.24	1.26	0.92	1.26	0.28	1.10
20:2n-6	0.13	0.35	0.18	0.11	0.14	0.36
20:3n-6	0.37	0.25	0.52	0.10	0.18	0.31
20:4n-6	**9.72**	**3.52**	**9.72**	**0.70**	**7.83**	**3.77**
20:3n-3	0.03	0.07	0.07	0.05	0.04	0.05
20:4n-3	0.14	0.12	0.29	0.13	0.19	0.12
20:5n-3	**2.01**	**1.05**	**3.17**	**0.38**	**1.52**	**1.04**
22:0	0.14	0.31	0.12	0.28	0.13	0.36
22:1	0.20	0.78	0.51	0.39	0.14	0.47
22:4n-6	1.39	0.59	1.53	0.20	1.40	0.35
22:5n-6	3.01	0.63	2.28	0.14	4.25	0.68
22:5n-3	1.20	0.81	3.73	0.52	0.98	0.49
22:6n-3	**22.04**	**4.08**	**16.92**	**0.59**	**28.32**	**4.30**
24:0	0.20	0.19	0.18	0.21	0.19	0.22
24:1	0.34	0.42	0.08	0.20	0.10	0.45
ΣSaturates	30.49	43.79	30.19	50.56	29.80	40.92
ΣMonoenes	16.83	34.93	18.31	33.62	13.91	32.34
Σn-6	17.15	8.22	16.87	4.36	15.17	9.68
Σn-3	25.78	6.93	26.08	4.14	31.63	6.97
Σn-3HUFA	25.40	6.14	24.11	1.62	31.06	6.00

1.2. Tropical Pelagic Fishes

For tropical pelagic fishes including coral reef waters, Table 18 shows the gonadal fatty acid composition of two *Trachurus* species and Tables 19-21 do the whole-body fatty acid composition of six pelagic species. Most important feature to be noted is that in the polar lipid fraction, EPA level ranging 4.1% to 9.6% was always higher than ArA level ranging 2.6% to 6.6% excluding the cases of *Trachurus* sp.-2 (testis) (Table 18) and *Rastrelliger* sp. (whole body) (Table 21). The polar lipid fraction had ArA level lower than EPA level even in the ovary of the two *Trachurus* species (Table 18). It should also be noted that DHA ranging 11.7% to 26.5% was always the top or second-top fatty acid component in the polar lipid fraction. Thus, there was a difference in the ArA distribution between the coral reef demersal fishes and the tropical pelagic fishes.

1.3. Possible Origin of ArA in Coral Reef

EPA and DHA in fish have been known to be derived from the pelagic food chain from phytoplankton to accumulate in higher order carnivores, especially in pelagic species and deeper offshore demersal species (Sargent and Whittle, 1981). Thus, planktonic microorganisms may be not the primary source of ArA even in tropical waters. This appears to reflect in the ArA distribution of the tropical pelagic fishes in the present study. On the other hand, all the demersal species investigated in the present study were coral reef-associated species. *Serranidae*, *Lujanidae*, *Lethridae* and *Labridae* are carnivores, which feed on smaller fishes, crabs, shrimps, cephalopods, polychaeta worms, gastropods and urochordates. *Sigaidae* are herbivores, which feed mainly on benthic macroalgae. Dunstan et al. (1988) found that in temperate marine fish from Southern Australian coastal waters, demersal omnivore species (macroalgae consumers) have relatively high ArA/EPA ratio (0.9) compared to demersal carnivores (0.6) and pelagic carnivores (0.2). ArA may be provided primarily from some organisms existing in/on benthic substrate and benthic detritus rather than pelagic organisms. Little information is available on fatty acid composition of benthic organisms as an ArA source in the tropical marine food chain. The present result, high ArA levels in coral reef demersal fishes, suggests that the existence of an ArA-rich food chain may be widespread in coral reef areas, and that the widespread existence of ArA-rich food chain may lead to comparatively higher ArA contents in the coral reef demersal fishes. However, the issue of ArA origin in the coral-reef food web is still unclear. In this connection, see another chapter of the present text book “Coral Reefs: Ecosystems, Environmental Impact and Current Threats.”

1.4. Rabbitfish

Tables 11-14 show fatty acid composition of ovaries of *S. guttatus* (wild-caught and hatchery-produced) and *S. canaliculatus* (wild-caught). Rabbitfish (*S. g.* and *S. c.*) ovaries also showed that in the polar lipid fraction, ArA levels were always higher than EPA levels, irrespective of the difference in species and in sample source (wild and hatchery). It should also be noted that *S. g.* ovary contained a relatively high linoleic acid (18:2n-6) level, irrespective of the source and lipid class (Table 12), compared to *S. c.* ovary. In wild *S. g.* and

S. c., linoleic acid levels of the ovarian polar lipids were 6.9 and 0.9%, respectively. The whole-body fatty acid composition (%) of wild-caught *S. g.* fry or hatchery-produced *S. c.* fry is shown in Tables 12 and 14, although direct comparison in fatty acid composition is not appropriate due to the difference in species and sample source. ArA, EPA, DHA levels and the ratios of wild *S. g.* fry were 4.0, 2.7, 23.9% and 1.5/1.0/8.8, while those of hatchery-produced *S. c.* fry were 1.4, 2.1, 13.4% and 0.7/1.0/6.5, respectively (Figure 4). ArA/EPA ratio of hatchery-produced *S. c.* fry was less than 1.0.

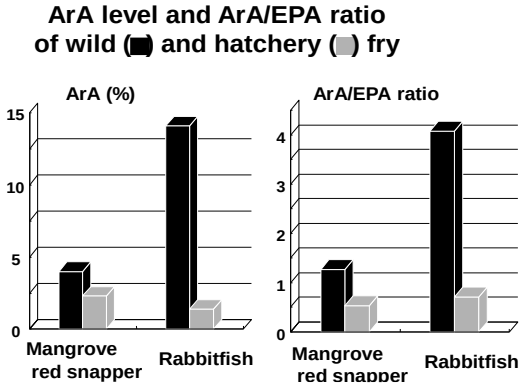

Figure 4. Comparison of ArA level and ArA/EPA ratio between wild and hatchery fry.

The information in the present study can be used as a guideline for development of appropriate broodstock and larval diets, to ensure high egg and larval quality of sustainable hatchery production in coral reef areas. We have conducted follow-up studies on the effects of dietary ArA on reproductive performance and larval/fry quality in coral reef rabbitfish.

2. Effects of Dietary Arachidonic Acid Supplementation on Reproductive Performance of Rabbitfish Broodstock

Feeding trials were conducted to investigate the effects of dietary ArA supplementation on reproductive performance of rabbitfish (*Siganus guttatus*) broodstock.

2.1. First Trial

Five females and five males (340g—810g) of rabbitfish broodstock were each stocked in 5-ton-concrete tanks at Tigbauan Main Station, the Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC/AQD) (Iloilo, Philippines). Fish have been fed each of the three test diets (Table 22) twice a day at a rate of 4% of biomass/day from July. Diet 1 was a basal diet with 1% of soybean oil + 6% cod oil, in diet 2 the soybean oil was replaced with 0.75% of ArA (VEVODAR CRUDE ARACHIDONIC OIL (DSM Food Specialties, Delft, the Netherlands)), and in diet 3 all of the soybean oil and a part of cod oil was replaced with 1.5% of ArA. Fish meal, soybean meal, acetes and squid meal were the protein sources and wheat flour and corn starch were the carbohydrate sources. The mixture was pelleted using a twin-screw extruder.

Table 11. Fatty acid composition (%) of *Siganedae*

Source	Ishigaki Japan		Ishigaki Japan		Ishigaki Japan	
Sample name	*Sigunus guttatus* ovary		*Siganus guttatus* ovary		*Siganus guttatus* testis	
Sample #	4		4		3	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	1.3	4.5	1.1	4.6	1.3	1.6
14:1	0.2	0.8				
15:0	0.7	1.3	0.6	1.1	0.3	0.7
16:0	29.5	33.6	27.0	29.0	27.0	26.2
16:1n-7	2.7	10.4	1.9	12.0	1.2	4.1
17:0	0.7	1.4	1.0	1.0	0.7	0.8
16:3n-6	1.0	1.4				
16:3n-3	1.0	0.9				
18:0	6.4	3.9	7.3	3.1	11.8	8.8
18:1n-9	7.5	8.1	7.4	12.6	8.3	10.4
18:1n-7	2.3	6.6	1.8	5.7	2.3	4.4
18:2n-6	1.5	1.7	0.9	2.2	3.2	5.5
18:3n-6	0.2	0.3	0.2	0.5	0.6	0.8
18:3n-3	1.5	3.7	0.2	1.3	1.5	2.9
18:4n-3	0.2	0.8		0.4	0.3	0.5
20:0		0.1			0.2	
20:1	0.2	0.6	0.2	0.3		0.2
20:2n-6	0.1	0.1	0.3		0.3	0.4
20:3n-6	1.0	0.4	0.5	0.7	0.8	0.9
20:4n-6	**9.0**	**1.9**	**12.7**	**4.3**	**6.7**	**10.0**
20:3n-3	0.4	0.5	0.1	0.3	0.5	0.8
20:4n-3	1.2	0.7	0.3	0.6	0.4	0.6
20:5n-3	**1.6**	**1.5**	**2.7**	**2.6**	**1.7**	**2.5**
22:0	0.1	0.8	0.2		0.4	
22:1	0.3	0.1				
22:4n-6	2.0	0.6	3.2	1.3	2.3	2.2
22:5n-6	1.5	0.2	1.7	0.6	1.7	1.4
22:5n-3	3.7	1.7	5.3	4.4	4.4	4.5
22:6n-3	**14.5**	**3.3**	**19.9**	**7.0**	**11.9**	**10.4**
24:0						
24:1						
ΣSaturates	38.7	45.7	37.0	38.9	41.7	37.0
ΣMonoenes	13.3	26.5	11.1	30.5	11.8	19.0
Σn-6	16.2	6.5	19.5	9.6	15.6	21.0
Σn-3	24.2	13.1	28.4	16.4	20.7	22.2
Σn-3HUFA	21.1	7.1	28.1	14.6	18.4	18.1

Table 12. Fatty acid composition (%) of *Siganedae*

Source	Igang		Igang AQD		Igang AQD		AQD	
Sample name	*S. guttatus* wild ovary		*S. guttatus* hatchery ovary		*S. guttatus* hatchery liver		*S. guttatus* hatchery fry	
Sample #	4		4		2		2 pooled	
Lipid class	PL	NL	PL	NL	PL	NL	PL	NL
14:0	1.29	2.98	1.64	3.29	1.13	3.74	2.1	3.6
14:1	0.07	0.14	0.15	0.09	1.04	0.09		
15:0	0.37	0.43	0.32	0.32	0.32	0.54	0.5	0.5
16:0	28.51	22.63	26.44	19.74	28.36	35.66	23.2	21.3
16:1n-7	1.89	7.30	2.08	5.10	1.54	4.30	2.0	3.6
17:0	0.13	0.21	0.28	0.23	0.09	0.24		
16:3n-6	0.24	0.42	0.29	0.29	0.39	0.57		
16:3n-3	0.11	0.31	0.17	0.31	0.08	0.25		
18:0	5.97	3.22	6.30	2.91	9.31	6.27	9.0	5.1
18:1n-9	10.90	28.83	13.35	29.62	12.29	24.08	15.1	22.7
18:1n-7	1.45	4.31	1.22	2.28	1.06	2.16	2.6	2.7
18:2n-6	6.89	11.44	5.66	12.69	5.38	7.83	9.2	15.7
18:3n-6	0.53	0.99	0.52	1.57	0.30	0.29		
18:3n-3	0.79	1.58	0.11	0.51	0.09	0.20	0.4	1.1
18:4n-3	0.14	0.86	0.03	0.11	0.04	0.06	0.1	0.3
20:0	0.14	0.13	0.06	0.11	0.09	0.15		
20:1	0.45	1.11	0.36	1.28	0.39	1.35	1.6	2.6
20:2n-6	0.45	0.36	0.37	0.53	0.40	0.47		
20:3n-6	1.91	0.66	2.09	1.31	1.66	0.80	0.7	0.4
20:4n-6	**5.48**	**0.59**	**5.82**	**0.98**	**6.73**	**1.14**	**1.4**	**0.6**
20:3n-3	0.12	0.18	0.05	0.08	0.06	0.07		
20:4n-3	0.53	0.50	0.30	0.16	0.08	0.50	0.4	0.5
20:5n-3	**1.48**	**0.47**	**0.79**	**0.43**	**0.85**	**0.28**	**2.1**	**1.1**
22:0	0.05	0.08	0.13	0.13	0.19	0.11	0.3	0.2
22:1	0.03	0.53	0.30	0.45	0.20	0.25	0.8	1.2
22:4n-6	1.18	0.19	0.90	0.45	0.65	0.32	0.3	0.1
22:5n-6	1.33	0.16	1.50	0.53	1.37	0.28	0.4	0.2
22:5n-3	2.46	0.88	1.43	1.36	1.02	0.68	1.8	2.1
22:6n-3	**20.43**	**2.47**	**21.97**	**7.22**	**20.66**	**3.00**	**13.4**	**5.7**
24:0					0.11	0.04		
24:1	0.11	0.13	0.37		0.49	0.04		
ΣSaturates	36.45	29.69	35.15	26.68	39.53	46.71	36.7	31.2
ΣMonoenes	14.78	42.27	17.71	38.87	17.00	32.23	22.4	33.0
Σn-6	15.48	14.44	14.75	17.37	16.88	11.68	12.6	18.6
Σn-3	25.97	7.22	24.85	10.18	22.87	4.96	18.6	11.2
Σn-3HUFA	25.00	4.49	24.54	9.26	22.67	4.48	17.8	9.7

Table 13. Fatty acid composition (%) of *Siganedae* (wild)

Source	Tangharan		Sagay	Ishigaki Japan	
Sample name	*S. guttatus* Muscle		*S. guttatus* Muscle	*Siganus canaliculatus* ovary	
Sample #	3		3	3	
Lipid class	PL	NL	PL	PL	NL
14:0	0.91	3.16	0.32	1.2	2.6
14:1					
15:0	0.41	1.33	0.40	0.7	1.3
16:0	20.16	33.82	21.34	31.1	32.2
16:1n-7	2.51	7.04	1.18	1.2	7.8
17:0	1.03	1.39	0.99	0.7	0.9
16:3n-6					
16:3n-3					
18:0	8.71	11.60	8.05	5.6	4.3
18:1n-9	7.82	10.34	6.42	5.8	15.4
18:1n-7	2.36	3.49	2.48	1.4	4.4
18:2n-6	1.22	1.37	9.93	0.7	2.5
18:3n-6	0.45	0.51	0.87	0.2	0.6
18:3n-3	0.40	0.57	7.22	0.2	1.3
18:4n-3	0.15		0.54	0.2	0.4
20:0	0.55	0.35			
20:1					0.2
20:2n-6	0.20	0.17	0.70	0.1	0.2
20:3n-6	0.37	0.34	1.14	0.8	1.0
20:4n-6	**8.38**	**4.73**	**7.14**	**9.9**	**3.9**
20:3n-3			1.01	0.1	0.4
20:4n-3	0.36	0.37	1.46	0.4	1.2
20:5n-3	**3.82**	**2.04**	**4.04**	**2.3**	**1.6**
22:0	0.31				
22:1					
22:4n-6	1.41	0.68	1.68	1.8	1.5
22:5n-6	3.51	1.23	2.34	1.3	0.9
22:5n-3	4.44	2.55	4.71	5.1	4.4
22:6n-3	**25.23**	**9.12**	**12.52**	**27.0**	**6.9**
24:0	0.33				
24:1	0.50				
ΣSaturates	31.72	51.66	31.11	39.3	41.3
ΣMonoenes	13.03	21.02	10.08	8.4	27.8
Σn-6	15.47	8.63	23.81	14.8	10.5
Σn-3	34.29	14.52	31.51	35.1	16.2
Σn-3HUFA	33.85	13.95	22.74	34.7	14.1

Table 14. Fatty acid composition (%) of *Siganedae*

Source	Igang-AQD		Igang		Tanghalan	
Sample name	*S. canalicilatus* ovary		*S. canaliculatus* wild ovary		*S. canaliculatus* wild fry	
Sample #	2		3		2 pooled	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	1.07	2.06	1.67	4.28	1.8	3.7
14:1			0.12	0.27		
15:0	0.57	0.82	0.69	1.05	0.8	1.3
16:0	30.60	27.85	31.87	30.43	24.2	26.9
16:1n-7	1.18	7.51	1.70	10.43	2.2	5.0
17:0	1.09	1.16	0.25	0.32		
16:3n-6			0.59	0.98		
16:3n-3			0.26	0.46		
18:0	6.65	6.60	6.83	6.64	12.7	12.0
18:1n-9	7.72	23.75	6.40	12.55	8.6	8.6
18:1n-7	1.77	5.17	1.36	4.02	2.2	3.8
18:2n-6	2.48	5.11	0.92	3.29	1.3	1.5
18:3n-6	0.25	0.80	0.10	0.45		
18:3n-3	0.20	0.62	0.19	1.32	0.3	0.5
18:4n-3			0.05	0.42	0.1	0.2
20:0			0.05	0.20		
20:1		0.55	0.13	0.90	0.1	0.6
20:2n-6	0.15	0.27	0.17	0.27		
20:3n-6	0.50	0.34	0.80	0.60	0.3	0.1
20:4n-6	**6.79**	**1.74**	**10.57**	**2.55**	**4.0**	**4.0**
20:3n-3			0.10	0.22		
20:4n-3	0.23	0.35	0.28	0.80	0.4	0.6
20:5n-3	**1.52**	**0.75**	**1.77**	**1.42**	**2.7**	**2.7**
22:0			0.06	0.09	0.4	0.4
22:1			0.26	0.36	0.1	0.2
22:4n-6	1.10	0.54	2.01	0.77	1.2	0.5
22:5n-6	2.43	1.00	2.76	0.51	Tr	Tr
22:5n-3	2.59	2.07	3.94	2.80	2.7	3.2
22:6n-3	**27.83**	**6.78**	**18.84**	**4.50**	**23.9**	**12.0**
24:0			0.09	0.03		
24:1	0.35					
ΣSaturates	39.97	38.48	41.44	43.03	40.8	45.4
ΣMonoenes	10.84	36.97	9.97	28.41	13.7	18.5
Σn-6	13.49	9.78	13.16	8.14	5.8	6.3
Σn-3	32.35	10.56	25.42	11.95	30.4	19.2
Σn-3HUFA	32.16	9.94	24.92	9.75	29.8	18.4

Table 15. Fatty acid composition (%) of *Siganedae* (wild)

Source	Igang		Ishigaki Japan		Ishigaki Japan	
Sample name	*S. canaliculatus* liver		*Sigunus virgatus* ovary		*Sigunus virgatus* testis	
Sample #	2		2		3	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	1.60	4.61	7.5	2.8	4.1	1.4
14:1	0.18	0.18				
15:0	0.65	1.32	0.9	0.7	0.6	0.6
16:0	38.49	57.87	23.4	29.1	28.1	26.0
16:1n-7	1.55	5.56	0.9	4.8	0.8	2.7
17:0	0.17	0.29	1.1	0.7	0.9	0.9
16:3n-6	1.15	1.54				
16:3n-3	0.10	0.16				
18:0	12.70	8.91	11.4	7.9	12.6	10.1
18:1n-9	7.27	6.34	6.4	16.6	9.0	12.5
18:1n-7	1.43	2.87	1.4	2.8	1.7	2.5
18:2n-6	0.50	0.85	0.7	1.3	1.0	1.7
18:3n-6	0.12	0.16	0.4	0.4	0.5	0.7
18:3n-3	0.13	0.25		0.5	0.3	0.5
18:4n-3	0.10	0.09		0.2		
20:0	0.10	0.42		0.3	0.3	
20:1	0.40	0.27		0.4	0.4	0.5
20:2n-6	0.13	0.09		0.2	0.3	0.3
20:3n-6	0.46	0.25	0.6	0.9	0.6	0.8
20:4n-6	**6.66**	**1.63**	**8.9**	**10.1**	**7.9**	**13.2**
20:3n-3	0.07	0.09		0.2		0.2
20:4n-3	0.13	0.14		0.9	0.3	0.3
20:5n-3	**0.68**	**0.36**	**0.9**	**1.7**	**1.3**	**2.1**
22:0	0.28	0.06	0.7		0.4	
22:1	0.21	0.07				
22:4n-6	0.69	0.24	3.3	2.6	2.6	3.4
22:5n-6	1.61	0.23	1.8	1.0	2.2	2.3
22:5n-3	1.81	0.67	4.7	5.6	4.7	6.1
22:6n-3	**13.56**	**1.57**	**7.9**	**6.0**	**9.6**	**9.7**
24:0	0.35	0.05	0.4			
24:1	0.49	0.03	1.8		0.9	
ΣSaturates	54.32	73.51	45.4	41.3	47.0	39.0
ΣMonoenes	11.51	15.26	10.5	24.6	12.8	18.1
Σn-6	11.29	4.98	15.6	16.3	15.0	22.4
Σn-3	16.59	3.32	13.5	14.9	15.8	18.5
Σn-3HUFA	16.26	2.83	13.5	14.2	15.7	18.2

Table 16. Fatty acid composition (%) of *Lethridae* (wild)

Source	Ishigaki Japan		Ishigaki Japan		Ishigaki Japan	
Sample name	*Lethrinus miniatus* ovary		*Lethrinus atokinsoni* ovary		*Lethrinus atokinsoni* testis	
Sample #	2		2		2	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	0.7	3.8	0.7	3.7	0.5	1.5
14:1						
15:0	0.8	2.1	0.5	1.4	0.6	1.1
16:0	21.4	27.3	19.7	30.9	19.1	26.2
16:1n-7	1.8	12.5	1.1	6.6	1.2	2.2
17:0	1.2	1.9	1.2	1.7	1.1	1.9
16:3n-6						
16:3n-3						
18:0	10.7	8.9	11.1	9.3	9.2	11.9
18:1n-9	6.7	15.7	8.0	13.4	12.0	8.2
18:1n-7	2.5	5.9	1.8	3.8	1.9	3.0
18:2n-6	1.4	1.6	1.5	3.2	1.4	1.7
18:3n-6	0.3	0.5	0.4	0.7	0.8	0.8
18:3n-3	0.2	0.7	0.3	1.3		
18:4n-3				0.4		
20:0	0.2	0.4			0.4	0.5
20:1	0.3	2.0	0.2	0.8	0.4	1.0
20:2n-6	0.3	0.4	0.4	0.6	0.9	1.3
20:3n-6	0.4		0.4	0.5	0.5	0.7
20:4n-6	**17.3**	**3.7**	**19.4**	**7.4**	**21.4**	**14.3**
20:3n-3			0.2	0.3		
20:4n-3	0.2		0.3	0.3		
20:5n-3	**4.8**	**1.7**	**4.8**	**3.1**	**1.1**	**1.3**
22:0	0.3		0.2		0.3	0.6
22:1						
22:4n-6	3.4	1.6	3.2	1.8	3.8	4.5
22:5n-6	1.5	0.4	1.6	0.5	1.5	0.8
22:5n-3	4.5	1.7	5.4	2.3	2.7	2.4
22:6n-3	**14.8**	**2.6**	**14.4**	**3.3**	**14.4**	**7.6**
24:0						
24:1	0.4		0.3		0.6	1.3
ΣSaturates	35.0	44.2	33.3	47.0	31.3	43.8
ΣMonoenes	11.2	36.1	11.2	24.5	16.1	15.7
Σn-6	24.6	8.2	26.8	14.6	30.3	24.0
Σn-3	24.5	6.6	25.2	10.8	18.1	11.3
Σn-3HUFA	24.3	6.0	24.9	9.0	18.1	11.3

Table 17. Fatty acid composition (%) of *Lethridae* and *Kyphosidae* (wild)

Source	Ishigaki Japan		Ishigaki Japan		Ishigaki Japan	
Sample name	*Lethrinus nebulosus* testis		*Lethrinus ornatus* testis		*Kyphosus vaigiensis* ovary	
Sample #	2		2		1	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	1.2	2.6	1.5	2.6	1.2	2.8
14:1			0.7	0.3		
15:0	0.9	1.1	0.1	0.4	0.4	0.8
16:0	17.5	24.8	19.3	27.3	21.2	30.2
16:1n-7	1.5	4.1	0.7	3.4	1.2	6.3
17:0	1.5	1.8	0.5	0.3	0.7	1.4
16:3n-6			0.2	0.5		
16:3n-3			0.1	0.3		
18:0	13.2	15.3	13.5	8.9	12.1	11.3
18:1n-9	6.8	12.0	7.8	10.6	9.3	20.9
18:1n-7	3.3	3.4	1.1	2.0	2.9	3.7
18:2n-6	1.6	2.0	0.8	2.1	1.5	2.1
18:3n-6	0.6	0.6	0.1	0.3		0.5
18:3n-3		0.6	0.3	2.2		0.8
18:4n-3			0.1	0.8		0.4
20:0	0.5	0.5	0.1	0.4	0.5	0.5
20:1		0.8	0.4	1.6		0.9
20:2n-6		0.4	0.2	0.5	0.4	0.6
20:3n-6	0.5	0.4	1.1	1.2	0.5	0.4
20:4n-6	**22.5**	**10.9**	**26.7**	**11.7**	**22.9**	**4.6**
20:3n-3			0.1	0.3		
20:4n-3			0.7	1.0		0.3
20:5n-3	**2.2**	**2.4**	**1.1**	**1.4**	**2.7**	**2.8**
22:0	0.8		0.2	0.5	0.7	0.5
22:1			0.1	0.6		
22:4n-6	4.7	2.6	5.7	3.5	3.9	1.3
22:5n-6	1.1	0.8	2.4	1.3	1.1	0.3
22:5n-3	3.6	2.3	2.3	1.6	3.0	1.4
22:6n-3	**4.9**	**3.8**	**4.7**	**2.6**	**4.5**	**1.6**
24:0			0.2	0.1		
24:1	1.3	1.6	1.6	0.9	1.3	
ΣSaturates	35.5	45.9	35.5	40.5	36.8	47.6
ΣMonoenes	13.0	22.0	12.4	19.4	14.7	31.7
Σn-6	30.9	17.5	37.3	21.0	30.2	9.8
Σn-3	10.8	9.1	9.3	10.0	10.2	7.3
Σn-3HUFA	10.8	8.6	8.7	6.5	10.2	6.1

Table 18. Fatty acid composition (%) of small-size pelagic fish (horse mackerel) (wild)

Source	Guimbal	Guimbal		Guimbal	
Sample name	*Trachurus* sp.-1 ovary	*Trachurus* sp-2 ovary		*Trachurus* sp-*2* testis	
Sample #	3	2		2	
Lipid class	PL	PL	NL	PL	NL
14:0	1.42	0.9	3.5	0.7	7.8
14:1		0.4	0.1	0.3	0.2
15:0	0.57	0.3	0.5	0.5	1.1
16:0	29.21	27.4	24.0	25.7	30.4
16:1n-7	2.81	2.6	12.1	2.6	9.3
17:0	1.18	0.2	0.3	0.4	0.5
16:3n-6		0.7	1.0	1.0	1.7
16:3n-3		0.3	1.1	0.3	0.3
18:0	5.18	8.9	7.5	8.5	13.4
18:1n-9	8.54	5.9	19.2	6.9	9.8
18:1n-7	1.98	2.3	7.4	3.5	2.9
18:2n-6	0.49	0.6	1.1	0.5	0.8
18:3n-6	0.23	0.2	0.3	0.3	0.5
18:3n-3	0.27	0.2	0.2	0.2	0.4
18:4n-3	1.04	0.2	0.1	0.1	0.3
20:0	0.28	0.2	0.5	0.3	0.4
20:1		0.2	0.7	0.4	0.7
20:2n-6		0.2	0.3	0.2	0.1
20:3n-6		0.2	0.2	0.1	0.1
20:4n-6	**2.71**	**6.6**	**2.7**	**5.1**	**4.9**
20:3n-3		0.0	0.1	0.1	0.0
20:4n-3		0.3	0.3	0.2	0.2
20:5n-3	**4.91**	**9.6**	**5.1**	**4.9**	**2.7**
22:0		0.2	0.1	0.2	0.4
22:1		0.2	0.1	0.0	0.0
22:4n-6		0.5	0.3	0.5	0.2
22:5n-6	0.95	1.3	0.4	1.3	0.1
22:5n-3		4.1	1.5	4.0	1.0
22:6n-3	**20.21**	**21.7**	**4.8**	**26.5**	**3.7**
24:0		0.1	0.0	0.3	0.4
24:1	0.68	0.0	0.0	0.4	0.0
ΣSaturates	37.36	38.0	36.5	36.3	54.1
ΣMonoenes	14.01	11.7	39.5	13.6	23.0
Σn-6	4.38	10.3	6.4	9.1	8.3
Σn-3	21.34	36.0	6.7	36.1	6.3
Σn-3HUFA	23.48	35.6	5.4	35.6	5.6

Table 19. Fatty acid composition (%) of tropical pelagic fishes (wild)

Source	Guimbal			Guimbal		
Sample name	*Cypselurus* sp. whole body			*Stolephorus andhraensis* whole body		
Sample #	2			2		
Lipid class	TL	PL	NL	TL	PL	NL
14:0	3.07	1.91	4.55	4.17	6.41	2.67
14:1	0.21	0.23	0.30	0.26	0.44	0.15
15:0	0.56	0.46	0.73	0.96	1.31	0.75
16:0	19.84	18.95	22.17	25.10	25.10	24.97
16:1n-7	4.19	2.95	6.67	4.31	8.33	3.05
17:0	0.56	0.42	0.50	0.45	0.68	0.65
16:3n-6	0.58	0.36	0.46	0.17	1.44	1.07
16:3n-3	0.41	0.60	0.24	0.39	0.29	0.43
18:0	13.21	14.00	10.22	7.53	7.26	7.60
18:1n-9	5.94	6.26	6.73	6.14	5.28	6.75
18:1n-7	3.22	2.86	3.91	2.66	2.80	2.52
18:2n-6	1.33	0.69	1.81	1.11	2.20	0.95
18:3n-6	0.29	0.17	0.33	0.26	0.45	0.22
18:3n-3	0.41	0.13	0.43	0.71	0.99	0.54
18:4n-3	0.72	0.12	0.54	0.69	1.21	0.43
20:0	0.28	0.08	0.27	0.35	0.49	0.26
20:1	2.27	0.58	2.84	0.92	0.80	0.47
20:2n-6	0.78	0.21	0.69	0.81	1.22	1.68
20:3n-6	0.14	0.08	0.18	0.28	0.25	0.20
20:4n-6	**2.66**	**3.28**	**2.84**	**2.57**	**2.62**	**2.72**
20:3n-3	0.55	0.11	0.03	0.06	0.00	0.13
20:4n-3	0.34	0.22	0.23	0.17	0.00	0.17
20:5n-3	**6.93**	**4.10**	**5.82**	**6.11**	**7.16**	**5.68**
22:0	0.44	0.65	0.39	0.42	0.50	0.27
22:1	2.66	0.22	1.24	0.28	0.45	0.23
22:4n-6	0.47	0.39	0.40	0.18	0.22	0.19
22:5n-6	0.80	1.64	0.65	1.16	0.74	1.36
22:5n-3	2.00	2.63	2.20	0.72	0.73	0.95
22:6n-3	**18.50**	**26.28**	**13.01**	**20.91**	**11.69**	**26.32**
24:0						
24:1						
ΣSaturates	37.96	36.47	38.84	38.99	41.75	37.18
ΣMonoenes	18.49	13.10	21.68	14.58	18.11	13.16
Σn-6	7.04	6.82	7.36	6.54	9.14	8.38
Σn-3	29.13	34.07	21.97	29.07	20.86	34.23
Σn-3HUFA	27.42	33.02	21.04	27.74	19.57	32.95

Table 20. Fatty acid composition (%) of tropical pelagic fishes (wild)

Source	Guimbal			Guimbal		
Sample name	*Sardinella* sp. whole body			*Gymnocaesio gymnoptera* whole body		
Sample #	2			2		
Lipid class	TL	PL	NL	TL	PL	NL
14:0	7.82	0.91	17.60	6.03	3.90	8.03
14:1	0.32	0.62	0.37	0.17	0.20	0.21
15:0	1.79	0.19	1.68	0.77	0.47	0.76
16:0	31.92	20.40	27.16	28.92	24.38	31.07
16:1n-7	7.04	1.98	9.47	8.61	4.57	11.41
17:0	0.61	0.15	0.64	0.35	0.38	0.35
16:3n-6	0.97	0.33	2.08	0.94	0.93	0.94
16:3n-3	0.32	0.19	0.35	0.34	0.45	0.54
18:0	6.28	9.25	6.24	9.14	11.57	7.20
18:1n-9	5.37	13.93	6.80	8.82	7.05	8.37
18:1n-7	4.01	2.65	4.09	3.54	3.38	3.72
18:2n-6	0.78	0.44	1.13	0.69	0.63	0.70
18:3n-6	0.36	0.13	0.49	0.22	0.30	0.19
18:3n-3	0.37	0.08	0.37	0.29	0.20	0.27
18:4n-3	0.23	0.04	0.18	0.29	0.10	0.43
20:0	0.27	0.15	0.57	0.34	0.27	0.28
20:1	1.40	1.77	1.31	0.84	0.71	0.77
20:2n-6	0.20	0.22	0.22	0.21	0.87	0.19
20:3n-6	0.13	0.13	0.33	0.10	0.11	0.17
20:4n-6	**1.88**	**3.44**	**1.19**	**2.93**	**4.08**	**2.71**
20:3n-3	0.05	0.41	0.00	0.05	0.08	0.02
20:4n-3	0.13	0.15	0.00	0.24	0.16	0.26
20:5n-3	**3.56**	**9.28**	**4.21**	**6.35**	**5.64**	**6.69**
22:0	0.38	0.08	0.46	0.44	0.49	0.33
22:1	0.41	0.12	0.34	0.17	0.17	0.31
22:4n-6	0.07	0.34	0.16	0.25	0.33	0.21
22:5n-6	0.43	0.49	0.31	0.72	1.27	0.42
22:5n-3	0.40	1.86	0.29	1.15	1.11	1.15
22:6n-3	**13.27**	**25.11**	**4.47**	**11.17**	**18.95**	**6.45**
24:0						
24:1						
ΣSaturates	49.07	31.13	54.34	45.98	41.46	48.02
ΣMonoenes	18.57	21.07	22.39	22.15	16.09	24.79
Σn-6	4.82	5.51	5.92	6.07	8.53	5.53
Σn-3	18.10	37.06	9.70	19.59	26.58	15.38
Σn-3HUFA	17.23	36.24	8.98	18.67	25.70	14.29

Table 21. Fatty acid composition (%) of tropical pelagic fishes (wild)

Source	Guimbal			Guimbal		
Sample name	*Rastrelliger* sp. whole body			*Pterocaesio pisang* whole body		
Sample #	2			2		
Lipid class	TL	PL	NL	TL	PL	NL
14:0	2.99	1.75	4.95	4.28	1.76	4.74
14:1	0.23	0.12	0.44	0.11	0.22	0.13
15:0	0.91	0.56	1.20	0.54	0.31	0.51
16:0	24.80	21.14	26.35	29.00	23.74	27.74
16:1n-7	3.91	2.20	6.59	8.49	3.42	9.89
17:0	0.70	0.52	0.84	0.36	0.30	0.31
16:3n-6	1.40	1.22	1.76	0.74	0.67	0.67
16:3n-3	0.43	0.41	0.34	0.31	0.44	0.40
18:0	12.98	12.56	12.49	7.66	10.50	6.13
18:1n-9	6.32	6.83	6.87	12.21	8.92	12.46
18:1n-7	2.91	2.66	3.66	3.18	1.44	3.13
18:2n-6	0.69	0.56	0.71	0.64	0.59	0.69
18:3n-6	0.54	0.46	0.48	0.19	0.25	0.13
18:3n-3	0.20	0.14	0.22	0.25	0.17	0.45
18:4n-3	0.14	0.11	0.22	0.38	0.06	0.53
20:0	0.57	0.39	0.77	0.33	0.21	0.29
20:1	1.32	1.31	1.72	0.73	0.67	0.75
20:2n-6	0.46	1.45	0.63	0.20	0.97	0.16
20:3n-6	0.22	0.25	0.16	0.12	0.16	0.18
20:4n-6	**4.75**	**5.29**	**3.99**	**2.60**	**4.07**	**2.57**
20:3n-3	0.06	0.13	0.03	0.06	0.10	0.07
20:4n-3	0.16	0.18	0.07	0.37	0.28	0.44
20:5n-3	**3.67**	**4.07**	**3.72**	**6.83**	**6.32**	**7.93**
22:0	0.37	0.35	0.54	0.43	0.38	0.30
22:1	0.60	0.24	0.76	0.48	0.21	0.30
22:4n-6	0.60	0.66	0.45	0.37	0.53	0.36
22:5n-6	1.87	2.47	0.78	0.76	1.57	0.62
22:5n-3	1.24	1.55	0.85	1.89	1.90	2.10
22:6n-3	**17.17**	**22.60**	**8.22**	**10.48**	**20.42**	**9.53**
24:0						
24:1						
ΣSaturates	43.34	37.27	47.14	42.60	37.20	40.02
ΣMonoenes	15.29	13.36	20.03	25.20	14.88	26.67
Σn-6	10.54	12.37	8.97	5.62	8.81	5.38
Σn-3	22.93	29.09	13.45	20.19	29.63	20.92
Σn-3HUFA	22.08	28.22	12.79	19.19	28.64	19.56

To induce natural spawning, pairs of one female and one male of each dietary treatment were separately transferred to spawning tanks just before first quarter moon when it is the time for *S. guttatus* to spawn (natural spawning of this species follows a lunar cycle) (Duray 1998). Since eggs spawned from *S. guttatus* are demersal and strongly adhesive (Duray 1998), and indeed the sticky eggs were attached to the wall of the spawning tank, we were not able to collect and count the number of spawned eggs. In the present report, we show only the number of spawning and the total number of hatched-larvae in each dietary treatment.

Table 22. Composition of test diets fed to rabbitfish broodstock (first trial)

Ingredients (%)	Diet 1	Diet 2	Diet 3
Fish meal (Peruvian)	25	25	25
Soybean meal	25	25	25
Acetes[1]	8	8	8
Squid meal	1.5	1.5	1.5
Soybean oil	1.0	0.25	0
Cod oil	6.0	6.0	5.5
Arachidonate		0.75	1.5
Lecithin (Soybean)	3.0	3.0	3.0
Rice bran	4.3	4.3	4.3
Wheat (Bread) flour	10	10	10
Corn starch	10	10	10
Dicalcium Phosphate	2	2	2
Vitamin mix[2]	1	1	1
V-22[3]	2	2	2
Vitamin C	0.2	0.2	0.2
CMC (Binder)	1.0	1.0	1.0

[1] Shrimp meal. [2] Imported vitamin. mixture. [3] vitamin mixture for poultry.

Table 23. Supplementation level of arachidonic acid (ArA) and reproductive performance of *S. guttatus* fed test diets

	No. of spawning	No. of hatching	Total No. of hatched-larvae
Diet 1 (+0%ArA)	3	1	500,000
Diet 2 (+0.75% ArA)	4	3	1,548,000
Diet 3 (+1.5% ArA)	3	0	0

The number of spawning, the number of hatching and the total number of hatched-larvae are shown in Table 23 without statistical treatment. The broodstock spawned three times for diets 1 and 3 and four times for diet 3 during the period from July to December. The total numbers of hatched-larvae were 500,000 for diet 1, 1,548,000 for diet 2 and 0 for diet 3. Diet 3 with 1.5% of ArA supplementation appeared to affect negatively the reproductive performance of *S. guttatus* broodstock, perhaps due to the excessive supplementation. Similar results were observed, that is, reproductive performance of Japanese flounder was improved by ArA supplementation with 0.6 g/100 g diet but was adversely affected by that with 1.2 g/100 g diet (Furuita et al., 2003).

2.2. Second Trial

Diet 1 was a basal diet with 1% of soybean oil + 6% squid oil + 4% cod oil (Table 24). The soybean oil was replaced with 0.3% (for diet 2) or 0.6% (for diet 3) of ArA (*Vevodar Crude Arachidonic Oil*, DSM Food Specialties, Delft, the Netherlands). Fish meal, soybean meal, acetes and squid meal were the protein sources and wheat flour and corn starch were the carbohydrate sources. The mixture was pelleted using a twin-screw extruder.

Two sources of broodstock, hatchery-bred broodstock and wild-caught broodstock, were employed in the 2nd feeding trial. The hatchery-bled broodstock were raised from eggs that were spawned at Tigbauan Main Station, and the wild-caught broodstock were purchased from local dealer. Three females and three males from each source were each stocked in six 5-ton-concrete tanks at Tigbauan Main Station (two sources per each dietary treatment x three dietary treatments and three pairs in each tank) (Table 25). Fish have been fed each of the three test diets (Table 24) twice a day, six days a week at a rate of 3% of biomass/day from March. To induce natural spawning, pairs of one female and one male of each dietary treatment were separately transferred to spawning tanks (18 tanks: three pairs x three dietary treatments x two sources) just before first quarter moon when it is the time for rabbitfish *S. guttatus* to spawn (natural spawning of this species follows a lunar cycle) (Duray, 1998). Since eggs spawned from *S. guttatus* are demersal and strongly adhesive, and indeed the sticky eggs were attached to the wall of the spawning tank, we were not able to collect and count the number of spawned eggs. Again, we show only the number of spawning and the total number of hatched-larvae in each dietary treatment.

The number of spawning and the number of hatching during the period of May through January of the next year are shown in Table 26. Table 27 lists the total number of hatched-larvae, the average number of hatched larvae per pair, the total number of normal larvae and the average number of normal larvae per pair. Table 28 also shows average % of normal larvae. There were no differences between hatchery-bled and wild broodstock in any indices.

The broodstock spawned 13 times for diet 1, 14 times for diet 2 and 17 times for diet 3 during the period May through Jan 2of the next year. The numbers of hatching were 10 of the 13 spawning for diet 1, 11 of the 14 spawning for diet 2 and 14 of the 17 spawning for diet 3, respectively. The total numbers of hatched-larvae were $3{,}818 \times 10^3$ for diet 1, $4{,}391 \times 10^3$ for diet 2 and $4{,}597 \times 10^3$ for diet 3. The average numbers±S.E. ($\times 10^3$) of hatched larvae were 636±216 for diet 1, 878±257 for diet 2 and 766±213 for diet 3, respectively. The total numbers of normal larvae were $3{,}322 \times 10^3$ for diet 1, $3{,}700 \times 10^3$ for diet 2 and $3{,}876 \times 10^3$ for diet 3. The average numbers±S.E. ($\times 10^3$) of normal larvae were 554±183 for diet 1, 740±243 for diet 2 and 646±183 for diet 3, respectively. Thus, broodstock fed diet 2 or 3 with ArA supplementation tended to show better performance in spawning and hatching, but with big variations. Percent of normal larvae (average±S.E.) was as: 76.3±6.4% for diet 1, 79.3±6.1% for diet 2 and 79.2±5.8% for diet 3. The % of normal larvae did not differ among dietary treatment. Considered together with the adverse effects of ArA over-supplementation (Furuita et al., 2003) and the results of the 1st trial, the optimum supplementation level of ArA in broodstock diets may be 0.5% to 0.7%.

Table 24. Composition of test diets fed to rabbitfish broodstock (second trial)

Ingredients (%)	Diet 1	Diet 2	Diet 3
Fish meal (Peruvian)	23	23	23
Soybean meal	23	23	23
Acetes[*1]	4	4	4
Squid meal	10	10	10
Soybean oil	1.0	0.70	0.40
Squid oil	6.0	6.0	6.0
Cod oil	4.0	4.0	4.0
Arachidonate[*2]		0.30	0.60
Lecithin (Soybean)	2.0	2.0	2.0
Rice bran	6.3	6.3	6.3
Wheat (Bread) flour	6.5	6.5	6.5
Corn starch	8	8	8
Dicalcium Phosphate	2	2	2
Vitamin mix[*3]	1	1	1
V-22[*4]	2	2	2
Vitamin C	0.2	0.2	0.2
CMC (Binder)	1.0	1.0	1.0

*1 Shrimp meal. *2 *Vevodar Crude Arachidonic Oil* (DSM Food Specialties, Delft, the Netherlands). *3 Imported vitamin mix. *4 vitamin mix for poultry (local vitamin mix.).

Table 25. Pairing and body weight (g) of rabbitfish broodstock

	Diet 1 (0% ArA)					
	hatchery-bled			wild		
	No. 1	No.2	No.3	No. 1	No.2	No.3
Female	508.9	414.6	600.0	468.1	338.6	476.6
Male	495.2	389.5	281.7	406.5	380.8	458.9
	Diet 2 (0.3% ArA)					
	hatchery-bled			wild		
	No. 1	No.2	No.3	No. 1	No.2	No.3
Female	716.1	615.9	476.0	490.2	473.7	579.5
Male	447.0	376.1	413.3	406.4	424.1	426.6
	Diet 3 (0.6% ArA)					
	hatchery-bled			wild		
	No. 1	No.2	No.3	No. 1	No.2	No.3
Female	968.7	578.6	511.7	574.5	460.8	420.4
Male	258.3	347.6	403.2	441.5	343.8	431.6

Table 26. Total number of spawning and total number of hatching during the period of May through Jan of the next year

	No. of pair	No. of spawning	No. of hatching
Diet 1 (0% ArA)			
hatchery-bled	3	7	5
wild	3	6	5
Total	6	13	10
Diet 2 (0.3% ArA)			
hatchery-bled	2	3	3
wild	3	11	8
Total	5	14	11
Diet 3 (0.6% ArA)			
hatchery-bled	3	8	7
wild	3	9	7
Total	6	17	14

Table 27. Total number of hatched larvae (x10^3), the average number of hatched larvae per pair (x10^3) (±S.E.), the total number of normal larvae (x10^3) and the average number of normal larvae per pair (x10^3) (±S.E.)

	Total No. of hatched larvae	No. of hatched larvae per pair	Total No. of normal larvae	No. of normal larvae per pair
Diet 1				
hatchery-bled	1,779	593±399	1,492	497±188
wild	2,039	680±271	1,830	610±242
Total	3,818	636±216	3,322	554±183
Diet 2				
hatchery-bled	1,569	785±189	1,424	712±7
wild	2,822	941±450	2,276	759±433
Total	4,391	878±257	3,700	740±243
Diet 3				
hatchery-bled	2,682	894±410	2,182	727±349
wild	1,915	638±204	1,694	565±196
Total	4,597	766±213	3,876	646±183

Table 28. Average % of normal larvae (±S.E.)

	No. of sample	% of normal larvae
Diet 1		
hatchery-bled	5	75.8±10.6
wild	5	76.8±8.5
mean	10	76.3±6.4
Diet 2		
hatchery-bled	3	91.9±1.7
wild	8	74.6±6.6
mean	11	79.3±6.1
Diet 3		
hatchery-bled	7	73.0±6.5
wild	7	85.5±4.3
mean	14	79.2±5.8

3. Effects of DHA/Arachidonic Acid-Enriched Rotifers on Survival and Growth of Rabbitfish Fry

Feeding trials were conducted to investigate the effects of DHA/ArA-enriched rotifers on survival and growth of rabbitfish (*Siganus guttatus*) fry.

3.1. Preliminary Studies

Our preliminary studies showed that rotifers (a live food) were enriched with emulsified triacylglycerol-type ArA, that EPA and DHA levels in the rotifers decreased as the increment of the amount of ArA in culture media, and that the optimum level ArA supplementation to the culture media appeared at 5% or lower.

3.2. Materials and Methods

Rabbitfish eggs used in this experiment came from rabbitfish broodstock maintained at Tigbauan Research Station of SEAFDEC/AQD. Rabbitfish eggs were naturally spawned. The eggs were incubated according to standard practice at SEAFDEC/AQD.

Rotifers *Brachionus sp*. were first cultured in 20 cubic meter outdoor concrete tanks fed with *Nannochloropsis sp.* for five days. The rotifers were harvested and were intensively cultured in four different 30 *l* cylindrical-conical fiberglass tanks by feeding an artificial rotifer-diet (Culture Selco (CS), Inve Aquaculture, Baasrode, Belgium) for 24 h. Subsequently, the rotifers were cultured with various combinations of artificial rotifer-diets and ArA. Rotifers in the first group were enriched with Culture selco, the second group was enriched with Culture Selco + 5% ArA, the third group were enriched with DHA Protein Selco (DHAPS) and the fourth group were enriched with DHA Protein Selco + 5% ArA. The enrichment of rotifers with each treatment was performed twice a day (9-10 AM and 7-8 PM), and the rotifers were harvested after 24 h. Prior to the enrichment, rotifer density in each tank was determined to calculate the amount of each supplement to the tank. For the second and fourth group an addition of 5% ArA by weight were made.

Newly hatched (Day 0) rabbitfish were stocked (30 larvae/*l*) in circular, conical-bottom fiberglass tanks filled with 200 *l* filtered seawater. Larval rearing protocol followed the method described by Gapasin & Marte (1990) with modifications.

The four treatments (in a completely randomized design with 4 replicates) were:

- Treatment I: larvae fed rotifers cultured on Culture Selco
- Treatment II: larvae fed rotifers cultured on Culture Selco + 5% ArA
- Treatment III: larvae fed rotifers cultured on DHA Protein Selco, and
- Treatment IV: larvae fed rotifers cultured on DHA Protein Selco + 5% ArA.

Treatment I was a control, which is standard procedure of rabbitfish larvae practiced at SEAFDEC/AQD. Larval culture for all treatments was conducted using greenwater (*Nannochloropsis sp*) with a density of 5-10 x 10^4 cells/ml. Temperature (27-29°C), salinity (29-31‰) and standing food count were monitored prior to water exchange and feeding.

3.3. Survival and Growth

The feeding trials were terminated on day 17. Larval survival was determined, and 10 samples from each tank were measured for total length and body weight.

Survival of D17 rabbitfish fry (Figure 5) was significantly improved. The average survival±S.E. was 16.4±4.1% for CS, 31.2±3.7% for CS+5% ArA, 23.9±7.4% for DHAPS, 44.4±4.5% for DHAPS+5% ArA. Surprisingly, of DHAPS+5% ArA group, the best survival value recorded 58.8%. The body length (mm) and the body weight (mg) were 5.44±0.38 and 2.0±0.3 for CS, 6.20±0.34 and 2.5±0.5 for CS+5%ArA, 6.05±0.53 and 2.6±0.5 for DHAPS, and 6.23±0.27 and 2.4±0.3, respectively. Growth was not different among treatments. Thus, although dietary ArA supplementation would help advance fry production technologies in coral reef fishes, it should be noted that excessive feeding of ArA also causes adverse effects

to fry and juvenile performance (Zeng et al., 1996; Xu et al., 2010). Since DHA level in rotifers is decreased by over-supplementation of ArA as shown in our preliminary studies, the dietary balance between DHA and ArA should be considered.

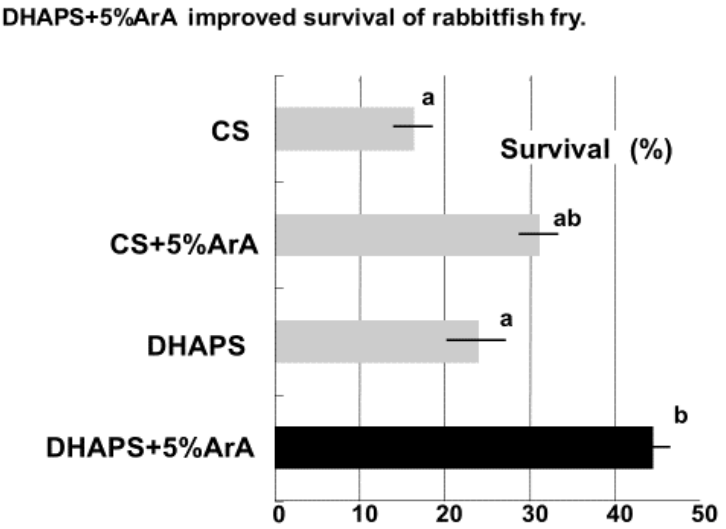

Figure 5. Survival and total length in larval rearing test – DHAPS+5%ArA improved survival of rabbitfish larvae (Day 17).

CONCLUSION

ArA was not a minor HUFA but an essential component in gonads of coral reef, demersal fishes with 2:1 of DHA-to-ArA ratio.

The number of spawning and the number of hatched larvae tended to be better in rabbitfish broodstock fed diets with ArA enrichment than in those fed a diet without ArA enrichment. The optimum level of ArA incorporation in broodstock diets appears between 0.5% and 0.7%.

Rabbitfish fry fed ArA-enriched rotifers showed significantly improved survival.

The present paper indicates that dietary ArA supplementation is very promising for the advancement of fry production technologies in coral reef fishes.

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Chapter 9

PROTEINS RESPONSIVE TO VARIABLE TEMPERATURE EXPOSURE IN THE REEF-BUILDING CORAL *SERIATOPORA HYSTRIX*

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ABSTRACT

Although reef-building corals engaged in mutualistic relationships with dinoflagellates of the genus *Symbiodinium* are threatened by global climate change, many anthozoan-dinoflagellate endosymbioses display a marked capacity for acclimation with respect to temperature changes. For instance, specimens of the Indo-Pacific reef coral *Seriatopora hystrix* from Southern Taiwan were found to readily acclimate to temperatures that fluctuated from 23 to 29ºC over six hours, a periodicity aimed to

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simulate local upwelling events that are common during boreal summer spring tides. To gain greater insight into the molecular mechanisms underlying this ability to acclimate to a variable temperature regime, proteins from corals exposed to both stable (26ºC) and variable temperatures for one week were electrophoresed across two dimensions, and differentially expressed proteins were sequenced with mass spectrometry. Seventy-five (64%) and forty-two (36%) proteins were expressed at higher levels by coral hosts and their *Symbiodinium* populations, respectively, of the stable temperature treatment. This suggests that a number of cellular pathways, including lipid body stabilization and metabolism in the *Symbiodinium* cells, are down-regulated upon exposure to variable temperature, and the potential shift in energy modulation implied by these findings may play a role in the restoration of homeostasis necessitated by exposure to such highly variable temperature conditions.

INTRODUCTION

Most current global climate change (GCC) models assume that reef-building corals are unable to acclimate to changes in their abiotic environment [1]. Although it is true that many corals are known to live near the upper threshold of their thermotolerance and readily bleach in response to sustained temperature increases [2-3], recent studies have revealed that not only can corals readily acclimate to elevated temperature, salinity, and pCO_2 [4-7], but they can thrive under such conditions [8-10]. For instance, corals from Houbihu, Taiwan (Figure 1A) are readily exposed to episodic, spring tide upwelling during the boreal summer, periods during which temperatures may change up to 9-10ºC within several hours [11]. Corals from these upwelling habitats have proven to be markedly resilient to both short- [12] and long-term [13] increases in temperature, as has been predicted to occur based on studies of intertidal organisms [14].

In order to gain insight into how corals from these upwelling sites acclimate to such dramatic temperature changes, an experiment was conducted in which corals from not only Houbihu, but also a nearby, non-upwelling site, Houwan, were exposed to either a variable (23-29ºC over a 6-hr period) or stable (26ºC) temperature profile for seven days [15-17]. *Seriatopora hystrix* (Figure 1B-C) was chosen as the model coral for such laboratory-based studies, given its 1) widespread distribution across the Indo-Pacific [18-19], 2) propensity for bleaching under periods of elevated temperatures [20], and 3) modest existing understanding of its molecular eco-physiology [21-22]. In general, even *S. hystrix* specimens that were never exposed to upwelling *in situ* readily acclimated to variable temperature conditions (Table 1), and an effort was made to develop both a physiological and a sub-cellular understanding of how such acclimation occurred in the samples from this "*Seriatopora hystrix* variable temperature study" (SHVTS; [15-17]).

Given recent success in employing molecular biology-driven approaches to answering an array of both fundamental [23-27] and stress/environmental biology [28] questions in the field of anthozoan-dinoflagellate endosymbiosis, the expression of a series of gene mRNAs was measured in samples of the SHVTS [15, 17]. Although several genes encoding proteins involved in photosynthesis were differentially expressed between the stable and variable temperature treatments (TT; [15, 17] and Table 1), the variation was generally modest, and it was, furthermore, unclear whether such changes in mRNA expression would actually lead to altered levels of translation of the respective proteins; indeed, in the few studies that have

looked at both gene and protein expression in the same anthozoan-dinoflagellate sample [7, 26], there was not always a significant, positive correlation between gene and protein expression [7]. Therefore, a whole-proteome-based approach employing two-dimensional (2D) electrophoresis followed by protein sequencing via mass spectrometry (MS) was taken herein in order to better unravel the molecular means by which *S. hystrix* and its endosymbiotic *Symbiodinium* populations acclimate to a variable temperature regime.

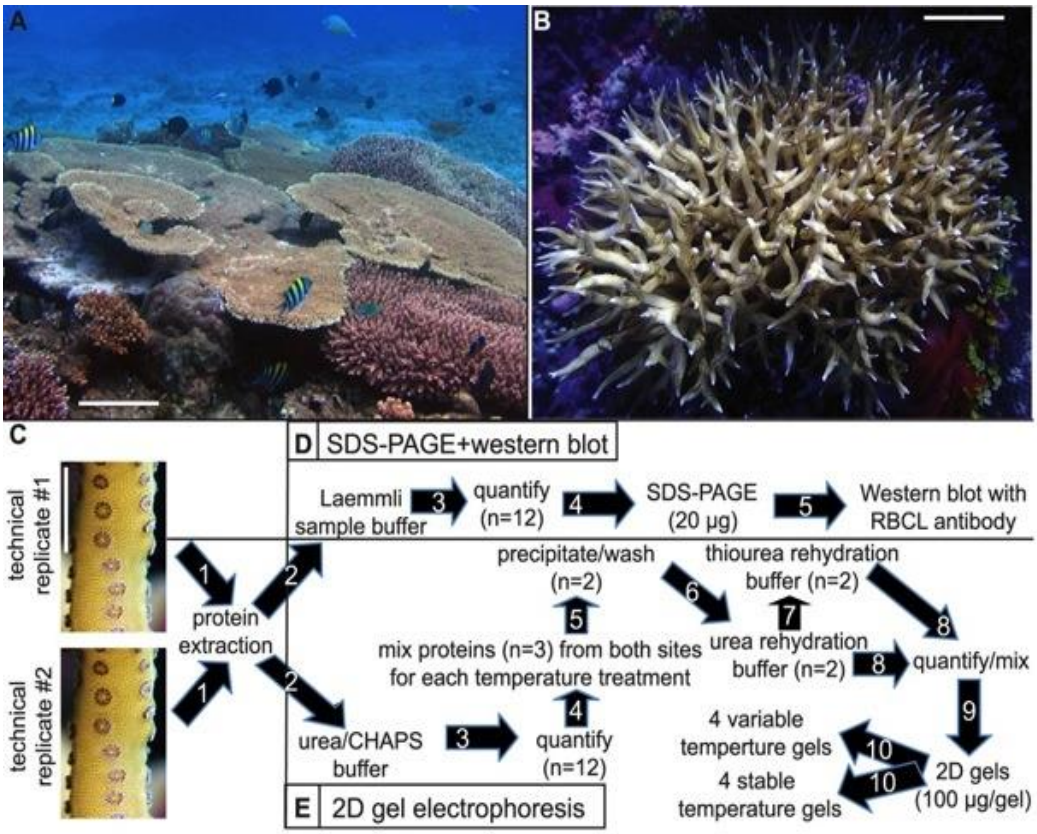

Figure 1. The upwelling field site Houbihu, the model coral *Seriatopora hystrix*, and an analytical flow-chart of the proteomic analyses. (A) Houbihu, the upwelling site from which half of the *Seriatopora hystrix* specimens used in the variable temperature study were sampled (photograph taken by Dr. Pi-Jen Liu, National Museum of Marine Biology and Aquarium, Taiwan). (B) An adult *S. hystrix* colony. (C) Proteins were extracted from each of two technical replicates (*i.e.*, nubbins) from each of the 12 experimental aquaria after a 7-d exposure to either variable (23-29ºC over a 6-hr period; n = 6 aquaria) or stable temperature (26ºC; n = 6 aquaria). (D) The expression of RBCL was quantified in the 12 samples dissolved in SDS-PAGE sample buffer. (E) For the second technical replicate from each aquarium, proteins were prepared for 2-dimensional gel electrophoresis as described in the text. Proteins were pooled across sites of origin (SO; *i.e.*, proteins from Houbihu were mixed with those of Houwan) for each of the two temperature treatments (TT) given the fact that *only* a TT effect on protein expression was of interest herein. The numbers on the arrows represent the respective experimental steps in E, and the scale bars in A, B, and C represent 500, 50, and 5 mm, respectively.

Table 1. Summary of results of the *Seriatopora hystrix* variable temperature study (SHVTS). Site of origin (SO), temperature treatment (TT), and interaction effects were deemed statistically significant at $\alpha < 0.05$ (denoted by "*" in the respective cells). "Upwelling site" and "non-upwelling site" refer to Houbihu and Houwan, respectively. NA = not applicable. Chl-a = chlorophyll a

Response variable	SO effect	TT effect	Inter-action effect	Major finding(s)	Reference
Host coral genotype		NA	NA		[17]
Symbiodinium genotype					[17]
Growth			*	Faster growth in non-"transplanted" corals	[15]
Symbiodinium density	*			Non-upwelling site > upwelling site	[15]
Chl-a concentration		*	*	Variable temperature > stable temperature Higher chl-a in non-"transplanted" corals	[15]
Maximum dark-adapted quantum yield of photosystem II (F_V/F_m)	*	*	*	Upwelling site > non-upwelling site Variable temperature > stable temperature Higher F_V/F_M in non-"transplanted" corals	[15]
Symbiodinium heat shock protein 70 (*hsp70*) genome copy proportion (DNA content)					[15]
RNA/DNA ratio					[15]
Protein/DNA ratio	*			Non-upwelling site > upwelling site	[15]
Symbiodinium ribulose-1,5-bisphosphate carboxylase/ oxygenase (*rbcL*) mRNA expression		*		Variable temperature > stable temperature	[15]
Symbiodinium photosystem I (subunit III; *psI*) mRNA expression	*	*		Upwelling site > non-upwelling site Variable temperature > stable temperature	[15]
Symbiodinium phosphoglycolate phosphatase (*pgpase*) mRNA expression		*		Variable temperature > stable temperature	[15]
Symbiodinium ascorbate peroxidase (*apx1*) mRNA expression					[15]
Symbiodinium hsp70 mRNA expression		*		Variable temperature > stable temperature	[17]
Symbiodinium nitrate transporter 2 (*nrt2*) mRNA expression					[17]
S. hystrix hsp70 mRNA expression		*		Stable temperature > variable temperature	[17]
S. hystrix α-tubulin (*tuba*) mRNA expression					[17]
S. hystrix tropomyosin (trp1) mRNA expression					[17]
S. hystrix β-actin *(actb)* mRNA expression		*		Variable temperature > stable temperature	[17]
S. hystrix ezrin mRNA expression					[17]
S. hystrix phospholipase *α-2 (cplap2)* mRNA expression			*	Higher mRNA expression in "transplanted" corals	[17]
S. hystrix transient receptor cation channel *(trcc)* mRNA expression					[17]
S. hystrix organic anion transporter *(oatp)* mRNA expression					[17]
Symbiodinium RBCL protein expression					herein
Protein expression (2D gel)		*		Stable temperature > variable temperature	herein

MATERIALS AND METHODS

SHVTS

The SHVTS was discussed in previous works [15-17]. Briefly, six *S. hystrix* colonies from both the upwelling (Houbihu; Figure 1A) and non-upwelling (control) sites (Houwan) were collected, acclimated in indoor aquaria to allow for recovery from transplantation [16], fragmented into nubbins, acclimated again for several weeks, and randomly assigned to 1 of 12 experimental aquaria: 3 for each site of origin (SO) x TT combination. For protein work, ~100 mg pieces/branches from each nubbin (n = 2 pseudo-/technical replicates per aquarium) were immediately immersed in TRIzol® (Life Technologies, USA) after a 7-d exposure to either a stable (26ºC, n = 6 aquaria) or variable TT (23-29ºC over a 6-hr period, n = 6 aquaria), with time 0 samples (~100 mg; n = 2 pseudo-replicated nubbins/aquarium) taken just before the temperature began to fluctuate in the variable temperature aquaria; such samples (n = 6 for each SO) were collected to uncover SO, rather than TT (the focus of this work), differences and are not discussed further herein. Samples were frozen in TRIzol at -20ºC until the day of extraction. The remainder of each of the 48 nubbins (2 nubbins/aquarium x 12 aquaria [3 for each of 4 SO x TT interaction groups] x 2 sampling times [t = 0 and 7 d]) was used for a variety of additional molecular and physiological analyses discussed in Table 1 and in prior works [15-17]. RNAs and DNAs were isolated from the same 100-mg fragments from which the proteins, discussed below, were isolated, as TRIzol permits the extraction of high quality RNA, DNA, and protein from the same biological sample [12].

Protein Extraction

Proteins were extracted from ~100-mg fragments from each of the 24 nubbins sampled at the t = 7 d sampling time (2 pseudo-replicates/aquarium x 12 aquaria) with TRIzol as recommended by the manufacturer except with 10-min sonications on ice between the washes with "protein wash I." As mentioned above, the respective RNAs and DNAs from each of the same 24 samples were already purified and analyzed [15-17]. For one of the two technical replicates from each aquarium (Figure 1C), the proteins were dissolved in 100-200 µl Laemmli sample buffer [29] without the additional of bromophenol blue (Figure 1D), boiled, spun at 12,000 *xg* for 10 min at 4ºC, and the supernatants were transferred to a new 1.5-ml microcentrifuge tube. Approximately 20-25 µl of protein were quantified with the 2D Quant kit (Amersham Biosciences, USA), as recommended by the manufacturer. For the second of the two technical replicates from each aquarium (Figure 1E), the proteins were purified as described above except they were dissolved in rehydration buffer (8 M urea, 2% CHAPS; *i.e.*, "urea/CHAPS buffer" of Figure 1E) at room temperature (RT) for 2-3 hr, with constant, vigorous agitation. Approximately 20-25 µl of protein were quantified as described above, and proteins were frozen at -80ºC.

Given that the temperature regime itself, rather than the SO, was found to have a greater influence on coral physiology based on previous analyses (Table 1), proteins to be electrophoresed across two dimensions were pooled across SO. Given the oceanographic

differences between Houbihu and Houwan, future work should, however, seek to look at SO differences in addition to TT alone, as was done herein, in order to uncover how environmental history drives the future physiological response to altered abiotic conditions. After several days of storage at -80ºC, proteins dissolved in the initial urea/CHAPs buffer were thawed, and three samples (one from each aquarium) from each of the two SO from the same TT (n = 6 protein aliquots/TT) were mixed in equimolar concentrations:

Sample 1: 3 Houwan-stable TT samples + 3 Houbihu-stable TT samples = *1 stable TT* sample pooled across SO to be analyzed by 2D + MS.

Sample 2: 3 Houwan-variable TT samples + 3 Houbihu-variable TT samples = *1 variable TT* sample pooled across SO to be analyzed by 2D + MS.

These two, pooled protein samples were precipitated with 2 ml acetone supplemented with 0.07% beta-mercaptoethanol (BME) at -80ºC for 1 hr. Protein pellets from the stable and variable TT samples were washed thrice with acetone-BME, dried on the benchtop at RT, and dissolved in 150 µl of the rehydration buffer recommended by Jacobs et al. [30]: 9.5 M urea, 2% CHAPS, 0.5% carrier ampholytes (GE Healthcare, USA), and 65 mM dithiothreitol (DTT). Samples in this "urea rehydration buffer" (Figure 1E) were vortexed vigorously for several minutes, spun at 12,000 *xg* for 10 min at 4ºC, and the supernatants were transferred to new tubes and quantified (20-25 µl aliquots) as described above.

The remaining, un-solubilized protein pellets were dissolved in 150 µl of the "thiourea rehydration buffer" (Figure 1E) described by Jacobs et al. [30]: 2 M thiourea, 7 M urea, 4% CHAPS, 0.5% carrier ampholytes, and 65 mM DTT. In general, this allowed the remaining proteins to be solubilized. Then, 20-25 µl of these proteins were quantified as described above. Because preliminary experiments found that urea and thiourea rehydration buffer-dissolved proteins presented different profiles on 2D gels (data not shown), approximately 200 µg protein from each TT and solubilization buffer were mixed to yield 400 µg protein in urea + thiourea buffer for each of the two TT, a sufficient quantity for running 4 100-µg 2D gels (*i.e.*, four technical replicates/sample) for each of the two pooled protein samples.

2D Gel Electrophoresis 1st Dimension-Isoelectric Focusing

Isoelectric focusing (IEF) was used for the first dimension of the 2D gel with the Ettan IPGphor IEF system (Amersham Biosciences). Four gels were run for each of the two TT: stable and variable, and approximately 100 µg protein were loaded into each of the eight gels. Samples, which represented a mix of proteins from samples of both SO, as well as a mix of both urea and thiourea-based buffers, were diluted to 125 µl with the addition of thiourea buffer and, if necessary, additional carrier ampholytes to where the latter was at a final concentration of 0.5%. Proteins of each of the two TT were focused at the same time on different IEF strips (*i.e.*, one of the four stable TT protein samples was run at the same time as one of the four variable TT protein samples). Along the center of the bottom of the IEF strip holder, proteins (100 µg/TT; 125 µl) were loaded evenly from left to right, while simultaneously ensuring that there were no air bubbles. The protective membrane was removed from the IEF strip (pH 4-7, 7 cm, Amersham Biosciences), which was then placed into the strip holder with the gel side down. Then, 200 µl of dry strip cover fluid were

aliquoted over the strip, and the lid was placed over the strip holder. The two strip holder units (one for each of the two co-run samples) were placed in the Ettan IPGphor IEF electrophoresis chamber (Amersham Biosciences), and the following program was run at 20ºC: 50 V for 12 hr (rehydration), 300 V for 60 V-hr along a gradient, 600 V for 120 V-hr along a gradient, 1000 V for 500 V-hr along a gradient, 2000 V for 1000 V-hr along a gradient, 5000 V for 6000 V-hr, and 50 V for 10 hr. The same protocol was used on the three additional pairs of stable and variable TT samples, which were electrophoresed on different days.

2D Gel Electrophoresis 2nd Dimension-SDS-PAGE

Chromatography paper (Whatman, USA) was cut to a 1 x 0.5 cm size and overlaid with 5 µl protein marker (Fermentas PageRuler™ prestained protein ladder, Life Technologies). Then, ~ 1 ml of 1% agarose was aliquoted onto a smooth sheet of plastic wrap, and the chromatography paper was placed over the agarose. An additional 1 ml of 1% agarose was then overlaid on the chromatography paper. After the agarose solidified, the chromatography paper was removed from the agarose to where a 1 mm distance was maintained around the paper. Meanwhile, the IEF strips were immersed in equilibration buffer (6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl [pH 8.8], 0.002% bromophenol blue, and 1% DTT) at RT for 15 min. Then, strips were transferred to the same buffer, except with 1% iodoacetamide (IAA) instead of 1% DTT, for 15 min at RT, washed with SDS-PAGE running buffer to remove residual IAA, and placed on top of a 5-14% stacking-separating Tris-glycine SDS-PAGE gel. Electrophoresis was conducted on ice at 70 V for ~30 min and 120 V for 1-2 hr in a Mini-PROTEAN® Tetra cell (BioRad, USA), with two samples (one stable and one variable) run at the same time. In total, eight 2D gels were run (four technical replicates for each of two TT), though only two gels were run at any given time (*i.e.*, four days were required to run all eight gels).

Each of the eight gels was fixed in 50% methanol and 7% acetic acid for 30 min after removing the stacking gel. Then, the gels were stained with SYPRO® Ruby (Life Technologies) on a shaker table in the dark overnight. The gels were then destained in 10% methanol and 7% acetic acid for 30 min and imaged with a Typhoon Trio™ scanner (GE Healthcare) at 312 nm (aperture = 2.8, exposure time = 2.4 s). In general, there were no unique protein spots between the stable and variable TT (Figure 2A-B), though this could be due to the low amount of protein loaded (100 µg per gel). Since *Symbiodinium* density was similar between TT (Table 1), the protein spot intensity values of each of the eight gels (data not shown) were *not* normalized to a genome copy proportion (GCP) prior to the subtraction step (described below). In contrast, the target protein (RBCL) data, described below, warranted the use of a GCP given that both SO and TT effects were tested in that analysis, and a difference in *Symbiodinium* density between SO was documented previously (Table 1 and [15]).

Image analysis software (ImageQuantTL) provided with the scanner was used to perform a "subtraction" whereby the gel image of one stable TT replicate sample was overlaid on the variable TT replicate run and processed simultaneously to better portray differentially expressed proteins (Figure 2C-D). This subtraction was performed on the four pairs of stable vs. variable TT run on four different days. Because no unique spots were evident in any of the

four pairs of gels, proteins found to be expressed at significantly higher quantities by ImageQuantTL in the stable temperature gel were instead targeted, and 10 protein spots that were found to be over-expressed in all four stable TT gels (*i.e.*, all technical replicates; Figure 2C) relative to their variable temperature counterparts were excised from a representative stable TT gel (Figure 2D) with sterilized 200-µl pipet tips and placed into 1.5-ml microcentrifuge tubes.

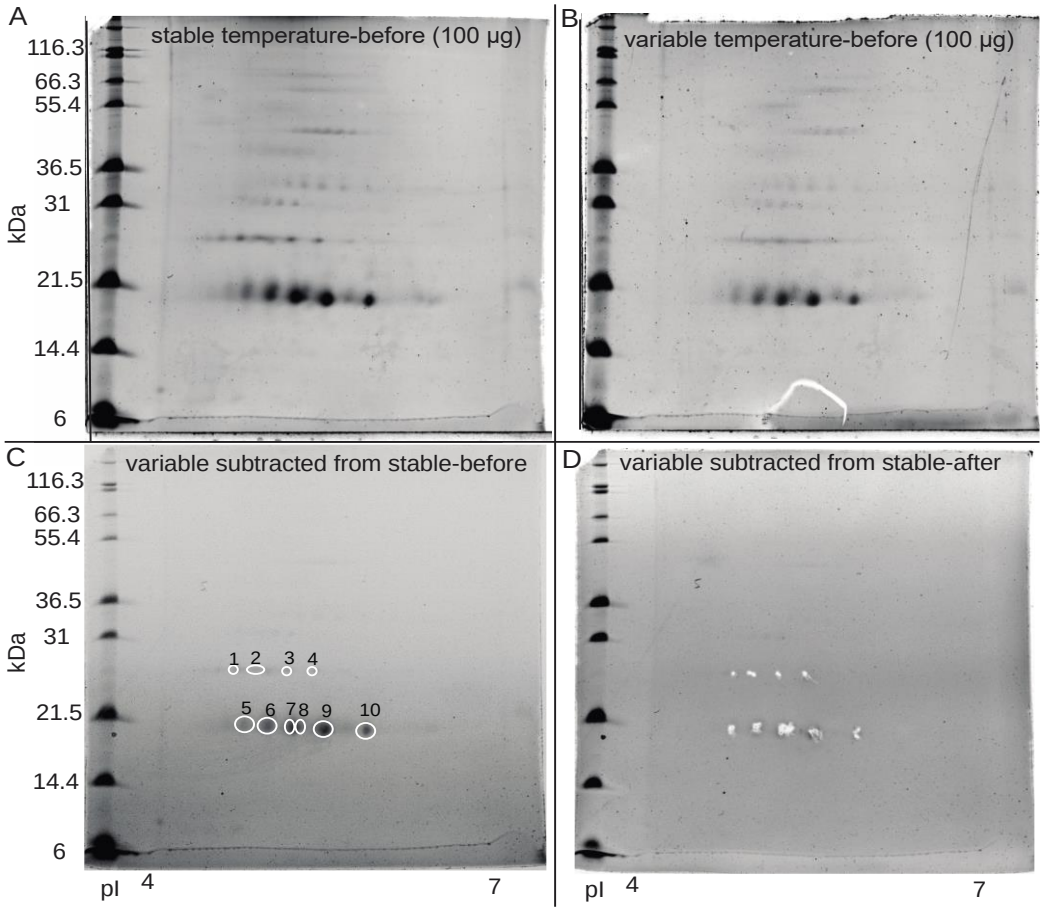

Figure 2. 2-dimensional gel electrophoresis of proteins expressed by *Seriatopora hystrix* specimens exposed to either a variable or stable temperature regime for seven days. Proteins dissolved in a urea + thiourea-based buffer were pooled between the two sites of origin (SO), Houbihu (upwelling site) and Houwan (non-upwelling site), and electrophoresed across two dimensions as described in the text. A representative 2D gel out of the four that were run for each temperature treatment (TT) has been shown for both stable (A) and variable (B) temperature specimens. Although no proteins appeared to have been solely expressed by one treatment, a total of 10 protein spots (circled in C) were found to be over-expressed by samples of the stable TT by image analysis software, and these protein spots were extracted from the gel with sterilized pipet tips (D), processed as described in the text, and submitted for sequencing by mass spectrometry (MS). The y-axis labels in (A) and (C) are shared with (B) and (D), respectively, while the x-axis labels in (C) and (D) are shared with (A) and (B), respectively. "Before" and "after" refer to before and after removing the protein spots, respectively, with sterilized pipet tips. pI = isoelectric point. kDa = kilodalton.

The ten excised protein spots were destained and in-gel digested as follows. First, the protein + gel slabs were washed in 50% acetonitrile in 25 mM ammonium bicarbonate (pH 8.5). Then, they were incubated in 100 µl of the same acetonitrile solution for 15 min and spun at 10,000 *xg* for 1 min. The supernatant was removed and replaced with 100 µl of 100% acetonitrile, and the samples were incubated for 5 min. The samples were spun again as above, and the supernatant was removed. The gel bits were allowed to dry for 5 min before incubation with 30 µl trypsin (a 2 µg aliquot that had been re-suspended in 1 ml water and 1 ml 50 mM ammonium bicarbonate) at 37ºC overnight. The next day, samples were centrifuged at 10,000 *xg* for 1 min, and the supernatant was transferred to a new microcentrifuge tube. Then, 50 µl of 50% acetonitrile and 5% trifluoroacetic acid (TFA) were added to the remaining samples, which were then sonicated 10 times (10 s each time). Samples were centrifuged again at 10,000 *xg* for 1 min, and the supernatant was combined with the supernatant from the first spin. Another round of 50% acetonitrile/5% TFA incubation followed by sonication/spinning/supernatant collection was conducted, and the third supernatant was combined with the previous two. The supernatant was dried for 1-2 hr prior to shipping to the MS facility at Kaohsiung Medical University's (KMU) Center for Research Resources and Development's Core Proteomics facility, where the 10 protein spots were analyzed by MS, as described below.

MS

After trypsin digestion, 2 µl of the digested peptides were injected into the nano-liquid chromatography (LC) system and detected by an LTQ Orbitrap Discovery Hybrid Fourier Transform Mass Spectrometer (FTMS; Thermo-Fisher, USA) at a resolution of 30,000 coupled with a nanospray source that was executed in the positive ion mode. The Nano-UPLC system (nanoACQUITY UPLC) was purchased from Waters (USA), as were the desalting (Symmetry C18, 5 µm x 180 µm x 20 mm) and analytical (BEH C18, 1.7 µm x 75 µm x 150 mm) columns. The peptide eluate from the column was directed to the nanospray source, and the MS was operated in positive ion, data-dependent mode.

MS Data Analysis

Raw data files (mascot generic format [.mgf]) were processed with Mascot distiller software (version 2.2, Matrix Science, USA) and then uploaded onto the Mascot server hosted by KMU. Several Mascot protein databases (Tables 3-4) were queried using Mascot's default search parameters. Comparison of MS data against the NCBI's nr database via Mascot yielded mainly common protein contaminants (human keratins, actins, etc.). However, upon comparing spectral data against the *Hydra magnipapillata* and *Acropora digitifera* (coral) proteomes, as well as a suite of others, a variety of both host coral and *Symbiodinium* peptides were identified. The *A. digitifera* proteome was conceptually translated previously and converted into a Mascot searchable database by Li et al. [31] and is referred to as "NMMBA" in Tables 3-4.

One of two criteria was required to have been met to determine verification of "presence" of a protein: either 1) 15 consecutive amino acid (AA) residues were sequenced or 2) two unique peptides mapping to the same protein were sequenced, and the total length of both

peptides was 15 AA or more. After using Mascot to determine the likely identity of each protein, individual peptide sequences were BLASTed (BLASTp) against the NCBI database to further verify the identities of the sequenced proteins. Proteins fulfilling the minimum criteria established *a priori* were assigned a functional category from the "Pfam" database, and 2-sample proportion tests were used to determine whether proportional differences existed between compartments (coral vs. *Symbiodinium*) in the functional categories in which the identified proteins were grouped. Two-sample proportion tests were also used to determine whether was compartment was over-represented in each of the 10 spots. Bacterial proteins were excluded from analysis, though should be more carefully considered in future works given the importance of probiotic microbes in maintaining coral health.

Western Blotting

Given that significant differences in ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene expression were documented across TT in the SHVTS [15], the respective protein, RBCL, was targeted herein for expression analysis with western blotting with a commercially available antibody from Agrisera (Sweden). Proteins (20 µg) representing one sample from each of the 12 experimental aquaria (n = 3 for each of the four SO x TT groups), as well as positive controls (30 µg protein from *Pocillopora damicornis* larvae exposed to ambient temperature and pCO_2 from Putnam et al. [7]), were electrophoresed on two 4-10% SDS-PAGE gels as in Mayfield et al. [12]; one SDS-PAGE gel was stained with SYPRO Ruby as described above for staining of the 2D gels and visualized on a Typhoon Trio scanner for assessment of protein quality. Proteins within the second gel were transferred to a polyvinylidene fluoride (PVDF) membrane on ice at 100 V for 75 min. Afterwards, the protein-laden membrane was stained with Ponceau S (Sigma, USA) according to the manufacturer's recommendations in order to visualize degree of protein transfer.

De-stained PVDF membranes were blocked in 5% skim milk (w/v) in Tris-buffered saline with Tween-20 (TBST; 100 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) for 1 hr at RT. The blocking buffer was decanted, and 10 ml of a 1:5000 dilution of a RBCL primary antibody (forms I and II, Agrisera) in 5% skim milk (w/v) in TBST were added to the membranes, which were then incubated for 2 hr with gentle agitation at RT. This antibody has been used successfully for detection of the RBCL of *Symbiodinium* [26], as well as other dinoflagellates [32-33]. Membranes were washed thrice (10 min each) with TBST and then incubated in 10 ml of a 1:5000 dilution of goat anti-rabbit secondary antibody (Millipore, Germany) in TBST for 5 min and washed with TBST as above. Membranes were then stained with 400 µl SuperSignal® West Pico Chemiluminescent Substrate Kit chemiluminescent reagent (Thermo-Scientific), and the chemiluminescent signal was immediately visualized on a Fusion FX7 (Vilber Lourmat, France) gel doc under the chemiluminescence setting.

ImageJ (National Institutes of Health, USA) was used to quantify RBCL protein band intensity, and values (arbitrary units) were first divided by the intensity of the positive control band on the respective gel. These gel-normalized values were then divided by the respective *Symbiodinium* GCP for each sample, which was previously calculated [15] and is routinely used to control for variable ratios in host: *Symbiodinium* biological material between samples [34]. The effects of SO, TT, and the SO x TT interaction on GCP-normalized RBCL expression were tested with a 2-factor ANOVA, which was performed with JMP® (ver.

11.1.1, SAS Institute, USA) after having log-transformed the data due to lack of normality. Then, JMP was used to determine the statistical significance of the correlation between *rbcL* mRNA expression (measured previously [15] in the same samples from which proteins were extracted herein) and the RBCL protein expression quantified herein. It was hypothesized that a significant, positive, linear relationship would be documented between expression levels of these two molecules for both TT (n = 6 protein samples/TT), as well as across the dataset as a whole (n = 12 protein samples), and an α level of 0.05 was set for all aforementioned statistical tests.

RESULTS

Differentially Expressed Proteins Uncovered by a 2D + MS-Based Approach

It is evident from Tables 3 and 4, as well as Figure 3, that a number of different cellular processes were affected by exposure to variable temperature. Of the coral host's differentially expressed proteome (DEP; Tables 3 and S1), 18 Pfam categories could be identified, and these groupings encompassed 62 of the 75 proteins identified (83%). The only functional categories in which more than five proteins were identified with confidence (Figure 3) were DNA binding/transcription factor (n = 8; 10.7%), metabolism (n = 8; 10.7%), and mRNA processing (n = 15; 20%). In contrast, only one transcription factor (2%) was under-expressed in *Symbiodinium* samples of the variable TT (Tables 4 and S2), and this zinc finger transcription factor was one of only two proteins found in the DEPs of both compartments (the other being the pre-mRNA splicing factor CWC22). However, the degree of homology of these proteins across compartments could not be ascertained due to the short nature of the sequenced peptides (17-20 AA).

There were only two functional categories that encompassed multiple proteins and were represented in each of the two eukaryotic DEPs of this association: metabolism and mRNA processing. Regarding the latter, although mRNA processing is surely a cellular process that could be hypothesized to undergo differential regulation during periods of temperature change, the fact that the majority of the proteins aligned most closely to published fungal proteins precluded the ability to confidently ascribe them to one compartment; in fact, many were equally homologous to bacterial proteins (see annotations in Tables 3-4.). Therefore, while these mRNA processing proteins are *likely* involved in the coral and/or *Symbiodinium* response to variable temperatures, their relative importance has been downplayed in this manuscript until longer peptide sequences can be obtained and the compartment of origin more confidently assigned.

Regarding the second functional category that featured multiple proteins for both eukaryotic compartments of this reef-building coral, eight and nine metabolism-targeted proteins were over-expressed in stable TT samples of the host coral and *Symbiodinium* compartments, respectively (Figure 3), and one such *Symbiodinium* protein, lipoxygenase, is known to play a role in lipid metabolism. Also pertaining to lipids/lipid metabolism, one process that was over-represented in the *Symbiodinium* DEP relative to the host coral one was lipid bodies (LBs); nearly 1/3 of the *Symbiodinium* DEP (Figure 3) was comprised of proteins involved in stabilization and metabolism of LBs, notably oleosins and caleosins.

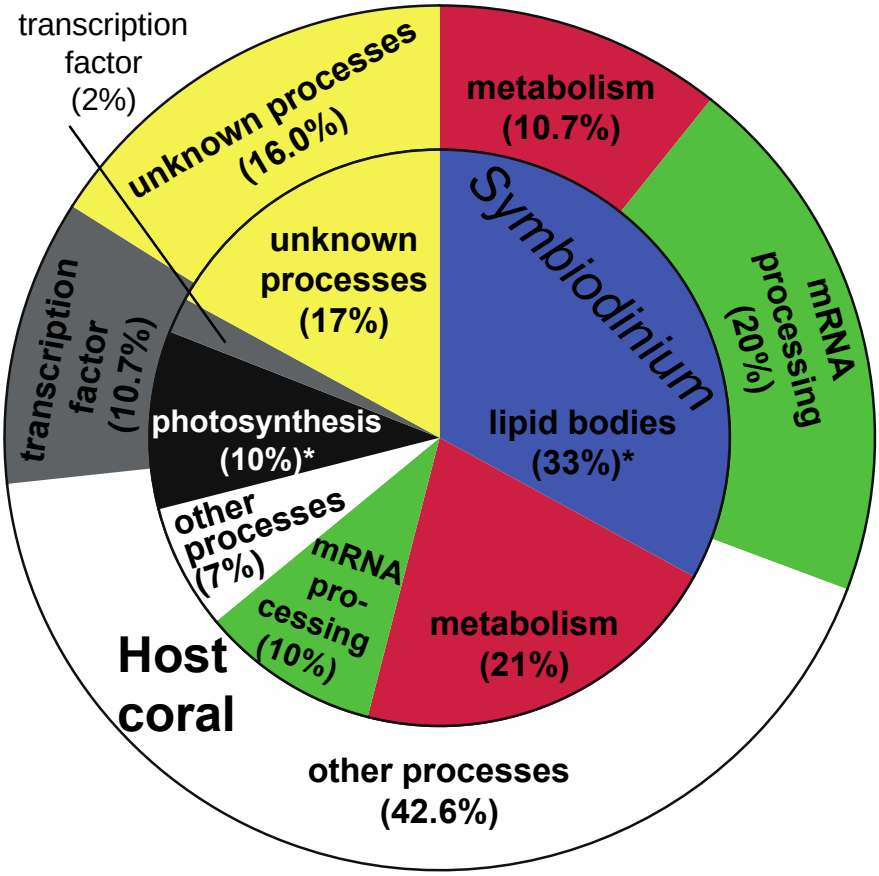

Figure 3. Functional distribution of the host coral and *Symbiodinium* differentially expressed proteins. Percentage breakdown of Pfam functional groups encompassing the 75 host coral (outer pie graph) and 42 *Symbiodinium* (inner pie graph) proteins that were over-expressed in samples exposed to a stable temperature regime. Functional categories that were over-represented in the *Symbiodinium* differentially expressed proteome (DEP) relative to the host DEP (2-sample proportion test, $p < 0.05$) have been marked with an asterisk (*).

RBCL Western Blot

The *Symbiodinium* populations (clade C only [15, 17]) housed within corals exposed to the variable TT for seven days expressed the RBCL protein (Figure 4) at similar levels between the four SO x TT groups (n = 3; Figure 4B-C), and, furthermore, there was no significant, positive correlation between *rbcL* mRNA and RBCL protein expression across the 12 samples of the SHVTS collected after seven days of treatment exposure (Figure 4D).

Table 2. A breakdown of the 10 sequenced protein spots by compartment of origin: coral host or *Symbiodinium*. Of the 117 unique peptides that were sequenced and met the minimal inclusion threshold criteria (described in the main text), 75 (64%) were from the coral host, *Seriatopora hystrix*, and 42 (36%) were from the dinoflagellate endosymbionts (genus *Symbiodinium*) living within the hosts' gastrodermal cells. Two 2-sample proportion tests were conducted to determine if one compartment (host coral or *Symbiodinium*) was over-represented in the partially sequenced proteome within each spot; for the first test, the raw proportions were compared (non-adjusted). For the second, the total number of *Symbiodinium* proteins was multiplied by 1.8 to adjust for the fact that the host contributed 75 of the 117 unique proteins (i.e., 64% host/36% *Symbiodinium* = 1.8) across all 10 spots. For the "Total/Average" row, the total number of proteins is given for the 3rd-5th columns while the average percentages are given for the "% host" and "% *Symbiodinium*" columns; error terms represent standard deviation for the latter. NS = not significant (2-sample proportion test, $p > 0.05$). NA = not applicable. kDa = kilodalton. pI = isoelectric point

Spot	Molecular weight (kDa)	pI	# host proteins	# *Symbiodinium* proteins	# total proteins	% host	% *Symbiodinium*	2-sample proportion test *p* (non-adjusted)	2-sample proportion test *p* (adjusted)	Conclusion
1	27.4	4.9	6	7	13	46	54	NS	NS	
2	27.3	5.0	12	7	19	63	37	NS	NS	
3	27.4	5.2	14	4	18	78	22	<0.001	<0.05	host > *Symbiodinium*
4	27.3	5.5	13	4	17	77	23	<0.01	NS	
5	20.8	4.8	16	11	27	59	41	NS	NS	
6	21.1	5.1	6	8	14	43	57	NS	<0.05	*Symbiodinium*>host
7	20.9	5.3	8	6	14	57	43	NS	NS	
8	20.7	5.3	10	8	18	56	44	NS	NS	
9	20.4	5.5	9	5	14	64	36	NS	NS	
10	20.0	5.9	6	8	14	47	53	NS	<0.05	*Symbiodinium* > host
Total/Average			100	68	168	59 ± 12	41 ± 12	NA	<0.0001	Host > *Symbiodinium*
Unique			**75**	**42**	**117**	**64**	**36**	NA	<0.0001	Host > *Symbiodinium*

Table 3. Seventy-five host coral proteins expressed at higher levels in specimens of the stable temperature treatment in the 2010 *Seriatopora hystrix* variable temperature study. For proteins found in multiple spots, the range of molecular weights (in kilodaltons [kDa]) and isoelectric points (pI) have been provided. The average total length of all peptides mapping to a unique protein was 43 ± 33 (standard deviation for this and all values henceforth) amino acids (AA), and ranged from 15 (the *a priori*-set, lower cut-off value) to 212 AA (serine/arginine repetitive matrix protein 2). Coverage (number of sequenced AA/total AA in the hypothesized, full-length protein x 100) averaged 7.4 ± 8.2% and ranged from 1 to 43%. Please see Table S1 for the associated peptide sequences

Protein name	Total length of all peptides	Spot(s)	Predicted/actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
Cell surface proteins (n = 4)									
fibrocystin	100	1	678/27.4	8.2/4.9	NMMBA	*Saccoglossus kowalevskii*	19	A7SPV0	1
integrin α	69	7	112/20.9	5.6/5.3	Acropora	*Acropora millepora*	17	ABY74498	6
integrin β2	36	5-6	85.0/20.8-21.1	5.3/4.8-5.1	Acropora	*A. millepora*	14	ABY74499	4
H6 G-protein coupled receptor	19	9	34.7/20.4	9.7/5.5	Acropora	*A. millepora*	15	ABI50929	6
Circadian rhythm (n = 1)									
cryptochrome photolyase (CRY2)	91	3, 6-7, 10	59.9/20.0-27.4	8.8/5.1-5.9	Acropora	*A. millepora*	16	ABP97099	17
Cytoskeleton (n = 2)									
ciliary dynein heavy chain	21	5	525/20.8	5.7/4.8	nr other metazoa	*Pediculus humanus corporis*	38	XP_002426312	1
stathmin	19	5	34.7/20.8	9.8/4.8	nr other metazoa	*Trichoplax adhaerens*	50	XP_002114295	5
Development (n = 2)									
hedgehog	30	2	90.9/27.3	6.4/5.0	NMMBA	*Acropora cervicornis*	41	GASU01035919	43
embryonic polarity protein dorsal	19	7	93.2/20.9	5.4/5.3	nr other metazoa	*Culex quinquefasciatus*	42	XP_001844078	2
DNA binding/transcription factor (n = 8)									
nuclear receptor AmNR2	63	3, 8	49.9/20.7-27.4	6.0/5.2-5.3	Acropora	*A. millepora*	34	AF323681_1	14
sex comb on midleg-like protein 2	50	7-8	117/20.7-20.9	8.3/5.3	*Hydra magnipapillata*	*H. magnipapillata*	34	XP_002160709	3
nuclear receptor 6	49	1, 2, 10	38.8/20-27.4	8.3/4.9-5.9	Acropora	*A. millepora*	18	AF323686_1	13
estrogen receptor-β	48	2, 4	59.2/27.3	8.8/5.0-5.5	Selenoprotein	*H. sapiens*	29	ESR2_HUMAN	8
topoisomerase (DNA) III β	36	8	153/20.7	8.6/5.3	*H. magnipapillata*	*H. magnipapillata*	23	XP_002155556	2
zinc finger protein	26	10	71.3/20.0	9.2/5.9	*H. magnipapillata*	*H. magnipapillata*	32	XP_012561483	4
nuclear receptor AmNR8	18	3	42.5/27.4	8.8/5.2	Acropora	*A. millepora*	16	AF323688_1	4
DM domain protein	17	2, 9	51.5/20.4-27.3	9.0/5.0-5.5	Acropora	*A. millepora*	16	AF530064_1	3
DNA Repair (n = 1)									
Fanconi anemia group J protein	50	2	125/27.3	6.3/5.0	NMMBA	*Danio rerio*	36	K7GJE0	4
Growth factor (n = 1)									
imaginal disc growth factor	18	3-4, 9	49.4/20.4-27.4	6.7/5.2-5.5	nr other metazoa	*Diaprepes abbreviates*	41	AAV68692	4
Glycoprotein (n = 1)									
mucin-17	98	9	574/20.4	5.4/5.5	*H. magnipapillata*	*H. magnipapillata*	26	XP_002160582	2
Immunity (n = 1)									
beta-defensin	22	2	11.8/27.3	9.1/5.0	Defensin	*Canis lupus*	26	Q30KS7	22

Protein name	Total length of all peptides	Spot(s)	Predicted/actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
Metabolism (n = 8)									
thioredoxin reductase (isoform 1)	54	3, 5	56.2/20.8-27.3	7.2/4.8-5.2	Selenoprotein	*H. sapiens*	14	AF044212_1	10
porphobilinogen deaminase	39	6	39.3/21.1	6.7/5.1	SwissProt	*H. sapiens*	24	HEM3_HUMAN	11
carboxylesterase 1C	37	3	61.0/27.4	5.0/5.2	SwissProt	*Mus musculus*	55	EST1C_MOUSE	7
carbamoyl-phosphate synthetase/aspartate transcarbamylase/ dihydroorotase	29	9	101/20.4	6.8/5.5	*H. magnipapillata*	*H. magnipapillata*	19	NP_001267868	3
alpha-L-arabinofurano-sidase B	27	9	35.6/20.4	8.9/5.5	*H. magnipapillata*	*H. magnipapillata*	28	XP_002165874	8
phospholipase D	21	4	104/27.3	8.5/5.5	nr other metazoa	*Schistosoma mansoni*	38	XP_002576773	2
cytochrome 450	19	4	56.9/27.3	9.0/5.5	nr other metazoa	*T. adhaerens*	38	XP_002112306	3
mannosyl-glycoprotein endo-beta-N-acetylglucosamidase[a]	15	1	37.7/27.4	9.3/4.9	*Staphylococcus aureus*	*S. aureus*	23	WP_000247512	4
Mitosis (n = 1)									
inner centromere protein	17	4	112/27.3	9.3/5.5	*H. magnipapillata*	*H. magnipapillata*	26	XP_002167890	2
mRNA Processing (n = 15)									
serine/arginine repetitive matrix protein 2	212	5	295/20.8	12/4.8	SRProtein	*M. musculus*	22	SRRM2_MOUSE	7
pre-mRNA-splicing factor CWC22	127	3	65.3/27.4	9.3/5.2	SRProtein	*Bombus impatiens*	36	XP_003494375	22
pre-mRNA splicing factor prp8[b]	67	4	326/27.3	8.4/5.5	SRProtein	*Trichophyton tonsurans*	28	EGE00188	2
pre-mRNA-splicing factor CDC5/ CEF1b	55	5	87.0/20.8	6.5/4.8	SRProtein	*Pseudogymnoascus destructans*	22	ELR07040	7
transformer-2 protein homolog beta-like	52	7	82.2/20.9	11.6/5.3	TRA2-beta	*Salpingoeca rosetta*	14	EGD83325	7
pre-mRNA-splicing ATP-dependent RNA helicase prp28[b]	51	5	88.3/20.8	8.8/4.8	SRProtein	*Neosartorya fischeri*	35	PRP28_NEOFI	6
ATP-dependent RNA helicase DHX8-like	51	3	137/27.4	8.5/5.2	SRProtein	*S. kowalevskii*	22	XP_002734274	4
CWC25 spliceosome-associated protein homolog	42	5	58.1/20.8	10.0/4.8	SRProtein	*S. kowalevskii*	17	XP_002732008	8
pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16	37	5	144/20.8	6.4/4.8	SRProtein	*Oryzias latipes*	27	XP_004067034	3
pre-mRNA-splicing factor SYF1[b]	32	4	41.8/27.3	5.5/5.5	SRProtein	*Rhizoctonia solani*	31	CCO31421	8
KH domain containing, RNA-binding, signal transduction-associated 3	29	5	38.3/20.8	6.5/4.8	*H. magnipapillata*	*Hydra vulgaris*	45	XP_002163769	8
pre-mRNA-splicing factor CWC25[b]	29	4	61.0/27.3	10.2/5.5	SRProtein	*Ustilago maydis*	21	EAK85329	5
myb-related protein cdc5[b]	20	4	87.7/27.3	6.4/5.5	SRProtein	*Claviceps purpurea*	17	CCE33636	2
serine/arginine-rich splicing factor 7 isoform 1	18	4	27.4/27.3	11.8/5.5	SRProtein	*M. musculus*	28	NP_666195	7
bud13[b]	16	5	32.5/20.8	9.5/4.8	SRProtein	*Zygosaccharomyces rouxii*	22	CAR30797	5

Table 3. (Continued)

Protein name	Total length of all peptides	Spot(s)	Predicted/actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
MUSCLE (n = 2)									
dumpy CG33196-PB	92	8, 10	456/20-20.7	5.3/5.3-5.9	*H. magnipapillata*	*H. magnipapillata*	31	XP_002167485	2
dystrophin (isoform 2)	18	5	206/20.8	7.3/4.8	SwissProt	*Drosophila melanogaster*	41	DMDD_DROME	1
Protein quality control (n = 4)									
UDP-glucose: glycoprotein glucosyltransferase 2	131	6, 8	175/20.7-21.1	6.4/5.1-5.3	Selenoprotein	*H. sapiens*	18	NP_064506	9
E3 ubiquitin protein ligase	43	2	59.4/27.3	7.1/5.0	NMMBA	*Meleagris gallopavo*	38	B3RW44	7
ubiquitin carboxyl-terminal hydrolase 17-like protein 10	36	2, 5	59.8/20.8-27.3	8.5/4.8-5.0	Defensin	*H. sapiens*	40	I3L0E4	7
small heat shock protein	35	1	26.4/27.4	5.8/4.9	H. magnipapillata	*H. magnipapillata*	21	T2MHG6	15
Signaling/hormones (n = 5)									
serine/threonine-protein kinase ATR	46	8	190/20.7	8.3/5.3	*H. magnipapillata*	*H. magnipapillata*	27	XP_002160018	3
tyrosine kinase receptor	45	6-9	200/20.4-21.1	5.9/5.1-5.5	*H. magnipapillata*	*H. magnipapillata*	33	XP_002169535	3
baculoviral IAP repeat-containing protein	19	1	512/27.4	5.8/4.9	nr other metazoa	*Nasonia vitripennis*	24	K7JAH7	<1
receptor-type tyrosine-protein phosphatase alpha	18	9	90.5/20.4	6.3/5.5	SwissProt	*H. sapiens*	31	78PTPRA_HUMAN	2
serine/threonine-protein kinase PLK4-like	15	9	76.5/20.4	8.9/5.5	nr other metazoa	*S. kowalevskii*	30	XP_002734025	2
Transcription (n = 1)									
RNA binding motif protein	69	2-3, 8	55.8/20.7-27.4	10.0/5.0-5.3	TRA2-beta	*H. sapiens*	14	P0DJD3	10
Translation (n = 2)									
ribosomal protein S4	39	6	29.4/21.1	10.3/5.1	*H. magnipapillata*	*H. vulgaris*	30	XP_002154886	15
eukaryotic translation initiation factor 3 subunit	17	10	109/20.0	5.3/5.9	SwissProt	*Vanderwaltozyma polyspora*	24	EIF3A_VANPO	2
Transport (n = 4)									
apolipoprotein	58	3	30.6/27.4	5.6/5.2	SwissProt	*M. musculus*	97	APOA1_MOUSE	21
ATP binding cassette sub-family A	54	4	204/27.3	9.2/5.5	*H. magnipapillata*	*H. magnipapillata*	20	XP_002157466	3
serotransferin	48	3	76.7/27.4	6.9/5.2	SwissProt	*M. musculus*	59	TRFE_MOUSE	7
SEC14-like protein 1	25	2, 10	106/20-27.3	8.5/5.0-5.9	*H. magnipapillata*	*H. magnipapillata*	29	T2M7V3	3
Unknown function (12)									
viral A-type inclusion protein[a]	95	5	332/20.8	5.1/4.8	*H. magnipapillata*	*H. magnipapillata*	61	XP_002156044	3
kinesin motor domain + hypothetical protein	86	1	148/27.4	4.9/4.9	NMMBA	*Bombyx mori*	21	Q237L2	6
retrotransposon-like	47	2	141/27.3	9.2/5.0	*H. magnipapillata*	*H. magnipapillata*	22	T2MJH3	4
uncharacterized protein (putative apolipoprotein)	45	2	477/27.3	8.1/5.0	*H. magnipapillata*	*H. magnipapillata*	24	T2MHF5	1
hypothetical protein	32	5	32.3/20.8	6.0/4.8	Acropora	*A. millepora*	16	ACJ64664	10
selenoprotein	30	8	10.5/20.7	9.7/5.3	Selenoprotein	*H. sapiens*	14	2Q2F_A	33
hypothetical protein	30	3	121/27.4	6.5/5.2	*H. magnipapillata*	*H. magnipapillata*	16	XP_002160564	2

Protein name	Total length of all peptides	Spot(s)	Predicted/actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
hypothetical protein with galactose binding lectin domain 1	26	4	24.8/27.3	7.5/5.5	Acropora	*A. millepora*	21	ACJ64660	11
hypothetical protein with galactose binding lectin domain 2	20	7	30.9/20.9	6.8/5.3	Acropora	*A. millepora*	14	ACJ64658	6
hypothetical protein	17	3, 5, 8	35.2/20.7-27.4	8.5/4.8-5.3	Acropora	*Acropora tenuis*	14	BAE46797	5
hypothetical protein	16	7	47.9/20.9	5.3/5.3	*H. magnipapillata*	*H. magnipapillata*	26	XP_002163478	3
B-cell CLL/lymphoma 11A	15	3	90.1/27.4	7.6/5.2	nr other metazoa	*Tribolium castaneum*	35	XP_975280	2

[a] May be of bacterial/viral origin. [b] May be of *Symbiodinium* origin.

Table 4. Forty-two *Symbiodinium* proteins expressed at higher levels in specimens of the stable temperature treatment in the 2010 *Seriatopora hystrix* variable temperature study. For proteins found in multiple spots, the range of molecular weights (in kilodaltons [kDa]) and isoelectric points (pI) have been provided. The average total length of all peptides mapping to a unique protein was 36 ± 21 (standard deviation for this and all values henceforth) amino acids (AA), statistically similar to that of the host coral (43 ± 33; student's *t*-test, p > 0.05), and the total number of mapped AA per protein ranged from 16 to 135 AA (polyADP ribose polymerase). Coverage averaged 10.2 ± 6.4%, statistically similar to that of the coral host (7.4 ± 8.1; p > 0.05), and ranged from 2 to 25%. Please see Table S2 for the associated peptide sequences

Protein name	Total length of all peptides	Spot(s)	Predicted/actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
DNA Binging/Transcription Factor (n = 1)									
zinc finger transcription factor PEI1	20	1	28.2/27.4	6.8/4.9	32010	*Boechera stricta*	16	Q6WEQ1	8
DNA replication/repair (n = 2)									
polyADP ribose polymerase	135	1	287/27.4	5.9/4.9	SRProtein	*Acanthamoeba castellanii*	45	L8HDR5	5
chromosome segregation protein[a]	20	10	9.4/20.0	4.2/5.9	*Staphylococcus aureus*	*S. aureus*	21	WP_000294226	24
Extracellular matrix protein (n = 1)									
tenascin precursor	18	1	58.5/27.4	9.8/4.9	NMMBA	*Hordeum vulgare*	25	A7RGF8	3
Lipid bodies (n = 14)									
caleosin-related[b]	70	5-7	36.5/20.8-21.1	5.2/4.8-5.3	32011	*Moniliophthora perniciosa*	14	EEB94283	19
Caleosin	53	2, 5	23.8/20.8-27.3	9.6/4.8-5.0	32011	*Arabidopsis thaliana*	16	NP_173738	25
caleosin-related	51	5, 8	30.1/20.7-20.8	8.7/4.8-5.3	32011	*Setaria italic*	29	XP_004985161	18
caleosin-related[b]	49	5	60.3/20.8	8.9/4.8	32011	*Mucor circinelloides*	16	EPB88832	8
Oleosin	33	2, 7, 9-10	17.5/20.0-27.3	9.7/5.0-5.9	32010	*Persea americana*	19	AGT63296	21
caleosin-related[b]	28	7, 9-10	25.5/20.0-20.9	7.2/5.3-5.9	32011	*Parastagonospor nodorum*	20	EAT77728	12
caleosin-related[b]	24	8	28.5/20.7	7.1/5.3	32011	*Sporisorium reilianum*	16	CBQ73148	9
Oleosin	23	10	17.5/20.0	9.7/5.9	32010	*P. americana*	16	AGT63296	15
18 kDa oleosin	22	9	28.2/20.4	11.2/5.5	32010	*Zea mays*	13	ACG48647	8
oleosin	22	1	23.6/27.4	11.3/4.9	32010	*Brassica oleracea*	16	AAD24547	10

Table 4. (Continued)

Protein name	Total length of all peptides	Spot(s)	Predicted/actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
oleosin S4-4	20	6	23.0/21.1	9.1/5.1	32010	*Brassica napus*	14	ACG69510	9
pollen coat oleosin-glycine-rich protein	19	1, 5, 7-8	21.8/20.7-27.4	9.7/4.8-5.3	32010	*Sisymbrium irio*	25	Q6V5I9	8
steroleosin-B	17	10	39.1/20.0	6.2/5.9	32012	*Oryza sativa*	13	AAT77030	5
18.2 kDa oleosin	16	5	17.9/20.8	9.3/4.8a	32010	*Gossypium hirsutum*	18	AAA18524	9
Metabolism (n = 9)									
peptide methionine sulfoxide reductase	54	2, 6, 8	28.4/20.7-27.3	8.9/5.0-5.3	32010	*B. napus*	19	P54151	21
fructose-bisphosphate aldolase, chloroplastic-like[a]	42	10	33.0/20.0	4.9/5.9	*S. aureus*	*S. aureus*	19	YP_005326917	14
threonine synthase[a]	42	5, 8, 9	37.8/20.4-20.8	6.0/4.8-5.5	*S. aureus*	*S. aureus*	28	WP_001581605	12
deoxyribose-phosphate aldolase 1[a]	33	5	23.5/20.8	4.7/4.8	*S. aureus*	*S. aureus*	26	WP_001617202	15
succinyl-diaminopimelate desuccinylase[a]	32	6	45.1/21.1	4.6/5.1	*S. aureus*	*S. aureus*	16	YP_041474	8
acetyl-CoA acetyltransferase[a]	31	5	41/20.8	6.2/4.8	*S. aureus*	*S. aureus*	23	WP_001070664	8
8-hydroxyquercetin 8-O-methyltransferase-like isoform 1	23	2, 6	40.6/21.1-27.3	5.3/5.0-5.1	nr plant	*Glycine max*	39	XP_003527405	6
hydroxyethylthiazole kinase[a]	22	8	28.4/20.7	4.6/5.3	*S. aureus*	*S. aureus*	28	WP_001108492	8
lipoxygenase	21	1, 6	95.3/21.1-27.4	7.2/4.9-5.1	nr plant	*O. sativa*	34	NP_001055143	2
mRNA processing (n = 4)									
pre-mRNA-splicing factor ATP-dependent RNA helicase PRP43	63	5	81.2/20.8	9.6/4.8	SRProtein	*Chondrus crispus*	37	CDF37591	9
mRNA processing-related protein	41	1, 3-5	65.3/20.8-27.4	5.7/4.8-5.5	SRProtein	*Cryptococcus neoformans*	19	P0CR52	7
putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16[a]	28	3	119/27.4	5.8/5.2	SRProtein	*Galdieria sulphuraria*	27	EME29213	2
pre-mRNA-splicing factor CWC22[a,b]	18	4	64.9/27.3	5.1/5.5	SRProtein	*Entamoeba dispar*	36	EDR21607	3
Photosynthesis (n = 4)									
light harvesting protein, isoform 1	54	4, 7	29.0/20.9-27.3	8.0/5.3-5.5	Acropora	*Symbiodinium sp.*	15	CBI83412	18
light harvesting protein, isoform 2	46	6	49.3/21.1	8.9/5.1	Acropora	*Symbiodinium sp.*	45	CBI83416	9
light harvesting protein, isoform 3	41	7-8	26.7/20.7-20.9	9.1/5.3	Acropora	*Symbiodinium sp.*	58	CBI83417	12
light harvesting protein, isoform 4	39	9	44.8/20.4	8.7/5.5	Acropora	*Symbiodinium sp.*	28	CBI83414	9
Unknown function (n = 7)									
predicted protein	54	2-3	121/27.3-27.4	8.0/5.0-5.2	NMMBA	*Triticum urartu*	38	AOTI010780946	4
hypothetical protein	31	4, 10	171/20-27.3	6.1/5.5-5.9	nr plant	*Physcomitrella patens*	42	XP_001783424	2
leucine-rich repeat-containing protein	30	2	138/27.3	6.8/5.0	NMMBA	*Capsaspora owczarzaki*	33	A0A086TKY0	5
hypothetical protein	30	10	61.7/20.0	9.4/5.9	nr plant	*Medicago truncatula*	45	XP_003603005	6
glycine-rich peptide	30	8	11.3/20.7	9.2/5.3	nr plant	*M. trunculata*	41	XP_003616789	23
agglutinin	28	2, 6	34.9/21.1-27.3	6.8/5.0-5.1	BSA	*Amaranthus caudatus*	16	1JLY_A	9
hypothetical protein	16	6	68.6/21.1	6.9/5.1	nr plant	*Populus balsamifera*	39	XP_002298703	3

[a] May be of bacterial/viral origin. [b] May be of host coral origin.

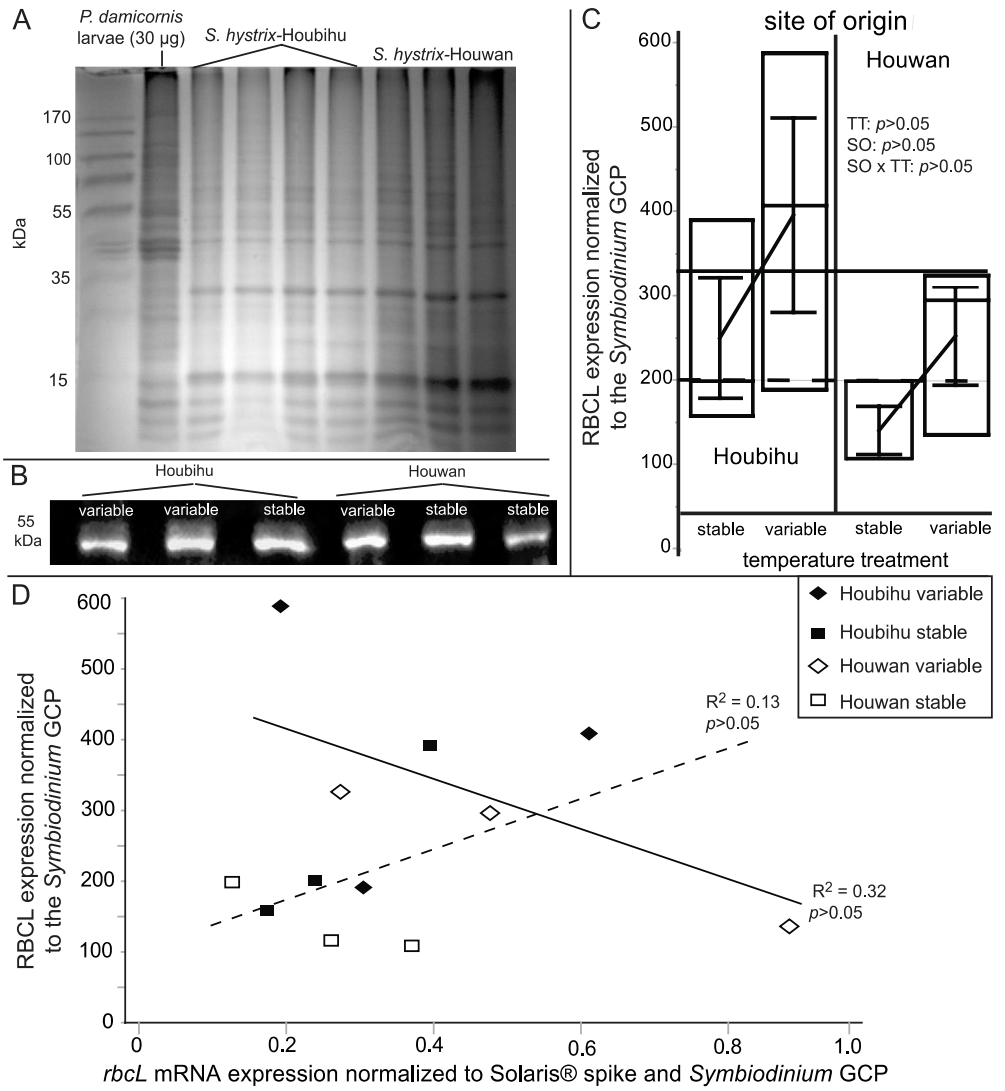

Figure 4. RBCL protein expression. Proteins were electrophoresed as described in the text, and a representative SDS-PAGE gel including a ladder, a positive control sample (30 µg of soluble protein from *Pocillopora damicornis* larvae [7]), and several proteins from each of the two sites of origin (SO) have been shown (**A**). A 55-kDa protein was detected with an RBCL (forms I and II) antibody, and representative bands from different SO and temperature treatments (TT) have been shown (**B**). The positive control sample also yielded a ~55-kDa band (data not shown). RBCL expression was normalized to a proxy for *Symbiodinium* density within samples, the genome copy proportion (GCP), and normal quantile plots (with averages connected by solid diagonal lines) for samples of both SO and TT have been shown (**C**). Error bars represent standard error of the mean (n = 3 for each SO x TT group), and the 2-way ANOVA (SO x TT) *p*-values have been presented on the figure. The global (pooled across SO) variable and stable TT expression levels have been plotted as horizontal solid and dotted lines, respectively. Expression of the RBCL protein (normalized to the *Symbiodinium* GCP) was plotted against expression of the respective *rbcL* mRNA (normalized to both the Solaris® [Thermo-Scientific] RNA spike and the *Symbiodinium* GCP), which was measured in a previous study [15], and best fit lines have been plotted for data of both the variable (solid line) and stable (dotted line) TT (**D**).

CONCLUSION

Curiously, no proteins were uniquely expressed by one treatment and not the other, nor were any proteins expressed at higher levels in samples of the variable TT. One explanation for this could be the low quantity of protein loaded; mini-gels (~10 x 10 cm) were used herein with 100 µg protein, and many spots on the gels were fairly faint. This may have limited the ability to detect proteins that were over-expressed by the variable TT samples, and future studies attempting to look at proteome-scale differences between experimental coral samples may consider loading larger quantities (*e.g.*, 500 µg) of protein into the gels. Furthermore, proteins were pooled across two SO of differing environmental history; although preliminary data revealed that the effect of TT led to greater variation in the *S. hystrix*-*Symbiodinium* physiological response than did the SO (Table 1; [15-17]), future work should nevertheless seek to determine the extent to which environmental history drives the ability of this common, widely distributed coral species to acclimate to future changes in temperature, using samples collected both before and after experimentation. Corals from Houwan, a site that never experiences upwelling *in situ*, were, in contrast to what had been hypothesized, readily able to acclimate to a temperature regime that fluctuated from 23 to 29ºC over a 6-hr period; it would be interesting to know if the protein-level acclimation response differed between these corals and those of Houbihu, which *do* experience such highly variable temperatures *in situ*.

Despite the potentially low resolution of the approach utilized herein, 117 proteins were nevertheless found to be expressed at higher levels in samples exposed to a stable TT for one week relative to those exposed to a variable TT for this same duration. The majority of these proteins (64%) were from the coral host, with the remaining 36% from the *Symbiodinium* populations housed within these samples. This ~2:1 ratio of host/endosymbiont agrees well with biological composition estimates made with other pocilloporids [13]. It should be noted that the fact that the coral host comprises a greater fraction of the holobiont means that differentially expressed proteins will be more readily documented for this compartment due to having loaded a larger quantity of coral protein *(i.e.*, ~64 µg coral host protein/100 µg total holobiont protein) into the gels.

Although *rbcL* mRNA expression was significantly higher in *Symbiodinium* populations harbored within *S. hystrix* colonies of the variable TT [15], the expression of the respective protein was not only similar between TT, but also between SO. As such, it was unsurprising that RBCL was *not* found to be differentially expressed between TT by 2D + MS analysis. Furthermore, *rbcL* gene expression did not correlate positively with RBCL protein expression to a significant degree across the 12 samples. This lack of correlation may suggest that inferring protein expression differences based only on mRNA-scale data, as is common in the coral biology field (e.g., [9-10, 35]), is risky. Additionally, the respective proteins of none of the differentially expressed genes identified in these same samples [15, 17], such as photosystem I (*psI*), were sequenced herein, further pointing to an absence of significant, positive correlation between gene and protein expression in this coral-*Symbiodinium* holobiont. As a final example, expression of no heat shock protein (*hsp*) mRNA was found to be affected by variable temperature exposure in these samples [15, 17], yet a small HSP was found by 2D gel electrophoresis to be down-regulated in host corals exposed to variable temperatures for seven days.

Although next generation mRNA sequencing has yielded marked insight into the molecular biology of cnidarian-dinoflagellate endosymbioses [13], the observation made herein that there is not always a positive association between mRNA and protein expression suggests that researchers should heir on the side of caution when attempting to use their mRNA data to make predictions about how corals will respond to environmental change. Rather than an end-all, such RNA Seq-based endeavors may be better seen as a means to target specific *proteins*, rather than gene mRNAs, for future, molecular characterization studies. Though not without their own limitations, 2D + MS-based methods yield direct insight into the molecules that actually carry out essential cellular processes; such proteins, of which several are discussed below, may better serve as biomarkers of the coral response to environmental changes, and notably GCC.

Upon a comprehensive look at the proteins that were down-regulated after seven days of exposure to variable temperature in the *S. hystrix-Symbiodinium* holobiont, it appears that different cellular processes were affected in each compartment. From an evolutionary perspective, eukaryotic cells are expected to respond similarly to changes in temperature [14, 36]. However, given the extensive evolutionary divergence between cnidarians and protozoans, it is unsurprising that some unique pathways were differentially affected by variable temperature exposure between the two endosymbiotic constituents. One group that merits further mention is the LB-associated proteins, oleosin and caleosin. The former is an abundant structural protein that acts to stabilize LBs, which are absent from asymbiotic or aposymbiotic cnidarians, by preventing their coalescence [37]. Caleosin is thought to play a role in the degradation of LB lipids in higher plants [38], though its ancestral role appears to be as another structural protein found on/in the coat of LBs [39]. As caleosin and oleosin are plant proteins, they were almost surely synthesized by the *Symbiodinium* cells. However, it cannot be ruled out that these proteins migrate alongside the LBs as they flow between compartments (discussed in more detail below). For the sake of argument, the remainder of this discussion will assume that these proteins are ultimately of dinoflagellate origin.

Endosymbiotic anthozoan LBs are thought to be involved in the metabolic dialogue between host anthozoans and their *in hospite Symbiodinium* populations [27, 37]; these organelles have been shown to flow back and forth between hosts and endosymbionts as a means of transferring lipids [24]. It seems reasonable to speculate, then, that the decrease in oleosin and caleosin expression in *Symbiodinium* within corals exposed to variable temperature for seven days may suggest that LB metabolism differed fundamentally between TT; perhaps the down-regulation of these two proteins under variable temperatures insinuates that LBs were being metabolized, since proteins that catabolize LB lipids could more readily interact with LBs upon the absence of these integral coat proteins. This presumed up-regulation of LB metabolism may have allowed for these *Symbiodinium* populations to sustain sufficient energy levels to maintain homeostasis under periods in which cellular energy demand could be hypothesized to be high due to, for instance, elevated rates of protein turnover brought on by these rapid temperature changes.

However, it should be noted that caleosin and oleosin are also known to have roles in lipid metabolism [40-41], and so their down-regulation, alongside a decrease in expression of another lipid-metabolizing protein, lipoxygenase, at variable temperature could, in contrast, suggest a decrease in LB metabolism. Indeed, the overall down-regulation of proteins involved in a variety of cellular pathways, notably metabolism, at variable temperatures in both compartments of this endosymbiosis may ultimately speak to this need to conserve

energy for growth, which was similar between temperature regimes (Table 1 and [15]), by suppressing certain metabolic pathways. As evidence for this, *Symbiodinium* have been found to actually *accumulate* lipids and LBs when deprived of nitrogen [42]. Future work should, then, seek to uncover the role of proteins involved in LB stabilization and metabolism in the response of *in hospite Symbiodinium* populations to environmental change; without further immuno-localization studies, it is premature to conjecture where these LB metabolic changes, in fact, took place, within the host coral or within the *Symbiodinium* cells. Such studies should also attempt to observe LB metabolism during stress events in order to determine whether they are more likely to be synthesized/accumulated, as when undergoing nutrient stress, or catabolized at such times. The ensuing data could help to develop a more comprehensive cellular model of how reef-building corals acclimate to highly variable temperature exposure both *in situ* and in the laboratory.

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AUTHOR CONTRIBUTION STATEMENT

A.B.M. conducted the experiment, processed the samples, analyzed the data, and wrote the manuscript. Y.-J.C. and C.-Y.L. ran the 2D gels and sequenced the proteins, respectively. C.-S.C. provided laboratory space, facilities, and reagents that were instrumental to the success of the project. A.B.M. declares that the authors have no competing interests.

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Table S1. Seventy-five host coral proteins expressed at higher levels in specimens of the stable temperature treatment in the 2010 *Seriatopora hystrix* variable temperature study. For proteins found in multiple spots, the range of molecular weights (in kilodaltons [kDa]) and isoelectric points (pI) have been provided

Protein name Total length of all peptides mapping to one protein	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
CELL SURFACE PROTEINS (n=4)									
fibrocystin 100	QVTVEVNSIPSTCSKR VTSFSKNNICCLTGAR ILIVDGGR QQPDRVAIAGGR VVQIVNALGDSRR ASDNDPSGGRAR LKESIPELR SMYNFSVIIKFASHK	1	678/27.4	8.2/4.9	NMMBA	*Saccoglossus kowalevskii*	19	A7SPV0	4
integrin α 69	YWAIVGAPLSNGSSGAEKFTR ADVVSGAPR YESGQFSVR NGYADVLVGAPYFTDVLDEGRVYIY LNDGK	7	112/20.9	5.6/5.3	Acropora	*Acropora millepora*	17	ABY74498	6
integrin β2 36	EITNITR QAYEKIAK AKPNQCLR DRDTGLLCGGPER	5-6	85.0/ 20.8-21.1	5.3/4.8-5.1	Acropora	*A. millepora*	14	ABY74499	4
H6 G-protein coupled receptor 19	LRAASVEK KIHDTATSSSR	9	34.7/20.4	9.7/5.5	Acropora	*A. millepora*	15	ABI50929	6
CIRCADIAN RHYTHM (n=1)									
cryptochrome photolyase (CRY2) 91	DLDTSLVECGSR ENGIEVISR QPEKPVPKVGR LDEIGGKFSK GCLSPRLLHQR GVSPPSELFVK SYGCVIGR QRAICVQRMQELAFKLASK	3, 6-7, 10	59.9/ 20.0-27.4	8.8/5.1-5.9	Acropora	*A. millepora*	16	ABP97099	17
CYTOSKELETON (n=2)									
ciliary dynein heavy chain 21	SQEAAAIVK ELKNAQELLDSK	5	525/20.8	5.7/4.8	nr other metazoa	*Pediculus humanus corporis*	38	XP_002426312	1
stathmin 19	HHEEQLIAK LEQSAENRK	5	34.7/20.8	9.8/4.8	nr other metazoa	*Trichoplax adhaerens*	50	XP_002114295	5

Table S1. (Continued)

Protein name Total length of all peptides mapping to one protein	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
DEVELOPMENT (n=2)									
hedgehog 30	KKPIPPTTNITLPR EMEKELK LLLTWNDLS	2	90.9/27.3	6.4/5.0	Acropora	*Acropora cervicornis*		GASU01035919	
embryonic polarity protein dorsal 19	ENMIFENELK SPDSPPNKK	7	93.2/20.9	5.4/5.3	nr other metazoa	*Culex quinquefasciatus*	42	XP_001844078	2
DNA BINDING/TRANSCRIPTION FACTOR (n=8)									
nuclear receptor AmNR2 63	NRCQYCRFNKCLAQGMLKEAVREDR SLAPRSADAPSSR LGKLLLCLPTLR ALENYVTLEFFGK	3, 8	49.9/ 20.7-27.4	6.0/ 5.2-5.3	Acropora	*A. millepora*	34	AF323681_1	14
sex comb on midleg-like protein 2 50	IQKAELATAR CNQDSRNPDK TTPMKPKTLNQAEVCINIECVCGPYIDVEK	7-8	117/ 20.7-20.9	8.3/5.3	*Hydra magnipapillata*	*Hydra magnipapillata*	34	XP_002160709	3
nuclear receptor 6 49	SSGFHYGVQSCEGCK CLSLGMLKEAVREDRAPGGRPRIK LPTLRHVSSK	1, 2, 10	38.8/ 20-27.4	8.3/ 4.9-5.9	Acropora	*A. millepora*	18	AF323686_1	13
estrogen receptor-β 48	SLEHTLPVNR CASPVTGPGSK SADEQLHCAGKAK IPGFVELSLFDQVR	2, 4	59.2/27.3	8.8/5.0-5.5	Seleno-protein	*H. sapiens*	29	ESR2_HUMAN	8
topoisomerase (DNA) III β 36	GLNPAQLKALYNSGYK MRQEQNK RSNPSEIPADDVK	8	153/20.7	8.6/5.3	*H. magni-papillata*	*H. magni-papillata*	23	XP_002155556	2
zinc finger protein 26	MHTVDANK HTTLDAARGR IATALDPR	10	71.3/20.0	9.2/5.9	*H. magni-papillata*	*H. magni-papillata*	32	XP_012561483	4
nuclear receptor AmNR8 18	CQGNGACPVDK EAVQCER	3	42.5/27.4	8.8/5.2	Acropora	*A. millepora*	16	AF323688_1	4
DM domain protein 17	SPPSQSPR SSTTPSIIK	2, 9	51.5/ 20.4-27.3	9.0/5.0-5.5	Acropora	*A. millepora*	16	AF530064_1	3
DNA REPAIR (n=1)									
Fanconi anemia group J protein 50	QVLTENHRALHVMTGSFLR NTQVMAGGWRR IVSEPRGGDK YTNGISKWVR	2	125/27.3	6.3/5.0	NMMBA	*Danio rerio*	36	K7GJE0	4

Protein name Total length of all peptides mapping to one protein	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
GROWTH FACTOR (n=1)									
imaginal disc growth factor 18	TLLESVESR EIQAGYLGK	3-4, 9	49.4/ 20.4-27.4	6.7/5.2-5.5	nr other metazoa	*Diaprepes abbreviates*	41	AAV68692	4
GLYCOPROTEIN (n=1)									
mucin-17 98	HSSNKSSGKSSSKSSSGK SSPSKSSSSK QNGSLLKR QHYPVYQLKPK AAAKVLFDINRPSIYATVENNYIETTS LPK SMLPTQTIDSSKIVTPSSK	9	574/20.4	5.4/5.5	*H. magni-papillata*	*H. magni-papillata*	26	XP_002160582	2
IMMUNITY (n=1)									
beta-defensin 22	VTEQLKRCWGEYIR ICRISEIR	2	11.8/27.3	9.1/5.0	Defensin	*Canis lupus*	26	Q30KS7	22
METABOLISM (n=8)									
thioredoxin reductase (isoform 1) 73	MAVALRGLGGR WRTQAVAGGVR WYDYDLIIIGGGSGGLAAAKEAAQL GRK VPDTRSLNLEK RSGLDPTVTGCUG	3-5	56.2/ 20.8-27.3	7.2/4.8-5.5	Seleno-protein	*H. sapiens*	15	AF044212_1	14
porphobilinogen deaminase 39	SIRGNLNTR MGWHNRVGQILHPEECMYAVGQGA LGVEVR	6	39.3/21.1	6.7/5.1	SwissProt	*H. sapiens*	24	HEM3_HUMAN	11
carboxylesterase 1C 37	AISESGVVINTNVGK APEEILAEK EGASEEETNLSK	3	61.0/27.4	5.0/5.2	SwissProt	*Mus musculus*	55	EST1C_MOUSE	7
carbamoyl-phosphate synthetase/ aspartate transcarbamylase/ dihydroorotase 29	KYFSDIVAR LALGIPLPKLK AKQMGFSDK	9	101/20.4	6.8/5.5	*H. magni-papillata*	*H. magni-papillata*	19	NP_001267868	3
alpha-L-arabinofuranosidase B 27	HQNFIFK LHSADFNSELYK IVKALNGR	9	35.6/20.4	8.9/5.5	*H. magni-papillata*	*H. magni-papillata*	28	XP_002165874	8
phospholipase D 21	NGEIFGKVFNCIPSSGLLTFR	4	104/27.3	8.5/5.5	nr other metazoa	*Schistosoma mansoni*	38	XP_002576773	2
cytochrome 450 19	LRSGTTLSPFNGAYVGLTR	4	56.9/27.3	9.0/5.5	nr other metazoa	*Trichoplax adhaerens*	38	XP_002112306	3
mannosyl-glycoprotein endo-beta-N-acetylglucosamidase[a] 15	EGLTTPEK QWIPTVK	1	37.7/27.4	9.3/4.9	*S. aureus*	*S. aureus*	23	WP_000247512	4

Table S1. (Continued)

Protein name Total length of all peptides mapping to one protein	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
MITOSIS (n=1)									
inner centromere protein 17	ESTPIEK LAEKDIAIGK	4	112/27.3	9.3/5.5	*H. magni-papillata*	*H. magni-papillata*	26	XP_002167890	2
mRNA PROCESSING (n=15)									
serine/arginine repetitive matrix protein 2 212	NLSLVRGR QIAPEPPKPYSLVRETSSSR SRSPQRPGWSR GRSGSSSER SNSPQPKVK TPSRQSCSGSSPR SRSISPCPK SISPCPKVDSR HSGSTSPYLK SEISTDPK VGLFSSQK SSSASPELKDGLPR SRSGSSPGLR ERSGSESSVEQK ALPRHSR TKSHTPPR SPTRQESSR TPLISRR SRSPLAIR SATPPATRNHSGSR TSPLMLDR	5	295/20.8	12/4.8	SRProtein	*M. musculus*	22	SRRM2_MOUSE	7
pre-mRNA-splicing factor CWC22 127	LIIQEFLKENIVR FSNIGHLILK MADGTLGR NSLKLIETSR AIKAILGMSK NITEGQIDK TPETFSR FISYLLYYLDTNK FFAHLLFTNSILGKVFCCIK FPFLSIDLVGLTDNLK HLKLYPESAIK	3	65.3/27.4	9.3/5.2	SRProtein	*Bombus impatiens*	36	XP_003494375	22
pre-mRNA splicing factor prp8[b] 67	IVKDIGDVSQK RSYLGALK LLKTYVLNELHK	4	326/27.3	8.4/5.5	SRProtein	*Trichophyton tonsurans*	28	EGE00188	2

Protein name Total length of all peptides mapping to one protein	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
	AFNIVSGEDR DVIEATTTNK QLTAVTTK AIATSNLR								
pre-mRNA-splicing factor CDC5/CEF1[b] 55	EAEQSSKR NIRALTETK KSQIETPNPMATPFR GEVSEEDAEIR DGSAVGAEIFDK	5	87.0/20.8	6.5/4.8	SRProtein	*Pseudogymnoascus destructans*	22	ELR07040	7
transformer-2 protein homolog beta-like 52	LQPLLSDHRR HYVLAPR GVHLSVSR SPTPRSR QDDADAAR LNGSELDGRSIR	7	82.2/20.9	11.6/5.3	TRA2-beta	*Salpingoeca rosetta*	14	EGD83325	7
pre-mRNA-splicing ATP-dependent RNA helicase prp28[b] 51	NSEVPTGPAAMRNK QKYMGTEK AREILEMER AAIPIALQSR SDDSSGFGNK	5	88.3/20.8	8.8/4.8	SRProtein	*Neosartorya fischeri*	35	PRP28_NEOFI	6
ATP-dependent RNA helicase DHX8-like 51	LEPEVTHVKDK FGCFVQLEGLRK QSLDLSPVR TLVDGQVVYIHPSSALFNR	3	137/27.4	8.5/5.2	SRProtein	*S. kowalevskii*	22	XP_002734274	4
CWC25 spliceosome-associated protein homolog 42	ELEEERAR DLLNNPVKMK SEWHKDSR ESTSPQRSR QTRSHSR	5	58.1/20.8	10.0/4.8	SRProtein	*S. kowalevskii*	17	XP_002732008	8
pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16 37	SKISSYK DWEEGKSDSGSDEEDDEE NK YGMVGCTQPR	5	144/20.8	6.4/4.8	SRProtein	*Oryzias latipes*	27	XP_004067034	3
pre-mRNA-splicing factor SYF1[b] 32	SVQAQYNTEVSFLAAQAQGAK VALEKELATAK	4	41.8/27.3	5.5/5.5	SRProtein	*Rhizoctonia solani*	31	CCO31421	8
KH domain containing, RNA-binding, signal transduction-associated 3 29	VVVPVKEYPK GRGFASAPIVR HASTAPDR	5	38.3/20.8	6.5/4.8	*H. magni-papillata*	*Hydra vulgaris*	45	XP_002163769	8

Table S1. (Continued)

Protein name Total length of all peptides mapping to one protein	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
pre-mRNA-splicing factor CWC25[b] 29	IREDPMLAIK LHDADDSRPASR QQARQLK	4	61.0/27.3	10.2/5.5	SRProtein	*Ustilago maydis*	21	EAK85329	5
myb-related protein cdc5[b] 20	GGVWTNIEDEILK MHEVALR	4	87.7/27.3	6.4/5.5	SRProtein	*Claviceps purpurea*	17	CCE33636	2
serine/arginine-rich splicing factor 7 isoform 1 18	SISRPRSSR SRSPSGSPHR	4	27.4/27.3	11.8/5.5	SRProtein	*M. musculus*	28	NP_666195	7
bud13[b] 16	SQETVFR EPVSLMGRK	5	32.5/20.8	9.5/4.8	SRProtein	*Zygosaccharomyces rouxii*	22	CAR30797	5
MUSCLE (n=2)									
dumpy CG33196-PB 92	DCPSGSYCPNR QTSCIICEAGR CALCSLGYRSNSNK GYYSHEK TYSNTEGATNNQDCR ISDCSPCPGGR ETGYGGVCPIGSFCK YGAKTASPR	8, 10	456/ 20-20.7	5.3/5.3-5.9	*H. magnipapillata*	*H. magnipapillata*	31	XP_012556696	2
dystrophin (isoform 2) 18	NGFQILDDR ELTEWVIRK	5	206/20.8	7.3/4.8	SwissProt	*Drosophila melanogaster*	41	DMDD_DROME	1
PROTEIN QUALITY CONTROL (n=4)									
UDP-glucose: glycoprotein glucosyltransferase 2 131	MAPAKATNVVR IAVNQHMR LFINGLR KAGASFYK MMDASVYLQREVFLGTLN DR INTLILR KLLFNALK EEIATAIYSGDK VDALMSSVPKR IINMKIK EDILTDEDEK FWLLKNYLSPTFK	6, 8	175/ 20.7-21.1	6.4/5.1-5.3	Selenoprotein	*H. sapiens*	18	NP_064506	9
E3 ubiquitin protein ligase 43	VCSTVEQYFKVIINALNNR IINDINHLGNIKEK IYVADSGNSR	2	59.4/27.3	7.1/5.0	NMMBA	*Meleagris gallopavo*	38	B3RW44	7

Protein name Total length of all peptides mapping to one protein	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
ubiquitin carboxyl-terminal hydrolase 17-like protein 10 36	ACLPGHKQVDR ALGVEDTDR FLQEQNK	2, 5	59.8/	8.5/4.8-5.0	Defensin	*H. sapiens*	40	I3L0E4	7
small heat shock protein 35	YYPLFNVGTGALAK VEGQTLEVSGKHR SQVQAPLK	1	26.4/27.4	5.8/4.9	*H. magni-papillata*	*H. magni-papillata*	21	T2MHG6	15
SIGNALING/HORMONES (n=5)									
serine/threonine-protein kinase ATR 46	IALKSIICLFNIMGAK CLGILGAIDPGR LIYSLGKTSCK EPILNMR	8	190/20.7	8.3/5.3	*H. magni-papillata*	*H. magni-papillata*	27	XP_002160018	3
tyrosine kinase receptor 45	RYSEVALR LSSSVFSKK EINLMKEIPYHK ERVDALER YTNPKYSK	6-9	200/ 20.4-21.1	5.9/5.1-5.5	*H. magni-papillata*	*H. magni-papillata*	33	XP_012562267	3
baculoviral IAP repeat-containing protein 19	ALQFDTYEMIVENPDGGFK	1	512/27.4	5.8/4.9	nr other metazoa	*Nasonia vitripennis*	24	K7JAH7	<1
receptor-type tyrosine-protein phosphatase alpha 18	QAGSHSNSKQAGSHSNSFR	9	90.5/20.4	6.3/5.5	SwissProt	*H. sapiens*	31	78PTPRA_HUMAN	2
serine/threonine-protein kinase PLK4-like 15	NAVVSISCLNLTQYR	9	76.5/20.4	8.9/5.5	nr other metazoa	*S. kowalevskii*	30	XP_002734025	2
TRANSCRIPTION (n=1)									
RNA binding motif protein 69	NRSPSGSLR MSYSRGLIPVK ATISSWR DEHSSRGYRNHR RHESYSR DYAPPHR NPPSLGR NRSPSGSLR	2-3, 8	55.8 20.7-27.4	10.0/ 5.0-5.3	TRA2-beta	*H. sapiens*	14	P0DJD3	10
TRANSLATION (n=2)									
ribosomal protein S4 39	LNAPKHWMLDK VGIITNRER DVTGNQFATRLSNIFLIGK	6	29.4/21.1	10.3/5.1	*H. magni-papillata*	*H. vulgaris*	30	XP_002154886	15
eukaryotic translation initiation factor 3 subunit 17	AQGPSASTEAPDDEGR	10	109/20.0	5.3/5.9	SwissProt	*Vanderwal-tozyma polyspora*	24	EIF3A_VANPO	2

Table S1. (Continued)

Protein name Total length of all peptides mapping to one protein	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
TRANSPORT (n=4)									
apolipoprotein 58	VAPLGAELQESAR LQELQGR LSPVAEEFR THVDSLR TQLAPHSEQMR SNPTLNEYHTR	3	30.6/27.4	5.6/5.2	SwissProt	*M. musculus*	97	APOA1_MOUSE	21
ATP binding cassette sub-family A 54	QGSEVAELPLLQNHAQSVICSPPRSK YGALGFGDLQSTITK VLSGGADNDLLR	4	204/27.3	9.2/5.5	*H. magni-papillata*	*H. magni-papillata*	20	XP_002157466	3
serotransferin 48	GTDFQLNQLEGK DGGGDVAFVK IPSHAVVAR VAQEHFGK LPEGTTPEK	3	76.7/27.4	6.9/5.2	SwissProt	*M. musculus*	59	TRFE_MOUSE	7
SEC14-like protein 1 25	SIISSLQLHK NPVNIEK QTVKAQNK	2, 10	106/ 20-27.3	8.5/5.0-5.9	*H. magni-papillata*	*H. magni-papillata*	29	T2M7V3	3
UNKNOWN FUNCTION (n=13)									
viral A-type inclusion protein[a] 95	ETQKLTIIEK EIHQFQKMGHTK EIESQIKALK QTLVSKLENIEK IVEITVEIKK ESELLINLELEKK DSLEDYLR ALLIKTQNEER IPSPRIDIR	5	332/20.8	5.1/4.8	*H. magni-papillata*	*H. magni-papillata*	61	XP_002156044	3
kinesin motor domain + hypothetical protein 86	TSLVVCVSPTMSDVSETKSSLYFGSR IIAKLEMDK KAMDVLEEPK LKAELTMSNIQTK ESVHEATRDRRAAASLLVSTRQERA ELNR	1	148/27.4	4.9/4.9	NMMBA	*Bombyx mori*	21	Q237L2	6
retrotransposon-like 47	ALEMIQSGASPK ARHFLLGR TPPYHPQSNGAAERMVET VK SDMSELR	2	141/27.3	9.2/5.0	*H. magni-papillata*	*H. magni-papillata*	22	T2MJH3	4

Protein name Total length of all peptides mapping to one protein	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon (top Mascot hit)	Score	NCBI accession	Coverage (%)
uncharacterized protein (putative apolipoprotein) 45	NCEGQENYERVEEILMSLK SINLNAKTVSR SNILSTAEELSKALK	2	477/27.3	8.1/5.0	*H. magni-papillata*	*H. magni-papillata*	24	T2MHF5	1
hypothetical protein 32	AAQQFIAKKPK EDESSLTTRVSK FTKEPPLK	5	32.3/20.8	6.0/4.8	Acropora	*A. millepora*	16	ACJ64664	10
Selenoprotein 30	IHHHHHHSSGR QEALAAAR KQEELNAQVEK	8	10.5/20.7	9.7/5.3	Seleno-protein	*H. sapiens*	14	2Q2F_A	33
hypothetical protein 30	NIDKANMVAK NSVSEMNPIREAK EATEYAK	3	121/27.4	6.5/5.2	*H. magni-papillata*	*H. magni-papillata*	16	XP_002160564	2
hypothetical protein with galactose binding lectin domain 1 26	KADTGLPR VIKINNAFWGR AALQRSR	4	24.8/27.3	7.5/5.5	Acropora	*A. millepora*	21	ACJ64660	11
hypothetical protein with galactose binding lectin domain 2 20	DDHVTCPK LCETSADNTIDK	7	30.9/20.9	6.8/5.3	Acropora	*A. millepora*	14	ACJ64658	6
hypothetical protein 17	MPAITLR NNVEHQQDPK	3, 5, 8	35.2/ 20.7-27.4	8.5/4.8-5.3	Acropora	*Acropora tenuis*	14	BAE46797	5
hypothetical protein 16	EIDIVIPK EKFGLQAK	7	47.9/20.9	5.3/5.3	*H. magni-papillata*	*H. magni-papillata*	26	XP_002163478	3
B-cell CLL/lymphoma 11A 15	DNNNSTSLTNQLKLR	3	90.1/27.4	7.6/5.2	nr other metazoa	*Tribolium castaneum castaneum*	35	XP_975280	2

[a] May be of bacterial/viral origin. [b] May be of *Symbiodinium* origin.

Table S2. Forty-two Symbiodinium proteins expressed at higher levels in specimens of the stable temperature treatment in the 2010 *Seriatopora hystrix* variable temperature study. For proteins found in multiple spots, the range of molecular weights (in kilodaltons [kDa]) and isoelectric points (pI) have been provided. The longest individual peptide lengths for the *Symbiodinium* and host coral compartments were 56 amino acids (AA; pre-mRNA-splicing factor ATP-dependent RNA helicase PRP43) and 31 AA (porphobilinogen deaminase), respectively

Protein name total number of AAs	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon	Score	NCBI accession	Coverage (%)
DNA BINDING/TRANSCRIPTION FACTOR (n = 1)									
zinc finger transcription factor PEI1 20	GGNGDGVAMRLDGEDYDTSR	1	28.2/27.4	6.8/4.9	32010	*Boechera stricta*	16	Q6WEQ1	8
DNA REPLICATION/REPAIR (n = 2)									
polyADP ribose polymerase 135	NALKSTIVAHGGK VVAAECRIGSDR ENTAANALPAK LSLAQLEK LELVQLMK HTIESTR NIFEVCR HGGSDVEVPLK NDSEHAIEAK HTLRTLAR GVPGARGGGFR GGFGGRGGSGGFR GGASLALGEDGFRGGR	1	287/27.4	5.9/4.9	SRProtein	*Acanthamoeba castellanii*	45	L8HDR5	5
chromosome segregation protein[a] 20	AQAFDEILEGMTNAIQHPVK	10	9.4/20.0	4.2/5.9	*S. aureus*	*S. aureus*	21	WP_000294226	24
EXTRACELLULAR MATRIX PROTEIN (n = 1)									
tenascin precursor 18	LAHHSSR CDNRGISKPDR	1	58.5/27.4	9.8/4.9	NMMBA	*Hordeum vulgare*	25	A7RGF8	3
LIPID BODIES (n = 14)									
caleosin-related[b] 70	MNTLSSGATSLGKGVGDGHPLDK GAGDAGQAAFNGAGNAASGAGDVGK EALQVAGKPPTFFDPDADGVVK	5-7	36.5/ 20.8-21.1	5.2/4.8-5.3	32011	*Moniliophthora perniciosa*	14	EEB94283	19
Caleosin 53	MSHQTVALASKAK GFSPLFPIDVKNSHLCMHGSDTDVYDDDGR NGLLSEKSVR	2, 5	23.8/ 20.8-27.3	9.6/4.8-5.0	32011	Arabidopsis thaliana	16	NP_173738	25
caleosin-related 51	TLQLVSSLPAR LAVPHLRR DSRGLSVLQQHAAFFDR	5, 8	30.1/ 20.7-20.8	8.7/4.8-5.3	32011	*Setaria italic*	29	XP_004985161	18

Protein name total number of AAs	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon	Score	NCBI accession	Coverage (%)
	FDAIFSK DKDGLLQR								
caleosin-related[b] 49	QPTSSFLKSHEESSIQK FEALFSKYAKSDTSAK DHALPRALR EHDDPSK	5	60.3/20.8	8.9/4.8	32011	*Mucor circinell-oides*	16	EPB88832	8
oleosin 33	MADQPKTIK DMAQQITKRVQNMGNSEILFSENR	2, 7, 9-10	17.5/ 20.0-27.3	9.7/5.0-5.9	32010	*Persea americana*	19	AGT63296	21
caleosin-related[b] 28	DGIISPYDTFIGFHR HGGDTGAYDNEGR	7, 9-10	25.5/ 20.0-20.9	7.2/5.3-5.9	32011	*Parasta-gonospor nodorum*	20	EAT77728	12
caleosin-related[b] 24	LVNGASKGEVAR CKHGSDSESYDR	8	28.5/20.7	7.1/5.3	32011	*Sporisorium reilianum*	16	CBQ73148	9
oleosin 23	DMAQQITK VQNMGNSEILFSENR	10	17.5/20.0	9.7/5.9	32010	*P. americana*	16	AGT63296	15
18 kDa oleosin 22	GARGPQDRAGRNGHPGQGAGGR	9	28.2/20.4	11.2/5.5	32010	*Zea mays*	13	ACG48647	8
oleosin 22	IIINRIK HLHRPNK IYDSETKK	1	23.6/27.4	11.3/4.9	32010	*Brassica oleracea*	16	AAD24547	10
oleosin S4-4 20	GSVPDQLEYAK GLETRTAAA	6	23.0/21.1	9.1/5.1	32010	*Brassica napus*	14	ACG69510	9
pollen coat oleosin-glycine-rich protein 19	FNIFLNLFSFLFPLLDVLK	1, 5, 7-8	21.8/ 20.7-27.4	9.7/4.8-5.3	32010	*Sisymbrium irio*	25	Q6V5I9	8
steroleosin-B 17	NANLVLVAR AAQQHKLE	10	39.1/20.0	6.2/5.9	32012	*Oryza sativa*	13	AAT77030	5
18.2 kDa oleosin 16	MAEVRDR EVGQKIENK	5	17.9/20.8	9.3/4.8a	32010	*Gossypium hirsutum*	18	AAA18524	9
METABOLISM (n = 9)									
peptide methionine sulfoxide reductase 54	AALSLSKRAKPTSPFPKTAR SPMNNLFTR QGNDVGTRYR SGIYFYTDEQEKLAR	2, 6, 8	28.4/ 20.7-27.3	8.9/5.0-5.3	32010	*B. napus*	19	P54151	21
fructose-bisphosphate aldolase, chloroplastic-like[a] 42	LTIPTEPNLYKDLAEHPNVVR EEFDKALGDAVESIYDASVNK	10	33.0/20.0	4.9/5.9	*S. aureus*	*S. aureus*	19	YP_005326917	14
threonine synthase[a] 42	IAICASTGNTSASAAAYAARAGLK VVAILTGNGLK DSIIDYIK	5, 8, 9	37.8/ 20.4-20.8	6.0/4.8-5.5	*S. aureus*	*S. aureus*	28	WP_001581605	12

Table S2. (Continued)

Protein name total number of AAs	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon	Score	NCBI accession	Coverage (%)
deoxyribose-phosphate aldolase 1[a] 33	ASELTKAAGADFVK IGTSAGVQIMQGLEADSDY	5	23.5/20.8	4.7/4.8	*S. aureus*	*S. aureus*	26	WP_001617202	15
acetyl-CoA acetyltransferase[a] 31	MNQAVIVAAK ETMIASMGIGGGLGNAALFIRF	5	41/20.8	6.2/4.8	*S. aureus*	*S. aureus*	23	WP_001070664	8
succinyl-diaminopimelate desuccinylase[a] 32	SEILKVNEHR DDIFVSALVGATDASSFLGDNK	6	45.1/21.1	4.6/5.1	*S. aureus*	*S. aureus*	16	YP_041474	8
8-hydroxyquercetin 8-O-methyltrans-ferase-like isoform 1 23	CKGVFSGLESLVDVGGGTGTMAK	2, 6	40.6/ 21.1-27.3	5.3/5.0-5.1	nr plant	*Glycine max*	39	XP_003527405	6
hydroxyethylthia-zole kinase[a] 22	AIVLANGSPLLAR IQEVEKYV	8	28.4/20.7	4.6/5.3	*S. aureus*	*S. aureus*	28	WP_001108492	8
lipoxygenase 21	ADLYGKPPQPAADARVMDELK	1, 6	95.3/21.1-27.4	7.2/4.9-5.1	nr plant	*O. sativa*	34	NP_001055143	2
mRNA PROCESSING (n = 4) **pre-mRNA-splicing factor ATP-dependent RNA helicase PRP43** 63	SLPVRQK LTNAGVLMSRLPVPPMLARCLLESLRV GCVESMIGVAAVLSVEGAILMSPTAKR	5	81.2/20.8	9.6/4.8	SRProtein	*Chondrus crispus*	37	CDF37591	9
mRNA processing-related protein 41	LANAASSASALLASSNER DRWNGYDPASYK ERESLAEQHLK	1, 3-5	65.3/20.8-27.4	5.7/4.8-5.5	SRProtein	*Cryptococcus neoformans*	19	P0CR52	7
putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16[a] 28	LMERDSK ELSRQEYLK SLHTDILMALVK	3	119/27.4	5.8/5.2	SRProtein	*Galdieria sulphuraria*	*27*	EME29213	2
pre-mRNA-splicing factor CWC22[a,b] 18	QSFMALK GKGLFVNSVIR	4	64.9/27.3	5.1/5.5	SRProtein	*Entamoeba dispar*	36	EDR21607	3
PHOTOSYNTHESIS (n = 4) **light harvesting protein, isoform 1** 54	RAEPVSAAAAAAAAAKAAAAAK KGDEAGFRNLRAAEIK AAMMAALGAVVQHYVK	4, 7	29.0/ 20.9-27.3	8.0/5.3-5.5	Acropora	*Symbiodinium sp.*	15	CBI83412	18

Protein name total number of AAs	Sequence(s)	Spot(s)	Predicted/ actual mass (kDa)	Predicted/ actual pI	Mascot database	Taxon	Score	NCBI accession	Coverage (%)
light harvesting protein, isoform 2 46	ASTASSSSGNPSR FADVPNGLAAISK VLTASDPAEK	6	49.3/21.1	8.9/5.1	Acropora	*Symbiodinium sp.*	45	CBI83416	9
light harvesting protein, isoform 3 41	KFSAMASK FADVPNGLAAISK VLTASDPAEK KLNAEIANGR	7-8	26.7/20.7-20.9	9.1/5.3	Acropora	*Symbiodinium sp.*	58	CBI83417	12
light harvesting protein, isoform 4 39	TASLAVAGVAMAALAAGGR VLTASDPAEK KLSAELANGR	9	44.8/20.4	8.7/5.5	Acropora	*Symbiodinium sp.*	28	CBI83414	9
UNKNOWN FUNCTION (n = 7)									
predicted protein 54	QSTSKLMATGR LKLDAQCK TAVGNTHSK MLSSYALDNSVFPSEDGTSVESLTLK	2-3	121/ 27.3-27.4	8.0/5.0-5.2	NMMBA	*Triticum urartu*	38	AOTI010780946	4
hypothetical protein 31	AFFNQIVVAPRAGLILVANAK SISPAKVTDK	4, 10	171/ 20-27.3	6.1/5.5-5.9	nr plant	*Physcomitrella patens*	42	XP_001783424	2
leucine-rich repeat-containing protein 30	NGLSVSDK GANSLSQALR VNTSLTSLDLSR	2	138/27.3	6.8/5.0	NMMBA	*Capsaspora owczarzaki*	33	EFW43177	5
hypothetical protein 30	LALQQDVDR MKLEHLEAASVNNANPNDSTK	10	61.7/20.0	9.4/5.9	nr plant	*Medicago truncatula*	45	XP_003603005	6
glycine-rich peptide 30	GGGGFGGGSGGGEGGGGGRGGGGYG GGGGR	8	11.3/20.7	9.2/5.3	nr plant	*M. trunculata*	41	XP_003616789	23
agglutinin 28	TAGLPVIMCLKSNNHQK DVFHVIDWK	2, 6	34.9/ 21.1-27.3	6.8/5.0-5.1	BSA	*Amaranthus caudatus*	16	1JLY_A	9
hypothetical protein 16	KEGSMSLADVCDFGHR	6	68.6/21.1	6.9/5.1	nr plant	*Populus balsamifera*	39	XP_002298703	3

[a] May be of bacterial/viral origin. [b] May be of host coral origin.

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Chapter 10

ARACHIDONIC ACID DISTRIBUTION IN SEAWEED, SEAGRASS, INVERTEBRATES AND DUGONG IN CORAL REEF AREAS

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ABSTRACT

Fatty acid composition was determined in seven species of seaweed, four species of seagrass, 17 species of invertebrates and dugong (mammal) sampled in coral reef areas in the Philippines. The data obtained indicated that Arachidonic acid (ArA) was not a minor component, and ArA distributes widely in coral reef organisms. Seagrass had high linoleic acid and linolenic acid levels with low ArA, EPA and DHA levels, while some species of seaweed had intermediate or high ArA levels (5% to 12%). In starfish, sea cucumber and some species of corals, ArA was the first major fatty acid (20% to 30%), but DHA levels were very low. Bivalves, abalone and shrimps had intermediate ArA levels. Total lipids of abdominal muscle and liver of dugong had respectively ArA levels of 7.8% and 11.0%, which were higher than EPA levels (2.4% and 1.6%), but DHA levels (0.4% and 2.3%) were low. It is clear that ArA is a major fatty acid in coral reef animals. Thus, intermediate or high ArA levels appear to be universally found in coral reef animals. However, the origin of ArA is not still clear. Micro-organisms on the bottom or in the soil and/or macro-algae may be the sources. Although it is highly speculative, the present results suggest that the existence of an ArA-rich food chain may be widespread in coral reef areas, and that the widespread existence of ArA-rich food

chain may lead to intermediate or high ArA contents in coral reef species. This speculation does not rule out the possibility that coral reef animals might have the ability to convert linoleic acid to ArA.

Keywords: fatty acid, arachidonic acid, DHA, EPA, coral reef, seagrass, seaweed, invertebrates, dugong

1. INTRODUCTION

Marine fish and invertebrate fatty acid signatures will be affected mainly by diet, as well as to some degree by the biosynthesis of certain fatty acids. Diets within and among fish and invertebrate species will in turn reflect characteristics of the prey field as well as morphological constraints and life style of the predators. Lipids, and especially fatty acids, have long been used as biological markers and general indicators of diet in marine ecology (Sargent et al. 1988). Animals can biosynthesize a relatively limited number of fatty acids (Cook 1985). These biochemical restrictions, coupled with the fact that fatty acids in the marine food web are exceptionally complex and diverse, provide the opportunity to use fatty acids for understanding food habits and nutrients requirements for marine fish.

Arachidonic acid (ArA) is an essential fatty acid but a minor component especially in cold and temperate water species. The abundance of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in marine animals has been emphasized in view of the importance in aquaculture, while little attention has been given to the existence and importance of ArA. However, preliminary studies, which aimed at developing advanced diets for improving fry production technologies of tropical fish, ovaries, eggs and fry of mangrove red snapper were unexpectedly found to have intermediate or high ArA levels and relatively low EPA levels (Ogata et al. 2004; Suloma and Ogata 2011). This phenomenon was also found in two species of rabbitfish, coral trout and striped jack sampled in the Philippines (Ogata et al. 2004). The result suggests that ArA may be much more important in fry production of coral reef associated species compared with cold/temperate species. Together with a series of feeding studies on dietary ArA and reproduction/larval quality, we have investigated the distribution of ArA in mangrove and coral reef. In the present chapter, data of fatty acids distribution in seaweed, seagrass, invertebrates and dugong in coral reef areas *will be presented to speculate the possible source of ArA in coral reef food-web.*

2. METHODOLOGY

Samples from invertebrates (Crustaceans, Mollusks, Echinoderms, Cnidarians and Poriferans) and aquatic plant (Seaweeds & Seagrass) were collected from coral reef areas in the Philippines. Table 1 lists common names, scientific names, sampling places and the time of samplings. Abdominal muscle and liver tissues of dugong were provided from a local fisherman who found a dead dugong on a beach (Pandan, Antique). Fatty acid composition of total lipids was determined for seaweed, seagrass, acetes and dugong. Fatty acid compositions of both polar (PL) and neutral (NL) lipids were determined for other invertebrate species. All the samples were freeze-dried and stored at-80℃ until lipid extraction. The extraction of

lipids from freeze dried samples was carried out with a mixture of chloroform and methanol (2:1, v/v) containing 0.01% butylhydroxytoluene (BHT). Total lipids were separated into polar and neutral lipids with a silica cartridge. Fatty acid methyl esters (FAME) were prepared and purified with thin-layer chromatography. FAME were analyzed on a gas liquid chromatography. Individual FAME were identified using reference standards.

3. FATTY ACID COMPOSITION

I. Invertebrates

In general, linoleic acid and linolenic acid in both neutral and polar lipids were trace components in all the invertebrate species examined. ArA in all invertebrate samples showed higher levels especially in PL, and ArA was superior to EPA in all samples, and in some samples superior to DHA. ArA levels ranged between 1.26 %- 30.18 % in polar lipid and 0.83%-5.63% in neutral lipids. High or intermediate ArA levels were found in polar lipids of jellyfish (9.3%), sponge (5.9%), starfish (28.7%), sea urchin gonads (10.0%) and sea cucumber (17.4%). ArA was the first major fatty acid in starfish, *Asterias sp*. In sea cucumber, EPA (not detected) and DHA (0.7%) levels were very low. ArA content is remarkably higher in tropical holothurians than in the temperate species (Svetashev et al. 1991). DHA was not detected in starfish, and DHA level was very low in sea urchin gonads (0.70%). These invertebrate species may be good sources of ArA, but it should be noted that the balance of DHA and ArA is poor in these species.

Corals

Two species of corals, *Sarcophyton* sp. (22.7%) and *Dendronephthya* sp. (30.2%), had very high ArA levels in their polar lipid fraction (Tables 3 and 4). By contrast, ArA levels in *Millepora platyphylla* (2.3%) and *Nephthya* sp. (3.5%) were not high. At present, we cannot explain the reason for the extreme difference in ArA level among the coral species. *Nephthya sp*. also had low EPA (0.9%) and DHA (0.9%) levels (Table 2). In other coral species, EPA and DHA levels ranged from 1.9% to 6.1% and from 6.1% to 9.4%, respectively.

Crustaceans

In four crustacean species (torpedo shrimp, mantis shrimp and swimming crab for polar lipids and *acetes* for total lipids), ArA levels (3.7% to12.3%) were always lower than EPA levels (9.5% to15.0%) (Tables 4 and 5). However, ArA levels in these crustacean species were not low compared to cold/temperate water species. Especially ArA level of swimming crab was 12.3%. DHA levels ranged from 6.5% to16.6% in polar lipid of these crustacean species.

In tropical paracalanid copepods, EPA level is higher than ArA level with DHA/EPA/ArA ratios of 14:3:1 for Acartia sinjiensis, 20:9:1 for Parvocalanus crassirostris and 25:6:1 for Bestiolina similes, respectively (McKinnon et al. 2003). Thus, planktonic crustaceans do not appear to be the primary source of ArA.

Shells

Abalone had a high ArA level (13.8%) and low EPA (2.3%) and DHA (0.4%) levels (Table 6) in polar lipids. Scallop had a high DHA level (22.0 %) and intermediate ArA (4.8%) and EPA (4.9%) levels in polar lipids. ArA, EPA and DHA levels were 6.5%, 5.8% and 11.6% for mussel in polar lipids. ArA levels were equivalent to or higher than EPA levels in the shells in coral reef areas.

Table 1. Sample list

Name	Scientific name	Source	Month	Remarks
Seagrass	*Cymodocea* sp.	Sagay	Nov	
	Thalassia sp.	Nogas	Jul	
	Enhalus sp.	Nogas	Jul	
	Halodule sp.	Nogas	Jul	
Seaweed	*Ulva faciata*	Igang	Mar	
	Caulerpa sp.	Palawan	Oct	
	Corallinaceae	Nogas	Jul	
	Padina sp.	Igang	Mar	
	Galaxauraceae	Nogas	Jul	
	Kapaphycus alvarezi	Tigbauan	Mar	cultured
	Kapaphycus alvarezi	Tigbauan	Mar	
	Kapaphycus alvarezi	Palawan	Oct	
	Gracilaria sp.	Roxas	Jun	cultured
	Gracilaria sp.	Tigbauan	Mar	cultured
	Gracilaria sp.	Tigbauan	Nov	cultured
Jerry fish	*Ulamaridae*	Guimaras	Jul	
Sponge	*Spongidae*	Igang	Mar	
Starfish	*Asterias* sp.	Nogas	Jul	
Seaurchin	*Tripneustes gratilla*	Igang	Mar	gonads
Coral	*Millepora platyphylla*	Igang	Nov	
	Nephthya sp.	Igang	Nov	
	Sarcophyton sp.	Igang	Nov	
	Dendronephthya sp.	Igang	Nov	
Cucumber	*Bohadschia* sp.	Sagay	Nov	
Acetes	*Acetes* sp.	Tigbauan	Mar	
Torpedo shrimp	*Parapennaeopsis maxillipedo*	Hamtic	Nov	
Mantis shrimp	*Lysiosquilla* sp.	Guimbal	Mar	
Swimming crab	*Portunus* sp.	Nogas	Jul	
Abalone	*Haliotis assinina*	Guimaras	Jul	
Scallops	*Amusium pleuronectes*	Tigbauan	Jul	
Mussel	*Perna viridis*	Tigbauan	Jul	
Clam	*Anodontia edentula*	Tigbauan	Jul	
Dugong	*Dugong dugong*	Panoan	Oct	muscle and liver

Table 2. Fatty acid compositions of corals

Source	Igang		Igang		Igang	
Sample name	*Millepora* sp.-1		*Nephthya* sp.-1		*Nephthya* sp.-2	
Sample #	2 pooled		2 pooled		2 pooled	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	1.42	0.69	1.60	0.69	2.70	1.07
14:1	1.52	0.36	2.65	0.36	3.03	1.69
15:0	0.62	0.65	0.53	0.65	0.94	0.62
16:0	20.76	30.69	24.28	30.69	20.17	26.16
16:1n-7	20.89	4.83	26.52	4.83	25.45	2.68
17:0	0.78	1.12	0.70	1.12	1.79	0.79
16:3n-6	0.66	1.83	0.70	1.83	0.87	0.41
16:3n-3	0.34	0.28	0.24	0.28	0.37	0.31
18:0	4.06	4.15	3.92	4.15	4.58	3.64
18:1n-9	5.88	5.77	4.12	5.77	7.15	4.10
18:1n-7	2.46	2.13	2.30	2.13	2.32	2.83
18:2n-6	1.48	0.89	0.64	0.89	1.64	0.88
18:3n-6	0.70	1.29	0.78	1.29	0.68	0.41
18:3n-3	0.59	2.22	0.52	2.22	0.67	2.65
18:4n-3	0.47	0.27	0.39	0.27	0.30	0.79
20:0	0.65	0.16	2.01	0.16	1.57	1.29
20:1	0.60	3.17	2.53	3.17	2.23	4.82
20:2n-6	0.99	0.25	1.71	0.25	0.24	1.82
20:3n-6	0.24	0.39	0.18	0.39	0.68	0.43
20:4n-6	2.33	2.17	1.26	2.17	3.47	2.83
20:3n-3	0.52	0.25	0.19	0.25	0.26	0.73
20:4n-3	0.50	0.78	0.28	0.78	0.51	0.85
20:5n-3	1.94	0.07	2.23	0.07	0.90	0.49
22:0	0.38	0.08	0.61	0.08	0.61	0.29
22:1	0.35	0.33	1.15	0.33	1.35	0.79
22:4n-6	1.26	13.21	1.09	13.21	0.63	12.17
22:5n-6	0.18	0.66	0.06	0.66	0.08	0.34
22:5n-3	0.49	0.05	0.20	0.05	0.36	0.50
22:6n-3	9.07	1.21	1.41	1.21	0.92	2.97
24:0	0.12	0.57	0.47	0.57	tr	0.48
24:1	nd	0.80	nd	0.80	nd	nd
ΣSaturates	28.79	38.12	34.12	38.12	32.37	34.35
ΣMonoenes	31.69	17.39	39.28	17.39	41.53	16.90
Σn-6	7.85	20.69	6.41	20.69	8.29	19.29
Σn-3	13.92	5.12	5.45	5.12	4.28	9.28
Σn-3HUFA	12.00	2.10	4.12	2.10	2.68	4.81

Table 3. Fatty acid compositions of corals

Source	Igang		Igang		Igang	
Sample name	*Sarcophyton* sp.-1		*Sarcophyton* sp.-2		*Dendronephthya* sp.-1	
Sample #	2 pooled		2 pooled		2 pooled	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	2.21	3.35	2.13	3.83	1.25	3.35
14:1	0.50	1.23	0.76	1.02	0.11	1.23
15:0	0.12	0.20	0.09	0.14	0.11	0.20
16:0	15.05	16.95	14.97	18.72	12.31	16.95
16:1n-7	1.19	0.67	0.95	0.71	0.83	0.67
17:0	0.31	0.49	0.64	0.79	0.65	0.49
16:3n-6	0.36	0.36	0.74	0.29	1.05	0.36
16:3n-3	0.21	0.33	0.37	0.31	0.54	0.33
18:0	4.44	10.15	4.33	9.83	3.69	10.15
18:1n-9	7.27	28.63	7.34	29.43	1.75	28.63
18:1n-7	0.52	1.44	0.54	1.42	0.26	1.44
18:2n-6	2.54	2.18	2.33	2.21	2.60	2.18
18:3n-6	0.88	0.80	0.77	0.72	8.82	0.80
18:3n-3	1.07	1.19	1.03	0.96	0.42	1.19
18:4n-3	8.02	0.12	7.32	0.20	6.70	0.12
20:0	0.33	1.35	0.15	1.20	0.46	1.35
20:1	4.52	1.65	4.69	1.68	2.10	1.65
20:2n-6	0.22	0.39	0.17	0.34	0.27	0.39
20:3n-6	0.35	0.19	0.47	0.25	1.03	0.19
20:4n-6	21.38	4.77	22.67	4.95	28.73	4.77
20:3n-3	0.15	0.07	0.19	0.04	0.19	0.07
20:4n-3	0.05	0.69	0.04	0.28	0.35	0.69
20:5n-3	5.80	1.47	5.16	0.91	5.85	1.47
22:0	tr	0.06	tr	0.03	0.12	0.06
22:1	tr	0.54	tr	0.06	0.13	0.54
22:4n-6	0.12	1.50	0.78	1.14	1.10	1.50
22:5n-6	0.09	0.04	0.01	0.05	0.00	0.04
22:5n-3	0.00	0.01	0.00	0.04	0.10	0.01
22:6n-3	9.71	5.70	9.38	5.78	6.08	5.70
24:0	tr	tr	tr	tr	tr	tr
24:1	nd	nd	nd	nd	nd	nd
ΣSaturates	22.46	32.54	22.31	34.54	18.60	32.54
ΣMonoenes	14.00	34.16	14.27	34.33	5.18	34.16
Σn-6	25.94	10.24	27.95	9.97	43.59	10.24
Σn-3	25.01	9.59	23.48	8.53	20.23	9.59
Σn-3HUFA	15.56	7.87	14.58	7.02	12.38	7.87

Table 4. Fatty acid composition of corals, *Acetes* (alamang) and shrimps

Source	Igang		Tigbauan	Hamtic	
Sample name	*Dendronephthya* sp.-2		*Acetes*	*Parapennaeopsis maxillipedo* (torpedo shrimp)	
Sample #	2 pooled		2 pooled	3 pooled	
Lipid class	PL	NL	TL	PL	NL
14:0	1.37	3.60	4.24	0.89	3.14
14:1	0.31	1.91	0.24		
15:0	0.12	0.20	1.03	0.82	1.88
16:0	13.17	32.41	23.40	14.93	26.45
16:1n-7	1.22	3.46	7.80	5.21	8.21
17:0	0.80	0.61	0.45	2.34	3.05
16:3n-6	0.76	0.47	1.62		
16:3n-3	0.52	0.14	0.62		
18:0	3.93	6.17	8.34	11.91	13.57
18:1n-9	1.35	5.77	8.52	9.71	10.23
18:1n-7	0.22	0.88	2.55	3.28	4.78
18:2n-6	2.82	3.66	1.49	1.57	1.5
18:3n-6	9.51	13.07	0.15	0.58	0.86
18:3n-3	0.14	0.90	1.08	0.44	0.35
18:4n-3	6.71	0.40	0.66		
20:0	0.49	0.59	0.69	0.54	0.94
20:1	1.87	0.98	0.71	0.58	1.03
20:2n-6	0.15	0.59	0.36	0.87	0.81
20:3n-6	0.36	1.31	0.13	0.22	
20:4n-6	30.18	5.63	3.70	7.48	2.77
20:3n-3	0.30	0.55	0.15		
20:4n-3	0.06	0.14	0.24		
20:5n-3	6.09	1.83	9.52	12.27	2.82
22:0	0.13	0.33	1.53	0.87	0.73
22:1	0.14	0.15	0.37	0.19	
22:4n-6	1.28	2.34	0.11	1.17	0.98
22:5n-6	0.00	0.04	0.72	1.30	0.5
22:5n-3	0.09	0.25	0.46	1.46	0.61
22:6n-3	6.08	1.97	11.32	10.12	1.84
24:0	tr	tr	0.56	0.17	
24:1	nd	nd	0.51	0.76	
ΣSaturates	20.01	43.91	40.22	32.47	49.76
ΣMonoenes	5.11	13.14	20.69	19.73	24.25
Σn-6	45.05	27.11	8.27	13.19	7.42
Σn-3	19.99	6.16	24.02	24.29	5.62
Σn-3HUFA	12.32	4.19	21.52	23.85	5.27

Table 5. Fatty acid composition of mantis shrimp and swimming crab

Source	Guimbal		Nogas	
Sample name	*Lysiosquilla* sp. Mantis shrimp whole body		*Portunus* sp. swimming crab juvenile whole body	
Sample #	2 pooled		2 pooled	
Lipid class	PL	NL	PL	NL
14:0	2.43	12.96	0.57	3.65
14:1				
15:0	0.43	0.97	0.31	0.7
16:0	19.63	29.85	15.49	35.48
16:1n-7	5.65	9.34	2.52	7.04
17:0	1.30	1.46	1.39	2.3
16:3n-6				
16:3n-3				
18:0	12.14	5.93	11.83	9.84
18:1n-9	8.05	5.83	9.98	13.32
18:1n-7	3.22	4.2	3.70	5.45
18:2n-6	1.35	0.81	3.66	2
18:3n-6	0.43	0.38	0.63	0.69
18:3n-3	0.48	0.35	1.60	0.57
18:4n-3				
20:0	0.15	0.42	1.47	0.92
20:1	0.22	0.34	0.64	1.75
20:2n-6	0.34	0.27	0.80	0.5
20:3n-6	0.17	0.14	0.00	
20:4n-6	6.53	1.84	12.29	1.5
20:3n-3			0.21	
20:4n-3	0.16	0.21		
20:5n-3	10.15	4.22	14.95	0.84
22:0	0.44	0.38	0.83	0.84
22:1		0.16		
22:4n-6	0.50	0.22	0.64	0.29
22:5n-6	0.86	0.5	0.32	
22:5n-3	0.93	0.65	0.83	
22:6n-3	16.62	9.7	6.48	0.34
24:0	0.34	0.34		0.27
24:1		0.97		
ΣSaturates	36.86	52.31	31.90	54.00
ΣMonoenes	17.14	20.84	16.83	27.56
Σn-6	10.18	4.16	18.33	4.98
Σn-3	28.34	15.13	24.07	1.75
Σn-3HUFA	27.86	14.78	22.26	1.18

Table 6. Fatty acid composition of coral reef associated shellfishes

Source	Guimaras		Tigbauan		Tigbauan	
Sample name	*Haliotis assinina* Abalone wild		*Amusium pleuronectes* scallop wild		*Perna viridis* mussel wild	
Sample #	2 pooled		2 pooled		2 pooled	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	0.96	4.33	1.20	3.96	0.67	7.08
14:1	0.21	0.53	0.12	0.22	0.14	0.23
15:0	1.05	3.28	0.76	2.26	0.58	2.05
16:0	19.40	34.82	19.79	33.16	15.09	34.00
16:1n-7	2.72	6.79	1.57	6.03	5.86	11.22
17:0	0.32	0.85	0.37	0.62	1.42	0.97
16:3n-6	2.11	4.17	2.61	4.40	1.42	2.21
16:3n-3	0.32	1.23	0.27	0.73	0.28	0.47
18:0	5.58	10.84	10.00	21.15	4.49	7.49
18:1n-9	3.40	10.15	1.95	7.55	1.96	3.59
18:1n-7	3.26	3.67	2.27	3.41	1.33	3.16
18:2n-6	0.45	0.36	0.50	0.67	1.79	1.42
18:3n-6	0.19	0.33	0.43	0.39	0.30	0.40
18:3n-3	1.21	0.34	1.83	0.25	3.14	0.80
18:4n-3	0.12	0.08	0.17	0.14	0.19	0.12
20:0	0.17	0.33	0.22	0.41	0.28	0.59
20:1	5.74	2.74	5.45	1.62	8.07	5.17
20:2n-6	0.26	0.22	0.46	0.32	0.66	0.26
20:3n-6	0.24	0.12	0.55	0.31	0.52	0.31
20:4n-6	13.56	0.83	4.70	1.25	6.47	1.50
20:3n-3	0.21		0.06		0.22	0.04
20:4n-3	0.15		0.09	0.02	0.24	0.07
20:5n-3	2.31	0.17	4.90	0.58	5.81	2.08
22:0	0.18	0.27	0.10	0.10	0.10	0.18
22:1	1.77	1.43	0.61	0.23	0.51	0.24
22:4n-6	6.49	0.46	3.61	1.17	3.44	0.28
22:5n-6	0.48	0.00	2.94	0.20	2.05	0.12
22:5n-3	10.75	0.25	0.84	0.07	1.72	0.22
22:6n-3	0.26	0.36	22.00	1.24	11.76	1.57
24:0						
24:1						
ΣSaturates	27.66	54.72	32.44	61.65	22.64	52.35
ΣMonoenes	17.10	25.32	11.97	19.07	17.87	23.62
Σn-6	23.78	6.48	15.79	8.69	16.64	6.50
Σn-3	15.34	2.44	30.17	3.02	23.37	5.38
Σn-3HUFA	13.47	0.79	27.84	1.91	19.54	3.94

Table 7. Fatty acid composition of coral reef associated invertebrates

Source	Igang		Nogas		Guimaras	
Sample name	*Spongidae* sponge		*Asterias* sp. starfish		*Ulamaridae* jellyfish	
Sample #	2 pooled		2 pooled		2 pooled	
Lipid class	PL	NL	PL	NL	PL	NL
14:0	0.94	2.25	3.33	6.01	1.01	3.39
14:1						
15:0		0.85	0.80	1.72		0.57
16:0	6.49	21.91	4.87	28.30	25.60	37.07
16:1n-7	4.59	6.80	1.01	9.01	2.14	6.82
17:0	1.21	1.49	1.01	1.79	1.24	0.96
16:3n-6						
16:3n-3						
18:0	9.22	19.45	9.55	10.12	11.30	9.74
18:1n-9	3.62	13.32	1.50	5.22	4.26	19.24
18:1n-7	3.49	7.01	1.58	5.32	1.75	3.54
18:2n-6	1.13	2.87	1.84	1.50	0.75	2.08
18:3n-6	1.42	2.09	0.51	0.65	5.93	
18:3n-3	0.82		0.39	1.03	0.73	
18:4n-3						
20:0	2.85	2.06	0.76	0.84		0.32
20:1	1.13	1.20	11.22	6.46	2.37	0.38
20:2n-6			1.77	0.87		
20:3n-6			0.26	0.29		
20:4n-6	5.91	2.84	28.73	3.85	9.29	2.81
20:3n-3			0.85	0.43		
20:4n-3						
20:5n-3	3.22	2.44	3.63	0.52	8.61	2.82
22:0	2.35	0.88	0.27	0.33		
22:1						
22:4n-6					1.24	
22:5n-6	1.94					
22:5n-3	19.49		0.49		0.68	
22:6n-3	7.56	2.06			8.62	4.29
24:0	6.88	1.49	0.30	0.54		
24:1	2.40					
ΣSaturates	29.94	50.38	20.74	49.20	39.15	52.05
ΣMonoenes	15.23	28.33	15.30	26.00	10.53	29.98
Σn-6	10.40	7.80	32.97	7.02	17.21	4.89
Σn-3	31.09	4.50	5.11	1.98	18.64	7.11
Σn-3HUFA	30.27	4.50	3.87	0.52	17.91	7.11

Table 8. Fatty acid composition of coral reef associated invertebrates

Source	Igang		Sagay	
Sample name	*Tripneustes gratilla* sea urchin gonad		*Bohadschia* sp. sea cucumber	
Sample #	8 pooled		2 pooled	
Lipid class	PL	NL	PL	NL
14:0	3.94	9.45		6.83
14:1	0.08	0.25		
15:0	0.29	0.83		2.44
16:0	17.08	34.02	3.22	25.70
16:1n-7	3.33	5.27	1.65	15.19
17:0	0.11	0.18	0.60	1.94
16:3n-6	0.38	0.36		
16:3n-3	0.20	0.17		
18:0	6.29	5.00	5.08	10.56
18:1n-9	1.23	3.37	1.80	3.55
18:1n-7	2.16	2.21	4.57	6.30
18:2n-6	0.40	0.30	2.38	0.63
18:3n-6	0.68	0.42	1.20	0.98
18:3n-3	0.64	1.17	0.98	
18:4n-3	0.19	1.36	0.61	
20:0	1.06	1.35	2.11	1.59
20:1	19.28	9.57		
20:2n-6	1.47	0.73	1.45	0.45
20:3n-6	0.81	0.45	1.36	0.60
20:4n-6	9.97	3.15	17.37	1.53
20:3n-3	1.89	0.75		
20:4n-3	3.39	1.53		
20:5n-3	12.30	2.52		0.33
22:0	0.11	0.07	1.22	0.93
22:1	1.59	1.27	0.64	
22:4n-6	0.41	4.25	3.28	1.12
22:5n-6	0.39	0.09		
22:5n-3	0.51	0.17		
22:6n-3	0.70	0.82	0.91	
24:0		0.12	0.42	
24:1	0.26		3.74	1.72
ΣSaturates	28.88	51.03	12.44	49.99
ΣMonoenes	27.94	21.94	11.58	26.76
Σn-6	14.52	9.76	26.31	5.09
Σn-3	19.83	8.49	2.50	0.16
Σn-3HUFA	16.91	5.04	0.91	0.16

Table 9. Fatty acid composition of coral reef associated seaweeds

Source	Igang	Palawan	Nogas	Igang	Nogas
Sample name	*Ulva faciata*	*Caulerpha* sp.	*Corallinaceae*	*Padina* sp.	*Galaxauraceae*
Sample #	2 pooled	2 pooled	2 pooled	2 pooled	2 pooled
Lipid class	TL	TL	TL	TL	TL
14:0	2.9	4.3	2.2	2.9	3.9
14:1	0.5	0.5	0.4	0.4	1.0
15:0	0.8	0.4	0.4	0.3	0.9
16:0	37.4	31.4	39.5	32.2	39.6
16:1n-7	7.6	11.3	18.3	20.6	15.5
17:0	1.6	2.2	0.4	0.3	0.2
16:3n-6	0.4	0.4	0.3	3.4	0.5
16:3n-3	0.4	0.1	0.1	0.1	0.3
18:0	4.5	2.4	1.6	4.7	2.6
18:1n-9	8.7	2.8	7.9	2.7	5.5
18:1n-7	13.0	1.9	2.4	4.6	3.7
18:2n-6	1.5	5.6	1.1	2.0	1.8
18:3n-6	0.2	0.7	0.5	1.7	0.3
18:3n-3	2.3	4.8	0.3	0.1	1.0
18:4n-3	0.9	0.7	0.1	0.1	0.3
20:0	0.4	0.3	0.1	1.5	0.3
20:1	0.5	0.8	0.3	2.0	0.7
20:2n-6	0.1	0.3	0.3	0.2	0.9
20:3n-6	0.1	0.3	1.3	0.3	0.6
20:4n-6	0.5	3.5	12.9	3.3	3.2
20:3n-3	0.0	0.1	0.0	0.1	0.1
20:4n-3	0.1	0.1	0.4	0.0	0.1
20:5n-3	1.1	3.0	0.6	0.1	3.2
22:0	1.3	0.3	0.1	1.1	0.4
22:1	0.1	0.2		0.2	0.1
22:4n-6	0.0	0.5		0.2	0.3
22:5n-6	0.1	0.2		tr	tr
22:5n-3	0.1	0.4		1.4	0.3
22:6n-3	0.2	1.3		0.0	0.5
24:0	0.3	1.7		0.3	0.6
24:1	0.2	0.1		0.2	0.4
ΣSaturates	49.2	41.2	44.2	43.2	47.9
ΣMonoenes	30.6	17.5	29.4	30.7	26.6
Σn-6	2.8	11.5	16.3	10.9	7.7
Σn-3	5.3	8.5	1.5	1.9	5.4
Σn-3HUFA	1.6	3.4	1.1	1.6	4.1

Table 10. Fatty acid composition of coral reef associated seaweeds

Source	Tigbauan	Tigbauan	Roxas	Tigbauan	Tigbauan
Sample name	*Kappaphycus alvarezi* cultured	*Kappaphycus alvarezi* wild	*Gracillaria* sp. cultured	*Gracillaria* sp. cultured	*Gracillaria* sp. cultured
Sample #	2 pooled	2 pooled	2 pooled	2 pooled	2 pooled
Lipid class	TL	TL	TL	TL	TL
14:0	2.8	2.6	1.6	1.8	1.3
14:1	0.1	0.2	0.6	0.1	0.2
15:0	0.4	0.5	0.6	0.3	0.4
16:0	32.9	31.8	49.1	53.3	65.7
16:1n-7	0.6	0.6	3.0	1.7	2.1
17:0	0.2	0.1	0.2	0.1	0.1
16:3n-6	0.1	0.2	0.5	0.2	0.1
16:3n-3	15.4	13.5	0.1	0.1	0.2
18:0	2.1	2.5	4.0	2.5	2.3
18:1n-9	4.5	4.5	12.4	15.8	10.5
18:1n-7	1.3	1.5	3.8	1.7	1.7
18:2n-6	0.3	0.5	1.2	1.4	0.5
18:3n-6	0.2	0.1	0.3	0.2	0.0
18:3n-3	6.2	8.5	0.1	0.1	0.4
18:4n-3	tr	tr	0.0	0.0	0.5
20:0	0.1	0.1	0.2	0.1	0.1
20:1	0.2	0.2	0.5	0.3	0.2
20:2n-6	0.1	0.2	0.1	0.1	0.1
20:3n-6	0.3	0.3	0.7	0.9	0.4
20:4n-6	5.8	5.1	5.7	6.8	6.4
20:3n-3	0.1	tr	0.1	0.1	tr
20:4n-3	0.1	0.1	0.1	0.2	tr
20:5n-3	3.7	1.8	6.3	5.6	3.0
22:0	0.2	0.1	0.2	0.1	0.1
22:1	0.1	0.1	0.1	0.1	0.0
22:4n-6			0.2	0.2	0.3
22:5n-6			0.2	0.0	0.0
22:5n-3			0.3	0.3	0.1
22:6n-3			1.5	0.4	0.1
24:0	0.2	0.1	0.3	0.2	0.8
24:1			0.4	0.1	0.3
ΣSaturates	38.8	37.8	56.2	58.5	70.9
ΣMonoenes	6.7	7.0	20.8	19.8	15.0
Σn-6	6.9	6.4	8.8	9.9	7.8
Σn-3	25.5	23.9	8.5	6.8	4.3
Σn-3HUFA	3.8	1.9	8.2	6.5	3.2

Table 11. Fatty acid composition of coral reef associated sea grass and dugong

Source	Sagay	Nogas	Nogas	Nogas	Pandan	Pandan
Sample name	*Cymodocea* sp.	*Thalassia* sp.	*Enhalus* sp.	*Halodule* sp.	*Dugong dugong* muscle	*Dugong dugong* liver
Sample #	2 pooled	2 pooled	2 pooled	2 pooled	1	1
Lipid class	TL	TL	TL	TL	TL	TL
14:0	1.6	2.4	1.5	5.4	1.68	0.41
14:1	0.2	0.7	0.8	0.6	0.05	0.04
15:0	1.6	0.4	0.6	0.5	0.09	0.04
16:0	21.3	23.8	21.9	29.5	17.77	16.84
16:1n-7	12.9	13.7	12.1	10.8	8.15	3.61
17:0	0.6	0.4	0.5	0.3	0.80	0.16
16:3n-6	0.3	0.4	0.4	0.3	0.20	0.22
16:3n-3	0.3	0.1	0.3	0.1	0.20	0.11
18:0	2.4	4.4	5.1	2.5	9.95	15.33
18:1n-9	3.7	2.4	0.3	3.3	26.23	20.21
18:1n-7	1.1	1.5	1.5	2.7	1.85	1.69
18:2n-6	22.1	12.0	22.2	7.5	5.21	8.30
18:3n-6	0.1	0.2	0.2	0.4	0.10	0.20
18:3n-3	15.8	16.5	14.1	13.9	1.36	1.21
18:4n-3	0.1	0.2	0.2	0.9	0.03	0.03
20:0	0.1	0.7	0.4	0.6	0.17	0.20
20:1	0.9	0.7	0.7	0.3	0.80	1.03
20:2n-6	tr	0.2	0.2	0.2	2.40	1.78
20:3n-6	tr	0.1	0.2	0.2	0.75	0.64
20:4n-6	0.3	0.5	0.6	2.3	7.77	11.02
20:3n-3	tr	0.1	0.1	0.1	0.13	0.14
20:4n-3	tr	0.1	0.1	0.1	0.21	0.11
20:5n-3	0.4	0.4	0.5	3.6	2.44	1.61
22:0	1.1	0.8	1.2	0.5	0.27	0.37
22:1	tr	0.2	nd	nd	0.33	0.10
22:4n-6	0.1	nd	nd	0.1	0.54	0.54
22:5n-6	tr	nd	nd	0.1	0.19	0.83
22:5n-3	0.1	nd	nd	0.2	2.64	3.83
22:6n-3	1.3	0.2	0.5	0.3	0.35	2.31
24:0	2.0	0.7	2.9	0.7	0.31	0.39
24:1	nd	nd	nd	nd	0.93	1.08
ΣSaturates	29.1	33.6	34.0	40.0	30.46	33.36
ΣMonoenes	17.7	19.2	15.4	17.7	38.00	26.68
Σn-6	21.5	13.3	23.8	11.2	17.16	23.52
Σn-3	17.0	17.4	15.6	18.4	7.33	9.33
Σn-3HUFA	1.7	0.7	1.1	4.2	5.64	7.87

II. Aquatic Plants

Fatty acid composition of aquatic plants samples showed low ArA, EPA and DHA levels compared to invertebrates samples. The seagrass samples had low levels of ArA compared to seaweeds samples.

Seaweed and Seagrass

Substantial levels of ArA (3.2 % to 12.9%) in the total lipids were detected in seaweed excluding *Ulva faciata* (Table 9). A species of *Corallinaceae* contained 12.9% of ArA in the total lipid. Aquaculture species, *Kapaphycus* and *Gracillaria*, had ArA levels ranging 5.1% to 6.8% and EPA levels ranging 1.8% to 8.5%, and very low DHA levels (Table 10). Linoleic acid (0.3% to 5.6%) and linolenic acid levels in seaweed were low compared to those of seagrass. *Kapaphycus* and *Gracillaria* might be useful as feed ingredients to provide ArA and EPA.

Four species of seagrass had high linoleic acid (LA: 7.5% to 22.2%) and linolenic acid (LNA: 13.9% to 16.5%) levels with low ArA (0.3% to 2.3%), EPA (0.4% to 3.6%) and DHA (0.2% to1.3%) levels in the total lipids (Table 11). Other major fatty acids in seagrass were 16:0 (21.3% to 29.5%) and 16:1n-7(10.8% to13.7%).

Renaud et al. (1999) investigated fatty acid composition of 18 species of tropical Australian microalgae. Only two pelagic species, *Nitzschia* sp. and *Fragilarias* sp. had relatively high ArA levels equivalent to EPA levels, but in the remaining 16 species, EPA was the major HUFA and ArA level was low. EPA was also the major HUFA in tropical phytoplankton sampled from coastal waters of the South China Sea during one year cycle (Shamsudin, 1998). Thus, planktonic microorganisms do not appear to be the primary source of ArA even in tropical waters. High ArA levels are found in some species of marine red and brown seaweeds from both temperate and tropical waters, although this phenomenon is not always limited to tropical areas (Johns et al. 1979; Dembitsky et al. 1991; Vaskovsky et al. 1996). Nevertheless, red and brown seaweeds may be at least one of the sources.

III. Dugong

The total lipids of abdominal muscle had linoleic acid of 5.2%, linolenic acid of 1.4%, ArA of 7.8%, EPA of 2.4% and DHA of 0.4%, respectively (Table 11). The total lipids of liver had linoleic acid of 8.3%, linolenic acid of 1.2%, ArA of 11.0%, EPA of 1.6% and DHA of 2.3%, respectively. Other major fatty acids in the muscle and liver were 16:0 (17.8% and 16.8%), 16:1n-7 (8.1% and 3.6%), 18:0 (9.9% and 15.3%), 18:1n-9 (26.2% and 20.2%) and 22:5n-3 (2.6% and 3.8%).

ArA–derived eicosanoids have been identified in many fish species and have a wide range of physiological functions including control of fluid and electrolyte fluxes, the cardiovascular system, reproductive function and control of the neutral system (Mustafa and Srivastava 1989 and Johnston et al. 1983). Suloma and Ogata (2011) suggested that coral reef, in particular demersal fish appear to have a comparable ArA/EPA ratio with freshwater fish rather than cold and temperate water marine fish. Suloma and Ogata (2011) reported that the

testes of the coral reef fish have higher ArA levels and ArA/ EPA ratios than those of cold and temperate water species. This may imply that ArA is more important for coral reef fish.

The present data provide an insight into fatty acids flow through the coral reef community. It is widely accepted that fatty acid composition of fish tissues reflects dietary fatty acid composition. Our previews results suggest that muscles and gonads from carnivore species tend to contain ArA acids more than those from herbivores species in coral reef waters (Suloma & Ogata 2011 and Ogata et al. 2004). Thus the fatty acids profile of invertebrate and aquatic plants samples in the present study might answer why there are differences in ArA levels of coral reef fish families. Lutjanida, *Lethrinidae*, *Serranidae* and *Labridae*, which are carnivore species, had higher ArA levels compared to Siganidae species which are a browsing herbivore.

Scarce data are available on fatty acid composition of benthic prokaryotes and eukaryotes, bacteria, fungi and protozoa towards the beginning of ArA source in tropical marine food chain. The present result, high ArA levels in coral reef fish, suggests that the existence of an ArA-rich food chain may be widespread in coral reef areas, and that the widespread existence of ArA-rich food chain may lead to comparatively higher ArA contents in the coral reef fish. However, the origin of ArA in the coral-reef food web is still unclear.

CONCLUSION

From our previous and present studies we found that in general ArA was high and superior to EPA in coral reef fish compared to cold and temperate fish in all tissues studied. However, the origin of ArA is not still clear. Micro-organisms on the bottom or in the soil and/or macroalgae may be the sources. Although it is highly speculative, the present results suggest that the existence of an ArA rich food chain may be wide spread in coral reef areas, and that the wide spread existence of ArA-rich food chain may Lead to intermediate or high ARA contents in tropical species. This speculation does not rule out the possibility that coral reef animals might have the ability to convert linoleic acid to ArA. The information in the present study would be suggestive for development of appropriate grower and broodstock and larval diets, to ensure high egg and larval quality of sustainable hatchery production in coral reef areas.

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Chapter 11

CORAL SKELETONS AS A RECORDER OF METAL POLLUTION: ENVIRONMENTAL MONITORING IN THE GULF OF THAILAND

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ABSTRACT

Coral skeletons provide useful information on aquatic environments in which corals grew, and they also offer to use as recorders of pollution history from urbanized hinterlands to coral reefs. In order to monitor pollutant discharges from urban areas to the Gulf of Thailand since the 1980s, we collected *Porites* corals from Khang Khao Island about 50 km southeast of Bangkok in 1985, 1998, 2001 and 2008. The coral collection periods since the 1980s coincided with a series of laws enacted by the Thai government to curb environmental pollution. To determine the skeletal growth of the samples, oxygen isotopes ($^{18}O/^{16}O$ as $\delta^{18}O$) in coral aragonite was measured by stable isotope mass spectrometry. A cyclical change in $\delta^{18}O$ is observed to record an annual change in seawater salinity, and then the coral growth rate is estimated at ~18 mm/year on average. Using the skeletal $\delta^{18}O$ method, the coral chronology was established in the Gulf of Thailand. Nest we used a recently developed laser ablation inductively coupled plasma

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mass spectrometry (LA-ICP-MS) method to assess the impact of metal pollution on coral skeletons taken from the Gulf of Thailand since the 1980s. The extent of anthropogenic contribution by riverine input to the gulf, including aerosol deposits, was assessed by comparing metal-calcium (Me/Ca) ratios of Khang Khao corals to those of Rukan-sho, a relatively unpolluted coral reef, Okinawa. In this comparison, high riverine inputs of Ba, V, Cd and Pb were observed from the Me/Ca values in the Thai coral samples. Since Ba concentration in seawater around Khang Khao Island largely depends on input from the rivers, especially the Chao Phraya River, the Ba/Ca ratios in Khang Khao corals reflect by the high concentrations of riverine input, showing cyclical variations like those of oxygen isotopes in the coral samples. The V/Ca ratios of Khang Khao corals showed a higher average value than that of the Rukan-sho coral, suggesting anthropogenic vanadium inputs due to fuel oil pollution in the Gulf since the late 1990s. Higher Cd/Ca ratios were observed in Khang Khao corals compared to that of Rukan-sho coral, indicating that the Cd concentration in the Gulf continuously suffered from anthropogenic input since 1983. The levels of Cd in the coral indicate a gradual decrease in the Gulf in the late 1990s, with a drastic drop in concentration from the 1980s. The historical variation in Pb/Ca ratios recorded in the coral skeletons suggests the Gulf of Thailand suffered from anthropogenic lead from 1985 to 2001. The Pb/Ca values recorded in Khang Khao Island corals suggest that the Gulf has been polluted by anthropogenic Pb from the early 1990s. After the use of leaded gasoline was banned in Thailand since 1995, the Pb/Ca in the Khang Khao Island corals showed a remarkable decrease, indicating that regulatory control has limited anthropogenic Pb inputs to the Gulf of Thailand. In conclusion, the coral archival record of the metals (V, Cd, Hg and Pb) strongly suggests the success environmental of the laws and regulations by the Thai Government introduced since the middle 1990s.

Keywords: coral skeleton, metal pollution, laser ablation, environmental monitoring, Thailand

1. INTRODUCTION

Sediments are chemical fossils that record global and local environmental changes in water media. However, the record from sediment data could be altered by human activities such as dredging and processes like remineralization and recrystallization. Compared to sediments, coral aragonite ($CaCO_3$) is a better proxy tool and remarkably provides a more faithful representation of environmental metal loads. Corals are deemed to be very useful indicators of pollution level because their skeletons assimilate records of certain metals over hundreds of years (Esslemont, 1999). The composition of coral skeleton reflects the extent of pollution caused by heavy metals or nuclear wastes (Scott, 1990). *Porites* corals, for example, can function as recorders of mining and environmental impacts (Fallon et al., 2002; David, 2003; Edinger et al., 2008). A comparison with the natural background of the area could then provide the basis for identifying influences of local human activities on coral reefs.

During the formation of $CaCO_3$, metal ions are taken up by the aragonite lattice, according to an ion-exchange reaction, i.e., $CaCO_3 + Me^{2+} = MeCO_3 + Ca^{2+}$. The metal-to-calcium (Me/Ca) ratio of coral aragonite is mainly controlled by three factors: (1) the distribution coefficient of the metal ion between aragonite and seawater, (2) the Me^{2+}/Ca^{2+} ratio of the surface oceanic water, and (3) biological effects. Biological effects are possibly negligible when the same coral species is used.

Certain Me/Ca ratios in corals have been well documented as a mean to evaluate aquatic environmental conditions in which the corals grew. For instance Sr/Ca in seawater is uniformly distributed in the world's oceans. Because Sr distribution coefficients are controlled by temperature, Smith et al. (1979) clarified that Sr/Ca ratio in coral skeleton could be used as a thermometer. This Sr/Ca thermometer is now currently used to track sea surface temperature (SST). There is also the promise of using the Mg/Ca ratio along with the Sr/Ca ratio because the former varies with SST change about four times greater than that of the coral Sr/Ca ratio (Mitsuguchi et al., 1996). As a coral recorder for pollution use, Shen and Boyle (1987) showed that Pb/Ca in coral skeleton increased in the 1940s due to the use of leaded fuels for motor vehicles. Bastidas and Garcia (1999) have also confirmed that Pb/Ca ratio could be applied as a recorder of marine pollution from anthropogenic activities. In order to assess lead contamination due to rapid development near a coastal area, Bangkok, Tanaka et al. (2010) measured Pb/Ca in *Porites* coral. Similarly, other Me/Ca ratios in corals might be used to extend the pollution assessment to other metals present in the ambient seawater (Tanaka et al., 2013).

As is well known, the ecological balance is disturbed by land-use changes and associated river discharges (West and van Woesik, 2001) that contain sediments contaminated by trace metals (Elbaz-Poulichet et al., 1984; Bastidas et al., 1999). Rivers are subject to discharges of wastewater as an inevitable result of urbanization. Untreated sewage coming from domestic and industrial sources is transported from the rivers to the estuary, carrying pollutants like heavy metals. The adverse effects of these pollutants is evident in the coastal environment, e.g., reduced biodiversity, diminished productivity and even the death of certain corals. Although there are marine species that can thrive under relatively high metal concentrations (Miao et al., 2001), the difference in species-specific response due to biochemical utility or toxicity (Esslemont et al., 2000) may prove deleterious to other life forms in which low metal concentrations can significantly affect the fertilization process (Reichelt-Brushett and Harrison, 1999). Pollution from sewage and heavy metals might contribute to the collapse of reef ecosystems adjacent to the river mouth and lead to the loss of estuarine habitats (Zann, 2000).

Riverine transport is regarded as an important pathway for input of nutrients to the coastal environment. Riverine nutrient loads of nitrogen, phosphorus and silica have been known to limit biological primary production in coastal areas. For example, phosphate deficiency in coastal areas could preclude the dominance of diatoms in the phytoplankton community (Egge, 1998). In some cases, nutrient input may generate eutrophication which enhances photoplankton growth. Because river discharge is a major source of both nutrients and heavy metal pollutants, it is appropriate to measure the chemical composition of rivers and estuarine areas, in the interest of pollution monitoring. Several studies have been carried out to assess the metal content of river water, colloidal particles and sediments in estuary (e.g., Banat and Howari, 2003; Ramos et al., 2004; Wijaya et al., 2012; 2013). To better understand the anthropogenic impact of metals in the coastal environment, it is therefore critically important to study river transport and discharge to the estuary and to monitor the metal contents in reefs adjacent to the river mouth.

Coral reefs are widely distributed along the coasts of the Gulf of Thailand. This region, which includes the capital of Thailand, Bangkok, has experienced rapid urbanization and industrialization in the last two decades. The impact of heavy metal pollution in Bangkok has been studied to assess the effects of urbanization and industrialization on the environment

(Mukai et al., 1993; Cheevaporn et al., 2004). Lead isotopes in airborne particles collected from Bangkok suggest that the main source of Pb has been the use of leaded gasoline (Mukai et al., 1993). Since four rivers discharge into the northern coast of the Gulf after running through the urbanized and industrialized areas, the impact of heavy metal pollution on aquatic environments in Thailand has also been investigated (Hungspreugs et al., 1989; Cheevaporn and Menasveta, 2003). The Chao Phraya River, the largest river in Thailand, is heavily affected by a variety of anthropogenic activities along its length. There are numerous sources of domestic and industrial effluents that lead to heavy metal enrichment in water and sediments. Indeed, at the mouth of the Chao Phraya River, which runs through Bangkok, a relatively higher heavy metal concentration was observed from river water and sediments (Hungspreugs et al., 1989; Wijaya et al., 2013).

To date, only a few studies have assessed the impact of metal pollution in Thai coral reefs (Hungspreugs et al., 2002; Tanaka et al., 2010; 2013). Corals are often chosen as samples in such studies as their colonies live for long periods of time and their aragonite skeletons often have growth bands that show a clear chronology of their lifetime. Using such skeletons, laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has been used to measure heavy metals to understand trends in historical pollution in coastal areas (Alibert et al., 2003; Edinger et al., 2008; Tanaka et al., 2013). The coralline archival approach is now viable because newly developed LA-ICP-MS methodology makes it possible to measure one-dimensional and two-dimensional trace metals' distribution in coral carbonates. Here we present recent changes in heavy metal distribution in corals from Khang Khao Island examining the period from 1982 to 2008. The coral archive provides a means to examine the health status of the current coastal environment in the Gulf of Thailand such that it can be preserved for the benefit of the Thai people.

2. MATERIALS AND METHODS

2.1. Study Location

The Gulf of Thailand is located in the southwest of the South China Sea (Figure 1a), with its upper portion bounded by the Thai coast (Figure 1b) and characterized by limited water exchange with the open ocean (Hungspreugs and Yuangthong, 1983). Four rivers (the Chao Phraya, the Mae Kong, the Ta Chin, and the Bang Pakong) flow into the northern coast of the Gulf; the largest among them being the Chao Phraya River, which has a drainage area of ~177,000 km^2 and an annual average flow rate of >1000 $m^3 s^{-1}$ (Hungspreugs et al., 1989) and it runs through both urbanized and industrialized areas in Bangkok before it discharges into the Gulf. The suspended matter concentration in the river mouth has been estimated to be over 100 mg/L (Cheevaporn and Menasveta, 2003). The sampling site, Khang Khao Island (13˚09′N, 100˚48′E), is located in the upper Gulf about 40 km southeast of the river mouth of the Chao Phraya and directly south of the island of Ko Sichang (Figure 1b). Sea surface temperature (SST) was measured using a standard mercury thermometer (minimum unit: 0.1°C, precision: ± 0.05°C) at the sampling site (water depth: ~0.5 m) of Khang Khao Island when we collected the coral samples from 1998 to 2008 (see Figure 1b). On 10 November 1999, a Tidbit temperature logger (Stow Away, USA) was also set up at the study site to

record *in situ* seawater temperature every 1 hour. The logger was collected on 15 March 2001 and the recorded SST's were downloaded at the laboratory.

To serve as a reference for the relatively pristine, un-contaminated coral community, a sample was also taken from Rukan-sho (26˚06′N, 127˚32′E; see Figure 1a), Okinawa, Japan. Rukan-sho, an isolated and beautiful Okinawan island, is a small atoll that has a rich coral reef fauna and flora (Ohde and van Woesik, 1999).

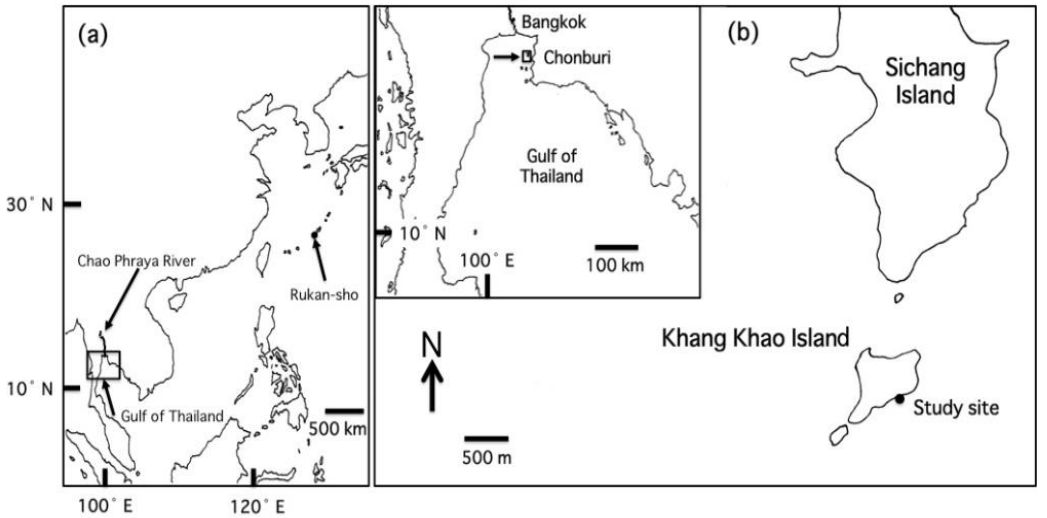

Figure 1. Map showing sampling locations. (a) The Gulf of Thailand and Rukan-sho, Okinawa, (b) the Chao Phraya River estuary and Khang Khao Island in the Upper Gulf of Thailand (inset) and the sampling site off of Khang Khao Island.

2.2. Sampling

The sampling site for both seawater and coral was located approximately 30 m off of the southern coast of Khang Khao Island (Figure 1b). This fringing reef was located underneath a cliff that sloped down towards the north side of the island, making groundwater contamination negligible. Seawater samples were collected from approximately 0.5 m below the surface and stored in 250 mL plastic bottles. Within several days, salinity was measured using a salinometer (601 MKIII, YEO-KAL Electronics, Australia) after calibrating it with an IAPSO standard seawater (Salinity: 34.996 psu, K_{15}: 0.99990) (precision: ±0.003). Four *Porites* coral samples were collected from the reef approximately 5 m below the surface, within an approximate 10 m radius of the sampling site. In order to check metal pollution as far back as the early 1980s, we collected a coral sample (size, ϕ: ~15 cm, h: ~10 cm) in the years of 1985, 1998, 2001 and 2008, hereafter referred to by sample numbers, KK-85, KK-98, KK-01 and KK-08, respectively. After being stored in polyethylene bags, the samples were washed several times using deionized water (MQ water). In preparation for analysis, the samples were cut into slabs of approximately 5 mm thickness along the growth axis using a diamond saw. For stable isotope and trace element analyses, the slab was cut into a small fragment (~7 x 40 mm) in order to fit into the experimental LA cells of the mass spectrometer

(LA-ICP-MS). It was then cleaned with MQ water in an ultrasonic bath before being dried in an air oven (~60ºC, 7 h).

For the purposes of registering baseline metal data, we used a relatively unpolluted *Porites* coral sample collected from the reef at Rukan-sho (Figure 1a) on 3 November 1987 (RS-87). In Japan, the use of leaded gasoline has been prohibited since 1980, while it was used up until the late 1990s in Thailand. Considering these time periods, we used a *Porites* sample collected from Rukan-sho in the year 1987 (RS-87).

2.3. Preparation of $CaCO_3$ Matrix Standard

A series of calcium carbonate (aragonite) matrix standards (Std-0, Std-1, and Std-2) were prepared for LA-ICP-MS by using a mixed solution of a multi elemental standard solution containing 100 mg/L of V, Cd, Ba, and Pb (SPEX, XSTC-22, USA) (see Table 1). This multi elemental standard and 1 M HCl (10 mL) were added to a 1 M $CaCl_2$ solution (40 mL), which was then diluted to 140 mL with MQ water and then heated. With gentle boiling, a 1.5 M Na_2CO_3 solution (60 mL) was added into the solution, producing a $CaCO_3$ (aragonite) precipitate. Use of higher temperature makes it possible to precipitate aragonite crystal from aqueous solution rather than calcite. After stirring and heating for 2 minutes, the suspension was then kept at 15°C for 4 hours, before the $CaCO_3$ was removed from the suspension through filtering. The parent solutions for synthesized Std-1 and Std-2 contained the metals (V, Cd, Ba and Pb) as concentrations of 1.19 mg/L and 2.91 mg/L at the initial stage of the experiments. The precipitate was dried at 50ºC for 14 hours, and then pressed into a pellet (ϕ: 10 mm, ~3 mm thickness). Calcium carbonate was also synthesized in the absence of the multi-elemental standard solution and served as a low concentration standard (Std-0). Calibration for V/Ca, Cd/Ca, Ba/Ca, and Pb/Ca in coral samples was based on the use of the synthetic $CaCO_3$ standards (Table 1). A coral aragonite reference material, JCp-1 (Okai et al., 2002), was pressed into pellet form to facilitate determination of B/Ca in the coral samples by LA-ICP-MS. These solid standards were then dissolved in 3% nitric acid and analyzed for V/Ca and Cd/Ca by ICP-AES (Spectro Arcos, Spectro, Germany) at Sheffield University, and for Ba/Ca and Pb/Ca by ICP-MS (HP-4500, Hewlett Packard, Japan) at Ryukyu University.

Table 1. Added amount of multi-elemental standard, XSTC-22, and count ratio for V/Ca, Cd/Ca, Ba/Ca and Pb/Ca of the standard measured by LA-ICP-MS. The parent solution of synthesized standard, Std-1 and Std-2, contained metals (V, Cd, Ba and Pb) of 1.19 and 2.91 ppm, respectively. Molar ratio of the standard was determined by ICP-AES and ICP-MS as described in the text

Code	Added amount	V/Ca		Cd/Ca		Ba/Ca		Pb/Ca	
	(mL)	($x10^{-3}$ count/ count)	(μmol/mol)	($x10^{-3}$ count/ count)	(μmol/mol)	($x10^{-3}$ count/count)	(μmol/mol)	($x10^{-3}$ count/count)	(μmol/mol)
Std-0	0	0.00857	0.430	0.0223	0.400	0.510	2.75	0.0575	0.214
Std-1	3	0.696	3.22	2.43	55.9	8.55	41.6	12.1	26.2
Std-2	7.5	1.98	9.46	5.07	138	16.1	110	25.3	70.0

2.4. LA-ICP-MS Analysis

The LA-ICP-MS system at Sheffield consisted of a Nd:YAG laser (UP266 MACRO, New Wave Research, USA) connected to an ICP-MS (HP-4500, Agilent Technologies, Japan). One-D and 2-D (imaging) analyses were carried out on coral skeletons and synthetic metal standards prepared in the $CaCO_3$ matrix. Main laser operating parameters were wavelength, 266 nm; beam diameter, 155 µm; laser energy, 2 mJ; repetition rate, 10 Hz. Prior to analysis, the coral section was first subjected to laser ablation along the line that would be used for laser sampling as a means of pre-treatment for the sample surface cleaning. Acquisition times for measurement of ^{11}B, ^{51}V, ^{111}Cd, ^{137}Ba, ^{202}Hg, and ^{208}Pb were 100 ms and 10 ms for ^{42}Ca. The isotope ^{42}Ca was used as the internal standard, as measurement precision was lower than that for ^{43}Ca. For coral samples, the sample translation stage was moved at a constant speed of 10 µm s^{-1} to create an ablation track along the growth axis of the skeleton. The LA tracks for samples KK-85, KK-98, KK-01, KK-08, and RS-87 were 36.5, 38.8, 42.3, 36.6, and 34.1 mm in length, respectively. For the synthetic metal standards prepared in the $CaCO_3$ matrix, each pellet was subjected to three consecutive and parallel line rasters of 9 mm in length with a spacing gap of 400 µm between each ablation track. The data obtained from the analyses were relatively noisy; hence a noise-reduction filtering procedure (Sinclair et al., 1998) was also utilized. The precisions (external) for the LA-ICP-MS measurement of B/Ca, V/Ca, Cd/Ca, Ba/Ca, and Pb/Ca, were 8.5%, 1.4%, 6.0%, 2.1%, and 6.0%, respectively.

2.5. Stable Isotopic Analysis

For the stable isotopic analysis of oxygen and carbon, a vertical bar (~40 x 4 x 3 mm^3) was cut along the major growth axis of the coral sample using a fine diamond saw. The sample was cleaned ultrasonically and dried up. Approximately 1 mm of aragonite coral was gradually shaved from the top of the bar as a sub-sample using a dental drill. A sub-sample depth was measured by an Absolute Digital Solar Caliper (CD-S20C, Mitutoyo, Japan) after shaving. The shaved sample (~0.15 mg) was dissolved with 100% phosphoric acid at 70ºC for isotopic analysis. The reaction occurred in an automated individual carbonate reaction device (Kiel device), and oxygen and carbon isotopes were measured using a mass spectrometer (Finnigan MAT251 or Delta V, Thermo Fisher, Germany) (Hossain et al., 2008). The isotope ratios are presented in conventional δ notation relative to the isotope ratio of CO_2 gas derived from the PDB standard through NBS-19. Isotope values for the coral skeleton are expressed in per mil (‰) as follows:

$$\delta^{18}O = 1000 \times \{(^{18}O/^{16}O)_{sample} / (^{18}O/^{16}O)_{standard} - 1\}.$$

Skeletal $\delta^{13}C$ data are measured simultaneously in term of the $^{13}C/^{12}C$ ratio. For seven replicate measurements, the precision of internal standard was calculated at 0.18 and 0.12‰ for $\delta^{18}O$ and $\delta^{13}C$, respectively.

In addition, $\delta^{18}O$ values for 15 seawater and 8 river water samples were measured using the same mass spectrometer (MAT 251). Most of the samples were measured twice. A 2 mL

of the sample was taken into a syringe and equilibrated with 4 mL of CO_2 of known $\delta^{18}O$ by shaking it at 25 ± 0.1 ˚C for 12 h. The equilibrated CO_2 gas was then pushed through the vacuum line of the spectrometer for CO_2 extraction and the value was measured. Seawater $\delta^{18}O$ are also expressed in per mil (‰) relative to the Vienna-Standard Mean Ocean Water (V-SMOW) standard as follows:

$$\delta^{18}O_w = 1000 \times \{(^{18}O/^{16}O)_{sample} / (^{18}O/^{16}O)_{SMOW} - 1\}.$$

3. RESULTS

3.1. Seawater Temperature, Salinity, and Oxygen Isotope

The salinity and SST data including seawater $\delta^{18}O_w$ observed at Khang Khao Island are given in Table 2. The SST was nearly constant between 27.1 and 31.0 ˚C, with an average SST (±1σ) of 29.4 ± 1.0 ˚C (Table 2). The SST for the location (11.5-13.5°N, 99.5-101.5°E) was also obtained from data acquired by NOAA (http://iridl.ldeo.columbia.edu) (see Figure 2c). Salinity varied largely between 21.129 and 32.515 psu. During the wet season (June - October), relatively lower salinity (21.129 – 30.412 psu) was observed, whereas higher salinity (31.260 - 32.515 psu) was observed during the dry season (November - April)(see Table 2). Similarly, seawater $\delta^{18}O_w$ varied between -1.609 and -0.232‰ (SMOW). As listed in Table 2, during the wet season (June - October), relatively lower $\delta^{18}O_w$ (-1.609 and -0.537‰) was observed, whereas higher $\delta^{18}O_w$ (-0.365 and -0.232‰) was observed during the dry season (November - April).

Table 2. Salinity, sea surface temperature (SST) and seawater $\delta^{18}O$ observed at Khang Khao Island from 1998 to 2008

Date	Salinity (psu)	SST (˚C)	$\delta^{18}O$ (‰, SMOW)(±1σ)
1998.11.17	31.973	28.4	-
1999.3.3	32.515	29.0	-
1999.8.26	28.431	30.2	-
1999.11.10	31.401	29.2	-
2000.1.6	32.405	25.9	-
2000.1.22	32.042	27.1	-0.300 (±0.030)
2000.2.22	32.352	27.5	-0.248 (±0.035)
2000.4.8	32.389	32.4	-0.232 (±0.026)
2000.5.5	31.645	30.5	-0.350 (±0.025)
2000.5.20	31.126	31.0	-0.464 (±0.022)
2000.6.10	30.412	30.5	-0.537 (±0.030)
2000.6.17	27.355	30.0	-0.938 (±0.025)
2000.7.20	27.541	29.2	-0.848 (±0.029)
2000.7.27	21.129	29.4	-1.609 (±0.025)
2000.8.29	26.362	29.4	-0.973 (±0.023)
2000.10.5	28.965	30.5	-0.724 (±0.040)
2000.10.19	30.383	30.1	-0.625 (±0.017)
2000.11.8	31.260	28.6	-0.365 (±0.025)
2001.3.15	31.879	29.0	-0.335 (±0.023)
2001.7.20	28.794	29.9	-0.700 (±0.040)
2002.9.25	25.442	29.9	-
2008.9.5	28.266	30.0	-

Sampling date (e.g., 2008.9.5: 5 September 2008)

In order to understand the annual variation in seawater $\delta^{18}O_w$ at Khang Khai Island, river water was collected from the Chao Phraya River near Shangri La Hotel, Bangkok in July, September and November, 2000, and July 2001. River water $\delta^{18}O_w$ varied between -6.60 and -4.10‰. Relatively lower $\delta^{18}O_w$ was observed at -6.14 and -6.60‰ in September and November 2000, while higher $\delta^{18}O_w$ was observed at -4.81 and -4.10‰ in July 2000 and July 2001. In addition, we also collected river water samples from the Mekon River near Golden Triangle, Thailand. The Mekon River water $\delta^{18}O_w$ showed lowest value at -11.0 (±0.070)‰ as $\delta^{18}O_w$.

3.2. Oxygen and Carbon Isotopes in Coral

The coral sample KK-98 collected on 17 November 1998 from the Khang Khao Island reef showed that two pairs of density bands were observed for a single year's growth (Hossain et al., 2008). This fact makes determining the chronology of the sample unreliable. In the absence of a clearly defined sclerochronology, the oxygen isotope data are used, instead, to determine the chronology and growth rate. The $\delta^{18}O$ data (Figure 2a) permitted the determination of the growth rate based on the cyclic changes of the sample, and this in turn contributed to the definition of the chronology. In addition, skeletal $\delta^{13}C$ values also shows the changes during the study (Figure 2b). The changes occur due to the changes in light intensity and water chemistry, especially carbon dioxide and nutrients in ambient seawater.

To examine the relationship between the coral skeletal $\delta^{18}O$ and precipitation, rainfall data in the periods of 1981 - 1985, 1996 - 2001, and 2006 - 2009 were acquired from the Thai Meteorological Department, and compiled for the Bangkok Metropolitan Area (Figure 2d) and the island of Ko Sichang (Figure 2e); Sichang is nearest to the Khang Khao Island sampling site (see Figure 1b). The total monthly rainfall data are presented for the years 1981 to 1985, 1996 to 2001, and 2006 to 2009 (see Figures 2d and 2e). From these data it is clear that precipitation around the Gulf of Thailand is generally cyclical in nature. The greatest amount of rainfall appears to occur in the months of August, September, and October, with the months of January, February, March, and December receiving little to no recorded rainfall in either Bangkok or Ko Sichang Island.

3.3. Me/Ca in Coral

Using the LA-ICP-MS data, the calibration curves for V/Ca, Cd/Ca, Ba/Ca, and Pb/Ca (Table 1) were calculated to have correlation coefficients (*r*) of 0.996, 0.995, 0.981, and 0.991, respectively, which were then used to determine the Me/Ca ratios in the coral samples. The measured values of B/Ca, Ba/Ca V/Ca, Cd/Ca, Pb/Ca (μmol/mol), and Hg/Ca (cps/cps) in the coral skeletons from Khang Khao Island are shown in Figures 3a-f, respectively, with the averages and standard deviations listed in Table 3. The average ratios found in the Rukan-sho sample (RS-87) are represented by a dashed line in the figures. In evaluating the Me/Ca values in the coral samples' tissue layer (~5 mm from the surface of the sample), the ratio values in the surface layer were calculated to be much higher compared to the ratios found in the remaining skeletal layers. As it has been found that corals seem to have higher concentrations of these trace metals in the surface layer due to an as yet unidentified factor,

the tissue layer ~~was~~ has not been discussed in this study. For our model, interspecies variations are considered negligible.

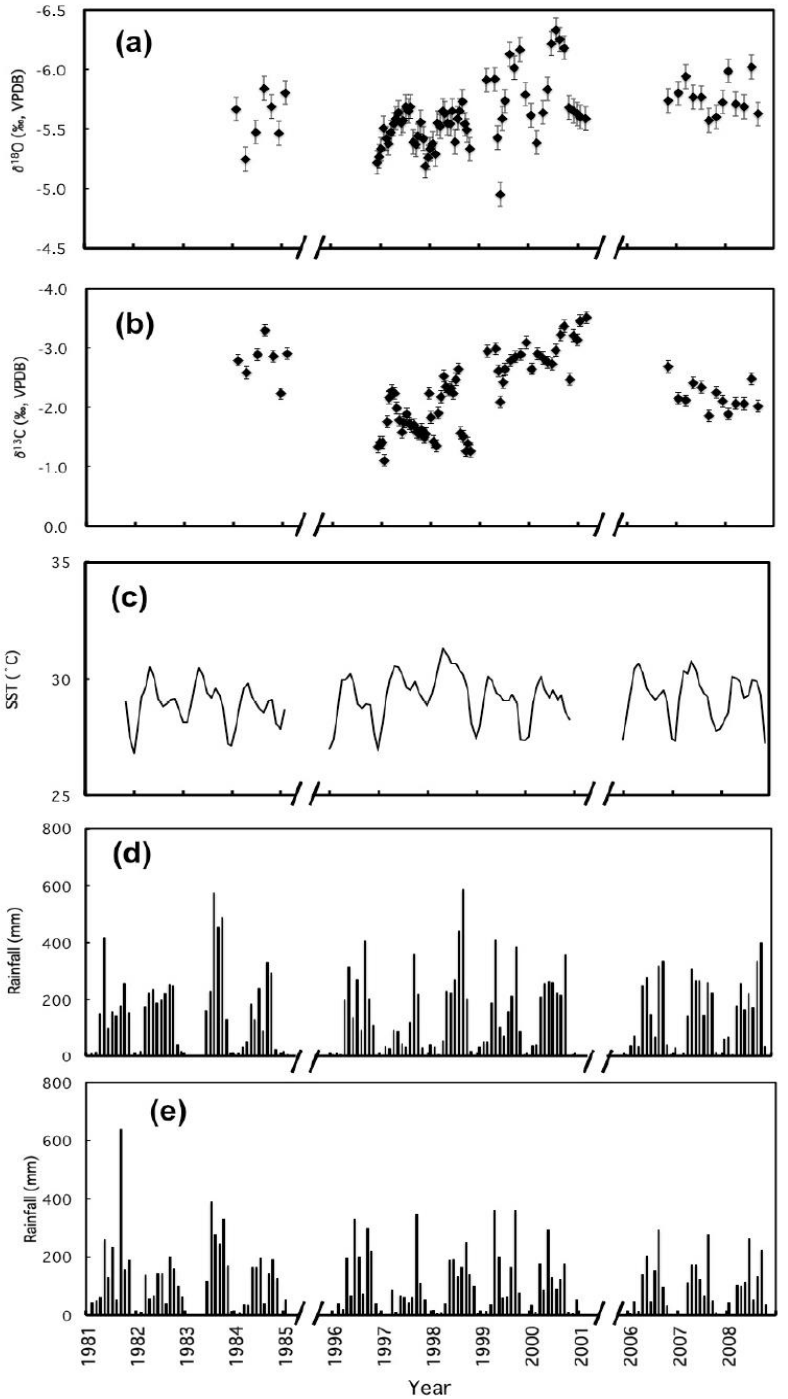

Figure 2. (a) Oxygen isotopes ($\delta^{18}O$) and (b) carbon isotopes ($\delta^{13}C$) in corals from Khang Khao Island, (c) SST (NOAA satellite data near the Khang Khao site described in the text), and total monthly rainfall for (d) Bangkok and (e) Sichang Island, Thailand.

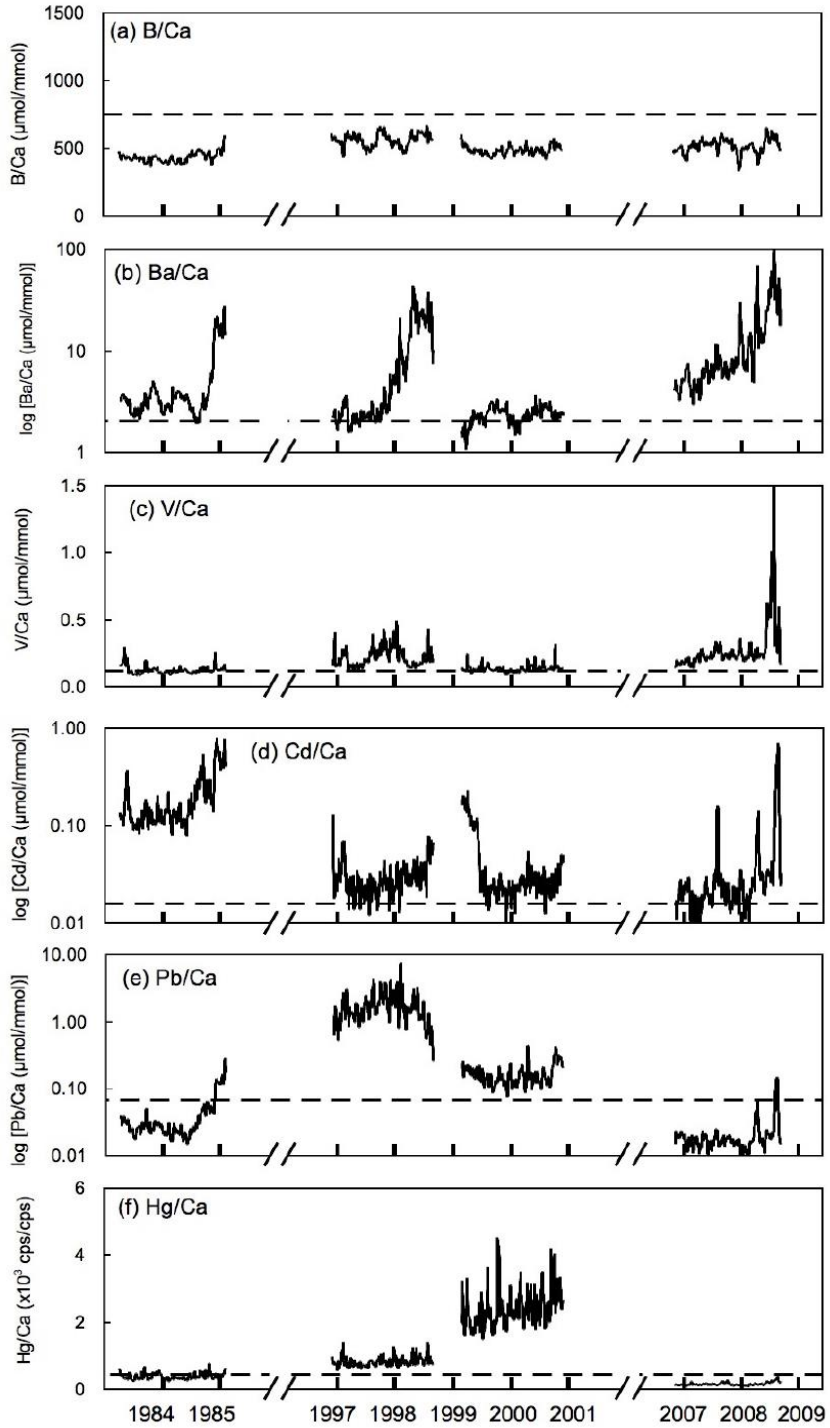

Figure 3. Metal-to-calcium molar ratio (cps/cps for Hg/Ca) in corals from Khang Khao Island. (a) B/Ca, (b) Ba/Ca, (c) V/Ca, (d) Cd/Ca, (e) Pb/Ca, and (f) Hg/Ca. Dashed line shows average value observed in the Rukan-sho sample.

4. DISCUSSION

4.1. Salinity and $\delta^{18}O_w$ in Seawater, and Oxygen and Carbon Isotopes in Coral

Salinity in the Gulf of Thailand is primarily dependent on the influx of freshwater as a result of the annual monsoon cycle. The wet season from late May to October causes a decrease in salinity, and the lack of rainfall in the dry season from November to April causes the salinity to increase (see Figures 2d-e). Table 2 clearly shows such a cyclical pattern to the salinity in the Gulf. The SST data (Figure 2c) show an annual cyclical change with a pair of quartic maxima. Such quartic maxima peaks (Figure 2c) may be explained from three seasons that include hot dry, hottest dry and hotter wet cycles of the annual climate change. These fluctuations in SST and salinity are then reflected in the $\delta^{18}O$ in the carbonate skeletons (Dettman et al., 2001). Sun et al. (2005) have previously measured the annual monsoon season's effects on *Porites* specimens, as the rainfall including river water input, enriches the local seawater with the lighter isotope (^{16}O), which is then reflected in the skeletons as a cyclical decrease in the $\delta^{18}O$.

Being a tropical area, the upper Gulf of Thailand usually receives a very heavy rainfall during the wet season. Since local precipitation and evaporation rate including river run-off affects salinity and eventually changes the seawater $\delta^{18}O_w$ values, the corals in Khang Khao Island are likely to grow up under a large variation (~11 psu) in sea surface salinity (Table 2). Moreover, there were often sudden outbreaks of floods along the Chao Phraya River in Bangkok. For example, severe flooding occurred from July to November 2011, which caused much damage and hardship to Thai people and the economy. Considering the annual seasonal change for rainfall (Figures 2d-e), there would be expected to be a correlation changes in salinity and skeletal $\delta^{18}O$ values. In order to verify such a correlation, a calibration was established for seawater $\delta^{18}O_w$ and salinity values using the data in Table 2. The linear correlation between seawater $\delta^{18}O_w$ and salinity is expressed as follows: $\delta^{18}O_w$ (‰, SMOW) = 0.1205 × Salinity – 4.1829 (r^2 = 0.979, n=26). The rainfall enriches the local seawater with the lighter isotope (^{16}O) as it decreases the surface salinity, which is then reflected in the skeletons as a cyclical decrease in the $\delta^{18}O$. In turn, when the temperature rises, the lighter isotope is also preferred in isotopic reactions, depressing the observed $\delta^{18}O$ (Gagan et al., 2012). The seasonality of these events results in a cyclical change in $\delta^{18}O$ expressed in the coral skeletons, with the monsoonal rainfall first decreasing salinity and therefore $\delta^{18}O$, and then the hotter monsoon season's increase in SST further depresses the $\delta^{18}O$ ratio.

On the other hand, skeletal $\delta^{13}C$ values show slightly less clear cyclical changes compared to those of $\delta^{18}O$ (Figures 2a and 2b). The photosynthetic rate of coral's symbiotic zooxanthallae increases with increasing in the light intensity and thereby $\delta^{13}C$ values increase due to the uptake of $^{12}CO_2$ in seawater instead of $^{13}CO_2$. Moreover, the sun photoperiod and nutrients influence $\delta^{13}C$ in coral skeletons. As shown in Figure 2b, high $\delta^{13}C$ values in the coral skeletons suggest the records during cool months (December to February) since low photosynthetic rate in the coral reef. The $\delta^{13}C$ data in Figure 2b somewhat indicate variations related to the climate change due to the alternating dry and rainy season, supporting the coral $\delta^{18}O$ record shown in Figure 2a. The $\delta^{13}C$ data might be controlled by the primary production associated with the sunlight intensity (cloud cover) and water chemistry (nutrients and

suspended matter). Nonetheless, variations in skeletal $\delta^{13}C$ values may be interpreted as the changes in light levels of the sample habitat during different seasons, but the $\delta^{13}C$ values are largely affected by metabolic activities for which the values are difficult to use as a tracer of the coral chronology.

We also measured SST every 1 hour for 16 months from November 1999 at the site in Khang Khao Island and the weekly average was used for the calibration of skeletal $\delta^{18}O$ – SST relationship. Monthly mean SST fluctuated from 26.0 to 30.60˚C and salinity varied from 21 to 33 psu during the study. The wet season in the upper Gulf of Thailand lasts from late May to October, and the dry season lasts from November to early May. The $\delta^{18}O_w$ values in ambient seawater are extremely correlated ($r^2 = 0.979$) with salinity values (Table 2), indicating that seawater $\delta^{18}O$ values are strongly affected by salinity.

As pointed out, $\delta^{18}O$ values are lighter in river water, and the rainfall also enriches localized seawater with the lighter isotope (^{16}O) given that it decreases surface salinity, which is then reflected in the skeletons as a cyclical decrease in the $\delta^{18}O$. In turn, when the temperature rises, the lighter isotope is also preferred in isotopic reactions, depressing the observed $\delta^{18}O$. The seasonality of these events results in a cyclical change in $\delta^{18}O$ expressed in the coral skeletons, with the monsoonal rainfall first decreasing salinity and therefore $\delta^{18}O$, and then the hotter monsoon season's increase in SST further depresses the $\delta^{18}O$ ratio. Therefore, we have used $\delta^{18}O$ for coral values to reconstruct the growth rate of the corals due to salinity and SST in the upper Gulf of Thailand.

Using a *Porites* coral from Khang Khao Island (KK-98), Hossain et al. (2008) pointed out two pairs of density bandings were observed in a *Porites* coral for one year (see Table 3 and Figure 2a). The growth rate of the *Porites* samples could not be estimated from the coral density bandings in the Khang Khao coral samples for this reason. In the absence of using the coral skeletons' density bands to determine the chronology, the cyclical manner of $\delta^{18}O$ has been used as an alternative method (Maier and Titschack, 2010). These isotopic data allow us to establish the chronology in scenarios where density banding is unreliable. Using the cyclical changes of the isotopes, we determined the growth rate of the *Porites* samples, which is reported in Table 3.

Another factor that aided in establishing the chronology and the growth rate of the coral samples was the fact that we specifically chose four different samples over the sampling period rather than a single long core. While a single long core is normally preferred in such studies, the density banding of these samples were so poor that using a single core would have made the chronology impossible to determine. The skeletal $\delta^{18}O$ in multiple colonies of *Porites* corals was measured to evaluate inter-colony variation of $\delta^{18}O$ that reported that inter-colony variation of the $\delta^{18}O$ which has reported to be negligible (Matthews et al., 2008; Hayashi et al., 2013). Cadmium contents measured in multiple *Porites* colonies showed similar value among the colonies (Matthews et al., 2008). Spatial distribution of Pb in the surface water of the Pacific Ocean was investigated by measuring Pb/Ca in *Porites* corals taken from different sites, assuming negligible inter-colony variation of the skeletal Pb/Ca (Inoue et al., 2006). Based on these studies, we assume that inter-colony variation of the skeletal $\delta^{18}O$ and metal contents is negligible in the corals taken from within a 10 m radius of the sampling site. The model used in this study to examine the heavy metals incorporated into the coral skeleton considers any such interspecies variations in ratio levels as being negligible, and instead relies on the observed cyclical differences and the observed levels of $\delta^{18}O$ (Figure 2) to estimate the chronology. In conclusion, the cyclic change in $\delta^{18}O$ has been

observed to record an annual change in seawater salinity, and then the coral growth rate is estimated at ~18 mm/year on average (Table 3). Using the skeletal $\delta^{18}O$ method, the coral chronology was established in the Gulf of Thailand in order to determine the horizontal axis shown in Figure 3.

Table 3. Coral sample number (sampling date), growth rate (mm/yr), average value of molar ratio and standard deviation (±σ) of B/Ca, Ba/Ca, V/Ca, Cd/Ca and Pb/Ca (μmol/mol) and count ratio of Hg/Ca (x103 cps/cps)

Sample No	Growth Rate	B/Ca	Ba/Ca	V/Ca	Cd/Ca	Pb/Ca	Hg/Ca
KK-85 (850201)	10.8	434±33	4.89±4.77	0.128±0.030	0.202±0.144	0.0427±0.0411	0.392±0.087
KK-98 (981117)	21.0	563±49	8.32±9.19	0.225±0.073	0.0303±0.0124	1.78±0.99	0.821±0.142
KK-01 (010315)	15.4	488±86	2.34±0.45	0.139±0.030	0.0442±0.2843	0.173±0.276	2.38±0.94
KK-08 (080905)	17.4	519±54	13.4±15.5	0.282±0.188	0.0431±0.0893	0.0211±0.0193	0.168±0.049
RS-87 (871103)	10	753±98	2.03±0.48	0.123±0.013	0.0161±0.0038	0.0679±0.0225	0.936±0.114

Source: Sampling date (e. g. 080905: 8 November 2008) 4.2. B/Ca in Coral.

4.2. B/Ca in Coral

The B/Ca ratios in the samples (Figure 3a) show clear cyclical variations during 1997-1998 in the KK-98 sample, with slightly cyclical variations observed in KK-85, KK-01, and KK-08. The average B/Ca value (±1σ) of the Khang Khao corals was calculated to be 514 ± 87 μmol/mol (434-563 μmol/mol) shown in Table 3. The B/Ca in Rukan-sho coral varied between ~600 - 1110 μmol/mol with an average of 753 μmol/mmol. The average value of B/Ca in Khang Khao corals (434-563 μmol/mol) was lower than the Rukan-sho value.

Using secondary ionization mass spectrometry (SIMS) (Hart and Cohen, 1996) and LA-ICP-MS (Sinclair et al., 1998; Fallon et al., 1999; 2003), a narrow range of seasonal variation in B/Ca was observed in *Porites* corals. Previous studies have subsequently suggested a strong temperature-dependence such that B/Ca in corals increases with decreasing sea surface temperature (Sinclair et al., 1998; Fallon et al., 2003; Ohde et al., 2011). We also pointed out that the distribution coefficient between the seawater borate ion and the aragonite crystal might have a temperature dependency during precipitation (Ohde et al., 2011). Based on the temperature-dependence of B/Ca, the relatively higher SST for the annual average at Khang Khao Island (29.4 ˚C) compared to Rukan-sho (25.0 ˚C) can explain the observation that B/Ca in Khang Khao corals are lower than that in Rukan-sho coral (see Table 3). Further, due to the relatively narrow range of SST at Khang Khao compared to Rukan-sho, the variation in B/Ca of Khang Khao corals was smaller than that of Rukan-sho coral (Table 2). These observations agree with the prediction based on the temperature dependence of B/Ca, which shows that the ratio in the Khang Khao corals is possibly controlled by seawater temperature and has a relatively small variation because of a narrow range of SST observed in the Gulf of Thailand (Table 2).

The data in Table 3 also indicate a range of average B/Ca ratios from 434 to 563 µmol/mol, showing a slight variation in values. The variation of SST and salinity within the monsoon cycle will possibly have an effect on the B/Ca ratio, as it is dependent on these two factors, as well as a biological effects. In spite of such differences amongst the four coral samples, our model does not take into account the biological variation. The cyclical variations found in the B/Ca values in the time periods when it is evident can likely be linked to the same cyclical variations for the $\delta^{18}O$ and precipitation values (see Figures 2a and 3a). The variation of SST and salinity within the monsoon cycle will possibly have an effect on the B/Ca ratio, as it is dependent on these two factors, as well as biological effects. In spite of such differences amongst the four coral samples, our model does not take into account the biological variation. When we use the same coral species, somewhat biological effects are possibly negligible and can be discounted.

4.3. Ba/Ca in Coral

The Ba/Ca in the Khang Khao corals varied in a range from approximately 1 µmol/mol to over 100 µmol/mol. The surface layer in the samples (KK-85, KK-98, and KK-02) was significantly more concentrated in Ba than the lower layers. Such a variation in the barium concentration has been reported previously (Sinclair et al., 1998; Alibert et al., 2003); it is thought that the living tissue is concentrated in Ba through some means, either through the seawater concentration or through the organism consuming Ba-rich food, and this barium is then precipitated into the skeleton (Sinclair, 2005). The values of Okinawa sample (RS-87) showed a narrow range of variation from ~1.5 to 3.7 µmol/mol with an average value of 2.03 µmol/mol (Table 3), which is lower than the Thai samples.

Both river water and deep seawater are enriched in barium due to silicate rock weathering and dissolution of barium-rich minerals, respectively. In surface seawater, Ba is mainly supplied from these river inputs and the upwelling of deep seawater. Because the Ba content in coral skeletons reflects the concentration in seawater (Lea et al., 1989), Ba/Ca in coral skeletons reflects both riverine input (McCulloch et al., 2003; Alibert et al., 2003; Wyndham et al. 2004; Horta-Puga and Carriquiry, 2012) and deep seawater (>1000 m) upwelling (Lea et al., 1989; Fallon et al., 1999). However, upwelling of barium-rich deep seawater is unlikely to occur in the Gulf of Thailand as the depth of the Gulf is much too shallow at ~45 m (Nozaki et al., 2001). Khang Khao Island is located about 40 km to the southeast of the mouth of the Chao Phraya River (Figure 1b) where Ba concentration varied from 250 to 430 nmol kg^{-1}, while Ba in the East China Sea was approximately 40 nmol kg^{-1} (Nozaki et al., 2001). In the upper Gulf of Thailand, seawater Ba concentration increased with decreasing salinity (Nozaki et al. 2001). Barium influx from the Chao Phraya River (250 - 430 nmol kg^{-1}) significantly contributes to the higher Ba concentration at the inner Gulf compared to the East China Sea (~40 nmol kg^{-1}), and with the higher Ba concentration being linked to increasing salinity (Nozaki et al., 2001). Thus the Ba concentration in seawater of the Gulf is formed through the mixing between the East China seawater and river water.

In addition to the Ba concentration in seawater, temperature can also influence the Ba/Ca values in coral aragonite; dissolved Ba^{2+} can be incorporated in coral aragonite as a solid solution. The temperature dependency of the distribution coefficient of barium (D_{Ba}) between seawater and aragonite has been studied, defined as $D_{Ba} = (Ba/Ca)_{aragonite} / (Ba/Ca)_{seawater}$

(Zachel et al., 2003; Dietzel et al., 2004). Assuming this relationship between D_{Ba} (Dietzel et al., 2004) and SST observed at Khang Khao (27.1-30.0 ˚C) (Table 2), the D_{Ba} value is calculated to be 1.37 and 1.27 at 27.1 ˚C and 30.0 ˚C, respectively. This variation in D_{Ba} results in a small variation in Ba/Ca in coral aragonite (less than 8%). Thus the temperature effect on the Ba/Ca values in Khang Khao corals is negligible.

Since Ba concentration in seawater around Khang Khao Island largely depends on input from the rivers, especially the Chao Phyraya River, Ba/Ca in Khang Khao coral reflects the high concentration of riverine input. In the light of such Ba influx from the rivers, the Ba/Ca ratios in the samples (KK-85 and KK-01) could show an annual cyclical change as evident in Figure 3b. Such changes might occur as a result of the monsoon cycle in Thailand.

4.4. V/Ca in Coral

The V/Ca values (Figure 3c) varied over a narrow range in the Khang Khao samples, with the average value being comparable to that of the Rukan-sho sample (Table 3). As shown in Figure 3c, the V/Ca in KK-85 varied in a narrow range (0.09 - 0.30 μmol/mol) with an average value of 0.128 μmol/mol. The V/Ca values in KK-98 (0.16-0.50 μmol/mol) and KK-01 (0.09-0.30 μmol/mol) were higher than that for KK-85. The V/Ca in KK-08 varied between ~0.17-0.30 μmol/mol except for the surface layer, in which the highest V/Ca in the Khang Khao corals (1.56 μmol/mol) was observed. The average value of V/Ca in the coral samples is given in Table 3. Although the average V/Ca in KK-85 (0.128 μmol/mol) is comparable with that in RS-87 (0.123 μmol/mol), the average V/Ca values in KK-98, KK-01 and KK-08 (0.255, 0.139 and 0.282 μmol/mol, respectively) are higher than that of RS-87 (0.10 μmol/mol).

Since vanadium is enriched in crude oil, it is suggested that the main source of anthropogenic input for this element is from refining operations and the burning of residual oil (Hope, 1997). Only a few studies have measured V in coral skeletons in an attempt to detect oil pollution in coral reefs (Shen and Boyle, 1988; Guzman and Jarvis, 1996). Shen and Boyle (1988) reported an almost constant V/Ca in a coral skeleton from Bermuda (~0.1 μmol/mol) and suggested that the variation related to anthropogenic vanadium was possibly swamped by the background concentration in seawater. Guzman and Jarvis (1996) analyzed the V/Ca ratio in a coral skeleton from a Caribbean reef and found an increasing trend from the 1960's (~0.15 μmol/mol) to the 1990's (~0.35 μmol/mol) due to oil pollution.

In order to assess the impact of oil pollution in the Gulf of Thailand, the concentration of petroleum hydrocarbons in marine, estuarine, and riverine environments has been studied (Wattayakorn et al., 1998; Cheevaporn and Menasveta, 2003; Boonyatumanond et al., 2006). The concentration of petroleum hydrocarbons in seawater was measured at 78 sites along the coast in the Gulf of Thailand from 1994-1995 (Wattayakorn et al., 1998). These authors found oil pollution in the coastal waters around oil refineries in Si Racha, which is located about 10 km to the east from Khang Khao Island, and suggested the sources of petroleum pollution might be spills from tankers and the waste derived from the oil refinery and associated industries in the coastal region. The concentration of petroleum hydrocarbons in sediments from the Chao Phraya River collected from 2003-2004 increased from the upper stream to Bangkok and composition profiles of hydrocarbons in sediments from the urban canals were similar to those in street dusts, indicating that street dusts in Bangkok are a

source of oil pollution in the Gulf (Boonyatumanond et al., 2006). In addition, petroleum hydrocarbon contamination was suggested by high vanadium concentrations observed in the seawater of the Gulf (Censi et al., 2006). In contrast, in the 1980s, the petroleum hydrocarbon contamination level in seawater of the Gulf of Thailand was lower than the baseline value of 100 µg/L, suggesting that oil pollution in the Gulf was negligible (Cheevaporn and Menasveta, 2003). Based on the previous studies, it is estimated that the Gulf suffered from oil pollution in the 1990s and 2000s, whilst not in the 1980s. This estimation of the historical change in oil pollution in the Gulf agrees with the variation in the average value of vanadium in corals from Khang Khao (see Figure 3c and Table 3). Therefore, the vanadium content in corals from Khang Khao Island is indicative of oil pollution in the Gulf of Thailand.

4.5. Cd/Ca in Coral

The Cd/Ca values (Figure 3d) in the Khang Khao corals show an unusually high level of Cd in the KK-85 sample, much higher than the other 3 samples, and also much higher than the Rukan-sho values (Table 3). In KK-85, Cd/Ca was significantly higher than in the other three coral samples from Khang Khao. The average Cd/Ca value of Khang Khao corals (KK-85, KK-98, KK-01 and KK-08) is calculated to be 0.202, 0.0303, 0.0442 and 0.0431 µmol/mol, respectively, and is higher than that of RS-87 (0.0161 µmol/mol) (Table 3).

Since cadmium concentration in surface seawater is mainly affected by anthropogenic cadmium input and upwelling of deep water enriched in Cd, coral Cd/Ca values reflect pollution and upwelling events (Shen et al., 1987; Lea et al., 1989; Matthews et al., 2008). As mentioned above, since upwelling is unlikely in the Gulf of Thailand, and as a consequence, Cd/Ca in the coral skeletons from Khang Khao mainly reflects anthropogenic cadmium input. Hungspreugs et al. (2002) measured the metal contents in a *Porites* coral core (corresponding to the growth of 1950-1984) from Khang Khao Island and reported that the Cd/Ca value (0.17 µmol/mol) in the coral after 1964 was higher than that in the coral bands before 1964 (0.08 µmol/mol). Figure 3d also shows that the Cd/Ca in Khang Khao corals had several spikes. The average Cd/Ca value in Khang Khao corals is extremely higher than that in Rukan-sho coral (Table 3). These observations indicate that the Cd level in the Gulf of Thailand was higher than that in Rukan-sho from 1983 to 2008. As the highest Cd/Ca ratio was observed in KK-85, this suggests considerable Cd input into the Gulf took place in the early 1980s and anthropogenic cadmium inputs have significantly decreased in the subsequent decades.

In Thailand, cadmium contamination in water and sediments has been monitored (Hungspreugs and Yuangthong, 1983; Polprasert, 1982; McLaren et al., 2004). A high Cd concentration was observed in the surface (~0-5 cm) of a sediment core from the Chao Phraya River estuary collected in 1982 (Hungspreugs and Yuangthong, 1983). The dissolved Cd in the Chao Phraya River water tended to increase from the upper stream to the river mouth (Polprasert, 1982). McLaren et al. (2004) reported low levels of cadmium in the waters of the Chao Phraya River, although Cd in the river water showed a temporal variation. These studies suggest that the estuary was contaminated by anthropogenic cadmium from riverine inputs in the early 1980s, but the cadmium level in the estuary seems to have become lower in the 2000s.

The release of Cd from particulate matter in estuaries has been investigated to understand the behavior of cadmium (Tang et al., 2002; Audry et al., 2007). Using estuarine waters of

different salinities (S =0, 15 and 31 psu), the mobility of cadmium into particulate matter such as urban dusts and river sediments was assessed by extraction experiments (Schäfer et al., 2009). They found the highest mobility for Cd in particle matter exposed to saline estuarine water (S = 31 psu), for which up to 90% of Cd was desorbed. Considering that salinity ranges from 21-33 psu with an average of ~30 psu at Khang Khao Island (Table 2), cadmium from rivers seems to be present as dissolved Cd^{2+} in seawater in the Gulf. Accordingly, the cadmium content of corals from Khang Khao possibly recorded the dissolved Cd^{2+} in the Gulf of Thailand, which was mainly affected by the anthropogenic cadmium inputs.

4.6. Hg/Ca in Coral

The count ratio of Hg/Ca is shown in Figure 3f. In KK-85, the Hg/Ca values varied over a narrow range with an average value of 0.392 x 10^{-3} cps/cps, lower than that of RS-87. The Hg/Ca values for KK-98 were relatively constant with an average value of 0.821 x 10^{-3} cps/cps. KK-08 showed spiky signals for Hg/Ca between 1.6-4.7 x 10^{-3} cps/cps with an average value of 2.38 x 10^{-3} cps/cps. In KK-08, the Hg/Ca was lower than the average Hg/Ca of RS-87. The average count ratio of Hg/Ca in Khang Khao corals is higher in the order of KK-01 > KK-98 > KK-85 > KK-08 (Table 3). These findings suggest that the Gulf of Thailand experienced relatively higher Hg contamination from 1996 to 2001, whilst for the periods 1983-1985 and 2006-2008, the Hg concentration in the gulf water was comparable with that in Rukan-sho.

Only a few studies have investigated the mercury content of coral skeletons (Guzman and Garcia, 2002; Ramos et al., 2009). These examined the correlation between Hg and tracers of terrestrial input, such as aluminum and iron content, in order to clarify the route of anthropogenic input. In this study, the highest Hg/Ca value was observed in KK-01, indicating a significantly higher Hg level in the early 2000s (Figure 3f). In KK-01, however, there is no correlation between Hg/Ca and Ba/Ca, the latter of which reflects riverine inputs into the Gulf of Thailand (Figures 3f and 3b), showing anthropogenic mercury has some other form of input into the Gulf of Thailand. In Thailand, natural gas production in the Gulf (Windom and Cranmer, 1998), amalgamation in gold mining (Umbangtalad et al., 2007), and sewage from urbanized and industrialized areas (Cheevaporn and Menasveta, 2003) would seem to be possible sources of anthropogenic mercury during 1984-2001. However, further studies are required to identify the route and source of this anthropogenic Hg into the Gulf of Thailand.

4.7. Pb/Ca in Coral

Figure 3e shows the Pb/Ca in Khang Khao corals. In 1984-1985 (sample KK-85), the Pb/Ca value was lower than the average of Pb/Ca in Okinawan coral (RS-87: 0.0679 μmol/mol) except for the upper layer in which Pb/Ca increased to more than 0.25 μmol/mol. The Pb/Ca in KK-98 varied between ~0.3-7.0 μmol/mol. In KK-98, the Pb/Ca ratio gradually increased from late 1996 and steeply decreased from early 1998. The Pb/Ca in KK-01 was observed to be higher than the Rukan-sho value, while the Pb/Ca level in KK-01 decreased compared to that in KK-98. The Pb/Ca in KK-08 showed lower values than the average of

RS-87 except for a single peak in late 2008. The average values of KK-85 (0.04 μmol/mol) and KK-08 (0.02 μg/g) are lower than that of RS-87, whilst the average values of KK-98 (1.78 μmol/mmol) and KK-01 (0.17 μmol/mol) are higher than RS-87. The variation in Pb/Ca of corals from Khang Khao suggests that the Gulf of Thailand suffered from anthropogenic inputs of Pb from the 1990s to 2003, but the Gulf might contain slightly less anthropogenic Pb during the 1983-1985 and 2006-2008 periods.

The variation in Pb/Ca of corals from Khang Khao suggests that the Gulf of Thailand was polluted by anthropogenic lead inputs from 1984 to 2001, but the Gulf might ~~contain~~ have experienced slightly less anthropogenic lead input during the periods 1982-1983 and 2006-2008 periods. As shown in Figure 3e, Pb/Ca in Khang Khao coral significantly decreased from the late 1990s to 2008. This trend agrees with the decreasing trend in the Pb content of airborne particles in Bangkok (Cheevaporn et al., 2004).

Global lead production dramatically increased after the Industrial Revolution (Nriagu, 1998). Moreover, lead was added to gasoline as an anti-knocking agent in the form of tetraethyl lead. Lead in corals is considered to record anthropogenic Pb inputs into coral reefs due to human activities including the use of leaded gasoline (Dodge and Gilbert 1984; Shen and Boyle 1987; Inoue et al. 2006). Hungspreugs et al. (2002) measured Pb in *Porites* coral collected from Khang Khao Island and reported Pb/Ca in the skeleton corresponding to the coral growth from 1964 to 1984 was ~0.07 μmol/mol. In addition, based on Pb isotopic composition and lead contents in airborne particles, the sources of anthropogenic lead were assessed in Thailand (Mukai et al., 1993; Cheevaporn et al., 2004). Mukai et al. (1993) measured the Pb isotopic composition of airborne particulate matter collected from Bangkok in 1989 and reported that leaded gasoline might be the main source of anthropogenic lead in Bangkok. The government of Thailand, however, banned the use of leaded gasoline by the end of 1995 (Lovei, 1998). The content of airborne particles was measured in Bangkok from 1990 to 2000 and a significant decrease in Pb was observed after the ban of leaded gasoline (Cheevaporn et al., 2004). Accordingly, we consider that Pb in KK-98 and KK-01 was derived from anthropogenic lead due to the use of leaded gasoline in Thailand. The Pb content of KK-08 is lower than the baseline value observed in RS-87 (Figure 2e). This observation suggests that the regulation of leaded gasoline in Thailand resulted in markedly reduced anthropogenic lead input into the Gulf of Thailand.

CONCLUSION

The following tentative conclusions have been deduced from the measurement of metal contents in corals by LA-ICP-MS. Heavy metal input into the Gulf has been assessed by comparing the metal content of corals from Khang Khao Island to those of Rukan-sho, Okinawa, taken as a reference for baseline metal concentrations. The metal contents of the corals from Khang Khao Island possibly reflect the impact of metal pollution from urbanized and industrialized areas into the Gulf of Thailand.

(1) Stable isotope mass spectrometry provided the oxygen isotope ($\delta^{18}O$) data which revealed cyclical variations along the coral growth due to an annual change in seawater salinity. Using the skeletal $\delta^{18}O$ method, the coral chronology was established in the Gulf of Thailand.

(2) LA-ICP-MS made it possible to determine linear and two-dimensional metal-to-calcium (Me/Ca) ratios along the coral growth for monitoring aquatic environments in which the coral grew.

(3) The average values for V/Ca in three samples (KK-98, KK-01 and KK-08) were relatively higher, suggesting discontinuous inputs of anthropogenic vanadium into the Gulf of Thailand from 1997 to 2008. The V/Ca values in the corals may reflect oil pollution since the 1990s.

(4) The Cd/Ca ratio in corals from Khang Khao showed higher values compared to Rukan-sho, indicating that the cadmium concentration in the Gulf was continuously higher than that in Rukan-sho for the period from 1982 to 2008. However not withstanding this, anthropogenic cadmium input to the Gulf decreased over time.

(5) The Pb/Ca values for Khang Khao Island corals suggested that the Gulf has been polluted by anthropogenic lead since the middle 1980s. After the use of leaded gasoline was banned in Thailand in 1995, the Pb/Ca in the Khang Khao Island corals showed a remarkable decrease since the late 1990s, indicating that regulatory control has limited anthropogenic lead inputs to the Gulf of Thailand.

(6) The coralline archival record of the metals (V, Cd, Hg and Pb) gives convincing evidence of success for the regulatory laws of the Thai Government since the middle 1990s.

We hope that our studies would contribute to an improved understanding of the aquatic environments in the Gulf of Thailand and they will aid in the preservation of future ecosystems.

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Chapter 12

THE RATIO OF CONSTITUTIVE AND REPARATIVE NEUROGENESIS IN PALLIUM OF JUVENILE MASU SALMON (*ONCORHYNCHUS MASOU*)

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ABSTRACT

The chapter considers the ratio of constitutive and reparative neurogenesis in pallium of juvenile masu salmon *Oncorhynchus masou*. Since salmonid juveniles are characterized by a high level of constitutive neurogenesis, we investigated the proliferative activity and neurodifferentiation in the dorsal pallial area of juvenile masu salmon. The study of proliferative activity in pallium of juvenile masu salmon *O. masou* has allowed us to identify the superficially located periventricular proliferative zone, which corresponds to the pallial periventricular zone (PVZ) of other fish species, including the dorsal, lateral, and medial compartments. The PCNA+ cells are also identified in parenchyma of masu salmon intact brain, and their maximum concentration is observed in the medial zone. In intact brain, solitary proliferating cells were identified in parenchymal regions with their maximum concentration in the medial zone. After a mechanical injury, the zones of reparative neurogenesis — neuroepitelial neurogenic niches and zones of reactive neurogenesis surrounded by radial glial fibers—appear in masu salmon pallium. An increase in the proliferative activity is characteristic of all compartments of the PVZ (especially of the medial zone), including its deep parenchymal layers. The PVZ of juvenile masu salmon contains clusters of undifferentiated HuCD+ neurons. A change in the HuCD+ cell topography is observed in the mechanically injured masu salmon pallium, in particular, reactive neurogenic niches in the lateral zone and an increase in the cell distribution density and cell migration patterns in the medial zone. A high level of constitutive neurogenesis is characteristic of juvenile masu salmon brain. Undifferentiated HuCD+ neurons form clusters in the PVZ of intact juvenile masu salmon brain. Neurons at different stages of maturation, differing in the level of HuCD

activity, were detected in the parenchymal layers of the dorsal region. A mechanical injury of masu salmon pallium is accompanied by a change in the topography of HuCD+ cells. Formation of reactive neurogenic niches is characteristic of the lateral zone, while the medial zone displays an increase in the density of immunopositive and negative cells, as well as a distinct pattern of cell migration. HuCD and PCNA immunolabeling patterns in intact pallium suggest intensive constitutive neurogenesis in the pallial PVZ of juvenile masu salmon. After a mechanical injury, the neurogenic activity spreads to deeper layers of parenchyma. The main sources of new neurons during the reparative neurogenesis are reactive neurogenic niches.

INTRODUCTION

Neuronal regeneration is the restoration of structural integrity of injured cells and fibers; it is limited by the CNS ability to produce new cells at an adult age (Kempermann, 2013). Bony fishes is a group of vertebrates that retain through adulthood the ability to produce new cells in the specialized brain regions, proliferative zones (Lema et al., 2005; Lindsey et al., 2007), which, unlike mammals, are present in many brain regions. The periventricular brain region displays the highest proliferative activity during embryogenesis, while the active neurogenesis in adults resides in the secondary matrix regions of the brain located near the external walls of different brain regions: optic tectum, cerebellum, and myelencephalon at different levels from mesencephalic isthmus to rhombencephalon (Kaslin et al., 2008).

Unlike other vertebrates, a specific everted type of hemisphere formation and development is characteristic of telencephalon in bony fishes: the intracerebral ventricles are absent, while the matrix zones are located near the external walls of the dorsal and ventral zones and are able to produce new cells both under normal conditions and after injuries (Dirian et al., 2014). The proliferative activity in pallium of adult *Danio rerio* concentrates in the dorsal proliferative zone, which corresponds to the pallial periventricular zone (PVZ), and the ventral zone, corresponding to the subpallial PVZ (Adolf et al., 2006). The preliminary studies have shown the importance of phylogenetic studies of the pallial and subpallial proliferative zones in the forebrain of the zebrafish and other fish species. Lindsey et al. (2014) demonstrated the presence of a glial-like phenotype of neural stem cells (NSCs) involved in the adult neurogenesis and the expression of various transcription factors. According to other data, PVZs contain slowly and rapidly proliferating cells generated by adult NSCs/precursor cells with radial glial-like phenotype (Adolf et al., 2006). Neuroepitelial progenitors were also recently discovered in the adult zebrafish pallium (Dirian et al., 2014). A major part of the adult pallial ventricular zone is occupied by radial glia that acts as constitutive NSCs.

The neurogenesis in adult vertebrate brain can be modulated by changing the level of cell proliferation, their survival, and/or differentiation. This is associated with different functional requirements to new neurons, such as provision of specialized functions related to sexual behavior during the mating season in songbirds (Kaslin et al., 2008; Kempermann, 2013) and emergence of new neurons in the reticular formation involved in the innervation of salmonid spinocaudal muscles, increasing in volume during individual development (Pushchina et al., 2012).

According to the lesion paradigm formulated by Zupanc et al. (1998), a high regenerative potential of the fish CNS is determined by a set of processes, including the CNS response to injury. It is currently known that the insight into regeneration-competent organisms gives a certain advantage in studying the biology of NSCs, their interaction with the cell microenvironment, and specific features of their metabolism (Berninger et al., 2006; Stocum, 2006). The neurogenesis in an adult mammalian and human brain takes place only in the subventricular zone of the lateral ventricular wall and in the subgranullar zone of the hippocampal dentate formation (Chojnacki et al., 2009; Kaneko and Sawamoto, 2009). Neurogenesis can be stimulated or inhibited by various factors, including physical activity (Kempermann, 2011), changes in environmental conditions (Lema et al., 2005), and stress (Hutton et al., 2015). These factors have multidirectional effects on the intensity of neurogenesis and determine the final success of this process.

Variations in the physiological status of the animal, beyond regenerative conditions, are important players controlling the activation state of germinal zones in the mammalian forebrain. These aspects remain understudied in fish brain. The lack of standardized animal maintenance conditions explaines the large degree of variability between animals that complicates reading of subtle effects. Several neurotransmitters and hormones were recently shown to impact on radial glia activation (Mouriec et al., 2009; Perez et al., 2013). In the paraventricular organ for example, serotonine (5HT) is necessary for proliferation of radial progenitors (Perez et al., 2013), and the projection of 5HT processes toward pallial germinal zones suggests that it may be the case in this location as well (Lillesaar et al., 2009). Aromatase B (AroB), which converts androgens to estrogen, is strongly expressed in zebrafish radial glia (Menuet et al., 2005). Blockage of the aromatase activity in adult zebrafish led to a trend increase in the number of proliferating progenitors in several brain areas, particularly in pallium, suggesting a lower proliferation of oestrogens (Diotel et al., 2013). However, it remains to be assessed whether they primarily target radial glia or nonglial proliferating progenitors. Along with a quiescence-promoting effect on radial glia, radial glia, maintaining AroB expression after injury, are not activated and presumably do not participate in the repair process (Diotel et al., 2013).

A study of the constitutive neurogenesis under natural conditions gives a large volume of information for comparative analysis (Than-Trong and Bally-Cuif, 2015). However, the balance between constitutive and injury-induced neurogenesis in brain of regeneration-competent organisms is rather vague. Recent works considered the effect of behavioral challenges or sensory stimuli on zebrafish adult neurogenesis (Lindsey and Tropepe, 2014). As the most interesting fact, it was found that sensory stimulation (chemosensory or visual stimulations) selectively affected the neurogenic niches in the brain areas involved in processing these stimulations (Lindsey et al., 2014). Changes in the social context, such as social isolation or social novelty, also decrease the number of proliferating progenitors in sensory niches and increase the number of newborn neurons (Lindsey and Tropepe, 2014). The number and complexity of neurogenic niches in teleosts, and the complex repertoire of behaviors that a fish can exhibit, suggest that much is to be learned from this model in terms of the environmental modulation of adult stem cell pools and neurogenesis. The goal of our chapter was to observe the proliferation and neurogenesis in pallium of juvenile masu salmon *Oncorhynchus masou* before and after a mechanical injury.

PCNA LOCALIZATION IN THE MASU SALMON PALLIAL PVZ IN NORM AND AFTER A MECHANICAL INJURY

PCNA was immunolocalized in the dorsal, lateral, and medial zones of pallium in juvenile masu salmon (Pushchina et al., 2017). The PCNA+ cell distribution patterns in these zones were somewhat similar: solitary proliferating cells or their small clusters were observed in the surface and subventricular layers (Figure 1A, C, E). The main specific feature in the distribution of immunopositive cells in the intact animals was the pronounced surface periventricular layer containing PCNA+ conglomerates of cells that spread over all zones of the dorsal region (Figure 1A, C, E). Another specific feature consisted in the presence of solitary PCNA+ cells in deep parenchymal layers beyond the surface proliferative zone (Figure 1A, C, E). Similar patterns of PCNA+ cell distribution have been observed in other fish species (Lindsey et al., 2007; Pushchina et al., 2007; Zupanc and Sîrbulescu, 2013). Solitary PCNA+ cells were observed in the dorsal and lateral zones (Figure 1A, C) of masu salmon pallium versus the medial zone with more abundant immunopositive cells (Figure 1E).

Earlier studies demonstrated that the proliferative activity in telencephalon of adult *D. rerio* usually concentrated in the dorsal proliferative zone, corresponding to the pallial PVZ, and ventral zone, corresponding to the subpallial PVZ (Adolf et al., 2007; Lindsey et al., 2007). Data of different researcher demonstrate the importance of phylogenetic studies of the pallial and subpallial proliferative zones in the forebrain of zebrafish and other fish species (Lindsey et al., 2007; Zupanc and Sîrbulescu, 2013). The presence of the NSCs involved in adult neurogenesis with a glial phenotype is demonstrated (Adolf et al., 2007; Kempermann, 2011). In particular, it is shown that the PVZ contains slowly and rapidly proliferating cells developing from adult NSCs/precursor cells with a radial glial phenotype (Adolf et al., 2007). A recent classification, based on detailed immunohistochemical labeling (März et al., 2010) and clonal analysis (Rothenaigner et al., 2011), distinguishes between three types of mitotically active cells (II, IIIa, and IIIb) and non-dividing type I cells. An examination of the pallial–subpallial zone of the zebrafish forebrain PVZ has shown that the precursor cells express various transcription and growth factors, such as Pax6 and FGF, and are putatively regulated by these factors (Adolf et al., 2007; Ganz et al., 2010). However, the ever-increasing number of studies on the forebrain pallial and subpallial PVZs still fail to sufficiently clarify certain basic aspects in this area, including anatomical boundaries of these zones, their ultrastructural composition, and specific features in their cellular and molecular organizations.

Analysis of regeneration of zebrafish telencephalon has been a subject of several recent investigations (Kishimoto et al., 2011; Kroehne et al., 2011; März et al., 2011; Baumgart et al., 2012; Kroehne and Brand, 2012). In these studies, telencephalon was chosen as the assay system because: (1) newborn neurons are not observed in the parenchyma of the telencephalic pallial subdivision under normal conditions, thus enabling an observer to distinguish between constitutive neurogenesis and regeneration, and (2) there is hope to get an insight into functional differences of teleost and mammalian telencephalic regenerative behaviour.

Since juvenile masu salmon are in the state of active growth, the constitutive production of new cells, according to our data, is rather intensive and is observed both in the brain proliferative zones and beyond them. We believe that the phylogenetic factors, demonstrating

that salmonids belong to an evolutionarily ancient group, the teleosts, for which the uncompleted eversion of telencephalon and retained embryonic brain structural traits are characteristic in the postembryonic development, can also play an important role (Wullimann and Muller, 2009). A similar characteristic, referred to as *fetalization*, is typical of phylogenetically ancient vertebrate groups, the brain of which contains many morphogenetic zones with an increased proliferative potential (Artyukhin, 2008; Zupanc and Sîrbulescu, 2013). Our studies suggest that the cell proliferative potential of the medial zone is considerably higher as compared with the other zones of pallium. One more specific feature of the masu salmon medial zone is a pronounced depthward cell migration from the surface layers of pallium. The stratification of cell layers in this region has been traced in individual cases. Thus, our data suggest that the medial zone is the most important contributor to the constitutive morphogenesis of masu salmon telencephalon as compared with the remaining zones of the dorsal region.

Currently, the data on the embryonic origin, associated with homogeneity of the NSCs in the adult fish brain, are scarce. The studies on zebrafish demonstrate that the proliferative zone of the definitive pallium develops from two separate subtypes of embryonic precursors and comprises two NSC types involved in the adult neurogenesis (Dirian et al., 2014). The dorsomedial NSCs emerge owing to active amplification of the neurogenic radial glia in the embryonic telencephalon. The NSC population of the lateral zone is formed through constant supplementation of the pallial band from the pool of discrete neuroepithelial progenitors of the telencephalon posterior roof, activated in the postembryonic period and retained throughout the lifecycle. This dual origin of the zebrafish pallial proliferative zone implies a time-dependent construction of pallial regions as mosaic and adjacent compartments. Taking into account the results obtained from zebrafish, the data on a nonuniform distribution of the proliferating and HuCD+ cells in the masu salmon periventricular proliferative zones, and the ratio of migrating and proliferating cells in the brain parenchyma, we believe that the masu salmon pallial PVZ has a complex multicomponent mosaic structure and is most likely comprised of several formations containing NSCs.

After a mechanical injury of masu salmon pallium, we observed considerable changes in the cell proliferative activity in both the pallium proliferative zones and parenchyma. The most pronounced changes in the topography of proliferating cells were associated with the structural remodeling of the integrated proliferative, superficially located in the periventricular layer, and formation of the local zones of induced neurogenesis (Figure 1B, D). It should be noted that the periventricular zone as a rule was not verified. Formation of local cell clusters containing both PCNA+ elements and numerous PCNA- cells in *reactive neurogenic niches*, as well as vast populations of migrating cells and weakly labeled radial glial fibers, were observed (Figure 1B, D). These changes appeared after a mechanical injury, suggesting that they can be regarded as repair rearrangements.

Another specific feature in the post-traumatic morphogenesis of masu salmon telencephalon was the formation of the *secondary neurogenesis centers* (reactive parenchymatic niches) in the medial zone, which also possesses an elevated proliferative potential in norm (Figure 1F). After the injury, numerous PCNA- cells surrounded by undifferentiated immunonegative cells were observed. The distribution density in such reactive clusters considerably exceeded that in the adjacent regions, suggesting that these clusters can be regarded as integrated functional complexes with an increased proliferative activity. According to the data by Savel'ev (2001), the centers of secondary neurogenesis are

formed in vertebrate brain when cells from the primary periventricular proliferative region migrate to deeper brain layers retaining their increased proliferative potential. Such cells continue proliferation even beyond the typical conditions of cell microenvironment in the proliferative zone and form the centers of secondary proliferation. In this process, a part of the cells continues proliferation, whereas the other part starts to differentiate. Such cell cluster outside the matrix zone typically has a higher density and heterogeneous cell composition and contains cells at different stages of differentiation. We observed these particular cell clusters in the medial zone of masu salmon telencephalon after injury.

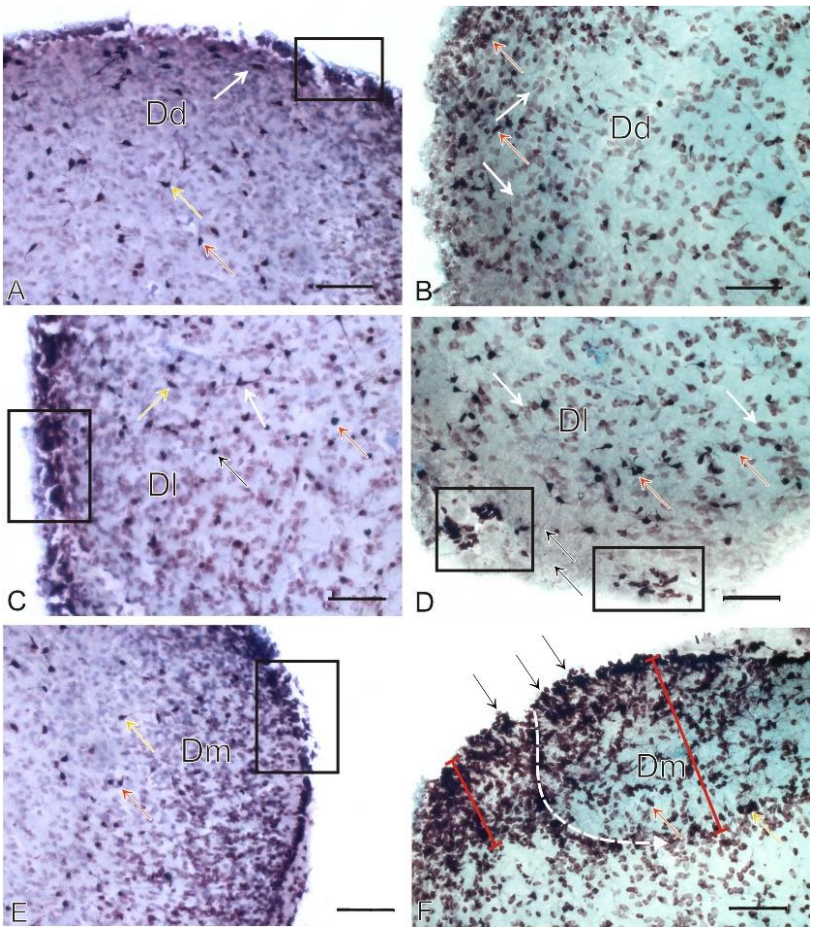

Figure 1. Distribution of the proliferating cell nuclear antigen (PCNA) in the pallial proliferative zone and deep layers of the dorsal telencephalon in juvenile masu salmon *Oncorhynchus masou* (A, C, E) in the norm and (B, D, and F) after mechanical injury: (A) dorsal (Dd), (C) lateral (Dl), and (E) medial (Dm) zones of the masu salmon dorsal telencephalon in the control (dashed line shows the boundaries of the proliferative zone; black arrows indicate solitary PCNA+ cells in the PVZ; red rectangle encloses PCNA+ cell clusters in the dorsal and medial zones; and red arrows indicate PCNA+ cells in parenchyma) and (B) dorsal, (D) lateral, and (F) medial zones after a mechanical injury (ovals enclose neurogenic niches; rectangle in panel (F), the zone of secondary neurogenesis; red arrows indicate PCNA+ cells in parenchyma and in panel (F), in the periventricular proliferative zone; yellow arrows indicate radial glial fibers; white arrows indicate clusters of migrating cells, and black arrows indicate PCNA– cells in parenchyma). Scale bar is 100 μm.

The studies on zebrafish have demonstrated that an injury of telencephalon induces a rapid proliferation of neural precursor cells in the PVZ of the injured telencephalon hemisphere as compared with the intact one. The NSC distribution, detected using BrdU and neurogenin, demonstrates that these cells migrate laterally and reach the injured site via subpallium and pallium (Kishimoto et al., 2011, 2012). Recent studies of the zebrafish NSCs have revealed new aspects in the physiology of the radial glia. In particular, it is shown that the radial glia is present in the fish brain proliferative zones (Grandel et al., 2006). A part of the cells forming the radial glial population possesses the NSC properties, which determines a high level of reparative neurogenesis (Zupanc, 2001; Grandel et al., 2006). Cells of the fish radial glia are spread all over the brain regions along the ventricular lumen and enhance successful regeneration of injured brain (Tozzini et al., 2012). It is known that the regenerative potential of somatic stem cells, as a rule, decreases with age of animals; thus, the intensity of constitutive neurogenesis in juvenile masu salmon individuals of different ages (Pushchina et al., 2012) suggests a proliferative potential of NSCs at different ages.

Studies on zebrafish have shown that neurogenesis and oligodendrogenesis in the ventricular zone, olfactory bulb, and parenchyma of the telencephalon weaken with aging (Edelmann et al., 2013). According to our data, pallium in juvenile masu salmon displays a high level of constitutive neurogenesis (Pushchina et al., 2017). We have earlier demonstrated that the periventricular region of masu salmon of different age cohorts have a large content of the radial glia, marked by tyrosine hydroxylase, NADPH diaphorase, and GABA (Pushchina et al., 2012). The radial glial cell population in the periventricular zone remains unchanged to a considerable degree, but immunohistochemical analysis demonstrates that such cells in more adult animals rarer enter mitosis and, consequently, produce fewer neuroblasts. The activity of neuroblasts remains rather constant with age (Adolf et al., 2006; Grandel et al., 2006); thus, neuroblasts produce the same number of postmitotic neurons. The decrease in the level of neurogenesis physiologically correlates with the increase in the radial glia quiescence (Edelmann et al., 2013). An injury activates the radial glia and induces its proliferation. However, the radial glia is to a considerably lesser degree involved in neurogenesis in adult animals as compared with juveniles, suggesting certain irreversible changes in the radial glial cells during brain aging.

Muller and Wullimann (2003) have shown that PCNA labeling of proliferative regions reflects a neuromeric organization of the brain, which has also been confirmed by autoradiography and immunocytochemical (BrdU) assays. PCNA expression in cells retains for 24 h after the completion of mitosis, but the level of its activity decreases by 30% (Wullimann and Puelles, 1999). PCNA is expressed in mitotic cells during the entire proliferative cycle and has a prevalent nuclear localization (Waseem and Lane, 1990); however, cytoplasmic localization of PCNA is also observable in some cases (Vriz et al., 1992). Specific methodical features of PCNA as a cell proliferation marker in the studies of different neurogenesis stages in fish brain has made it possible to demonstrate that this marker at the early developmental stages in zebrafish (days 1–4 after fertilization) identifies neuroepithelial cells that have a rather short cell cycle in this period (Muller and Wullimann, 2003). Nonetheless, PCNA is a convenient cell proliferation marker when studying later postembryonic neurogenesis in fish (Candal et al., 2005), since the cell cycle at subsequent stages is considerably (manifold) longer as compared with the early neurogenesis.

Various lesion paradigms have been elaborated for studying the nervous tissue regeneration of fish brain and its functional repair after CNS injury. Many studies of telencephalon injuries focus on injuries of olfactory bulbs or the dorsal region in one of the telencephalon hemispheres (Zupanc, 2001; Zupanc and Sîrbulescu, 2013). In some cases, the neural repair was studied in the lateral and dorsolateral parts of telencephalon (Ayari et al., 2010). A better insight into the bony fish brain regeneration capacities requires the knowledge of the sources for neuron regeneration in injured brain. Genetic studies involving an injury of dorsal pallium demonstrate that most of regenerating neurons originate from radial glia-type progenitor cells, while differentiation of nonneurogenic cells plays a minor role in the brain repair (Kroehne et al., 2011). A systematic neuroanatomy examination of zebrafish allowed distinguishing of 16 constitutive neurogenic domens located along the brain axis (Adolf et al., 2006; Grandel et al., 2006; Kaslin et al., 2008). As has been shown, the adult NSCs are mainly associated with the ventricular system (Tozzini et al., 2012). The NSCs in fish telencephalon have radial glia morphology and express certain molecular markers (vimentin and nestin) characteristic of the mammalian NSCs (Ganz et al., 2010; März et al., 2010). The pial and subpallial constitutive neurogenic niches, identified in fish, are now regarded as homologs of the subgranullar and subventricular proliferative zones in mammalian brain (Adolf et al., 2006; Mueller and Willimann, 2009).

Telencephalon of zebrafish mostly recovers histologically by 30 dpl and becomes indistinguishable from a non-injured telencephalon after 1 year. Besides the rapid reduction of cell death, gliosis and inflammation, the regenerative/reactive neurogenesis commences with the initial proliferative response of radial glia in the endogenous proliferation domain that produces new neurons in the damaged hemisphere (Kroehne et al., 2011). As early as at 4 dpl, pulse-chase experiments show many newborn pallial neurons in the periventricular zone (Kroehne et al., 2011; März et al., 2011), and newborn parenchymal neurons become evident in the damaged brain from the first week post injury (Kroehne et al., 2011; Baumgart et al., 2012). The parenchymally located newborn neurons, which arise from the ventricularly located radial glial cells as determined by Cre-lox lineage tracing technology, home into the lesion site only after a very long chase time (21–90 dpl) (Kroehne et al., 2011). Pallial radial glia are under the control of notch signalling (Ganz et al., 2010; Kishimoto et al., 2012) which can be monitored by transgene expression under the control of the her4.1 promoter. A transgenic conditional Cre-lox recombination approach has been used to genetically mark pallial radial glia and their progeny (Kroehne et al., 2011).

Thus, PCNA immunolabeling of masu salmon pallium demonstrates the presence of the superficially located proliferative layer and individual proliferating cells with spatially specific number and localization (Pushchina et al., 2017). In general, the distribution pattern of PCNA+ cells in the dorsal telencephalon matches the corresponding patterns of other fish species; it should be noted that an important specific feature consists in organization of the medial zone, the main characteristic of which is an increased proliferative potential. Taking into account the data on the incomplete eversion of salmonid telencephalon, we tend to believe that the medial part of telencephalon is the particular region that houses the most of cell proliferation and migration events that are decisive in telencephalon formation during persistent neurogenesis. The zones of induced neurogenesis appear exclusively in the postinjury process and, in our view, are related with the brain repair.

HuCD Localization in the Proliferative Zones and Parenchyma of the Masu Salmon Pallial PVZ in Norm and after a Mechanical Injury

Most researchers regard the cells of the PCNA- brain regions as stimulated to differentiation (Candal et al., 2005) because of the presence of neural determination markers, such as Pax6, Zash-1a, Zash-1b, neurogenin, and neuroD, and neural differentiation marker HuCD (Mueller and Willimann, 2009). The newborn neurons in zebrafish pallium also expressed more mature region-specific neuronal markers such as parvalbumin and Prox1 in an appropriate manner and appeared integrated into the neural circuitry as visualized by dendritic (MAP2a+b) and synaptic markers (SV2, mGlu2). The results of HuCD labeling of pallium of juvenile masu salmon confirm these data. A comparison of the distributions of HuCD+ neurons in the dorsal, lateral, and medial zones of masu salmon pallium demonstrates that the definitive cells with a mature neuron-like phenotype, most likely involved in formation of neuronal networks, display different degrees of HuCD immunostaining. The results obtained from juvenile masu salmon differ from the data on HuCD immunolocalization after a mechanical eye injury in adult trout (Pushchina et al., 2016). The trout pallial proliferative zone also displayed a high level of HuCD activity as compared with the definitive neurons in the dorsal regions (Pushchina et al., 2016). However, neither typical neurogenic niches nor structural changes in the dorsal pallial zone, observed after an injury of pallium in juvenile masu salmon, were detectable after an optic nerve injury in adult trout. Thus, we believe that the region of brain injury, as well as the animal age, influences the pattern of postinjury repair. In particular, an injury of trout eye and optic nerve activates neurogenic niches in the optic tectum and cerebellum, but no typical neurogenic niches were detected in telencephalon (Pushchina et al., 2016). The control masu salmon juveniles displayed rather intensive constitutive neurogenesis in different parts of the pallial proliferative zone. The superficially located HuCD+ cells either formed clusters or, in some cases, even a superficial cell layer. The distribution density of immunopositive cells in the lateral zone was higher as compared with the dorsal zone; however, the most superficial layer was formed of negative cells. A high density of both HuCD+ and HuCD– cells and a moderate density of cell distribution in the proliferative region were characteristic of the medial zone.

The densitometric data for the postinjury period make it possible to distinguish between two HuCD activity levels in masu salmon pallium: intensive and moderate (Pushchina et al., 2017). Intensively labeled definitive neurons were prevalent in all the regions of the control animals. The HuCD+ neurons were surrounded by undifferentiated immunonegative cells varying in number in different zones. According to the classification by Edelmann et al. (2013), we referred these cells as to the cell population stimulated to neural differentiation but not yet expressing neural determination markers. Our studies allowed us to distinguish four types of HuCD+ cells differing in their morphology and density of HuCD immunolabeling. We believe that the differences in densitometric characteristics of HuCD+ cells may reflect the differences in the distribution pattern of this protein in the neuron cytoplasm and correspond to different degrees of neuronal differentiation of the cells formed during reparative neurogenesis. Nonetheless, we should emphasize that masu salmon brain contains neurons displaying different degrees of HuCD immunolabeling irrespective of their

differentiation stage and also the described types of neurons. We also assume that different densitometric characteristics of HuCD+ cells may result from methodological aspects, in particular, the ability of antibodies to penetrate into different depths in brain sections. However, the most apparent and grounded assumption explaining the differences in HuCD labeling intensity during reparative neurogenesis, in our view, is that the produced cells are at different stages of neuronal differentiation.

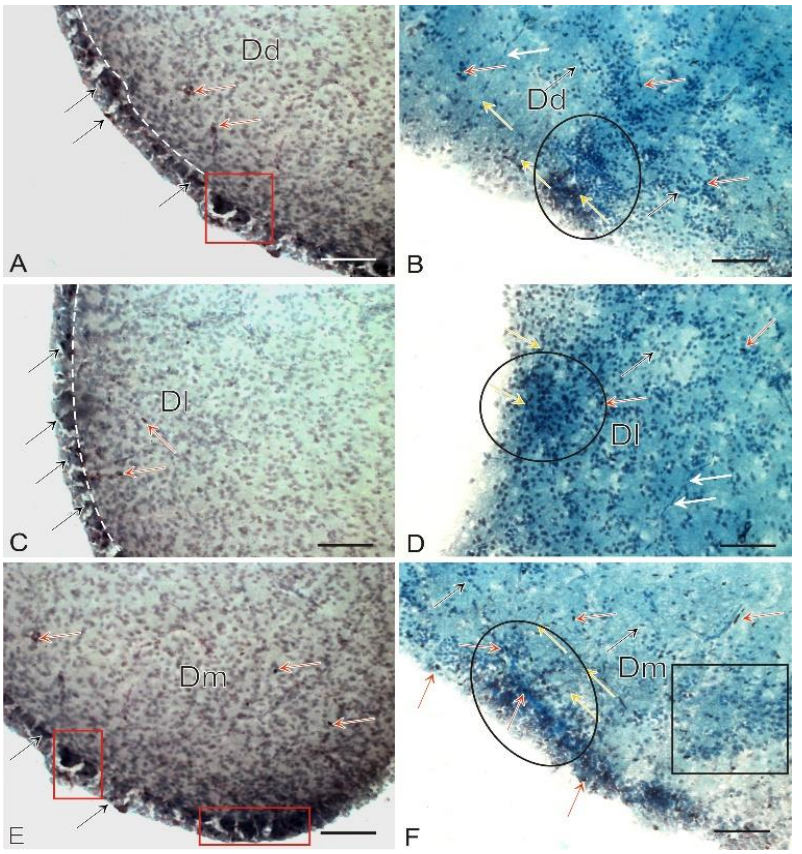

Figure 2. Distribution of the HuCD neural protein in the pallial proliferative zone and deep layers of the dorsal region in juvenile masu salmon *Oncorhynchus masou* (A, C, E) in norm and (B, D, F) after a mechanical injury: (A) dorsal (DD), (C) lateral (DL), and (E) medial (DM) zones of masu salmon telencephalon in the control (rectangles enclose regions of the periventricular zone and arrows of different colors indicate cells of types 1–4) and (B) dorsal, (D) lateral, and (F) medial regions after a mechanical injury (red arrows indicate intensively labeled neurons; white, weakly labeled; in panel (D), black arrows indicate radial glial fibers; rectangles enclose neurogenic niches containing newly formed neurons; black arrows in panel (F) indicate intensively labeled neurons in the pallial periventricular zone; white dashed line is direction of cell migration, and red line segments show different thicknesses of neurogenic layer). Scale bar is 100 μm.

The expression of HuCD protein in the pallial proliferative zones of masu salmon yearlings is rather pronounced, which agrees with the data on zebrafish (Mueller et al., 2011). The cells newly formed in dorsal telencephalon of zebrafish after an injury start to express HuCD on days 3–4 post-injury (Ayari et al., 2010; Kishimoto et al., 2011). The newly formed neurons appear both near the injury site and in the regions distant from this site. As is shown,

a small population of zebrafish cells, formed within 2 days before the injury, is involved in the repair of the injured brain region (Kroehne et al., 2011). The number of HuCD expressing cells gradually increases in the subsequent days, especially in the injured zone. An analysis of the spatiotemporal pattern of HuCD+ cell distribution in zebrafish demonstrates that the newly formed cells acquire the phenotype characteristic of the neurons during migration to the injured zone (Lam et al., 2009; Edelmann et al., 2013). These data suggest that the interactions between proliferating cells in an intact fish brain also change after the injury.

Both intensively and moderately HuCD immunolabeled cells were observed in the deep layers of the masu salmon dorsal zone on day 3 post-injury, which considerably differs from the immunolabeling pattern in the control (Figure 2A, B). In our opinion, a decrease in the HuCD immunolabeling intensity in most of definitive neurons against the background of retained intensive labeling in 25% of the cells is a manifestation of the adaptive response to a mechanical injury of pallium. A redistribution of the HuCD+ cells, forming the layer of irregular clusters with retention of the immunonegative surface layer in control animals, was observed in the proliferative region of the dorsal zone.

The ratio of intensively to moderately HuCD-labeled definitive neurons in the lateral zone was 1:2.5, differing from that in the dorsal zone. After injury, the proliferative zone underwent considerable changes (Figure 2C, D). Two zones of induced neurogenesis were observed as neurogenic niches of different morphologies and, possibly, different origins. After an eye injury in adult trout, local clusters of HuCD+ cells were observed in the lateral proliferative zone (Pushchina et al., 2016). Neurogenic niches in the lateral zone of juvenile masu salmon individuals were surrounded by weakly immunolabeled radial glial fibers. Formation of such structural complexes producing new neurons is a consequence of activated proliferation in NSCs induced by injury (Lam et al., 2009; Edelmann et al., 2013). NSCs are able to produce neurons and glia both *in vivo* and *in vitro*, although the neuronal or glial specialization is determined by the conditions of cell microenvironment and other dynamic factors (Adolf et al., 2006; März et al., 2010; Kishimoto et al., 2011). Similar to mammals, the precursor cells in zebrafish give rise mainly to neurons rather than glial cells (Hinsch and Zupanc, 2006; Mueller et al., 2011). The causes of this phenomenon in zebrafish are still unclear; however, a limited production of astrocytes in adult brain correlates with fits their total low content in brain of bony fish.

According to Kishimoto et al. (2012), the NSCs that emerge after an injury of zebrafish telencephalon differentiate into mature neurons during 1 week post-injury. Most of these cells express Tbr1, suggesting a normal adaptation of the neurons in this region, Thus, the ventricular zones of zebrafish telencephalon are involved in the repair of nervous tissue after a telencephalon injury in adult fish.

A considerable increase in the number of intensively labeled HuCD+ cells, localized to the proliferative zone and forming migratory flows of cells with neuronal specialization, was observed in the medial zone of masu salmon pallium (Figure 2E, F). This overproduction of neurons in the medial zone after injury generally agrees with the increased proliferative potential of this zone, which we revealed by PCNA labeling. As for adult trout, we observed an increased density of HuCD+ cells in the dorsal zone after an eye injury (Pushchina et al., 2016). We believe that the increased production of neurons during the postinjury period in different telencephalon zones is a species-specific feature of salmonids and may possibly reflect certain specific phylogenetic features of the repair process characteristic of this fish group.

CONCLUSION

Stab injury experiments were conducted by inflicting a damage to telencephalon with a needle. This type of traumatic brain injury initiates a cascade of events. An immediate response to the stab is the formation of an oedema and a wave of neuronal cell death, both of which rapidly recede at 7 dpl and 3 dpl, respectively. Another reaction to a stab injury is the onset of gliosis and inflammation in the damaged hemisphere of masu salmon pallium. In the parenchyma of the damaged pallium, from 4 dpl on, a marked hypertrophy of GFAP-positive and vimentin-positive radial fibres is observed.

L-plastin-positive cells in pallium of adult zebrafish consist of a heterogeneous mixture of blood-derived leukocytes and resident microglia that become more numerous after a stab wound (Kroehne et al., 2011; Baumgart et al., 2012). Proliferation levels of different cell types also increase after damage to telencephalon in adult zebrafish, peaking at 3 dpl in the endogenous ventricular proliferation domain, as well as ectopically in the damaged parenchyma. In the endogenous ventricular proliferation zone, radial glial cells expressing GFAP, vimentin, nestin and glutamine synthetase specifically upregulate their proliferation upon a stab injury. In the parenchyma of zebrafish telencephalon, mostly L-plastin-positive cells contribute to the proliferating population (Kroehne et al., 2011); however, a small fraction of oligodendrocyte precursors proliferate as well (Kroehne et al., 2011; März et al., 2011). Also, proliferating endothelial cells can be observed (Kroehne et al., 2011). Slightly elevated proliferation rates can still be detected at 2 weeks post-injury but decline subsequently. Likewise, the initial early inflammatory response does not persist and neither hypertrophic radial glial processes nor a surplus of L-plastin-positive cells are detected by 30 dpl. It is important that the glial scar does not form (Kroehne et al., 2011). This is in marked contrast to the reaction of mammalian telencephalon to a damage where regeneration is hampered by gliosis, inflammation and subsequent formation of the glial scar that inhibits regeneration of neural tissue (Fitch and Silver, 2008; Sofroniew, 2009).

The study of proliferative activity of pallium in juvenile masu salmon *O. masou* has allowed us to identify the superficially located periventricular proliferative zone, which corresponds to the pallial periventricular zone (PVZ) of other fish species, including the dorsal, lateral, and medial compartments. A superficially located periventricular proliferative area with PCNA+ cells, which corresponds to the PVZ of other fish species, including its dorsal, lateral, and medial compartments, has been discovered in pallium of juvenile masu salmon. The PCNA+ cells are also identified in the parenchyma of intact masu salmon brain, and their maximum concentration is observed in the medial zone. In intact brain, solitary proliferating cells were identified in parenchymal regions with their maximum concentration in the medial zone. After a mechanical injury, the zones of reparative neurogenesis — neuroepitelial neurogenic niches and zones of reactive neurogenesis surrounded by radial glial fibers — appear in masu salmon pallium. An increase in the proliferative activity is characteristic of all compartments of the PVZ (especially of the medial zone), including its deep parenchymal layers. The PVZ of juvenile masu salmon contains clusters of undifferentiated HuCD+ neurons. A change in the HuCD+ cell topography is observed in a mechanically injured masu salmon pallium: reactive neurogenic niches in the lateral zone and an increase in the cell distribution density and cell migration patterns in the medial zone. A high level of constitutive neurogenesis is characteristic of juvenile masu salmon brain.

Undifferentiated HuCD+ neurons form clusters in the PVZ of intact brain in juvenile masu salmon. Neurons at different stages of maturation, differing in the level of HuCD activity, were detected in the parenchymal layers of the dorsal region. A mechanical injury of pallium in masu salmon is accompanied by a change in the topography of HuCD+ cells. Formation of reactive neurogenic niches is characteristic of the lateral zone, while the medial zone displays an increase in the density of immunopositive and negative cells, as well as a distinct pattern of cell migration. HuCD and PCNA immunolabeling patterns in intact pallium suggest the intensive constitutive neurogenesis in the pallial PVZ of juvenile masu salmon.

The colocalization of newborn cells with the neuronal marker HuCD showed that her4.1-positive radial glia generates neurons. A two-third of the newborn neurons were located in a periventricular position close to the pallial proliferation zone that corresponds to their regular target area. A one-third of all HuCD-positive newborn neurons were found in a parenchymal position that was usually not attained by newborn neurons neither in undamaged brains nor in the control hemisphere.

Thus, the damage site specifically attracts newborn neurons from the endogenous proliferation domain that are rerouted to populate and integrate into this site (Kroehne et al., 2011). It will be important to decode the signals that emanate from the damage site, which act to trigger the regenerative response. Chemokines and their receptors have been suggested as possible candidates. They have been reported to localize to radial glia in telencephalon (Diotel et al., 2010), where their overexpression enhances the proliferative response after an injury (Kizil et al., 2012). Other, still unidentified, pathways may also funnel into a regenerative response to trigger there generative pathway at different angles (Kyritsis et al., 2012). This observation confirms the long-lasting suggestion (Kirsche and Kirsche, 1961) that periventricular progenitors of the “matrix zones” produce neuronal progeny, which are engaged in regeneration.

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Chapter 13

NEURAL STEM CELLS IN THE CEREBELLUM OF JUVENILE MASU SALMON (*ONCORHYNCHUS MASOU*) AFTER MECHANICAL INJURY

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ABSTRACT

The objective of this chapter was to study proliferation processes and the role of radial glia and neural stem cells in the event of injurious action on cerebellum of masu salmon *Oncorhynchus masou* juvenile. Using the immunoperoxidase staining of the glial fibrillary acidic protein (GFAP), doublecortine (DC) and proliferating cells nuclear antigen (PCNA), processes of proliferation and gliogenesis after mechanical trauma of cerebellum of juvenile masu salmon *O. masou* were studied. After the trauma, the intensity of proliferation and migration processes varies in different zones. Proliferation processes decrease after the trauma in lateral and basal zones, and migration increases. In the dorsal zone, on the contrary, migration processes significantly decrease and proliferation increases. In the dorsal matrix zone of cerebellum, intense cell proliferation was detected. In the dorsal, lateral, and basal zone of the molecular layer of cerebellum after traumatic injury, neurogenic niches containing PCNA and cells, as well as a heterogeneous population of PCNA- cells, were identified. At the location of neurogenic niches, fibers of radial glia and small single, intensely or moderately labeled GFAP cells were discovered. As a result of injury, GFAP+ fibers of radial glia, which form differently directed, radially oriented bundles, appeared in the dorsal matrix zone. Such structural formations were not discovered in intact animals. We suppose that, after the trauma, a structural reconstruction, connected with partial spatial reorientation of the radial glia fibers, and formation of specific directions for cells formed in this zone occur in the dorsal matrix zone. As a result of the trauma, elements of the radial glia, including both the cells possessing typical morphology and the cell fragments represented by long

radially oriented processes or cell body, containing initial fragments of radial fibers, appeared in masu salmon's cerebellum.

INTRODUCTION

Fish is quite an interesting subject for investigation of neurogenetic processes, since, during the period of their postembryonic development, the formation of brain, growth, and differentiation of integrative centers are very active (Zupanc, 2006). The peculiarity of adult neurogenesis in teleost fish is the ability to form large amounts of new cells. Studies on the fresh-water fish, *Danio rerio* and *Apteronotus leptorhynchus*, have shown that proliferation rate in these species is by 1–2 orders of magnitude higher than in mammals (Zupanc and Zupanc, 2006). A mass increase of intensity of cell proliferation is observed in bony fish as a response to brain damage. Complete recovery of neural tissue occurs due to colonization of the injured area with new neurons. In mammals, the long persistency of newborn cells, as a rule, is weakened, and their brain is restricted in its ability to recover (Zupanc and Sîrbulescu, 2011).

Cerebellar morphogenesis of zebrafish has been repeatedly investigated in recent years with a focus on progenitor migration during embryonic and early larval stages (Chaplin et al., 2010; Wullimann et al., 2011). However, since adult teleosts add large numbers of new cells to the cerebellum (Zupanc and Horschke, 1995; Grandel et al., 2006; Hinsch and Zupanc, 2006; Zupanc and Sirbulescu, 2011), it is clear that teleost cerebellar morphogenesis continues after embryogenesis. Recently, Kaslin et al. (2009) have studied the continued generation of new granule neurons during adult stages with the aim to determine the identity of progenitor/stem cells and their progeny.

The basis of this is postnatal neurogenesis, the process beginning with division of precursor cells, their subsequent migration, differentiation of newly formed cells and ending with integration of new neurons in the neural network (Kempermann, 2008). The phenomenological manifestations of neurogenesis in adult fish are similar to those in embryonic brain of birds and mammals (Levine et al., 1994). It is supposed that adult neurogenesis plays an important role in quantitative harmonization and coordination of CNS neurons in motoric brain structures. In mammals, body growth occurs due to the increase in size of muscle fibers rather than in their number. Number of muscle fibers increases throughout the lifecycle of fishes, and this probably requires corresponding increase in neuron units to control the muscle activity and for the quantitive harmonization of central neurons and peripheral sensor elements. In bony fishes, production of new neurons in association with a sufficiently long survival of their significant fraction leads to continuous growth of brain and its structures. In contrast to mammals, where central parts of sensory systems become completely formed and equivalent to the quantity of sensory receptors by the moment of birth and/or shortly after it, the growth and development of sensory projections in fish brain can continue throughout the lifecycle because of the necessity of adaptation to continuous significant increase in body size and amount of incoming sensory information. The continuous formation of new neurons in adult fish is provided by the presence of large amounts of cerebral radial glia (Onteniente et al., 1983; Pellegrini et al., 2007), which are known to perform a function of neural stem cells during embryonic neurogenesis in mammals (Noctor et al., 2002; Kriegstein and Alvarez-Buylla, 2009). In mammals, new neurons form

mainly in the following two zones of brain: (1) in the frontal part of the subventricular zone of the lateral ventricle, from which immature neurons migrate in the composition of the so-called "rostral migratory stream" to olfactory bulbs where they differentiate into granular neurons and periglomerular interneurons (Curtis et al., 2007), and (2) in the area located in the subgranular zone of the dentate gyrus from which new cells migrate for short distances to the granular layer of hippocampal cells and develop into mature granular neurons (Seri et al., 2004). In mouse brain after inflicting mechanical damage, proliferation of resting progenitor cells in cortex parenchyma was discovered (Ahmed et al., 2012). There is an increase in numbers of endogenic stem cells forming neurospheres when grown *in vitro*. Secondary spheres in case of differentiation yield three types of neural stem cells (NSC), which indicate their multipotency in the cortex and hippocampus alike (Ahmed et al., 2012).

Mature bony fish display high neurogenic potential in many cerebral areas. Zones of initial proliferation in fish are located along the brain ventricle and form 16 accumulations along the rostro-caudal axis of the brain (Ekström et al., 2001). The brain areas, where quite intense cell division takes place in adult individuals, are considered as zones of secondary neurogenesis being localized in telencephalon, optical tectum, and cerebellum, as well as in thalamus and brain axis (Zupanc and Ott, 1999).

Fish are able to regenerate neural tissue after a mechanic or chemical damage in cerebellum, telencephalon, olfactory bulbs, and retina, and this is supposedly closely allied to the processes of adult neurogenesis (Takeda et al., 2008; Kroehne et al., 2011; März et al., 2011; Diotel et al., 2013; Zupanc and Sîrbulescu, 2013). It has been proven than the neural tissue of fish regenerates after damage within a few weeks (Zupanc et al., 2003). Replenishment of a large number of cells lost as a result of damage occurs from different sources, such as radial glia, centers of initial and secondary proliferation, and neurogenic niches (Zupanc, 2011).

Evolutionary ancient groups like salmon fish (Salmonidae) are characterized by high concentration of undifferentiated elements in the matrix zones of brain and parenchyma alike (Pouwels, 1978). Cerebellum is a generally accepted brain zone for the study of morphogenetic processes, since it increases the proliferative activity both under normal conditions and in case of traumatic impact (Pouwels, 1978; Zupanc and Sîrbulescu, 2013). Among poikilotherms, cerebellum retains the embryonic properties only in mature bony fish, which is also typical for other areas of their brain (Margotta et al., 2004).

Recently, it has been established that the glial fibrillary acidic protein (GFAP) is one of the markers of NSC (Kriegstein and Alvarez-Buylla, 2009; Ahmed et al., 2012). The proliferating cell nuclear antigen (PCNA) is used in the study of proliferative cell populations (Candal et al., 2005). The objectives of the chapter were to study the proliferation processes and the role of the glia and neural stem cells in the event of damage to cerebellum of juvenile masu salmon *Oncorhynchus masou.*

COMPARATIVE ASPECTS OF ADULT NEURAL STEM CELL ACTIVITY IN AMPHIBIANS AND FISH

During the time of Altman's initial studies of rodents, reports on proliferation zones in brain of adult amphibians and fish were also published (Kirsche, 1967). In Amphibia,

proliferation and neurogenesis are more widespread than in amniotes and are localized in telencephalon, preoptic region, hypothalamus, thalamus, midbrain and cerebellum in urodeles and anurans (Margotta et al., 2004; Raucci et al., 2006). Unfortunately, there are no many reports on constitutive neurogenesis in amphibians, but a recent study confirms constitutive neurogenesis in the forebrain and midbrain of the adult bullfrog *Rana catesbeiana* discovered by pulse-chase experiments using co-labelling of newborn cells with a marker of early neurogenesis (Simmons et al., 2008). On the contrary, the aquatic salamander *Notophthalmus viridescens* does not show constitutive neurogenesis in the midbrain. With a specific lesion of midbrain dopaminergic neurons, however, quiescent ependymoglial cells can be activated in this species to proliferate and regenerate the lost cells (Berg et al., 2010).

In teleosts, the initial reports on proliferation zones in ventricular regions of the adult brain were subsequently extended to other teleost species and uncovered the presence of constitutive proliferation zones/neurogenic zones all along the rostro-caudal axis of the brain (Kuroyanagi et al., 2010; Tozzini et al., 2011). The widespread neurogenesis in both anamniote groups has been attributed to the protracted, perhaps lifelong, growth of the body and, notably, sensory organs. In both groups, freshly hatched animals are considerably smaller than adults. Thus, the constitutive neurogenesis observed in growing teleosts and amphibians may be attributable to a growing demand of CNS processing power in conjunction with increasing sensory input (Kaslin et al., 2008). This is nicely exemplified by the growing retina and optic tectum in amphibians and fish (Cerveny et al., 2012) and the increasing number of taste buds and growth of the chemosensory lobes in medulla in cyprinids (Komada, 1994).

It is important to note that brain proliferation zones in adult zebrafish reflect a more general proliferation pattern along the ventricular walls of teleost brain (Grandel and Brand, 2013). The teleost proliferation zones are not ill-defined but distinctly localized in all the subdivisions of brain along the rostro-caudal axis. From 12 to 16 distinct proliferation zones have been recognized as distantly related between teleost species such as stickleback *Gasterosteus aculeatus*, brown ghost *Apteronotus leptorhynchus*, and zebrafish *Danio rerio*. With the exception of minor differences, the discrepancy in number reflects the investigators' interpretation rather than an actual lack or increase of zones in these species (Zupanc and Horschke, 1995; Ekström et al., 2001; Zupanc, 2006). The partially characterized brain proliferation zones of medaka *Oryzias latipes* and *Nothobranchius furzeri* likewise correspond to the general proliferation pattern; however, observations on the posterior hindbrain in these species are missing (Kuroyanagi et al., 2010; Tozzini et al., 2011). Thus, zebrafish suits well to study adult teleost brain proliferation, especially with the opportunity to elucidate the organization of proliferation zones on a cellular level, the contribution of newborn cells to normal brain function, the fate of restriction of progenitor/stem cells and, hence, their potential to repair brain parts after an injury of brain tissue.

PROLIFERATION AND MIGRATION OF CELLS IN CEREBELLUM OF FISH

The largest formation containing PCNA with cells was a dorsal matrix zone of *O. masou* cerebellum. It is located in the dorsomedial part of cerebellum's body, at the boundary

between the molecular and granular layers, and the probability density of PCNA with cells in it was higher compared to the adjoining areas of the molecular layer. According to previously conducted investigations, in the composition of this zone, we identified four types of cells in a normal state and after the damage (Stukaneva et al., 2015).

In the lateral and basal zones, proliferation processes noticeably decrease after the damage and migration processes increase (Figure 1A, B). We explain it by the fact that newly formed cells begin to radially and tangentially migrate in large amounts within two days after the damage, and this process can intensify in the following days. Within the boundaries of the lateral zone, we discovered dense clusters of intensely labeled PCNA cells separated from each other by immune- negative zones. Such clusters were identified as neurogenic niches (Figure 1C). After mechanical injury, proliferation and migration processes change their activity in different zones (Figure 1D).

Thus, results of PCNA immune labeling indicate that the proliferative activity in *O. masou* is typical mostly for the dorsal matrix area of cerebellum that agrees with the data on other fish, such as *D. rerio* (Zupanc, 2006) and *Apteronotus leptorhynchus* (Sîrbulescu et al., 2015). The results of our observations allow us to assume that the structural changes detected have a direct relation to processes of reparative neurogenesis. Thus, the obtained results are a demonstration that, after the damage, the main volume of cell proliferation is found in the cerebellar dorsal matrix zone.

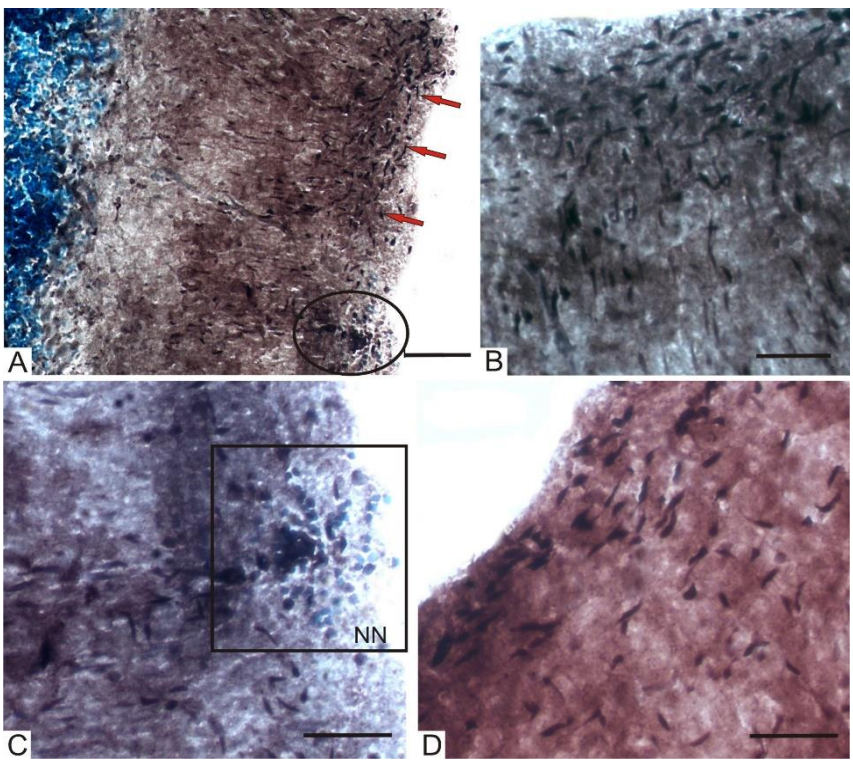

Figure 1. Localization of proliferative cell nuclear antigen (PCNA) in cerebellum of *Oncorhynchus masou* 2 days after stab-wound injury of cerebellum: (A) patterns of tangential migration (red arrows) and neurogenic niche (in oval) in the molecular layer of cerebellum; (B) radial migration of PCNA+ cells; (C) neurogenic niche (in square); (D) tangential migration of PCNA+cells. Scale bar: A, 100 μm; B–D, 50 μm.

Short-term bromodeoxyuridine (BrdU) labeling experiments or PCNA staining showed widespread proliferation in the cerebellar molecular layer of zebrafish. Newborn cells migrate radially along vimentin/ GFAP/BLBP-expressing radial fibres towards the surface of the molecular layer, where they migrate tangentially and disperse laterally to finally dive in a radial direction through the cerebellar molecular layer into the granular layer, where they differentiate into granule neurons (Grandel and Brand, 2013). Migrating progenitors and differentiated cerebellar granule neurons express the transcription factors Zic1, Zic3, Pax3 and Neurod (Kaslin et al., 2009). Migration through the molecular layer occurs rapidly. Within 6 days, the molecular layer of zebrafish cerebellum becomes devoid of labeled cells as they reach the granular layer (Grandel and Brand, 2013). This behavior is also observed with most cells above the dorsal matrix zone, except for label-retaining cells that remained after up to 6 weeks of chase and still showed the initial S-phase marker plus either pH3 or PCNA immunoreactivity, indicating that they were still proliferating. Such cells are considered the presumptive stem cells in other systems (Doetsch et al., 1999). Thus, Kaslin et al. (2009) determined their molecular characterristics. Tg (nestin-GFP) animals showed transgene expression in most proliferating cells above the granular layer "cap". Tg (nestin-GFP) was also found to co-localize with label retaining cells at this position. In addition, these cells maintained expression of Sox2, Meis and Musashi 1 that were shown to localize to stem or progenitor cells in other systems (Morales and Hatten 2006). Tg (nestin-GFP)-expressing progenitors also were distinctly polarized, displaying apical adherence junctions and expressing apical markers such as ZO1, β-catenin, γ-tubulin and aPKC (Grandel and Brand, 2013). As in the case of the subpallial proliferation domain and in the periventricular margin of optic tectum, the cerebellar progenitors displayed a neuroepithelial phenotype.

To underscore this finding, Tg (nestin-GFP)-positive cells did not co-stain with the radial glia markers vimentin/GFAP/BLBP. Radial glia in the cerebellar midline consists of a non-proliferating population and may serve to guide the migrating progeny of the neuroepithelial progenitor population. How do neuroepithelial granule cell progenitors become situated within *corpus cerebelli*, apparently far off from the ventricular system? Kaslin et al. (2009) studied the development of the cerebellar stem cell niche during embryonic and larval stages which showed that cerebellar progenitors were derived from the upper rhombic lip as in rodents and birds.

Within the first 10 days, the rate of cell proliferation in the area of brain trauma in bony fish increases several times compared to other areas of cerebellar body. Experiments with labeling by BrdU have shown that cells formed 2 days before the trauma take part in the regeneration process (Zupanc and Ott, 1999). This observation presupposes a presence of a link between continuous cell proliferation in an intact brain and restoration of the area damaged due to injury. A retrograde tracing in combination with BrdU labeling of S-phase have shown that new granular neurons are projected into the molecular layer of cerebellum (Zupanc and Ott, 1999). This fact allows us to assume that these neurons integrate into the already existing neuron network of cerebellum.

In zebrafish, however, a distinct morphogenetic process during embryonic and early larval stages (24 h to 7 days) displaces medially located rhombic lip progenitors and a small portion of the fourth ventricle into cerebellar corpus to form an anterior protrusion, which has been termed as cerebellar recessus (Kaslin et al., 2009). The latter grows further anteriorly thereby carrying the progenitors from the upper rhombic lip deeper into *corpus cerebelli*. Dye labelling experiments revealed that the lumen of cerebellar recessus extends across the

cerebellar midline and remains in contact with the fourth ventricle even during adult stages. This shows that the nestin-expressing neuroepithelial-like precursors in the cerebellar stem cell niche stay in contact with the ventricular system with their apical sides, much like the precursors of subpallium and the periventricular tectal margin.

In our investigations, we verified the zones after injury with the neurogenic activity, the neurogenic niches located in the molecular layer of the dorsal, lateral, and basal areas of masu salmon's cerebellum. We linked their appearance to intensification of genetic proliferative programs in neutral stem cells and formation of local neurogenic niches as a response to damage. When damage is inflicted to neurogenic zones, active proliferation and differentiation of new cells begins, and they migrate to the damaged area, restoring the injured tissue.

According to Zupanc's data, in case of damage to *A. leptorhynchus* brain, intense neurogenesis processes start in the basal zone of cerebellum (Zupanc, 2011). Thus, this area can serve as a source of cells for reparative neurogenesis. In the basal zone of cerebellum in the area of granular eminences of juvenile masu salmon, on the contrary, the intensity of proliferation is high enough in the control and significantly decreases after a damaging effect. It is possible that the decrease of proliferation processes in this area of cerebellum is replaced by cell migration processes.

Appearance of neurogenic niches in the area of dorsal, lateral, and basal zones of the cerebellar molecular layer is the next stage of reparative neurogenesis. Neurogenic niches appear *de novo* after a traumatic impact and contain PCNA+ cells, as well as a heterogeneous population deprived of a proliferation marker. Immunolabeling of GFAP has shown that fibers of radial glia, as well as isolated small intensely and moderately labeled cells, which are phenotypically corresponding to neural stem cells described in cerebellum of other bony fish, are detected in the area of neurogenic niches (Hinsch and Zupanc, 2006; Kaslin et al., 2013; Sîrbulescu et al., 2015).

The investigations conducted allow us to conclude that, after inflicting an injury to cerebellum of juvenile masu salmon, processes of reparative neurogenesis are added to the processes of constitutive neurogenesis: active proliferation and differentiation of new cells begin, and they migrate to the damaged area, repairing the tissue. The main source of new neurons is neurogenic niches that are formed during reparation of neural tissue.

GFAP, DOUBLECORTINE AND OTHER MARKERS OF NSCS IN CEREBELLUM OF FISH

In cerebellum of juvenile masu salmon in normal state, we discovered a significant level of GFAP-positivity detected in fibers of systems of the spinocerebellar tract: as a part of cerebellar peduncles located in the basal part of cerebellar body, in fibers of radial glia, and in a heterogenous population of rounded (intensely labeled) and elongated (moderately labeled) cells (Stukaneva et al., 2017).

In the lateral zone, we discovered a small amount of DC+ cells of the following two types: small cells of 5.99 ± 0.11/2.76 ± 0.12 μm in size and larger ones of 9.07 ± 0.26/2.76 ± 0.34 μm in size. The number of intensely DC-labeled cells per visual section was no more than 15 (Figure 2A). They had a rounded or elongated shape, were deprived of process, and

located solitary or formed small clusters (Figure 2B). In the granular layer of the basal zone, the probability density of differently directed, thin, DC+ radial glia fibers was moderate (Figure 2B). In a visual field, there were, on average, 14 DC+ cells and 24 DC+ radial glia cells. In the area of white matter of the basal zone (cerebellar peduncles), there were, on average, 30 DC+ cells and 48 GFAP+ fibers per a relevant test-field of 28 000 μm^2 (Stukaneva et al., 2017). In this area, fibers are prevailing GFAP+ elements, whose quantity differs significantly ($p < 0.05$). After injury, on the surface of the dorsal and basal zones and deep in the molecular layer of the lateral zone, we detected small local dense accumulations of GFAP- cells, whose morphological parameters correspond to those of cells of the first type labeled with PCNA (Stukaneva et al., 2017). We consider such GFAP+/DC+ cells conglomerates as neurogenic niches emerging in masu salmon's cerebellum as a response to damaging effect (Figure 2C, D). In dorsal, basal, and lateral zones, we found niches of 3192 ± 427, 6650 ± 721, and 1393 ± 653 μm^2, respectively (Stukaneva et al., 2017). In all the above-mentioned zones, parameters of proliferative PCNA+ cells coincide with the initial morphological parameters of GFAP+/DC+ cells.

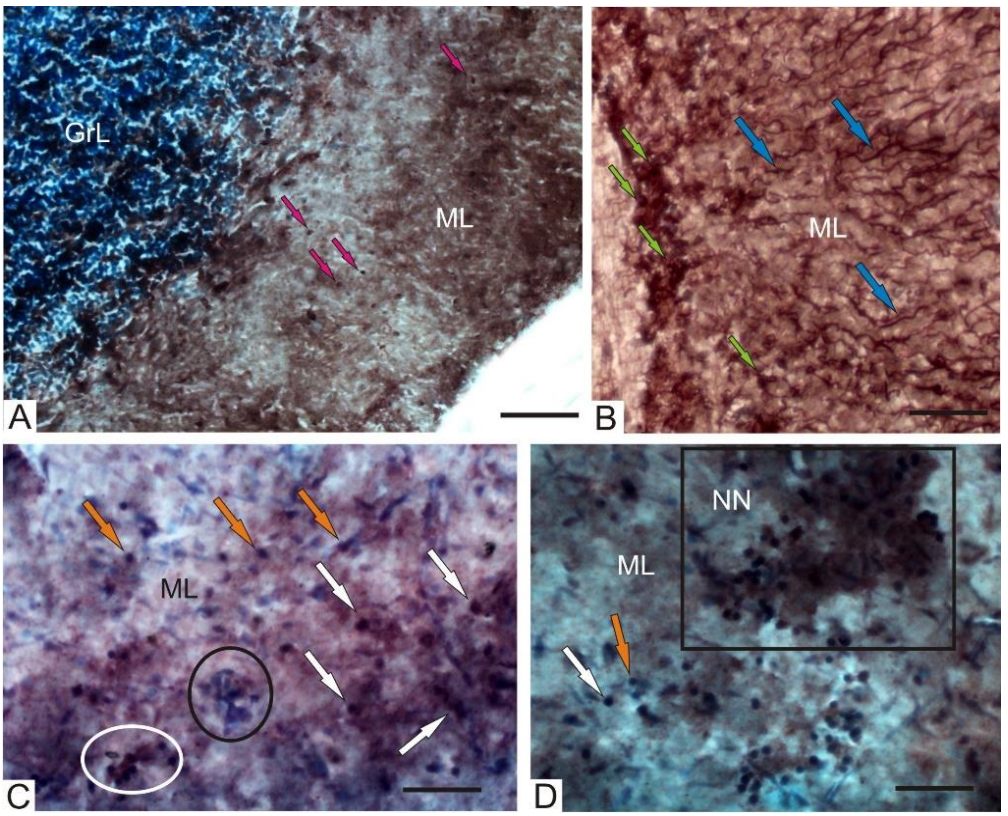

Figure 2. Expression of doublecortin (DC) in cerebellum of *Oncorhynchus masou* at 2 days post-injury: (A) general view of *corpus cerebellum*, (DC) immunopositive (ip) cells are indicated by pink arrows; (B) DC-ip cells (green arrows) and radial glia (blue arrows) in the basal part of *corpus cerebellum*; (C) DC-ip group of cells (in white oval), DC-ip single cells (white arrows) and DC- immunonegative (in) group of cells (in black oval) and DC-in single cells (orange arrows) in molecular layer of cerebellum; (D) a neurogenic niche containing DC-ip cluster of neural stem cells (in rectangle) in the molecular layer. Scale bar: A, 100 μm; B–D, 50 μm.

Comparative studies of the ratio of GFAP+ cells to fibers in the dorsal and basal zones have been conducted. At one day after inflicting a damaging effect, we observed changes of cell composition and fibers compared to the intact state. After the damage, the number of GFAP+ cells and fibers increased in the dorsal and basal zones. We suppose that the increase in number of GFAP+ cells is connected with appearance of activated astrocytes as a result of the damage (Stukaneva et al., 2017).

As a result of the damage in the dorsal matrix area, we recorded appearance of GFAP+ fibers of radial glia forming differently oriented bundles (Figure 3). Such structural formations have not been detected in intact animals. We suppose that, after the injury in the dorsal matrix area, there is a structural reconstruction, connected with appearance of additional labeled fibers in the area of the damage, that leads to twofold increase in the probability density of GFAP+ fibers in this area. We link such effects to intensification of spatial reorientation of radial glia fibers and formation of specific tracks for cells formed in this area.

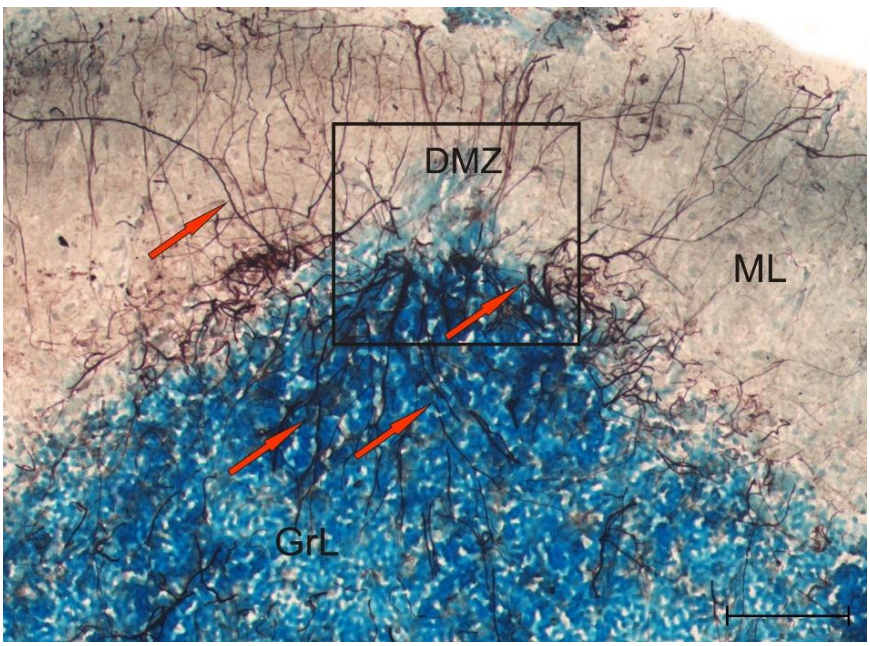

Figure 3. Localization of glial fibrillar acid protein (GFAP) in the cerebellum of *Oncorhynchus masou* at 2 days post-injury. Dorsal matrix zone is outlined by black squares; GFAP-immunopositive fiber is indicated by red arrows; DMZ, dorsal matrix zone; ML, molecular layer; GrL, granular layer. Scale bar: 100 μm.

In brain of adult mammals, cells of radial glia are located in restricted neurogenic areas, such as the subgranular zone of hippocampal dentate gyrus (Morrens et al., 2012; Dimou and Goetz, 2014). In mice, GFAP-labeled glia disappears from the beginning of myelinization (Nakahara et al., 2003), whereas in fish, GFAP-labeled fibers of radial glia are identified in different segments of the brain, including cerebellum, throughout their life (Zupanc and Horschke, 1995).

We suppose that the existence of GFAP+ and DC+ radial glia in an intact brain of juvenile masu salmon reflects processes of persistent neurogenesis. The morphology of GFAP+ structures, their topography, and interactions with other structural components allow us to assume that they obviously are elements of threadlike fibers of masu salmon's cerebellum. This is, in particular, indicated by the morphology of these structures and the presence of microcytocsculpture along fibers and their terminals.

In mammal brain, GFAP is a classical marker of astrocytes with numerous radially oriented processes and terminal pedicles that are often formed on the surface of vessels (Doetsch and Scharff, 2001). It has recently been shown that GFAP is one of the markers of neural stem cells (Kriegstein and Alvarez-Buylla, 2009; Ahmed et al., 2012). However, as a rule, their phenotype is more complex and, besides GFAP, contains vimentin, nestin, S-100, Sox2 (Adolf et al., 2006; Sîrbulescu et al., 2015). In studies of several areas of brain of adult bony fish, we identified precursor cells possessing characteristics of radial glia (Chapouton et al., 2007; Rothenaigner et al., 2011). In this case, they were capable of self-renewal and creation of different cell types, which demonstrate characteristics of genuine stem cells (Rothenaigner et al., 2011). It is possible that at least some of the radial glial cells in the brain of adult *A. leptorhynchus* fish act as these precursors. Since GFAP+ astrocytes can participate in recovery of cells lost in case of injury in the subventricular zone (Doetsch et al., 1999), it is obvious that these cells have the capability of self-repair. According to the data of Ahmed et al., (2012) cortex astrocytes in mouse brain stop fission by the tenth day of postnatal development, and GFAP expression after injury becomes more pronounced compared to proliferation processes.

GFAP+ cells of masu salmon's radial glia located in the surface layer of molecular layer have small-sized somas and long unbranched processes. Thus, GFAP+ cells and fibers of radial glia are both structural and morphological components in the composition of cerebellum of juvenile masu salmon. Our data support the results obtained for *Austrolebias affinis*, *A. charrua* and *A. reicherti* (Fernandez et al., 2011), and *A. leptorhynchus* (Clint and Zupanc, 2001) concerning the presence of GFAP+ radial glia in cerebellum of bony fish. We are inclined to attribute the few identified GFAP+ cells to neural stem cells. This assumption is based on a combination of morphological and topographic properties of neural stem cells, their connection with neurogenic zones, and relationships with fibers of radial glia.

In zones of secondary neurogenesis of masu salmon's cerebellum, we identified GFAP+ fibers and cells of radial glia. Data obtained from different species of bony fish is the evidence that radial glia is the prevalent type of GFAP-immunolabeled elements remaining in brain of adult fish. In addition to these fibers, whose numbers increase after injury, cell migration also takes place (Clint and Zupanc, 2001).

Data of Ahmed et al., (2012) have shown that, after a mechanical damage to mouse brain, endogenic GFAP+ progenitor cells are activated. Approximately 50% of cells forming neurospheres in hippocampus and 75% in cortex originate from GFAP-expressing cells. According to data of Sîrbulescu et al., (2015) GFAP in cerebellum of *A. leptorhynchus* labels neural stem cells/precursor cells (Sîrbulescu et al., 2015). This agrees with a hypothesis that such cells are supposedly a source of neurospheres after the damage (Ahmed et al., 2012). However, not all neurospheres originate from GFAP+ cells: their smaller part is precursors of GFAP- cells.

CONCLUSION

Neurogenesis during adult stages is found in species of all vertebrate classes, as it serves different needs. Adult neurogenesis is considered in the context of protracted or constitutive growth or neuronal turnover. Adult neurogenesis is observed within the same class or order, even within one species, to serve these different needs. Are there fundamental differences in the generation of new neurons that show different phenotypes and serve different functions? From a morphological viewpoint, most stem cell populations retain contact to the ventricular system. Structurally, they appear as neuroepithelial cells, radial glial or astroglial cell types. The different shapes of these progenitors have been suggested to be a secondary consequence of the architecture of the developing parenchyma overlying the ventricular stem cell zone of the embryo (Kriegstein and Alvarez-Buylla, 2009). We suggest that an important step in characterizing the degree of difference between various stem cells pools is to identify the pathways that control their activity. This could be a key to our understanding of stem cell diversity and a valuable cue to the development of therapies based on yielding activation of active or dormant populations.

Certainly, in this scheme, SGZ radial astrocytes of mammalian brain are an odd element, as they are described as having no contact with the ventricle, yet they express nestin as do their cerebellar counterparts in zebrafish. This difference may be related to the late appearance of dentate gyrus in evolution which can explain the lack of a directly homologous cell type in other vertebrate groups (Kempermann, 2012). Progenitors of *eminentia granularis lateralis* have lost the ventricular contact also. It can be argued that they "only" serve the protracted cerebellar growth; however, SVZ astrocytes also serve the protracted growth of the olfactory bulb initially.

The differences between various stem cells on the level of gene expression may highlight their restriction and differences with respect to their prospective progeny. It will be interesting to find whether distinct stem cells can be forced to produce unusual progeny if needed. Such a scenario and, hence, a broader prospective potential of adult neural stem cells are confirmed by the lesion experiments in zebrafish cerebellum, which show, at least topologically, that newborn neurons will populate regions within the granular layer that are otherwise not attained. On the other hand, it still remains unclear if all the cell types that are lost after the injury are subsequently replaced indeed.

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Chapter 14

CATALASE AND PEROXIDASE IN BLACK SEA TELEOSTS: EVOLUTIONARY, SYSTEMATICAL, AND PHYSIOLOGICAL PECULIARITIES

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ABSTRACT

Metabolism of the living organisms is associated with oxygen consumption. Microbes, plants and animals use oxygen in the major physiological and biochemical pathways. However, O_2 has two unpaired electrons and the univalent reduction of the molecular oxygen produces reactive oxygen species (ROS), which lead the oxidative stress and damage biomolecules, such as lipids, proteins, DNA, cells and tissues. Living organisms have antioxidant defense to counteract the toxic effects of ROS, and it plays a key role in inactivation of ROS, and thereby controls of oxidative stress as well as redox signaling. Among antioxidant enzymes catalase (CAT) (EC 1.11.1.6.) which catalyzes the reduction of hydrogen peroxide to water and total peroxidase (PER) (the sum of SeGPx (EC 1.11.1.9) and selenium-independent GPx (EC 1.11.1.9) activities), which reduces both hydrogen peroxide and organic hydroperoxides are the main antioxidant enzymes. The study of these enzymes in different taxonomic groups of the living organisms is very important for the understanding of the mechanisms of the evolutionary process and adaptations of the species for environmental conditions. Antioxidant status of the fish depends on many biotic and abiotic factors, such as temperature, season, diet, salinity, oxygen concentration and many others, as well as on their taxonomic position and life cycle. It reflects the adaptive strategy of fish species to oxidative stress and their ability to cope with the environment. At the other side, antioxidant status is attributed with phylogenetic position and ecological peculiarities of the organism, and these parameters in primitive life are differed compared to birds and mammals. The levels of antioxidant enzymes activity are ranged in fish tissues, and they depend on the tissues specificity and physiological function. The interspecies variations of the enzymes may reflect the specific adaptations to the oxidative stress and the protective mechanisms

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against ROS damage in fish, belonging to different ecological groups and inhabiting different geographical locations. Studies of antioxidant enzymes in different species are very important for the understanding the response mechanisms against pollution in fish of various taxonomic and ecological groups as well as for aquaculture purposes, and for receiving the information of repair mechanisms and adaptation to unfavorable factors. The combination of specific phylogenetic, physiological and ecological features of fish species may modify antioxidant status, and it is important for development of monitoring programs. The aim of the present work is to analyze catalase and peroxidase enzymes in different Black Sea teleosts related to their taxonomic, physiological and ecological position and for the purpose of the use of these antioxidant enzymes in fish tissues as biomarkers for the anthropogenic impact on marine environment.

1. INTRODUCTION

The introduction of molecular oxygen into Earth's atmosphere billions of years ago has allowed animals to produce energy more efficiently (Falkowski et al., 2004). Metabolism of the living organisms is associated with oxygen consumption. Microbes, plants and animals use oxygen in the major physiological and biochemical pathways. Living organisms obtain and use oxygen, and evolved over hundreds or millions of years together with the evolution of oxygen-delivery systems. A steady supply of oxygen to the existence of organism was very important, because its metabolism depends on oxygen as a primary source of fuel (Fisher & Burggren, 2007). However, O_2 has two unpaired electrons and the univalent reduction of the molecular oxygen produces reactive oxygen species (ROS), which lead the oxidative stress and damage biomolecules, such as lipids, proteins, DNA, cells and tissues. Living organisms have antioxidant defense to counteract the toxic effects of ROS, and it plays a key role in inactivation of ROS, and thereby controls of oxidative stress as well as redox signaling. A number of mechanisms have evolved to defend against oxygen deprivation (hypoxia and anoxia) and for the hyperoxic conditions. At the other side, ROS can play an essential role in the evolution of living organisms, because the growth of oxygen level in the atmosphere in the combination with the increased mobility and food intake, led to increase cellular level of free radicals, ROS interacted with DNA of the primitive life, enhancing mutations and providing new regulatory mechanisms and biodiversity (Yang et al., 2018).

Recent years the role of oxidative stress in the life history has been received much attention, caused the effects of the climate changes and anthropogenic pollution, and their consequences for environment and biota. However, generally the researchers focus their studies on high taxonomic groups, while the information about fish and invertebrates is less (Birnie-Gauvin et al., 2017).

Antioxidant system presents in all living organisms and it includes low molecular weight scavengers (vitamins A, E, K and C, carotenoids, SH-containing substances (glutathione) and small-molecule antioxidants namely uric acid, urea, and etc.), and special adopted enzymes (catalase, superoxide dismutase, peroxidases, especially selenium-dependent glutathione peroxidase (SeGPx) (EC 1.11.1.9), glutathione reductase (GR) (EC 1.8.1.7) and some others) (Winston, 1991). Heme peroxidases and catalases are the key enzymes of hydrogen peroxide metabolism and signaling, which present in all kingdoms of life (Zamocky et al., 2014). Among them catalase (CAT) (EC 1.11.1.6.) and total peroxidase (PER) (the sum of SeGPX

and selenium- independent glutathione peroxidase (GPx) (EC 1.11.1.9) activities), are the key antioxidant enzymes.

CAT catalyzes the reduction of hydrogen peroxide to water:

$2H_2O_2 \rightarrow 2H_2O + O_2$

Heme peroxidases are the most abundant family of peroxidase catalyzing the H_2O_2-dependent oxidation of a wide kinds of substrates. They involved in innate immune response, hormone and prostaglandin synthesis crosslinking of protein extracellular matrix (Øvergård et al., 2017). Additionally, they play a role in the process of the fish eggs fertilization. Total PER (SeGPx and GPx) reduces both hydrogen peroxide and organic hydroperoxides:

$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$ (SeGPx)
$H_2O_2 + 2\ GSH \rightarrow 2\ H_2O + GSSG$

GPx eliminates the peroxides produced as effect of reactions of biomolecules with ROS. There are several types of GPx, which was isolated and cloned in various organisms (Liu et al., 2018).

Therefore, study of these enzymes and their activity in different taxonomic groups of the living organisms is very important for the understanding of the mechanisms of the evolutionary process and adaptations of the species to environmental conditions. Fishes are highly distributed in various water bodies with great variability of their conditions, including salinity, oxygen concentration, temperature, and etc. (Birnie-Gauvin et al., 2017). They play an important role in the aquatic ecosystems and for people, because they are the main source of seafood, which has been recognized as a high-quality, healthy and safe food type. Fish tissues are rich of the antioxidants, and some of them are present in seafood in high concentrations. Therefore, they are the main sources of these essential components for people diet and one of the most important food commodities consumed worldwide. At the other side, antioxidant enzymes namely catalase and peroxidase are good biomarkers and they are used as a tool for the evaluation of stress conditions in the wild and aquaculture fish populations (Mukherjee et al., 2017; Alti et al., 2016).

Antioxidant status of fish depends on many biotic (food consumption, reproductive strategy, aging and senescence, belonging to different ecological groups), and abiotic factors such as temperature, season, salinity, oxygen concentration in water, and many others as well as on their taxonomic position and life history. It reflects the adaptive strategy of fish species to oxidative stress and their ability to cope with the environment. At the other side, antioxidant status is associated with phylogenetic position and ecological peculiarities of the organisms, and these parameters in primitive life are differed compared to birds and mammals. The levels of the antioxidant enzymes are ranged in fish tissues, and they depend on the tissue specificity and physiological function. The interspecies variations of the enzymes may reflect the specific adaptations to the oxidative stress and protective mechanisms against ROS damage in fish, belonging to different ecological groups and inhabiting different geographical locations and biotops. Thus, the comparative studies of antioxidant defense in teleosts are very important for the understanding the main ways of evolutionary process and the mechanisms of adaptation to aerobic conditions (Filho & Boveris, 1993; Filho et al., 1993; Filho, 1996; Sole et al., 2009).

Study of the antioxidant enzymes in different species is very important for the understanding the defense mechanisms against pollution in fish of various taxonomic and ecological groups, as well as for aquaculture purposes, and for receiving the information of repair mechanisms and adaptation to unfavorable impact. Combination of specific phylogenetic, physiological and ecological features of fish species may modify antioxidant status, and it is important for the creation of monitoring programs. The aim of the present work is to analyze the key antioxidant enzymes catalase and peroxidase in different Black Sea teleosts, belonging to different taxonomic groups related to their phylogenetic position, physiological, developmental and ecological peculiarities and anthropogenic impact.

2. ENZYME ACTIVITIES AND FISH PHYLOGENIC POSITION

Tested Black Sea fish species were grouped according their phylogenetic position (Table 1).

Table 1. Phylogenic position of tested Black Sea teleosts (the classification is present according Boltachev and Karpova, 2017)

Order	Family	Fish species
Gadiformes	*Gadidae*	*Merlangius merlangus euxinus* (Nordmann, 1840)
	Lotidae	*Gaidropsarus mediterraneus* (Linnaeus, 1785)
Ophidiiformes	*Ophidiidae*	*Ophidion rochei* Muller, 1845
Mugiliformes	*Mugillidae*	*Liza aurata* (Risso, 1810)
Perciformes	*Sciaenidae*	*Sciaena umbra* Linnaeus, 1785
Perciformes	*Sparidae*	*Diplodus annularis* (Linnaeus, 1785)
Perciformes	*Centracanthidae*	*Spicara flexuosa* Rafinesque, 1810
Perciformes	*Labridae*	*Symphodus tinca* (Linnaeus, 1785)
Perciformes	*Carangidae*	*Trachurus mediterraneus ponticus* Aleev, 1956
Perciformes	*Gobiidae*	*Neogobius melanostomus* (Pallas, 1814) *Mesogobius batrachocephalus* (Pallas, 1814)
Perciformes	*Mullidae*	*Mullus barbatus ponticus* Essipov, 1927
Perciformes	*Uranoscopidae*	*Uranoscopus scaber* Linnaeus, 1785
Scorpaenoformes	*Scorpaenodae*	*Scorpaena porcus* Linnaeus, 1785
Pleuronectiformes	*Pleuronectidae*	*Platichthys luscus* (Pallas, 1814)

The most ancient orders are recognized as *Gadiformes* and *Ophidiiformes*, the following is *Mugiliformes*, then *Perciformes*, the orders *Scorpaenoformes* and *Pleuronectiformes* are relatively recent taxonomic groups. We studied CAT and PER activity in fish red blood cells (RBC) and in the liver related to their phylogenetic position. CAT activity in the RBC of tested Black Sea teleosts tended to increase in the species, belonging to the order *Perciformes* and *Scorpaenoformes* as compared to the species of ancient orders namely *Gadiformes* and *Ophidiiformes* (Figure 1). At the other side, CAT activity in *Platichthys luscus*, belonging to very specific order *Pleuronectiformes* was lower as compared with the species of *Perciformes.* Additionally, we could note that enzyme level varied significantly among the various species of *Perciformes*, which could depend on their biological and ecological peculiarities (see below).

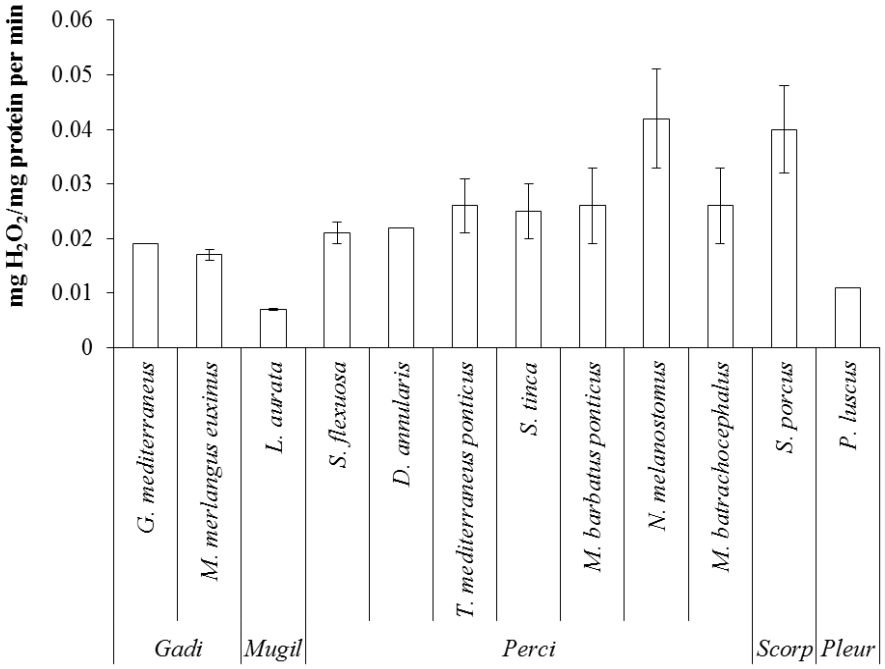

Figure 1. CAT activity in RBC of tested Black Sea fish species belonging to different orders: Gadi – *Gadiformes*, Mugil – *Mugiliformes*, Perci – *Perciformes*, Scorp – S*corpaeniformes*, Pleur – *Pleuronectiformes*.

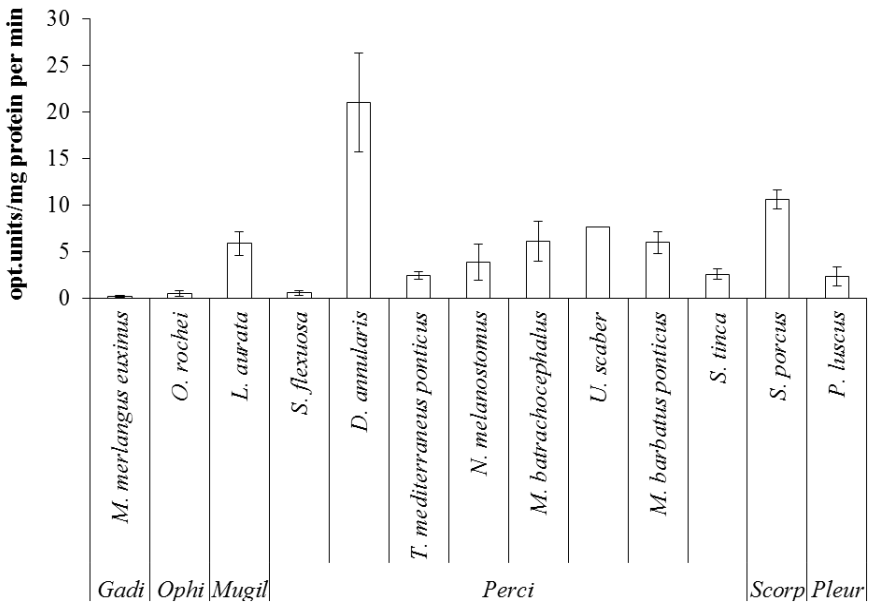

Figure 2. PER activity in RBC of tested Black Sea fish species belonging to different orders: Gadi – *Gadiformes*, Ophi – *Ophidiiformes*, Mugil – *Mugiliformes*, Perci – *Perciformes*, Scorp – S*corpaeniformes*, Pleur – *Pleuronectiformes*.

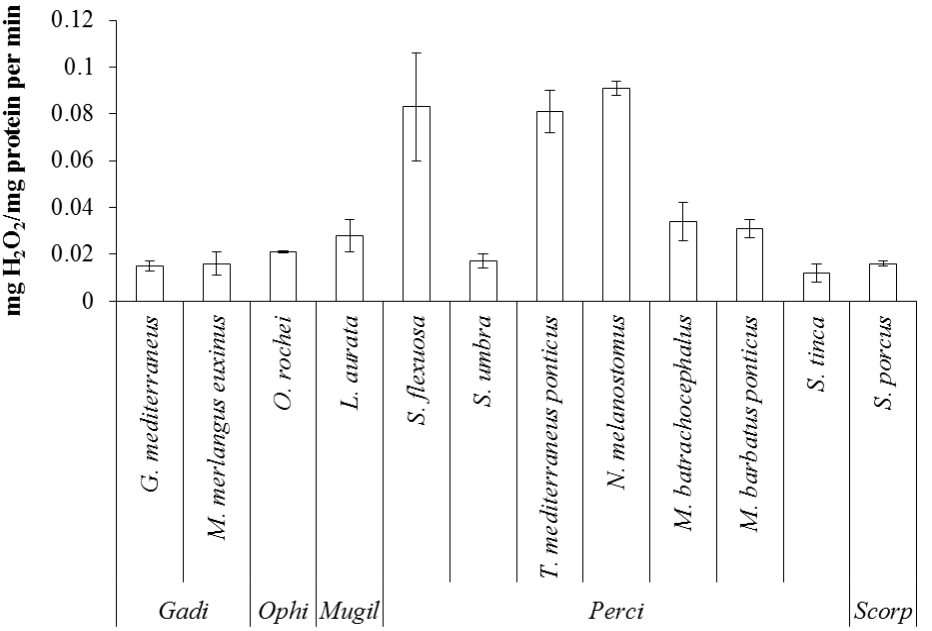

Figure 3. CAT activity in the liver of tested Black Sea fish species belonging to different orders: Gadi – *Gadiformes*, Ophi – *Ophidiiformes*, Mugil – *Mugiliformes*, Perci – *Perciformes*, Scorp – S*corpaeniformes*, Pleur – *Pleuronectiformes*.

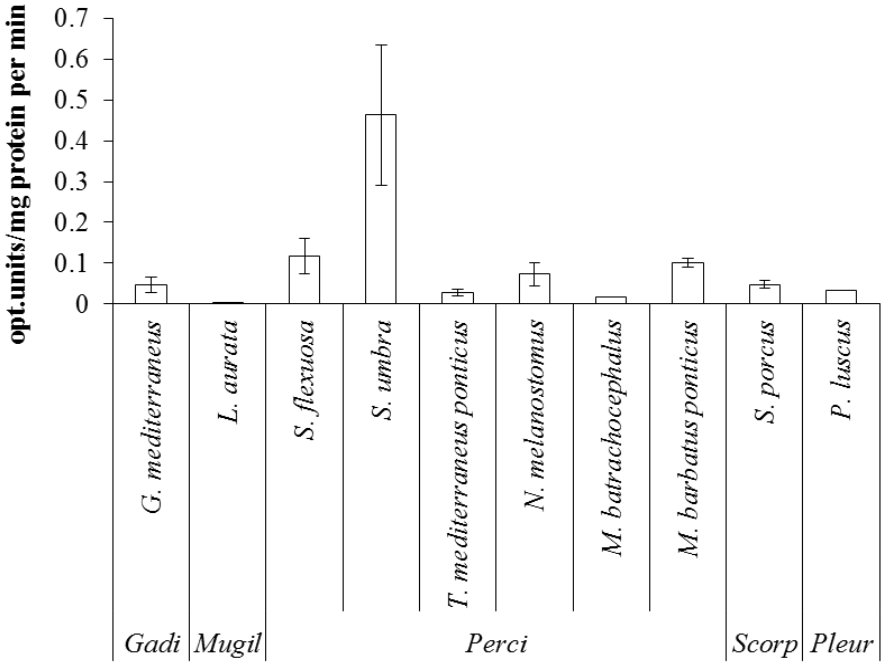

Figure 4. PER activity in the liver of tested Black Sea fish species belonging to different orders: Gadi – *Gadiformes*, Ophi – *Ophidiiformes*, Mugil – *Mugiliformes*, Perci – *Perciformes*, Scorp – S*corpaeniformes*, Pleur – *Pleuronectiformes*.

The lowest PER activity was indicated in the RBC of examined fish species belonging to *Gadiformes* and *Ophidiiformes*, in the other tested teleosts enzyme activity varied significantly, especially in the animals of the order *Perciformes* (Figure 2).

In the liver high CAT activity was observed in the *Perciformes* species (Figure 3), and it varied widely. Enzymatic activity in the RBC of the species belonging to the other examined orders was lower, however, it was comparable with the *Perciformes* fishes. PER activity in the liver ranged widely and we could not find any phylogenetic-related trends (Figure 4).

In our previous studies we showed the lack of CAT activity in the RBC in elasmobranch *Squalus acanthias* related to several examined Black Sea teleosts. We proposed that low molecular weight antioxidants, especially SH-containing substances play an important role in the antioxidant defense in elasmobranch, which compensate the lack of CAT and low activity of superoxide dismutase (SOD) and glutathione reductase (GR). As we documented previously, enzymatic activity was less in elasmobranch tissues as compared with the teleost species, excepted PER activity in muscle, which was the greatest among the all examined fish (Rudneva, 1997a; 2012).

A comparative study of hepatic antioxidant defense enzymes in teleosts and elasmobranchs demonstrated, that their content in primitive fish species was lower than that in teleosts, and seems to follow the overall metabolic oxygen consumption or activity level from each fish major taxonomic group (Filho & Boveris, 1993; Filho et al., 1993). Decrease of antioxidant enzymes level and high content of low molecular weight antioxidants in elasmobranch tissues related to teleosts was reported by other investigators (Martinez-Alvarez et al., 2005; Sole et al., 2009). Therefore, our findings provide that more primitive species such as elasmobranch and species, belonging to ancient teleost orders have more primitive antioxidant system in which non-enzymatic compounds play an important role in defense organism against oxidative stress.

The differences between CAT and PER activities can be linked with the differences of the enzymes functions: PER is non-specific enzyme as compared with CAT, and it reduces both H_2O_2 and organic peroxides (see section *Introduction*), and it could compensate the lack of CAT concentration. In general, our findings demonstrated that antioxidant enzyme levels in tested Black Sea teleost species depending on their taxonomic position, however, despite this, we could suggest that biological and ecological peculiarities of fish species may play more important role in antioxidant response than phylogenetic ones.

3. BIOLOGICAL PECULIARITIES OF FISH

3.1. Early Development and Ageing

Antioxidant enzyme activity was found in developing eggs of the fish. Ovoperoxidase was detected in the eggs, and it uses hydrogen peroxide as substrate, which is generated NADPH-oxidase system on the surface of egg shell (Takahashi et al., 1989; Winston, 1991). The authors demonstrated the changes in antioxidant enzyme activities in various fish species during early life. However, the trends of CAT and PER activities in various fish were not uniform and depended on species and developmental stage. Oxygen availability is one of the factor effecting hatching. Hypoxia or agents that cause hypoxia (low sea water dissolved

concentration of cyanide or high concentrations of dissolved hydrogen), stimulate hatching whereas hyperoxia may suppress hatching. Therefore, antioxidant enzymes level varied during fish embryogenesis and in hatching larvae.

In our studies we found, that during embryogenesis of several Black Sea gobies *Neogobius melanostomus*, *Proterorhinus marmoratus* and blenny *Blennius sanguinolentus*, majority of enzymes, including CAT and PER, tended to increase in developing eggs and especially in hatching larvae, while the levels of low molecular scavengers decreased (Rudneva, 1999; 2014). Our results agree with the data of other investigators, who reported the similar trends of CAT and PER levels in fish early development at the case of *Solea senegalensis* (Sole et al., 2004), *Scophthalmus maximus* (Peters & Livingstone, 1996), *Dentex dentex* (Mourente et al., 1999), *Paralichthys olivaceus* (Cao et al., 2010), *Lates calcarifer* (Kalaimani et al., 2008). Among the H_2O_2 -removing systems in *Salmo iridaeus* embryo development CAT seems to be the only enzyme presents at significant level and plays more important role in preventing H_2O_2 damage in fish embryo as compared with GPx. During further fish development, activity of antioxidant enzymes CAT and GPx increased with the maximum at stage 33, when the formation of the swim bladder as well as pigmentation has occurred. At the end of the development the activity of glutathione-containing enzymes increased progressively and values were found to be about 85-312 fold higher than in early stages, while the variations of CAT activity was lower (Aceto et al., 1994).

Therefore, we could conclude, that the increasing rate of oxygen uptake may elevate pro-oxidant processes such as ROS generation, and increase in CAT and SeGPx activities with development of embryos to larvae. They would indicate a progressive need to degrade H_2O_2 and peroxides from tissues (Peters & Livingstone, 1996). The increase levels of CAT and GPx may be in response to other metabolic changes occurring at this time, possibly leading to change sources of oxidative stress.

Further aging changes can be attributed to development, genetic defects, the environmental unfavorable factors and diseases. The accumulation of the negative changes and the loss of the ability to repair or detoxify them increase the risk of death (Harman, 2003). The study of aging strategy, both endogenous and exogenous factors, which it modulate in different species is very important for the understanding of the key mechanisms of aging and their early prevention. Several studies have shown that oxidative stress led the environmental factors is involved in age-related processes and modulate them. Oxygen stress relates to immune system dysfunction seems to have an important role in senescence, in agreement with the oxidation/inflammation theory of aging (De La Fuente et al., 2005).

There are many publications on the effect of age on antioxidant enzyme activities in laboratory animals including various mammalian and bird species, and perhaps for aquaculture fishes, while the information of wild species is very limited. However, the aging processes in wild fish populations are associated with many environmental factors, such as anthropogenic impact and the abilities to adapt to the changing living conditions, especially in recent decades, caused with climate changes. The life span of species might be influenced by its metabolic rate and its increase correlates with the high radical production and with oxidative damage (Zelinski & Portner, 2000). Whereas these aspects have been amply studied in mammals and especially in humans, information on fish and other aquatic animals is very limited.

Fish erythrocytes are an excellent model for the investigations of ROS and antioxidant status, especially for age-related studies, because in these cells mitochondria and nucleus were shown (Filho, 2007). In our previous studies (Rudneva et al., 2010a) we found, that the trends in antioxidant enzyme activities in blood of Black Sea teleosts were not uniform, and we showed three types of responses with aging increase, which depended on the enzyme specificity and fish species: 1. enzymatic activity increased with age; 2. enzymatic activity decreased with age and 3. enzymatic activity was not changed with age. For instance, we observed strong correlation between CAT activity and fish age in *M. merlangus euxinus* and *T. mediterraneus ponticus*, significant values was shown in *G. mediterraneus* and moderate in *M. barbatus ponticus*, and low in *S. flexuosa*. The highest negative correlation coefficients were shown between PER activity and age in *G. mediterraneus* and *T. mediterraneus ponticus*, in *S. porcus* it was significant and moderate in *M. merlangus euxinus*. CAT activity showed no age dependence in blood of *N. melanostomus*, *S. porcus* and *S. flexuosa*. Age changes in CAT level were not evident in rainbow trout *Oncorhynhus mykiss* and black bullhead *Ameiurus melas* also (Otto & Moon, 1996). *G. mediterraneus* and *T. mediterraneus ponticus* enzymatic activity decreased with the age, while in *M. merlangus euxinus* the opposite tendency was observed. In *M. barbatus ponticus* CAT activity elevated in the middle-age fish, then decreased progressively in the elder ones. The similar tendency was found in the blood cells of sturgeon *Acipenser naccarii* (Martinez-Alvarez et al., 2005). Our findings agree with the results of the other studies of age-related CAT activity in various animals. The investigators documented that CAT activity has been decreased (Semsei et al., 1989) or unchanged with the age in brain of albino rats (Yargicoglu et al., 1999). Age- and size-related changes of CAT activity was shown in mollusk *Perna viridis* (Lau & Wong, 2003).

High negative correlation was indicated between PER activity and fish age in the RBC of *G. mediterraneus* and *T. mediterraneus*, in *S. porcus* and *M. merlangus euxinus* the values were positive and moderate, while in the other examined species the correlations were not observed. In this case we could agree with the opinion of several authors who proposed, that CAT and PER activity in the brain and in the liver of freshwater murrel *Channa punctatus* were mostly maturation-related rather than senescence-related (Nayak et al., 1999, cited by Martinez-Alvares et al., 2005).

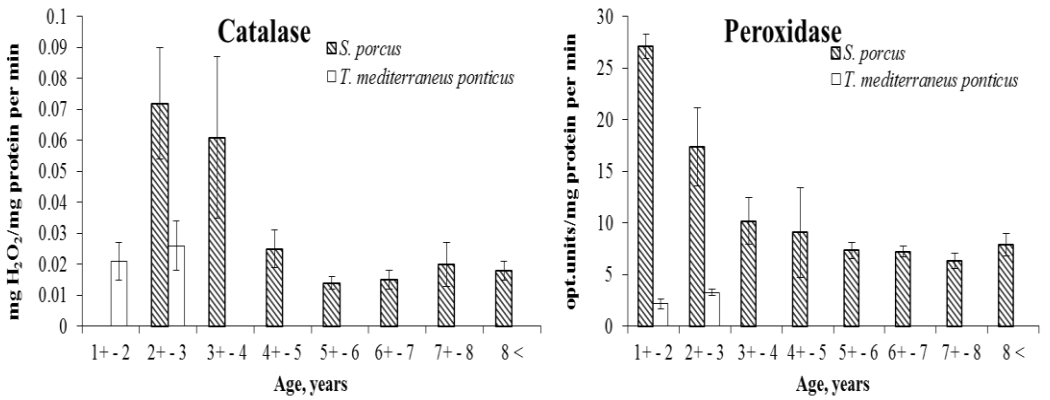

Figure 5. Age-related CAT and PER activity in RBC of Black Sea teleosts *S. porcus* and *T. mediterraneus ponticus*.

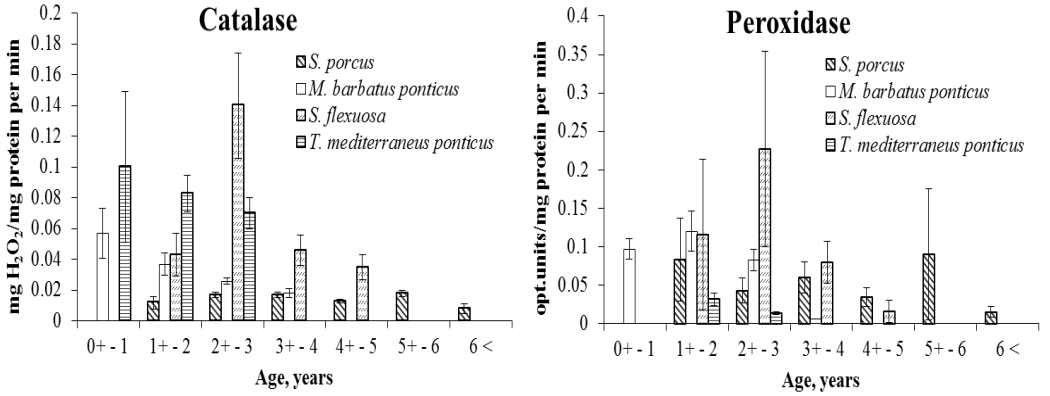

Figure 6. Age-related CAT and PER activity in the liver of Black Sea teleosts *S. porcus, M. barbatus ponticus, S. flexuosa, T. mediterraneus ponticus.*

Therefore, the age alterations of antioxidant enzyme activities in fish blood are the similar as compared with the other animals including terrestrial species. It may be the result of detrimental effect of life span that is conclude with the opinion of other researchers (Zelinski & Portner, 2000; Ahmed, 2005).

Our recent studies showed that in RBC of the several Black Sea teleosts (*S. porcus* and *T. mediterraneus ponticus*) CAT and PER level did not vary uniform related to age (Figure 5). Low correlation was detected between age and CAT activity in the blood of scorpion fish (r = 0.32), correlation between PER and fish age was greater (r = 0.62). We noted high age-related relationships between CAT and PER level in RBC of *S. porcus* (r = -0.73). Therefore, activity progressively decreased in the RBC of scorpion fish *S. porcus,* while in horse mackerel *T. mediterraneus ponticus* the values did not change. At the other side, the ratio of CAT/PER activity ranged between 0.002-0.007 in RBC of scorpion fish and 0.009-0.008 in horse mackerel.

In the liver of tested Black Sea teleosts CAT and PER activity varied related to age, tended to decrease in the major of tested fish species (Figure 6).

Age-related correlation of CAT activity was estimated as r = 0.31 in the liver of *S. porcus,* in red mullet *M. barbatus pointicus* the corresponding value was higher r = 0.93. No age-related correlations we found in hepatic CAT activity in high body pickerel *S. flexuosa* and horse mackerel *T. mediterraneus ponticus.* PER activity in the liver of tested teleosts demonstrated low age-related correlation (r = 0.17), at the case of scorpion fish and high body pickerel, in the liver of red mullet *M. barbatus ponticus* it was higher (r = 0.50). Great age-related correlation (r = 0.75 and r = 0.90) was observed between CAT and PER activity in *S. porcus* and *S. flexuosa* correspondingly.

These findings agree with the data of other investigators, who described the tendency of decrease of enzymatic activity in the erythrocytes and in the liver of old fish as compared with younger ones (Otto & Moon, 1996). It was linked with accumulation of oxidative products in organism and decrease of its defense abilities. At the other side, the antioxidant system in long-lived fish species has evolved to increase its capacity with age, while the other species have constant enzyme activities, or they decline throughout their life. These differences could play important role in the regulation of the mechanisms of the lifespan of teleost fish species.

However, we could note the significant differences in antioxidant enzyme responses in examined fish species, which were attributed with the peculiarities of their ecological and physiological characteristics (see section 3.2). For instance, in our previous publication, we suggested that age alterations were much more significant in benthic and suprabenthic fish species, compared with suprabenthic/pelagic and pelagic ones. We could propose that benthic and suprabenthic forms live in more contaminant environment, because many pollutants accumulate in bottom sediments and low water layers and they impact aquatic organisms (Rudneva et al., 2010b).

3.2. Reproductive Strategy and Gender Peculiarities

Aerobic organisms, in the process of the evolution have adapted to use their living resources (e.g., energy, time, nutrients, etc.) for reproduction. They have evolved different strategy of breeding frequently, fecundity, egg hatching rate and larval survival. The costs associated with reproduction have been attributed with the increase of metabolic rate and high production in ROS (Alonso-Alvarez et al., 2004), because spawning is a highly demanding activity which elevates metabolic rate for an extended period of the time. At the case of single breeding event and further individual death, fish may not invest resources into generating antioxidant defense, and all resources have been allocated to reproduction as, for instance, of semelparous fish species namely salmonides (Wilson et al., 2014), while iIteroparous species are characterized by multiple breeding events throughout life.

Fish may reduce investments in their own antioxidant defense in favour of eggs and embryos antioxidant protection, which is essential for hatching success of embryos and survival of new hatching larvae (Fontagné et al., 2006; Taylor et al., 2015). As we described previously, high concentrations of low molecular weight antioxidants were detected in the gonads of Black Sea teleosts, while antioxidant enzyme activities, including CAT and PER were not high (Rudneva, 2012). Additionally, antioxidant enzyme activities were very low in fish gonads, while high concentrations of low molecular weight antioxidants play an important role in antioxidant defense of fish reproductive system against ROS and oxidative stress.

We could also found the differences in CAT and PER activity in the RBC and in the liver of tested fish male and female (Figure 7).

No significant differences of CAT and PER activities in RBC in the majority of tested fish species were observed, with the exception of red mullet *M. barbatus ponticus*, in which PER activity was significantly higher as compared with female value. At the other side, we could note, that in female enzyme activities tended to increase related to male.

Generally, similar trends of CAT and PER activities related to fish gender were displayed in the liver (Figure 8). However, CAT activity in *M. barbatus* and *T. mediterraneus ponticus* was significantly greater in male ($p < 0.05$).

Therefore, we could conclude, that CAT and PER level did not differ significantly in fish male and female in the majority of tested Black Sea fish species.

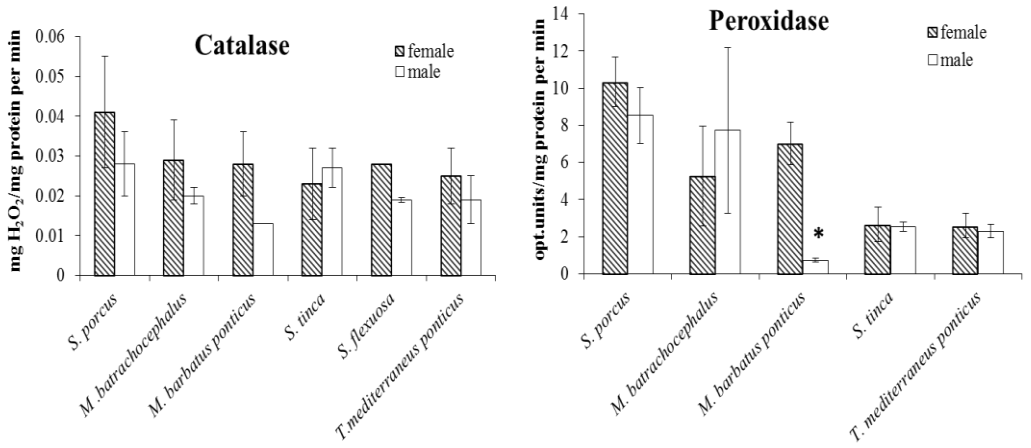

Figure 7. Gender-related differences of CAT and PER activities in RBC of Black Sea teleosts.
* - significant difference.

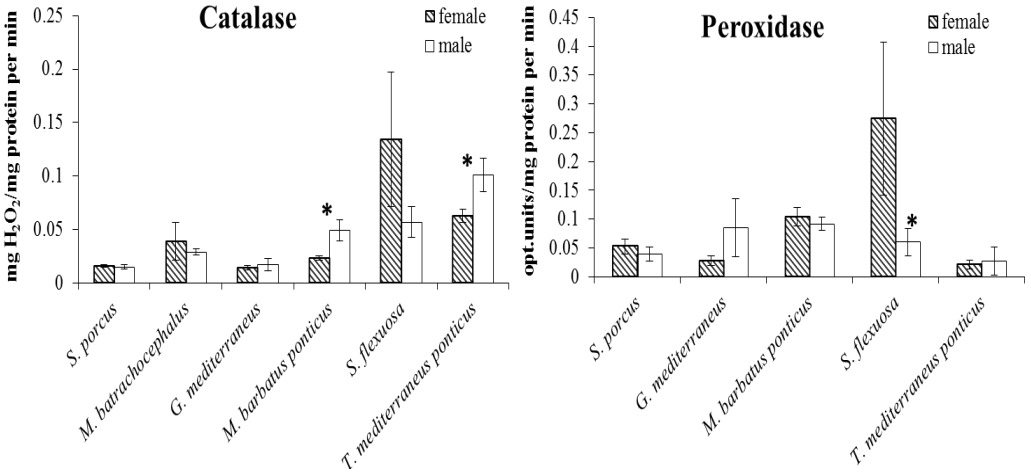

Figure 8. Gender-related differences of CAT and PER activities in the liver of Black Sea teleosts.
* - significant difference.

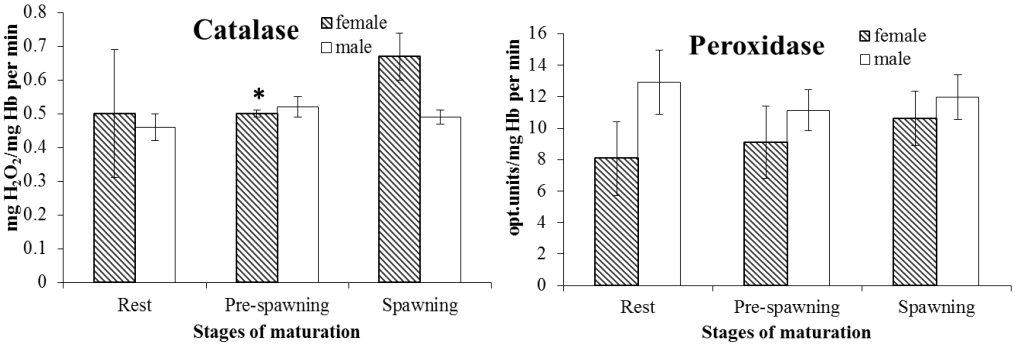

Figure 9. CAT and PER activities in RBC of *N. melanostomus* in different stages of reproductive cycle.

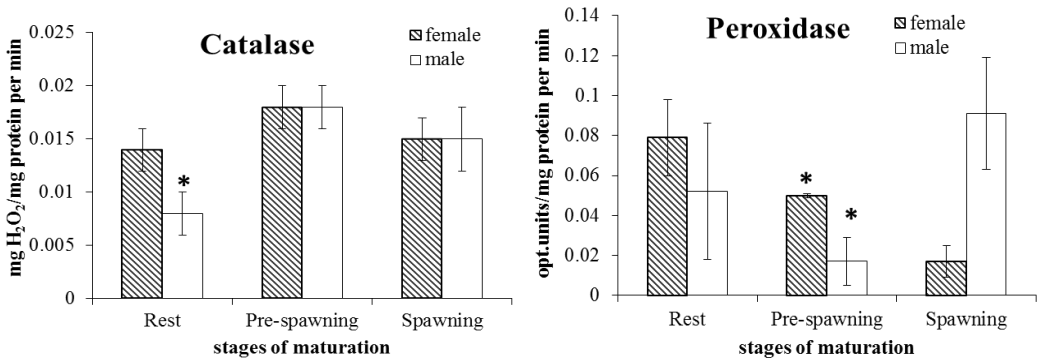

Figure 10. CAT and PER activities in the liver of *S. porcus* in different stages of reproductive cycle.

Because iteroparous species are characterized by multiple breeding events throughout their life, they are less depend to the resource-based trade-off associated with reproduction and antioxidant defense changes during maturation and reproduction of fish. There are several publications of the correlation between fish reproduction cycle and antioxidant defense status. The authors reported, that reproduction in zebra finches decreases antioxidant defense, which may suggest that oxidative stress is a cost of reproduction (Alonso-Alvarez et al., 2004). In our study we found the increase of CAT and PER activity in the RBC of the female of the round goby *N. melanostomus* in the spawning time (Figure 9). However, in male of the round goby CAT activity decreased, and we could propose that paternal care was negatively correlated with oxidative stress resistance, which agrees with the data of the other investigators (Wilson et al., 2012). In the liver of scorpion fish *S. porcus* we showed the increase of CAT activity in pre-spawning and spawning period both in female and male, while hepatic PER level increased in male in spawning time and decreased significantly in female (Figure 10). Therefore, enzyme activities depend on the period of reproduction, fish gender, interspecies features and specificity of tissue. Additionally, the cumulative effect of oxidative stress across the reproductive lifespan of fish has been attributed with the age-associated decline of antioxidant defense. At the other side, differences in oxidative stress levels during reproduction can be correlated with the specificity of diet and food deprivation, as well as to migration of the fish species.

3.3. Diet and Food Deprivation

Antioxidant status of the organism depends on food composition, because many kinds of essential antioxidants are contained in the diet. For instance, antioxidants namely vitamin E, carotenoids are strictly obtained as a nutritional antioxidants in the majority of fish species, while the others (for instance, vitamins C, A) are synthesized only in few fish species. These components play an important role in their adaptation to the changing environment. Unlike mammals, fish may survive long time of starvation, however, without food they change their metabolism and evolved metabolic adaptations that involve the up- and down-regulation of the major biochemical and physiological pathways (Morales et al., 2004).

Antioxidant status of fish is linked with fish trophic level, feeding behavior and nutrition factors, which also may affected antioxidant enzymes including CAT and PER (Martinez-Alvarez et al., 2005; Sole et al., 2009). Fish with different diets may cope with oxidative stress in different ways. Several essential microelements, which play a role in many physiological functions, including antioxidant defenses (such as selenium, copper, iron, and etc.) fish obtain with their diet. Several of these micronutrients are the components of the active centers of the antioxidant enzymes, namely CAT (iron), PER (iron), GPxs (Se). The authors have documented, that antioxidant enzyme activities was generally higher in herbivorous fish (grass carp *Ctenopharyngodon idella* and silver carp *Hypophthalmichthys molitrix)* than in omnivorous species barbel *(Barbus barbus), crucian carp (Carassius carassius)* and common carp *(C. carpio)* (Radi et al., 1985). The authors suggested, that plant-based diet contains the components, which are absent in other food or the prooxidants in this type of food contains stimulated ROS production, and the change of prooxidant/ antioxidant balance, attributed with the induction of enzyme activities. CAT activity was significantly higher in the liver of Senegalese sole fed high lipid diet as comparted with fish fed low lipid diets (Rueda-Jasso et al., 2004)

In our previous studies (Rudneva, 1997b; 2012) tested Black Sea teleosts species were grouped in pelagic plankton feeders (*T. mediterraneus ponticus*), omnivorous (*S. smaris*), carnivorous (*M. barbatus ponticus, N. melanostomus*) and predators (*M. merlangus euxinus, S. porcus, P. lucsus*). No clear relationships between feeding groups and CAT and PER activities in the RBC and in the liver were found. However, carnivorous fish species demonstrated approximately similar enzymatic activity in the liver. It could be explained that benthic invertebrates (mollusks, crustacean and worms) and fish, which are the preferable prey for this group might accumulate pollutants from the bottom sediments and transfer them via trophic nets to fish with the effect of concentration. Hepatic antioxidant enzyme activity in pelagic plankton feeder horse mackerel and omnivorous suprabenthic/pelagic *S. smaris* was approximately similar and lower than in carnivorous forms, which was explained the similarity of their high metabolic rate and diet consumption.

Food deprivation was also attributed with oxidative stress in fish. The researchers demonstrated the changes of prooxidant/antioxidant balance in the fish at the case of lack of food or deprivation, which was correlated with the increase of lipid peroxidation and CAT and GPx activities in common dentex (*Dentex dentex)* (Morales et al., 2004). Food consumption and oxidative stress, associated with food quality and deprivation play a role in fish migration and physical activity.

3.4. Swimming Activity

Physical activity plays an important role in antioxidant defense status in fish. Our study supports the great interspecies differences in antioxidant system in tissues between various fish species, characterizing different swimming activities. Our findings agree with the results of other researchers, who reported about high variability of antioxidants level in the fish (Winston, 1991; Filho et al., 1993; Sole et al., 2009). Interspecies differences in blood, muscle and liver in Black Sea teleosts were more significantly than in gonads, which showed more homogenous response (Rudneva, 2012).

The interspecies variations of antioxidant defense may reflect the specific adaptations to the oxidative stress and protective mechanisms against ROS damage. Therefore, activity of antioxidant enzymes in fish blood correlated with their swimming capacity. In our previous study we found that CAT activity in fast swimming pelagic horse mackerel *T. meditrraneus ponticus* and high body pickerel *S. smaris* were significant higher than those in blood of slow swimming gobies, scorpion fish and flounder (Rudneva, 1997a, b). Other researchers reported also, that CAT content in blood of more active forms was higher as compared to more sluggish species (Filho et al., 1993; Filho, 1996). They suggested, that antioxidant defense in marine species in the liver and blood may be related to the oxygen consumption of the tissues and of the whole organism, while in freshwater fish it may be related to physical and chemical characteristics of the living conditions rather than to physical activity (Filho, 1996). At the same time, the changes in the sturgeon blood prooxidant-antioxidant status, as a consequence of adaptation to marine conditions, were not reflected in the liver and other tissues (Martinez-Alvarez et al., 2005).

Liver of the vertebrates exhibits a high metabolism and oxygen consumption, and it is the main organ of xenobiotic detoxification. Fish liver displayed the highest levels of the key antioxidant enzymes CAT (Filho et al., 1993; Filho & Boveris, 1993; Rocha-e-Silva et al., 2004). CAT level in the liver appears to indicate that the most active species of teleosts had greater enzyme activity compared with low mobile forms (Filho et al., 1993; Filho & Boveris, 1993). The higher activity of antioxidant enzymes in the liver of active fish correlated with higher oxygen consumption in fast swimming species and their high metabolic rate (Martinez-Alvarez et al., 2005; Filho, 2007). Animals with high metabolic rate exhibit the high rates of free radical production and show the induction of antioxidant defense mechanisms (Zelinski & Portner, 2000). Physical activity of fish is often associated with fitness, reproduction or predator avoidance. Therefore, the link between oxidative stress and physical activity may appear to be more complex.

Additionally, the majority of our tested teleosts migrate in different distances, while the others are settled (gobies, scorpion fish, flounder). Therefore, their antioxidant status depend on the specificity on abundance and complex of living conditions in biotope.

4. ABIOTIC CONDITIONS

The researchers suggest, that oxidative stress in various aquatic organisms can correlate with several types of stressful conditions, such as chemical pollution, hypoxia/anoxia, microbial and viral infections and parasitic invasion, dietary influence, feed deprivation, acute temperature changes, algal blooms, UV-irradiation, and etc. (Ferreira Pereira, 2014). In this section we focus on the relationships between the ecological conditions in biotops and CAT and PER activities in tested teleosts.

4.1. Ecological Specificity of Abundance

Ecological conditions of the habitats play an important role in fish antioxidant status and their adaptation to habitats. Previously we described enzyme activities in various tissues in

several Black Sea teleost and elasmobranch species (Rudneva, 2012). Here we focus on the main trends of CAT and PER activities in the RBC and in the liver of tested Black Sea teleosts, which grouped in four ecological classes namely benthic, suprabenthic, suprabenthic/pelagic and pelagic. The highest CAT activity in the RBC was indicated in benthic forms *S. porcus* and *N. melanostomus,* in the species belonging to the other ecological groups the enzyme activity was comparable with the exception of *P. luscus* and *L. aurata,* which showed the low values (Figure 11). PER activity of examined teleosts did not vary uniform, and we can't find any trends in enzyme activities of species belonging to tested ecological groups. Similar observations we showed in the liver of checked teleosts, in which CAT and PER activity varied widely (Figure 12).

Therfore, the obtained results support the great interspecies differences in antioxidant activity in various tissues between examined species, which agrees with the opinion of many researchers studying fish antioxidants (Ciornea et al., 2009; Alti et al., 2016; Mukherjee et al., 2017). In our previous studies we showed the differences of low molecular weight scavengers in fish blood. For instance, glutathione content was relatively higher in pelagic active fish *T. mediterraneus ponticus* and in sluggish benthic forms, while in other species belonging to suprabenthic and suprabenthic/pelagic groups the values were intermediate (Rudneva, 2012). We found also, that SOD activity in RBC was significantly less in sluggish forms as compared to fast moving ones, which could indicate that the oxygen transport capacity of fish blood follows the same evolutionary trend of SOD level concentration in blood (Filho et al., 1993). Similar tendency was shown in CAT activity, PER level was not uniform and varied unclearly, while GR activity demonstrated the opposite trend: it was less in pelagic fish than in animals belonging to the other groups. Therefore, the results of the present study agree with our previous data.

Wide antioxidant enzyme fluctuations in the liver within the ecological group, especially in suprabenthic/pelagic class, in which enzyme activities differed in 2-7 fold, were showed in our studies. This was explained by the specificity of fish biology and life cycle related to temperature, oxygen concentration, pressure and other abiotic conditions.

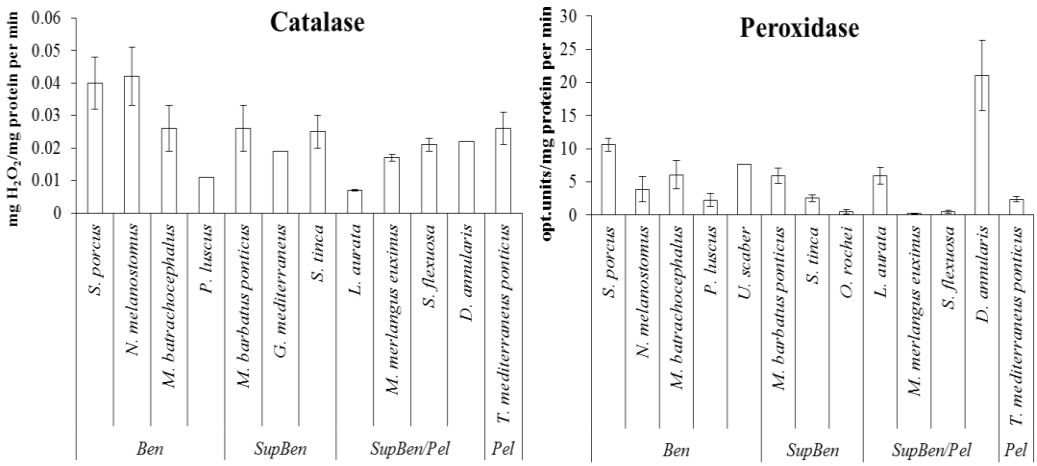

Figure 11. CAT and PER activity in RBC of Black Sea teleosts. Ecological groups:
Ben – benthic, SupBen – suprabenthic, SupBen/Pel – suprabentic/pelagic, Pel – pelagic.

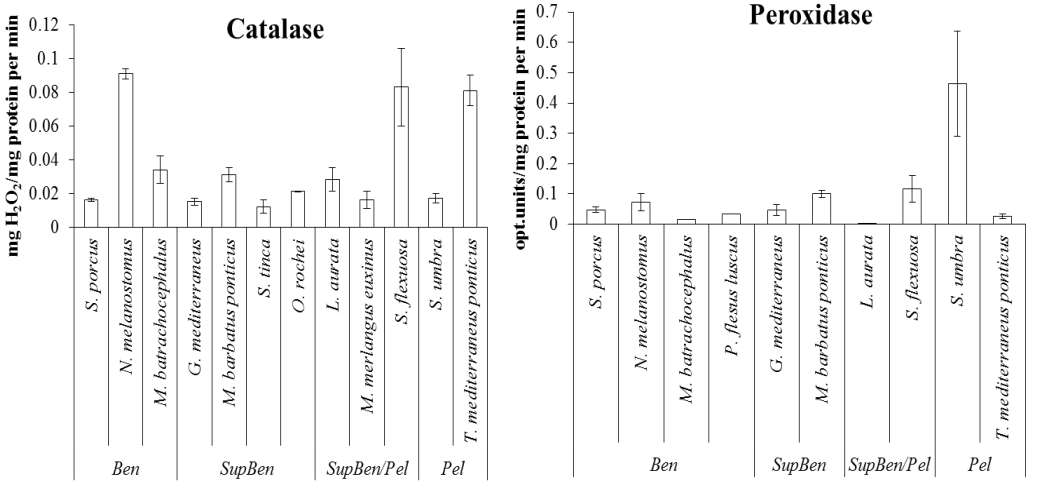

Figure 12. CAT and PER activity in the liver of Black Sea teleosts. Ecological groups: Ben – benthic, SupBen – suprabenthic, SupBen/Pel – suprabentic/pelagic, Pel – pelagic.

Due to the highly variable environment (absorption of solar radiation, including its UV-spectra, fluctuations of salinity, depth, oxygen concentration, presence of hydrothermal vents), marine fish species impact on abiotic factors greater related to freshwater forms. These factors and their fluctuations can cause the generation of ROS.

4.2. Salinity

Due to their life history many teleost fish species were adapted to salinity fluctuations, especially in spawning period, when they migrate from freshwater rivers into marine waters (e.g., salmonids). Because salinity changes the metabolic rate of the fish associated by osmoregulatory demands and physiological stress, the ROS production changes also and fluctuations of salinity induce of oxidative stress in fish (Zeng et al., 2017). In experimental conditions the researchers indicated the increase of GPx and CAT activities in *Litopenaeus vanna*, exposed to seawater for a 48-hr period (Liu et al., 2007) and in the olive flounder *Paralichthys olivaceus* (Choi et al., 2008). Therefore, the researchers postulated, that salinity fluctuations and especially the fish transition from freshwater to marine water conditions induce oxidative stress and the changing of prooxidant/antioxidant balance. At the other side, fish respond to oxidative stress with the increase of antioxidant defense, including CAT and PER activities, and they will cope their life with changing environmental conditions.

In our studies we compared age-related trends of CAT and PER activities in the RBC of round goby *N. melanostomus* from Black Sea and Azov Sea, characterizing different salinity (Figure 13). Water salinity in the Black Sea is estimated as 18‰, while salinity in Azov Sea varies between 11.4 and 12.1‰. CAT activity in the RBC was lower in the fish from Black Sea as compared with the animals from Azov Sea. However, CAT activity decreased in the RBC of the middle-age round goby, then increased in elder ones from Black Sea, while in the animals from Azov Sea CAT activity varied lower. PER activity in the RBC of fish from Black Sea was higher related to the values of the animals from Azov Sea. It showed the

opposite trend with aging as compared with CAT level, while PER activity in the RBC of round goby from Azov Sea varied insignificantly.

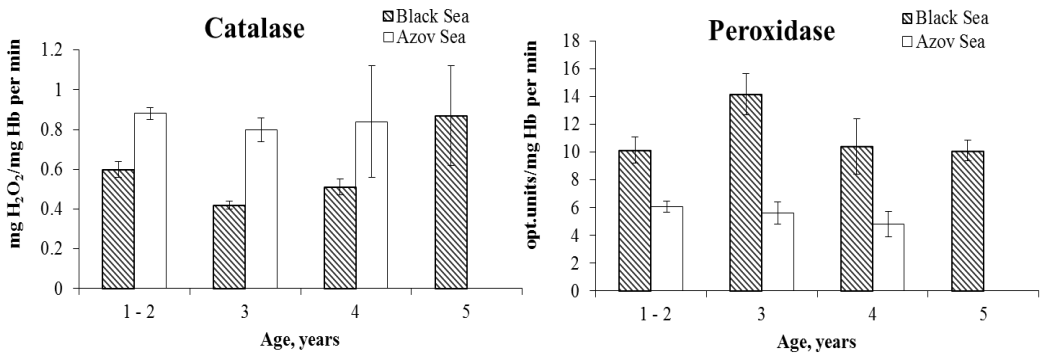

Figure 13. Age-related CAT and PER activity in RBC in *N. melanostomus* from Black Sea and Azov Sea.

Therefore, we could conclude, that CAT and PER activity of fish depends on living conditions in the abundance, especially salinity, which is the result of the evolutionary process and their adaptation to the specificity of biotope.

4.3. Pressure and Deep Sea Conditions

Deep sea biotops are characterized by reduced residual light, decreasing temperature, available nutrients, the low-oxygen environment or increased pressure. The researchers proposed, that physical and chemical conditions of this dark, poorly oxygenated environment, the meso- and bathypelagic waters of the oceans might be considered as refuges against oxidative dangers in the fish (Janssens et al., 2000). The study of antioxidants in the tissues of 16 species belonging to meso- and bathypelagic groups, which abundance was estimated between the surface and a depth of 1300 m indicated the differences of their antioxidant system status. The levels of SOD and GPx decreased in parallel with the exponential reduction in the metabolic activity in fish tissues, while CAT activity was affected neither by the metabolic activity nor by the depth of occurrence of the fishes. High levels of metabolic and antioxidant enzymes were observed in the light organs of bioluminescent species. The authors suggested that the adjustment of the activity of SOD and GPx to the decreased metabolic activity in deep-sea fish are used primarily against metabolically produced ROS, whereas the maintenance of CAT, remained unchanged while metabolic rates declined with increasing depth of occurrence (Janssens et al., 2000). The researchers postulate, that CAT and GPx activities in fish tissues were linked with the depth of their abundance (Ribata et al., 2015). Additionally, the lower metabolic rates of deep-sea animals are functionally adaptive for life in the high depth and the oxygen minimum layer and are not specifically evolved adaptations to the low food availability or low O_2 levels in these habitats (Section 4.4.). The declines of metabolic rate of the deep-sea aquatic organisms reduced metabolic requirements and this is attributed with the decrease of SOD and GPx activities. However, higher level of pollution of sediments and deep water layers is associated with the prooxidants and ROS generators (Porte et al., 2000).

In experimental conditions the researchers investigated the effects of exposure to 10.1 MPa (equivalent to 1000 m in depth) hydrostatic pressure on silver European eel (*Anguialla anguilla,* Anguillidae) oxidative stress and ROS production, no harmful oxidative damage was observed in the fish. Among our tested Black Sea teleosts we have not deep-sea forms and we *can't* discuss the influence of pressure on the CAT and PER enzymes of Black Sea fish.

4.4. Oxygen Availability

Marine environments are characterized by high oxygen concentrations in the euphotic zone and by a decrease to lower oxygen levels in the deep sea. Since oxygen is essential for aquatic organisms, they developed adaptations to oxygen concentrations and able to survive extreme oxygen level in deep sea regions (Janssens et al., 2000; Welker et al., 2013). In experimental conditions the researchers found an increase in fish antioxidant defense under hypoxia/anoxia, as "preparation for oxidative stress" to enhance their ability to degrade ROS production upon return to usual oxygen concentration (Hermes-Lima et al., 1998; Lushchak & Bagnyukova, 2007).

In deep-sea fish species SOD and GPx activity increased with depth, while CAT activity was not changed (Janssens et al., 2000), and the authors proposed that deep sea fish species demonstrated less oxidative stress with the decrease of their metabolic rate and low ROS production. Therefore, fish have evolved special mechanisms of regulation of prooxidant/antioxidant balance in changing oxygen conditions. Both hyperoxic and hypoxic conditions can induce oxidative stress in fish and increase antioxidant enzyme level, including CAT and GPx in tissues (Lushchak, 2011). However, oxygen availability is species-dependent, because fishes have evolved to different living conditions and they inhabit different biotops, where the oxygen level varies widely. For instance, pelagic fish species (*T. mediterraneus ponticus*) abundance is characterized high oxygen level, while benthic forms (*N. melanostomus, S. porcus*) live in the water, where oxygen concentration is lower. In addition, metabolic rate of horse mackerel is higher related to benthic forms, and we can propose, that antioxidant activity in pelagic forms is greater, than in bottom ones. In this context hepatic CAT activity in pelagic horse mackerel is significant higher, than in benthic and suprabenthic fishes, however, PER activity was very low (Figure 11). We could conclude, that characteristics of antioxidant defense in fish depended on the complex of living conditions in biotope and relationships among them and fish species, which was demonstrated at the case of temperature.

4.5. Temperature

Global climate change may cause stress to aquatic ecosystems and organisms, damage their health, growth and reproduction. Climate changes are accompanied with the temperature increasing caused the emission of greenhouse gases (CO_2, CH_4, SO_2, nitrogen oxides, and etc.). Seawater temperature of 2-3^oC above long-term average summer temperature results stress in aquatic organisms, led oxidative damage. High temperature elicits a series of physiological responses, such as increasing of standard body metabolism. Temperature has

been known to alter metabolic rate, enzyme activities, oxygen consumption and oxidative stress, attributed with the changes of prooxidant/antioxidant ratio of the organism (Dubey, 2013). It is predicted that global climate change will increase water temperature by 2°C by the end of the 21st century (IPCC Report, 2013) and biochemical alterations including oxidative stress in aquatic organisms will also elevate.

An increase in temperature is associated with an increased metabolic rate and oxygen consumption, attributed with an enhancing of ROS production (Almroth et al., 2015). The authors suggested, that although stenothermal fish can modulate their antioxidant capacity, they can only do so in a transient manner, the latter which remains insufficient in degradation of increased ROS production accompanying the enhance in metabolic rate with increased water temperature. Further accumulation of oxidative products in the cells and tissues can lead to a decrease in fitness and damage physiological functions in the organisms, which is important at the case of prediction of global warming consequences.

In experimental conditions the researchers indicated, that the activity of both SOD and CAT displayed only small changes across treatments and tissues during long-term acclimation of three notothenioid fish species to decreased seawater pH and increased temperature (Enzor & Place, 2014). The oxidative stress and antioxidant defense changes attributed with the water temperature fluctuations, were highly depended on tissues, and the researchers postulated, that basal antioxidant protection is kept high in those tissues that may have a stronger impact on health and fitness (Luschak, 2011).

We study the season changes of CAT and PER activity in the RBC and in the liver of several Black Sea teleosts. Our findings indicated, that in warm season the activity of tested enzymes increased in the majority of Black Sea teleosts, which could explain both water temperature increasing and the enhancing of fish metabolic rate (Figures 14, 15, 16).

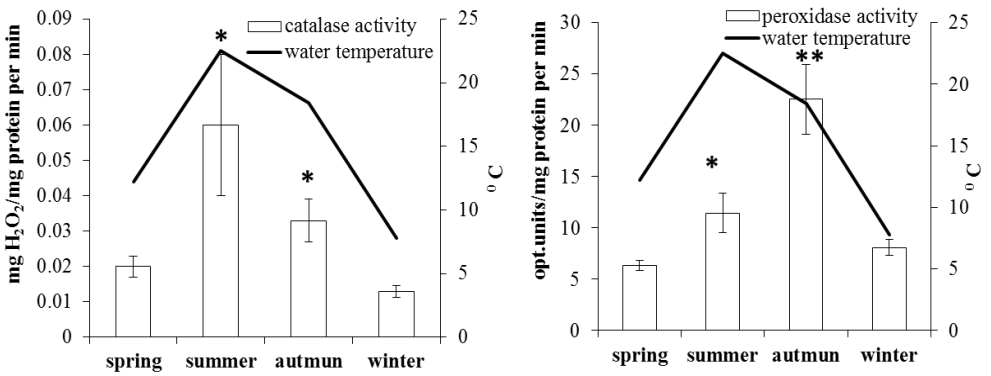

Figure 14. Seasonal changes in RBC CAT and PER activity of Black *S. porcus*.
* - significantly difference related to spring and winter values ($p < 0.05$).

In summer at high water temperature CAT activity was significantly greater in the RBC and in the liver of scorpion fish related to the values of the other seasons, while the highest PER level was indicated in autumn, when the water temperature was decreased insignificantly. In cold seasons, at the beginning of spring and winter, the enzyme activities in the RBC and in the liver of *S. porcus* was significantly lower (Figures 14, 15). However, in the RBC of round goby *N. melanostomus* high CAT activity was observed in spring and summer time, then it significantly decreased in autumn and in winter (Figure 16), while the

highest PER level was indicated in winter. Our results agree with the opinion of the other investigators, who documented, that temperature-related differences of the antioxidant enzymes demonstrate tissue- and species-dependent peculiarities and evolved in the complex of fish physiological and metabolic adaptations to the living conditions (Alti et al., 2016; Mukherjee et al., 2017).

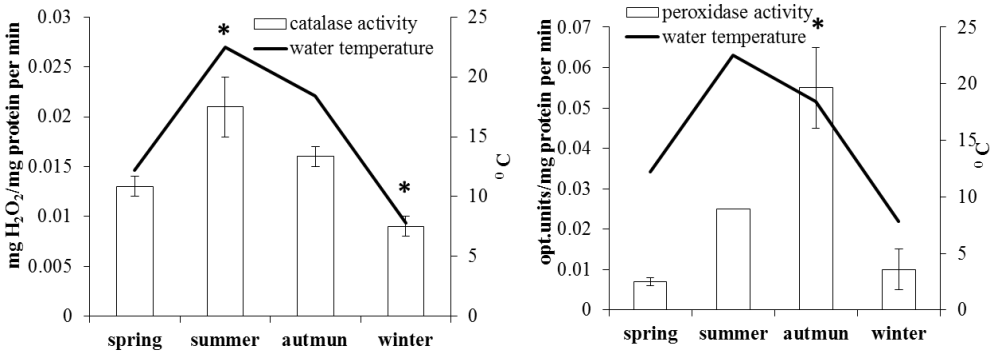

Figure 15. Seasonal changes in hepatic CAT and PER activity of Black Sea *S. porcus*.
* - significantly difference related to spring and winter values ($p < 0.05$).

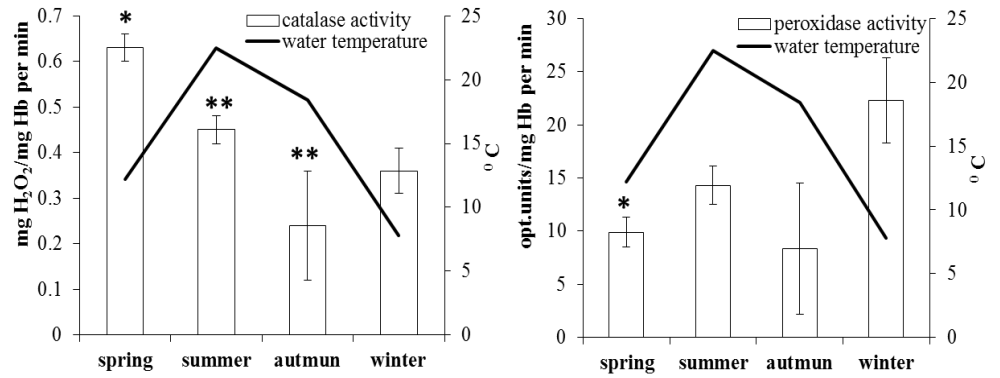

Figure 16. Seasonal changes in RBC CAT and PER activity of Black Sea *N. melanostomus*.
* - significantly difference related to spring and winter values ($p < 0.05$).

Finally, present data demonstrated that CAT and PER levels showed variations depending on the physical environment, but the changes are exclusively site as well as tissue and species specific. These findings also suggest that the effects of environmental factors on oxidative stress mechanisms may be tissue-and species-dependent and that species may have evolved different mechanisms to deal with the oxidative challenges induced by environmental stress of selection underlying adaptive divergence in these fish species. Because CAT and PER enzymes play a role in the adaptation of the fish to changing environmental conditions, they may modify their activities as a function of water temperature, pressure, salinity, oxygen concentration and the presence of various pollutants, they may use in ecotoxicological studies as biomarkers of fish response on unfavorable living conditions and anthropogenic impact.

5. ANTHROPOGENIC IMPACT

ROS production can be induced by external factors. Environmental pollution may be one of the strong cause of the ROS generation in the organisms. Chemicals represent in the environment (transition metals Cu and Fe, which catalyze the production of hydroxyl radicals through Fenton reaction, toxic elements Cd, Hg, Ni, biphenyls, quinones, nitroaromatics and drugs) can also induce production of superoxide by redox cycling and via biotransformation reactions (Winston, 1991; Livingstone, 2001; Lesser, 2006). The response on oxidative stress in fish is tissue-dependent and species-dependent. Fish in polluted habitats showed changes of CAT and PER activity, however, the trends were not uniform in the tissues and examined species. Finally, the results obtained provide evidence, that exposure to heavy metals in fish can unsettle the redox balance and alter antioxidant defenses. Aquatic environments are affected by various anthropogenic contaminants like pesticides, oil, polychlorinated biphenyls (PCBs), nanoparticles, microplastic, human pharmaceuticals, which can induce oxidative stress in fish. Coastal marine ecosystems are the main recipients of waste waters and effluents, containing high concentrations of various pollutants, which modify water hydrological properties, biodiversity and community structures. The indicators of oxidative stress in fish are very sensitive to this impact and can reflect the ecological status of habitats, as we described previously (Rudneva et al., 2012; Rudneva et al., 2016a, b; 2017). The present study also demonstrates the fluctuations of CAT and PER activities as markers of antioxidant defense in fish caught in polluted and non-polluted marine areas (Figure 17). We studied CAT and PER activities in the RBC of scorpion fish collected in the Sevastopol bays with different pollution levels as following (from the most polluted to reference area): Streletskaya→Alexandrovskaya→Karantinnaya→ Kazach'ya. High CAT activity was indicated in the fish from polluted waters of Streletskaya Bay and Alexandrovskaya Bay, while in reference area Kazach'ya Bay and less polluted Karantinnaya Bay the values were lower. However, at the case of PER activity in scorpion fish RBC we found the highest level in the fish caught in Alexandrovskaya Bay, while the enzyme activity in fish RBC from the other tested bays were lower or the similar.

Furthermore, the study of CAT and PER activity in the blood of round goby from three Sevastopol bays, characterizing different levels of pollution in two periods (2003 and 2012), also demonstrated significant differences of enzyme activities (Figure 18). CAT activity was higher in the RBC of round goby in 2012 as compared with 2003 in three tested bays, similar trend was shown in PER activity with the exception of the fish from Karantinnay Bay. We could conclude, that in recent years, when anthropogenic impact on marine water was increased, the fish responded the increase of antioxidant defense, including the induction of CAT and PER level in the RBC.

At the other side, the response of antioxidant system and antioxidant enzymes can be different in the tissues and depend on fish species (Figure 19). Hepatic CAT and PER activity was higher in the majority of fish from Alexandrovskaya Bay, with the exception of horse mackerel *T. mediterraneus ponticus,* while enzyme activities in the examined fish species collected in Karantinnaya Bay were lower. Therefore, the response of CAT and PER activity in the fish on pollution impact in the tested areas was not uniform and depended both on the contamination level, specificity of the enzyme, tissues and fish species. It is very important for the understanding the main mechanisms of teleosts adaptations to changing environmental

conditions, including the anthropogenic impact which is one of the main factor of the evolution at recent decades.

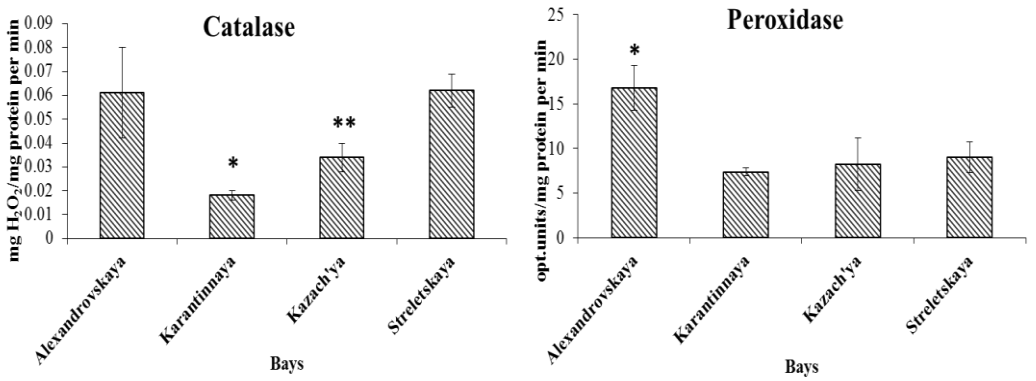

Figure 17. CAT and PER activity in RBC of *S. porcus* from different bays. * - significantly difference related to other bays ($p < 0.05$), ** - significantly difference related to Alexandrovskaya and Streletskaya bays ($p < 0.05$).

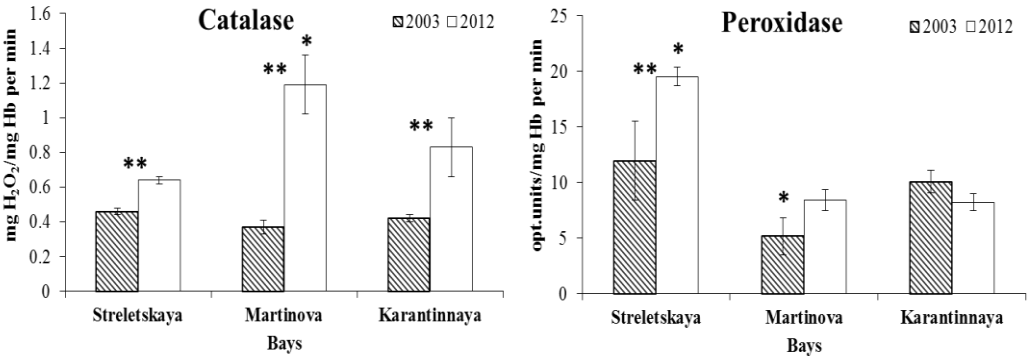

Figure 18. CAT and PER activity in RBC of *N. melanostomus* from different bays at the period of 2003 and 2012. * - significantly difference related to other bays ($p < 0.05$), ** - significantly difference related to periods ($p < 0.05$).

These findings suggest that in general, pollution of aquatic environments reflected physiologically in fish by perturbing normal free radical processes, leading to increase in oxidative damage and disturbance of antioxidant defenses. It would therefore be advisable to consider oxidative ecology in fisheries science and monitoring, given the extent of pollution in many waterbodies and the fish species that live within them (Birnie-Gauvin et al., 2017).

Recent decades, the new pollutants, namely microplastic, nanoparticules and human pharmaceuticals are highly distributed in the aquatic environments, and they are accumulated in the animals tissues. The information of their biological effects is very limited and there is an increasing need to assess wild populations subtle alterations that may indicate the presence of environmental stressors (Amerand et al., 2010; Oliveira et al., 2017a, b). Antioxidants and key antioxidant enzymes CAT and PER may be used for these purposes as a good biomarkers.

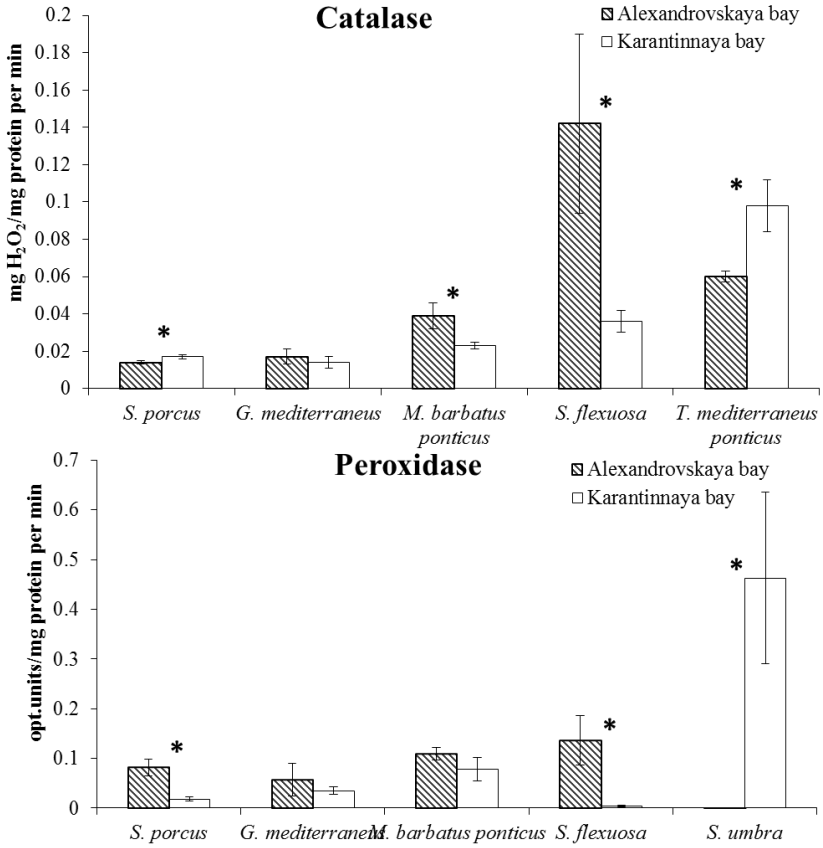

Figure 19. CAT and PER activity in the liver of Black Sea teleosts from different bays.
* - significantly difference between tested bays ($p < 0.05$).

CONCLUSION

On the basis of the data described above, we can conclude, that key antioxidant enzymes catalase and peroxidase in tested Black Sea teleosts may provide information about interspecies differences and the mechanisms of their adaptation to environmental conditions, including the complex of biotic, abiotic and anthropogenic factors. Moreover, the global climate changes in recent decades can directly and indirectly influence on fish antioxidant status and together with the local factors may be suitable for the understanding of the mechanisms of fish evolution and adaptation to variety of ecological conditions in marine ecosystems. At recent decades, anthropogenic impact on marine environment has become one of the important factor of ecosystem transformation and could use in the evolutionary process of fish. Therefore, fish are good monitors for the understanding of this process and their biomarkers may successfully use in monitoring programs.

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Chapter 15

THE TELEOST RETINA AS A MODEL FOR REGENERATIVE MEDICINE

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ABSTRACT

The retina of teleosts has become a powerful model system in which to study different aspects of development and regeneration. While retinal development is completed during embryogenesis in most classes of vertebrates, it continues in teleosts throughout much of the animal´s life. The mature retina grows from two specialized niches that sustain retinal stem cells (RSC): (i) the ciliary marginal zone (CMZ), a narrow ring of tissue located in the most-peripheral region of the retina that is composed of stem cells and progenitor cells that generate new neurons added to the periphery of the growing neural tissue, and (ii) the Müller glial cells, the radial glia of the neural retina, which generate neuronal progenitor cells that continue to proliferate and migrate to the photoreceptor layer, where they become precursor cells that are committed to differentiating into rod photoreceptors. Additionally, in the teleost retina, Müller glia have neurogenic capacity and mediates regeneration when stimulated by acute damage, by re-entering the cell cycle and re-establishing retinal cell types.

It has been suggested that the regenerative response of retinal tissue to injuries diminishes through the course of evolution from fish to mammals. Thus, the addition of new neurons from RSC is diminished in chickens compared to that observed in fish and amphibians. In contrast, the neuro-regenerative capacity of RSC in mammals is minimal

or non-existent and therefore, degenerative retinal diseases in humans are irreversible. There are many genes, including structural proteins, signaling pathways and transcription factors that are expressed by normal RSC in teleosts that may be related to maintaining progenitor-like properties. Understanding the combinatorial expression of genes involved in the stimulation of the formation of neurogenic RSC in teleostean experimental models might lead to new genetic treatments for retinal degenerative diseases in humans.

ADAPTATIONS OF THE FISH EYE

The fish eye has morphological, structural, and physiological adaptations to the aquatic medium (Bejarano-Escobar et al., 2014) (Figure 1). Fish normally present lateral eyes (Figure 1A) and they usually have a flattened cornea, usually thinner than that of terrestrial vertebrates. Most of the fishes cannot control the amount of light entering the eye due to the absence of eyelids and a fixed pupil aperture. They also have a highly developed reflecting surface in the choroid that is called the *tapetum lucidum*, and a high refractive spherical lens (Figure 1B,C). The lens in teleosts is pulled back towards the retina by muscles called retractors, whereas in some chondrychthyes it is pulled away from the retina by muscles called protractors. Fish vision also shows adaptation to particular photoenvironments. Thus, deep sea fishes have an all-rod retina or a rod-dominated retina with a few cones sustaining a monochromatic vision. On the contrary, cones are generally found in diurnal active species. However, most of the fish species have color vision and generally have rod cells and cone cells.

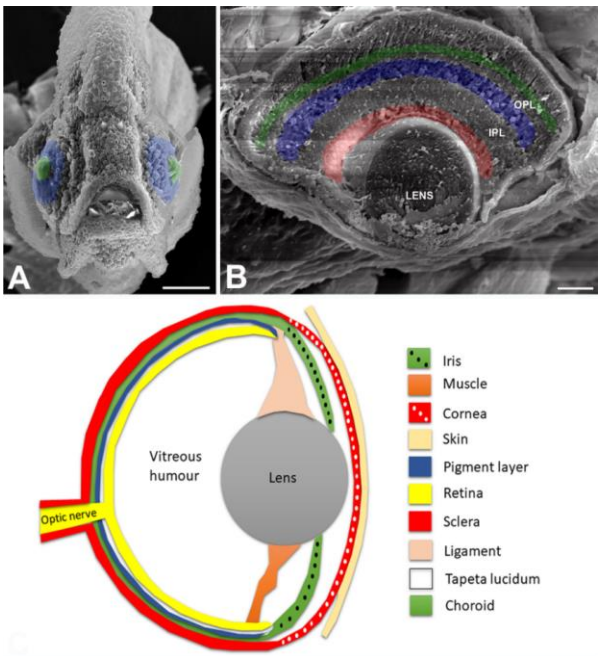

Figure 1. Structures and adaptations of the fish eye.A) Scanning electron microscopy of a *Solea senegalensis* symmetrical premetamorphic larvae. B) Section of the eye of *S. senegalensis* that shows the typical multi-layered structure of the retina. C) Scheme of the structures of the fish eye. One of the most notorious adaptations in the fish eye is the shape of the lens. As it can be observed in picture B, the lens has a spherical shape. *IPL, inner plexiform layer; OPL, outer plexiform layer.* Scale bars: 200 µm (A), 100 µm (B).

The Fish Retina

The fish retina is an excellent model in which to study developmental and physiological events. The neural retina is a thin layered structure whose main function consists on transducing light into nerve signals. It is located at the back of the eye upon which the visual image is focused by the cornea and lens. The retina is part of the central nervous system (CNS), originated as an outgrowth of the embryonic prosencephalon (see below). The neural retina is organized into different layers (Figure 2) which are, from the outermost to the innermost region: the outer nuclear layer (ONL), that is located next to the retinal pigment epithelium (RPE); the outer plexiform layer (OPL); the inner nuclear layer (INL); the inner plexiform layer (INL), the ganglion cell layer (GCL) and the optic fiber layer (OFL) (Figure 2). Nuclear layers are those containing cell bodies whereas plexiform layers are those ones where synaptic contacts between cells occur. The retina is constituted by six different types of neuronal cells that are: photoreceptors (cones and rods), bipolar cells, horizontal cells, amacrine cells and ganglion cells. Moreover, one type of radial glial cell can be found, the Müller glia (MG) (Figure 2B). Accessory glial cell types, such as microglial cells, can also be found in the teleost retina (Bejarano-Escobar et al., 2012b).

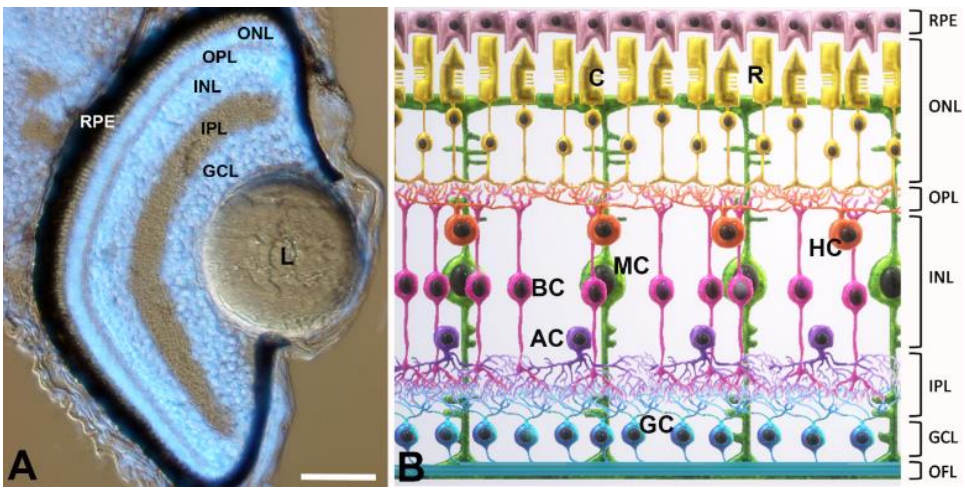

Figure 2. Histology and cell types of the fish retina. A) Retinal section that shows the multi-layered structure of mature retina. Notice the spherical lens. *GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; L, lens; ONL, outer nuclear layer; OPL, outer plexiform layer; PE, pigmented epithelium.* B) Schematic representation of retinal cell types. *AC, amacrine cell; BC, bipolar cell; C, cone; GC, ganglion cell; HC, horizontal cell; MC, Müller cell; R, rod.* Scale bar: 100 µm.

Fish Eye Development and Retinogenesis

Eye development follows a common pattern in all vertebrate animals (Figure 3). Eye morphogenesis occurs early in the developing fish embryo. It starts with a bilateral evagination of the diencephalon on the early neurula that leads to the formation of the optic vesicles (Figure 3A). These extend towards the surface ectoderm through the adjacent mesenchyme. Then, the vesicles come into contact with the ectoderm, displacing the

mesenchyme. Concomitantly, the surface ectoderm thickens forming the lens placode (Figure 3B). Both lens placode and optic vesicle invaginate, becoming the lens vesicle and a doubled-layered structure called the optic cup, respectively (Figure 3C). The inner layer of the optic cup forms the neural retina, while the outer layer becomes the RPE (Figure 3D).

Concerning the process of cell differentiation, RPE cells synthetize melanin. On the other hand, proliferating neuroepithelial cells are found in the presumptive neural retina (Figure 4A,E). The nuclei of these retinal stem cells migrate in an apical-to-basal manner and in phase with the cell cycle (Figure 4E). Thus, abundant apical mitoses are always found at the ventricular surface of the retina by these initial stages of eye development. As development proceeds, asymmetrical cell divisions produce migratory neuroblasts that lose contact with the ventricular surface of the retina and migrate radially to their final location, in which they differentiate (Figure 4D). During stages previous to the beginning of cell differentiation, retinal rudiment is composed of a neuroblastic layer (NbL) (Figure 4B). As cell differentiation occurs, the emergence of the retinal layers can also be observed (Figure 4C), increasing in thickness with the progression of retinal maturity (Figure 4D).

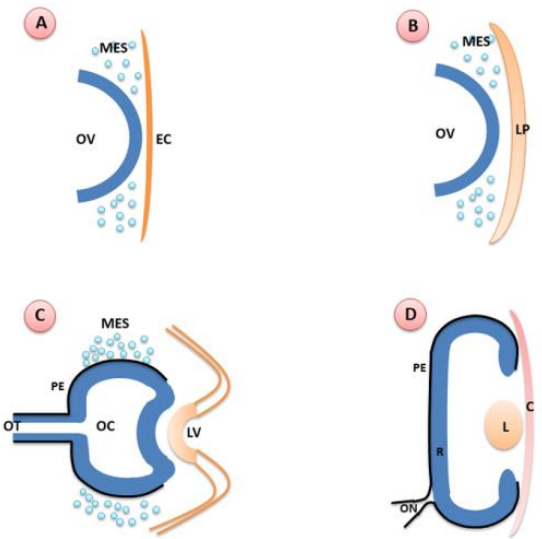

Figure 3. Development of the fish eye. Morphogenesis of the optic vesicle (A, B), the optic cup (C), the lens vesicle (C) and the mature lens and the differentiated retina (D). *C, cornea; Ec, ectoderm; L, lens; LP, lens placode; LV, lens vesicle; Mes, mesenchyme; OC, optic cup; ON, optic nerve; OT, optic tract; OV, optic vesicle; PE, pigmented epithelium; R, retina.*

This change in the potential of retinal cells is likely to be due to a combination of intrinsic and extrinsic factors, such as transcription factors and signaling molecules, respectively (Xiang, 2013). The onset of cell differentiation can be determined by using retinal markers to show the cessation of proliferative activity in the retinal tissue, or by employing cell-type-specific markers (for a review, see Bejarano-Escobar et al., 2014). This process will result in the formation of all neural retinal cell types described above (Figure 4E).

Fish species differ in their mode of development. Whereas in precocial species the specimens skip the larval period and exit the egg in a juvenile state, altricial prolarvae exit the egg in an undeveloped state. Concerning to visual system, retinogenesis is completed during

the embryological period in precocial species, such as goldfish (Sharma and Ungar, 1980), brown trout (Candal et al., 2005), medaka (Kitambi and Malicki, 2008), and several species of chondrichthyes (Harahush et al., 2009; Ferreiro-Galve et al., 2010; Bejarano-Escobar et al., 2012a; 2013) (Figure 5A,B). However, altricial species are born blind, and the acquisition of the full adult complement of cells does not occur until several days, weeks or even months after hatching, as occurs in the tench (Bejarano-Escobar et al., 2009), Senegalese sole (Bejarano-Escobar et al., 2010), or the gilthead seabream (Pavón-Muñoz et al., 2016) (Figure 5C, D).

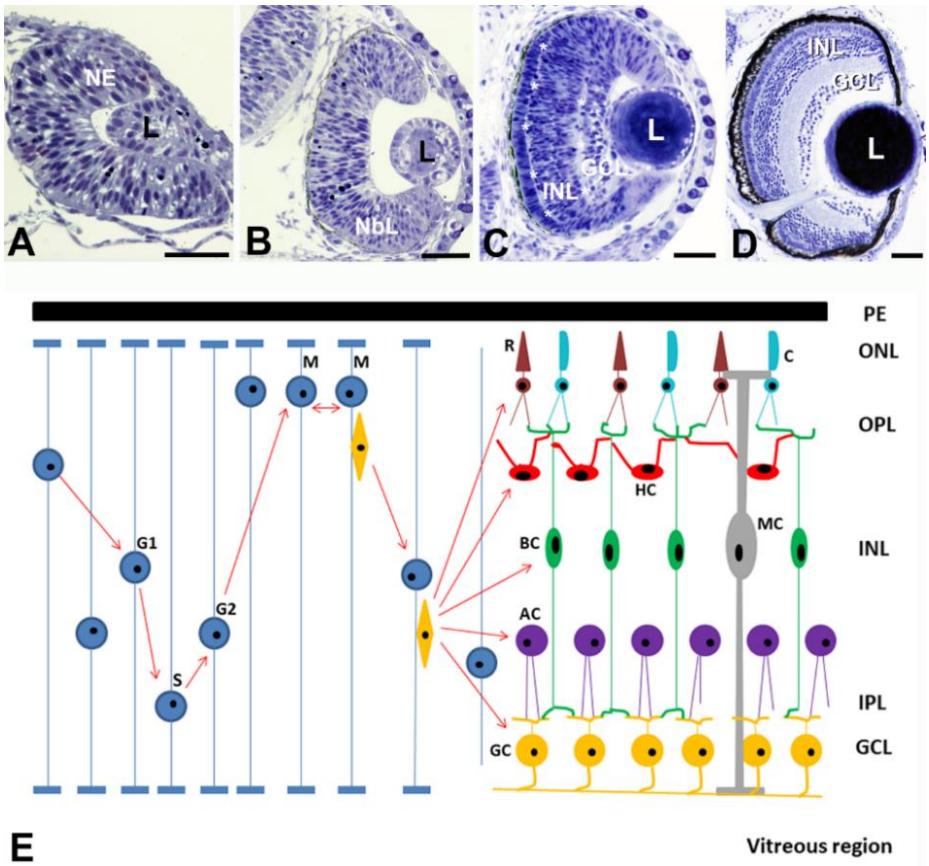

Figure 4. Fish retinogenesis. A-D) Toluidine-blue stained sections show the different phases of development of the retina. A) The retina is composed of a pseudostratified epithelium of multipotent progenitor cells. B) The retina is constituted of a neuroblastic layer. At this stage, neuroblastic cells migrate along the tissue to be located on its final position. C) Emergence of the nuclear and plexiform layers (asterisks). D) Mature retina shows a typical multi-layered structure. E) Cell differentiation. Migratory neuroblasts move from the vitreous (S phase) to the ventricular (M phase) region. When these cell types leave the cell cycle, the process of differentiation begins. *AC, amacrine cells; BC, bipolar cells; C, cones; GC, ganglion cells; GCL, ganglion cell layer; HC, horizontal cells; INL, inner nuclear layer; IPL, inner plexiform layer; L, lens; MC, Müller cells; NbL, neuroblastic layer; NE, neuroepithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; PE, pigmented epithelium; R, rodes.* Scale bars: 100 µm.

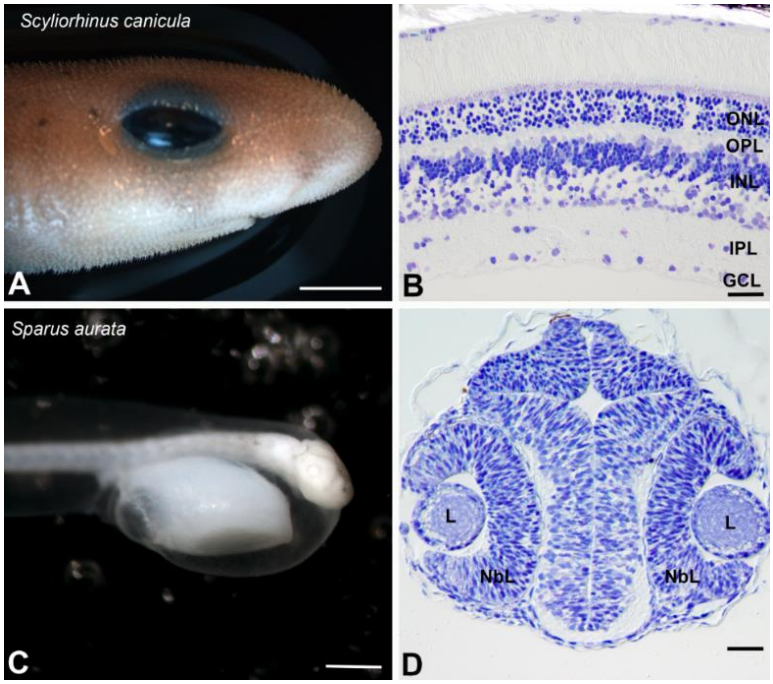

Figure 5. Retinal maturity at hatching in precocial (A, B) and altricial (C, D) fish species. At the hatching day, the retina of altricial species is still undifferentiated and is composed of a neuroblastic layer (A, B) while the retina of precocial species shows a completely differentiated tissue (C, D). Scale bars: 10 mm (A), 500 µm (C), 100 µm (B, D).

In any case, the retina of both precocial and altricial teleost species presents a very intense postnatal neurogenesis. Thus, as the fish grows, the retina continues to grow throughout its life and new neurons are constantly added to the mature retinal tissue. It is now widely recognized that retinal stem cells persist in specialized "niches" in the adult teleost retina where they generate a large number of different types of neurons (Raymond et al., 2006, Stenkamp, 2011; Lenkowski and Raymond, 2014).

Stem Cell Niches in the Teleost Retina

Healthy Retina

The proliferating cells in the mature fish retina are shown to accumulate ^{3}H thymidine (Raymond and Rivlin, 1987) or BrdU (Julian et al., 1998; Otteson et al., 2001). They can also be identified immunohistochemically with antibodies against the proliferating cell nuclear antigen (PCNA) (Bejarano-Escobar et al., 2009; 2010; 2014; Pavón-Muñoz et al., 2016) (Figure 6B,D,F,H) and against phospho-histone H3 (pHisH3) (Pavón-Muñoz et al., 2016) (Figure 6C,D,G,H). Proliferative activity is mainly detected in the ciliary marginal zone (CMZ), a circumferential ring of cells located at the periphery of the neural retina (Figure 6). Furthermore, proliferative Müller cells are also detected in the INL. These cells continue to divide and likely give rise to the precursor cells that migrate to the ONL and, after several cycles of cell divisions, differentiate into new rod photoreceptors (Figure 6). These stem cell niches are detailed below.

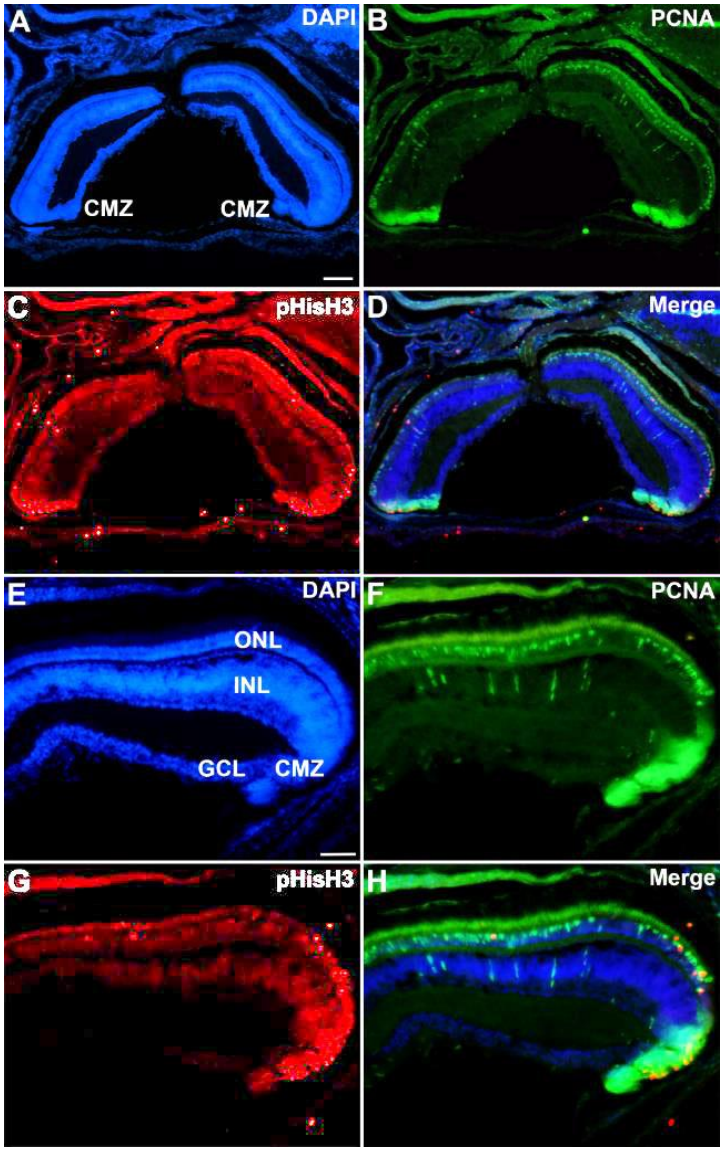

Figure 6. Niches of stem cells in mature retina.pHisH3 and PCNA-immunoreactivities of *Sparus aurata* retina. Magnifications of A-D are shown in E-H. Sections were counterstained with DAPI.
The presence of the CMZ can be observed at the periphery of the retina (asterisks in B,D,F,H). At this region, CMZ is composed of retinal stem cells and retinal progenitor cells that generate retinal neurons. Anti-PCNA antibody shows the presence proliferative photoreceptors (arrows in B,D,F,H) that originate from proliferative Müller cells (B,D,F,H). Anti-pHisH3 antibody shows the presence of mitotic activity in the CMZ and in the photoreceptor layer (arrowheads in C,D,G,H). Scale bars: 200 µm in A (A-D), 100 µm in E (E-H).

Ciliary Marginal Zone (CMZ)

The CMZ is also known in other vertebrates as *ora serrata*, *ora terminalis*, or circumferential germinative zone (CGZ) (Amato et al., 2004; Fischer et al., 2014). The CMZ is a remnant of the embryonic neuroepithelium preserved at the retinal margin following cell differentiation and lamination centrally (Raymond et al., 2006). It has been shown that new

rings of cells are constantly added from the teleost CMZ to the mature tissue. Therefore, cells situated more peripherally are younger than central ones. The teleost CMZ is able to generate an enormous number of progeny, since the total number of retinal cells generated far exceeds the number of CMZ cells present at the end of embryogenesis (Figure 7). However, reptilian and avian retinal histogenesis takes place almost exclusively during embryonic stages (Figure 7). Proliferative activity is detected in the CMZ of younger chicks (<30 days) and few retinal tissue is generated in the peripheral region in hatchlings (Fischer and Reh, 2000). The proliferation of these undifferentiated cells located in the CMZ can be greatly enhanced post-natally by intraocular injections of EGF, IGF-I, or insulin (Fischer and Reh, 2000). On the contrary, mammals do not show evidence of proliferative activity at the peripheral edge of the normal retina at birth, suggesting that mammals do not have CMZ (Figure 7).

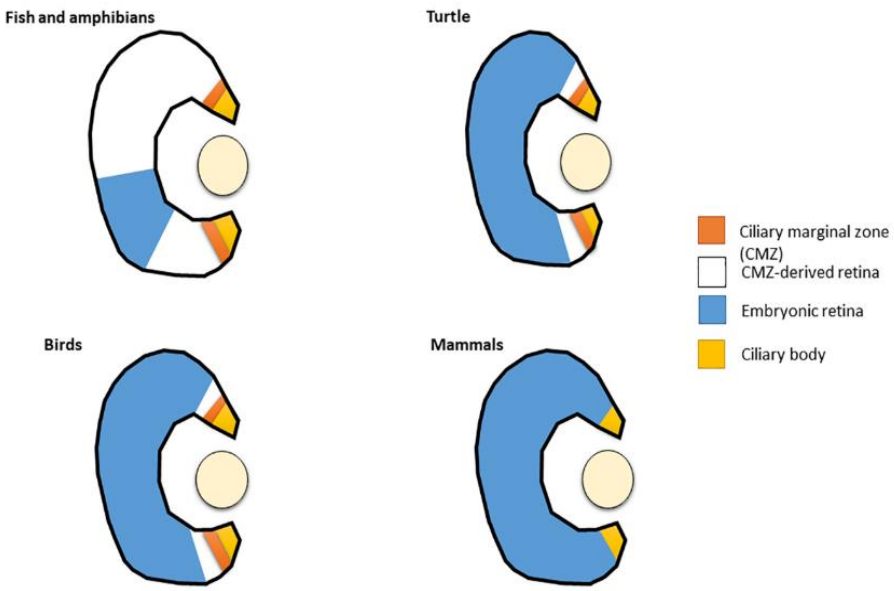

Figure 7. Schematic representation of the presence and absence of the CMZ among the vertebrates and its contribution to retinal growth. Neurogenesis continues after embryonic stages. In fish and amphibians, most of the retina derives from the CMZ, which contributes to the regeneration of this tissue. The presence of the CMZ is conserved in turtles and birds but its contribution to the generation of the retina is minimal. Most of this tissue is embryonic. In mammals, the CMZ is absent and the whole retina originates from embryonic ectoderm. (*Adapted from Moshiri et al., 2004)*.

In order to determine the histological organization of the CMZ, Raymond et al., (2006) systematically examined the expression patterns of selected genes involved in retinogenesis by using *in situ* hybridization. They subdivided the zebrafish CMZ into four anatomical regions: peripheral, middle, central-outer, and central-inner. Retinal stem cells are located in the peripheral sub-region and more restricted retinal progenitors lay closer to the differentiated retina. Thus, the definitive GCL derives from the middle sub-region of the CMZ. Progenitors at the central-inner CMZ give rise to cells located in the INL, and the central-outer sub-region generates cone photoreceptors in the ONL (Raymond et al., 2006). More recently, the clonal analysis and imaging studies showed that, as the retina develops, retinal stem cells located in the extreme peripheral edge of the CMZ divide asymmetrically to

generate a new retinal stem cell that remains in the stem cell niche, and a second daughter cell that is pushed centrally, where it becomes a more restricted retinal progenitor cell (Wan et al., 2016). Therefore, the CMZ is able to generate all the retinal cell types with the exception of rods (Figure 8) that are generated by the Müller glia (see below).

Müller Glia

In the normal developing and mature healthy retina, Müller cells have been characterized as the "radial glia" of the retina. Based on functional similarities, many of those authors considered MG as astrocyte-like cells. These cells are clearly defined by structure, function, and gene expression patterns (Reichenbach and Bringmann, 2013; Gallina et al., 2014; Lenkowski and Raymond, 2014). MG are involved in homeostasis, retinal innate immunity, retinal diseases and regeneration of the visual system, and phagocytosis of cell debris and diverse foreign bodies under physiological, pathological, and experimental conditions (Reichenbach and Bringmann, 2013; Gallina et al., 2014; Goldman, 2014; Gorsuch and Hyde, 2014; Lenkowski and Raymond, 2014; Hamon et al., 2016; Vecino et al., 2016; Bejarano-Escobar et al., 2017). However, within the past years, MG have been identified as a source of retinal cells in the healthy (Bernardos et al., 2007) and injured teleost retina (Fausset and Goldman, 2006). Thus, it has been shown that MG of teleosts continue to divide slowly and give rise to rod progenitors which form clusters in the INL that align in radial columns. These rod progenitors migrate away from the INL and into the ONL, where they continue to divide and differentiate into new rod photoreceptors (Julian et al., 1998; Bernardos et al., 2007) (Figure 8).

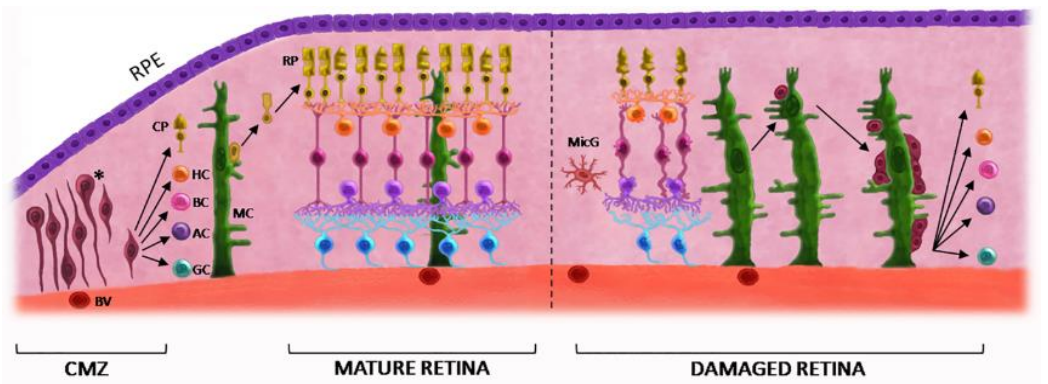

Figure 8. Neurogenesis of the fish retina during growth and regeneration. The CMZ is composed of retinal stem cells and retinal progenitor cells. RPCs are located in the peripheral retina and they will differentiate into retinal neurons. Müller cells are retinal progenitor cells that give rise to rodes. When the retina is damaged, microglial cells are activated and remove cellular debris. Nuclei of Müller cells move to the apical surface and divide asymmetrically. This process results in the formation of RPCs that migrate to the right position to generate the typical retinal neurons. (*Adapted from Lenkowski and Raymond, 2014*).

Injured Retina: Regeneration

It has been shown that adult teleosts can regenerate their retinas after different methods of injury such as cytotoxic lesion by injection of neurotoxins (Fimbel et al., 2007; Sherpa et al., 2008), surgical approach (Hitchcock et al., 1992; Fausset and Goldman, 2006), localized heat (Raymond et al., 2006), laser or light damage (Vihtelic and Hyde, 2000; Bernardos et al.,

2007; Thummel et al., 2008; Bejarano-Escobar et al., 2012b). In the case of constant intense-light exposure, cone-diurnal retina of albino fish (Allen et al., 1999; Vihtelic and Hyde, 2000; Allison et al., 2006), or rod-dominated retina of larval epibenthonic teleosts (Bejarano-Escobar et al., 2012b), are suitable models because of their relatively high susceptibility to light damage. In any case, after cell degeneration in the teleost retina, regeneration is conducted by the stem cells present at the niches described above. Thus, it has been demonstrated that retinal damage in teleosts causes increased cell proliferation at the peripheral edge, suggesting that the CMZ contributes to retinal regeneration (Raymond et al., 1988; Hitchcock et al., 1992). There is also evidence that after neurotoxic treatment, cones regenerate with a different pattern and density at the periphery than in the central goldfish retina (Stenkamp et al., 2001). This suggests that two cone populations arise via two spatially and cellularly distinct mechanisms. These authors suggest that the CMZ is the major source of the regenerated peripheral retina, but not of the central one. It was recently demonstrated that after rod cell ablation, CMZ stem cells generate all retinal cell types with the exception of rod photoreceptors (Wilson et al., 2016). It remains unclear why the proliferative response of the CMZ increases in rod degenerative fish models since they do not contribute to rod neurogenesis.

Although under normal physiological conditions, MG generate only rod photoreceptors, it is well established that a subset of Müller cells are the source of central retinal regeneration in teleosts (Fausset and Goldman, 2006; Bernardos et al., 2007; Thummel et al., 2008). Thus, after retinal damage, MG reenter the cell cycle and act similar to retinal progenitor cells during embryonic development, exhibiting inter kinetic nuclear migration apically to undergo a single asymmetric mitosis to self-renew and to produce a proliferating retinal progenitor (Nagashima et al., 2013). Nuclear migration is facilitated by the actin cytoskeleton and Rho-associated coiled-coil kinases (Rocks) and it is necessary for photoreceptor regeneration in the adult zebrafish retina (Lahne et al., 2015). The newly generated cell divides rapidly and repeatedly and a cluster of progenitor cells is raised. The vitreal or scleral migration of dividing progenitors and post-mitotic neural precursors is a necessary step to replace the population of cells that has been lost and restore the functional vision (Figure 8). Thus, the selective ablation of photoreceptors recruits progenitors to the ONL (Vihtelic and Hyde, 2000). In contrast, the ablation of cells located in the inner half of the retina retains the retinal progenitors in the INL to replace amacrine or ganglion cells (Fimbel et al., 2007) (Figure 8).

Different cytokines secreted after retinal damage in teleosts promote MG de-differentiation and proliferation by the expression of several proneural transcription factors. Thus, the pro-inflammatory cytokine TNFα produced by apoptotic neurons initiates MG proliferation (Nelson et al., 2013). Stat3, as well as members of the MAPK and Akt signaling pathways, increase their expression in MG prior to their de-differentiation and proliferative response (Kassen et al., 2007). Stat3-dependant signaling pathway is stimulated by ciliary neurotrophic factor (CNTF) (Kassen et al., 2009). The expression of the proneural basic-helix-loop-helix transcription factor Achaete-scute homolog 1a (Ascl1a) and the subsequent induction of lin-28, a pluripotency mRNA binding protein, is necessary for teleost MG and retinal regeneration (Ramachandran et al., 2010). These authors also showed that Müller glial cells respond by re-expressing pluripotency genes such as *c-myc, nanog, nox2, and oct4.* Other studies have shown that Ascl1a-Dkk-Wnt signaling pathway, in conjunction with Pax6, Stat3, and Hspd1, regulates MG de-differentiation and progenitor proliferation (Kassen et al.,

2009; Qin et al., 2009; Thummel et al., 2010; Ramachandran et al., 2011). A recent transcriptomic analysis has revealed that several other categories of genes/signaling, such as nuclear factor-κB (NF-κB), clock genes, and prostaglandin metabolism, rapidly up-regulate in reactive MG (Sifuentes et al., 2016). More recently, it has been shown that sex determining region Y-box 2 (Sox2) (Gorsuch et al., 2017), and atonal homolog 7 (Atoh7) (Lust et al., 2016) are re-expressed in Müller cells after injury and lead them to proliferate and generate new retinal neurons. Additionally, Sox2 has the capacity to induce Ascl1a and Atoh7 expression, which are able to activate lin-28 expression themselves. It has also been shown that Notch signaling-inhibition collaborates with Ascl1a and Lin28a to stimulate widespread MG proliferation in the uninjured retina (Elsaeidi et al., 2018). Recently, microRNAs (miR) have also been identified to control the generation of new neurons derived from teleosts MG (Madelaine and Mourrain, 2017). Thus, the depletion of miR-9 increases the number of undifferentiated progenitors in the teleost retina. One of the transcription factors mentioned above, *lin-28*, which is involved in inducing proliferation of neural retinal stem cells after damage, is a target of miR-9 (La Torre et al., 2013). Other authors have shown that microglial cells and macrophages are critical for proper rod cell regeneration, stimulating the activation of retinal MG to a stem cell-like state (White et al., 2017).

Therapeutic Applications

As we have previously shown, the retina of the teleost fish has a remarkable potential to regenerate new neurons after various types of injury. Our understanding of the molecular mechanisms that regulate retinal regeneration from MG in fish has advanced rapidly during the last decade. Currently, the MG is recognized as the primary source of new neurons after retinal damage also in birds (Gallina et al., 2014).

Mammals do not generally show evidence of proliferation at the peripheral edge of the neural retina, suggesting that this group of vertebrates do not have CMZ. Therefore, the attempts to stimulate retinal regeneration in the mammalian retina have focused on the MG. Retinal injury results in reactive gliosis and glial scaring in mammals (Bringmann et al., 2009) and traditional studies have failed to identify any regenerative potential in the mammalian retina. Thus, degenerative retinal diseases in mammals can lead to vision loss. However, retinal injury in either fish or mammals induces the up-regulation of many mitogenic factors (Kassen et al., 2009; Wan et al., 2012; 2014). Therefore, the initial steps in the response to injury that lead to regeneration in teleosts are also conserved in the retina of mammals. Thus, *in vitro* studies have demonstrated that mammalian Müller cells could act as progenitors of bipolar (Pollak et al., 2013) and photoreceptor cells (Giannelli et al., 2011). More recently, it has been shown that forced expression of Ascl1 promotes MG proliferation and differentiation into amacrine, bipolar, and photoreceptor neurons following retinal injury in young mice (Ueki et al., 2015). The combination of Ascl1 expression with a histone deacetylase inhibitor increases the differentiation of MG into new neurons in the mouse adult retina (Jorstad et al., 2017).

Therefore, our understanding of the molecular mechanisms that regulate retinal regeneration from stem cells in teleosts could bring insights into the molecular mechanisms that either sustain or prevent neural cell replacement and therefore help to design therapies to stimulate retinal regeneration in mammals.

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Chapter 16

IMPORTANCE OF BENTHOS IN THE TROPHIC STRUCTURE OF THE ICHTHYOFAUNA OF LOS FRAILES REEF, GULF OF CALIFORNIA, MEXICO

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ABSTRACT

The Gulf of California comprises a great diversity of benthic ecosystems. The western coast in particular is dominated by rocky and coral reefs that shelter a wide diversity and abundance of species. Despite the importance of benthic ecosystems for the structure and organization of associated communities, few studies have focused on the importance of benthos in the trophic organization of the fish community. In this chapter the fish communities of four areas of the Los Frailes rocky reef differing in benthic structural complexity (BSC) were evaluated through visual censuses. According to the BSC two low benthic structural complexity (LBSC) areas and two high complexity (HBSC) areas were identified. The HBSC areas had a higher number of cavities, greater depth and greater substrate heterogeneity. However, despite differences in structural complexity, the trophic organization of the ichthyofauna did not show significant changes

in terms of formation of functional trophic groups (FTG) between the HBSC (21 FTG) and LBSC (19 FTG) locations. The biomass flow diagram of the different prey species of carnivorous, omnivorous and herbivorous fish present in the reef showed variable connections and biomass flow, with dominance of benthic prey species, which highlights the importance of benthic invertebrates as a source of energy for the reef ichthyofauna.

INTRODUCTION

Reef systems represent the interdependence between the physical environment and organic activity. Physical factors are decisive in reef formation and in the specific composition of the benthic community that lives there (Jones & Endean, 1977). It has been shown that the factors that determine the benthic community structure of reef systems are caused by physical parameters that dictate the structure in shallow areas, and by biological parameters that are decisive in deeper areas, where competition for food and space is an important factor in this type of community (Glynn et al., 1972; Liddell & Ohlhorst, 1987; Borges-Souza, 2003).

The composition of the fish species assemblage associated to rocky and coral reef systems is the result of the interaction of several processes, including biotic factors associated with recruitment, predation and competition, and abiotic factors such as habitat structure (Ebeling & Hixon, 1991). Reef fish can respond to several aspects of habitat structure (substrate type, size and shape of cavities, crevices, rocks, algal growth, coral growth, etc.) that influence their distribution, abundance, specific richness and diversity (Álvarez-Philip, 2004). Several studies have examined the effect of different habitat characteristics and different attributes of the community (Luckhurst & Luckhurst, 1978; Roberts & Ormond, 1987; Öhman & Rajasuriya, 1998). Some of these studies reported a positive relationship between structural habitat complexity and ecological community indices such as fish diversity and rugosity (Risk, 1972; Roberts & Ormond, 1987). However, other studies report no such relationship (Sale & Dybdahl, 1975; Luckhurst & Luckhurst, 1978).

These differing results could be due to not all structural characteristics of the habitat having the same influence on each attribute of the fish community and influencing differently each of the species that comprise it (Roberts & Ormond, 1987; Grigg, 1994; McClanahan, 1994; Gratwicke & Speight, 2005). Depending on the structural complexity of the habitat, a greater or smaller number of microhabitats can be established that impact the degree of competition among species and individuals, as well as the persistence of the predators and their prey, which in turn impacts directly the different attributes of the community (abundance, species richness and diversity) (Crowder & Cooper, 1982; Wooton, 1990; Gerking, 1994).

To analyze jointly the functioning and feeding organization of the ichthyofauna within a community context it is necessary to investigate the effect of the habitat structural complexity and existing trophic relationships (functional groups) (Elliott et al., 2007). In this chapter we analyzed the effect of the habitat complexity on the structure and trophic organization of the conspicuous ichthyofauna of the Los Frailes reef, B.C.S., in order to determine the ecological role of the habitat structural complexity (as one of the main factors) in the variability of benthic fish associations in this area of the mouth of the Gulf of California (GC).

The Los Frailes reef is located at 23º25´N and 109º30´W (Figure 1). It is part of Cabo Pulmo National Park, at the entrance to the GC (Robinson & Thomson, 1992; Reyes Bonilla, 1997). The Los Frailes Bay is located in a transition zone between the Eastern Tropical Pacific and the Temperate Eastern Pacific, where three water masses converge: (1) cold low-salinity water (34.6 PSU; practical salinity units) of the California Current; (2) warmer medium-salinity water (34.65-34.85 PSU) from the southeast, carried by the Costa Rica coastal current; and (3) warm high-salinity water (>34.9 PSU) from the GC (Álvarez-Borrego & Lara-Lara, 1991).

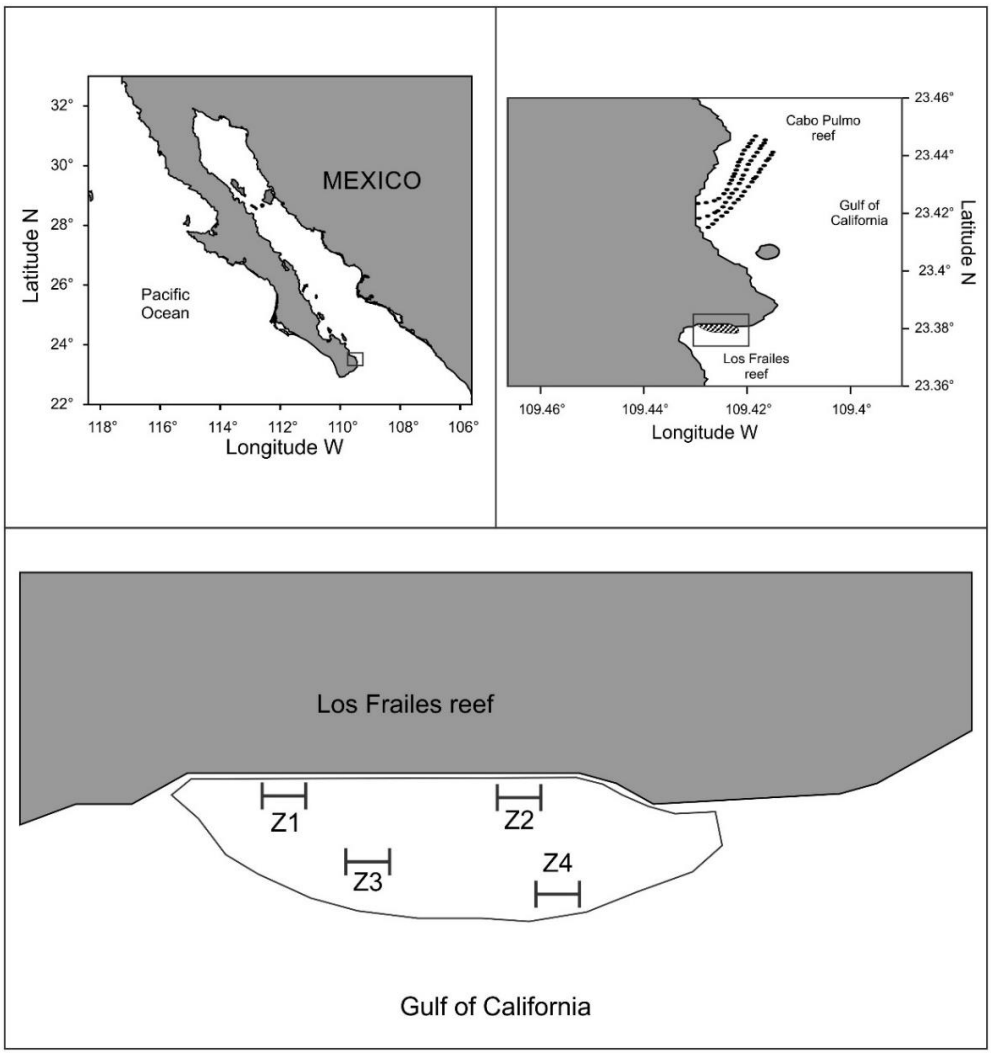

Figure 1. Los Frailes reef. Locations Z1 and Z2 have low benthic structural complexity (LBSC) and locations Z3 and Z4 have high benthic structural complexity (HBSC).

There are no studies to date on oceanographic variables at Los Frailes reef, only isolated measurements of some variables taken in Cabo Pulmo reef (Trasviña-Castro et al., 2013). The mean temperature on the reef is 24.9ºC, but there are temperature records of 17ºC in February 1988 and of 32ºC in August 1997 (Anaya-Reyna, 1993). Salinity remains relatively constant

at 35 PSU year-round. Water in the area has low turbidity and precipitation is low at 200 mm year^{-1}, although two creeks flow into the Los Frailes area and provide an important volume of fresh water and terrestrial sediment. The rainy season ranges from July to September (Anaya-Reyna, 1993). Large structures and rocks can be observed in this area from the intertidal zone to 15 m depth, with presence of sandy patches (Moreno-Sánchez, 2009). Four sampling areas were chosen at locations Z1 and Z2, found at a shallow depth of between 1 and 3 m. These two locations are characterized by high energy waves, by rocky material that ranges in size from pebbles (20 to 30 cm) to blocks over 3 m in diameter, and by a not very steep beach slope. Both locations had 80 percent rock cover and presence of coral heads.

The Z3 and Z4 locations were situated at an intermediate depth of 7 to 10 m. At these locations there were fewer pebbles, the slope was steeper, there was 70% percent cover of rocky material, with up to 20% sand cover. These two locations were at the transition between the rocky part of the reef and the sandy ocean bottom (Figure 1). The submarine Los Frailes canyon, with a depth of over 1000 m, is located near the reef. This bathymetric feature enables the presence of pelagic species and of local events such as upwelling (Fiedler, 1992; Reyes Bonilla, 2001). To characterize the benthic structural complexity (BSC) the following characteristics were taken into account at each location:

Characteristic	Measurement
Depth	Depth was measured at 3 points along the transect using a standard scuba depth gauge, and a mean depth was calculated for each sampling area.
Substrate type cover	Substrate cover (rock, coral and sand) was calculated from 250 1m^2 photographs taken at each transect, using the program Coral Point (V3.4)
Number of cavities	All cavities were counted at each location
Rugosity index (RI)	The method of contour distance to linear distance was used (McCormick, 1994). This was calculated as the proportion between the final length of a chain (10 m long; 0.5 cm links) placed over the substrate topography and the total linear distance (10m).
Rock size	The size of rocks at each location was categorized according to diameter (Ø), in the following way: A = (Ø) < 30 cm (pebble; B = 30 cm < (Ø) > 1m; C = 1m< (Ø) < 3m; D = L (Ø) 3m (Aburto-Oropeza, 1999).

Sampling trips were carried out monthly from November 2004 to October 2006 at four locations (Z1 and Z2 at shallow depths, Z3 and Z4 at intermediate depths), within the Los Frailes reef, B.C.S. The method of visual censuses by scuba and free diving was used, which is a method commonly used in fish ecology studies of this type of community (Bortone et al., 1991). Censuses were carried out along transects. This technique is recommended when a quantitative estimate of abundance is needed.

In this chapter transects 50 m long by 5 m wide were used (Elorduy-Garay & Jiménez-Gutiérrez, 2000). Censuses were carried out during daylight hours between 10:00 and 16:00, which is when illumination is best. Transects started from a fixed point along the shore where coordinates were taken using a global positioning system (GPS). All fish species and abundances were recorded on acrylic sheets using graphite pencils. Photographs and videos were also taken for later reference, comparing with specialized bibliography (e.g., Fischer et al., 1995; Thomson et al., 2000; Gotshall, 2001; Robertson & Allen, 2008).

With the recorded information a systematic list of conspicuous fish at the Los Frailes reef was assembled. The composition was evaluated counting the number of species present in each sample at each location (Brower & Zar, 1977).

In order to obtain a hierarchical location of species within the community, species were grouped into four categories according to their frequency and relative abundance at each location in the following manner (Villegas-Sánchez et al., 2009):

- Rare species: relative abundance under 0.1%
- Common species: relative abundance under 1% and over 0.1%
- Frequent species: relative abundance over 1% and under 10%
- Abundant species: relative abundance over 10%

To evaluate the diversity of functional trophic groups defined as a set of permanent or temporal polyphyletic species sharing morphological characteristics and carrying out equivalent functions in the ecosystem (Naeem & Li, 1997; Blondel, 2003), a search of ecological attributes and morphological characteristics was carried out. The ecological attributes taken into account were diet, trophic level, species residency (frequency and abundance), and position in the water column, whereas the morphological characteristics taken into account were length (cm), average weight, form of the caudal fin, position and type of mouth. These attributes and characteristics were selected based on studies by several authors who selected them as key characteristics associated to the function of a species within an ecosystem (Sánchez-Gil & Yáñez-Arancibia, 1997; Mathieson et al., 2000; Dumay et al., 2004; Álvarez-Filip & Reyes-Bonilla, 2006). The information was obtained from books, scientific articles and the FISHBASE data base (Froese & Pauly, 2006; http://www.fishbase.org).

Excepting diets, all data were standardized using the square root transformation. Diet was recorded as the percent weight of each prey species. A cluster analysis was carried out starting from a similarity matrix (calculated using the Bray-Curtis similarity index with complete linkage). The samples were successively joined in groups according to the highest similarity value. This process ended when one unit containing all samples was obtained (Clarke & Warwick, 2001). Results were represented in a dendrogram, where the "y" axis represented all groups and the "x" axis defined the similarity level at which all groups or samples were deemed to be joined. The trophic categories (TC) were obtained using the program PRIMER 6.1.6. The selection of the cut-off point in the dendrogram was arbitrary, selecting a similarity level that would produce a manageable number of groups and would at the same time maximize the biological coherence among the members of each TC (Simberloff & Dayan, 1991; Petchey & Gaston, 2002).

To determine the importance of benthos in the trophic organization of the fish community the differences between the number of species and the functional trophic groups (FTG) were evaluated. The biological consistency of the functional groups was analyzed using the species that integrated a FTG in particular and presented similar characteristics in habitat use. We also determined whether several species carried out the same ecological role within the community (ecological redundancy). To estimate the importance of the benthos in the trophic organization of the fish community of the Los Frailes reef the FTG were integrated into three feeding categories: herbivores (diet composed of over 70% algae), omnivores (diet composed

of 30% algae and 70% animals), and carnivores. On this basis a biomass flow diagram was designed. This type of analysis allows the visualization of how the different prey and predator species are connected, as well as the number of connections within the reef.

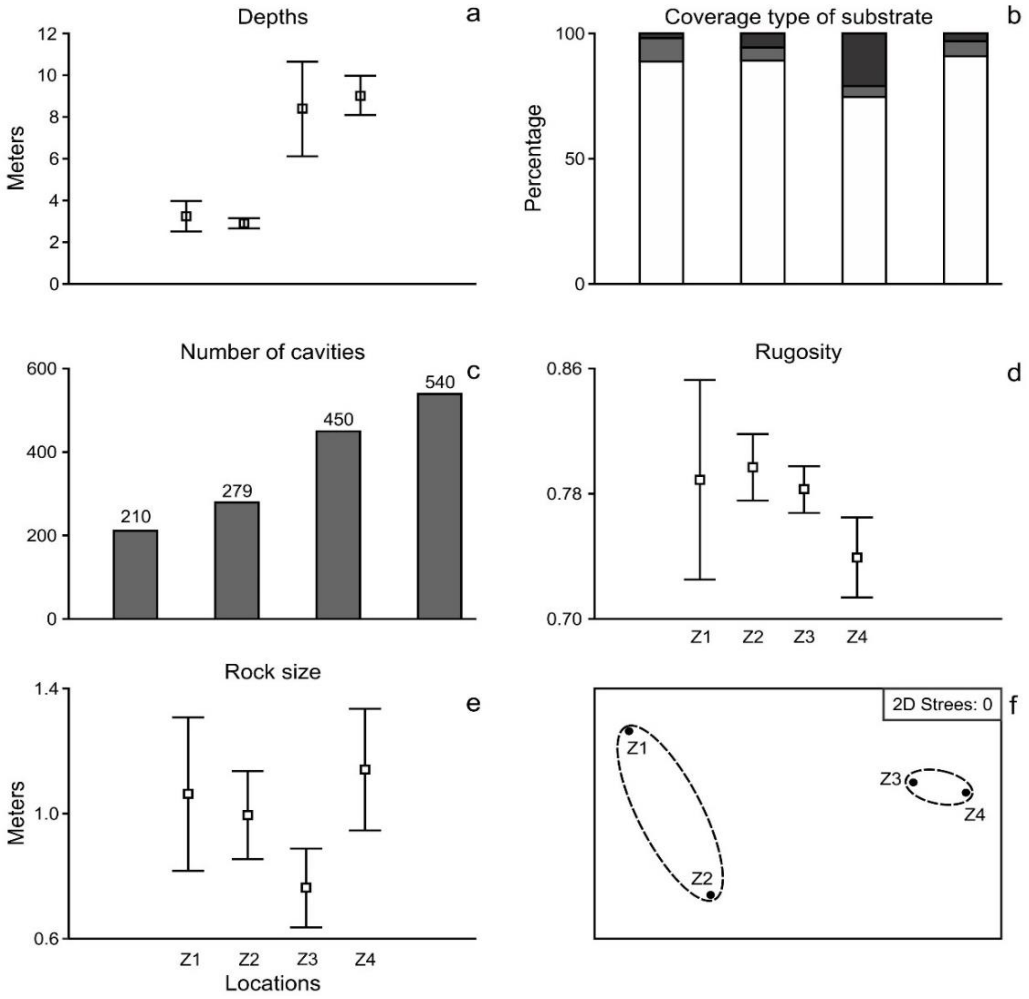

Figure 2. Habitat characteristics at each location. Z1 northwest, Z2 northeast, Z3 southwest, Z4 southeast. (a) average depth (□) and standard deviation (T); substrate cover, (b) white: rock cover, grey: coral cover, black: area cover, (c) number of cavities; (d) rugosity – average (□) and standard deviation (T); (e) rock size, average (□) and standard deviation (T).

The Z3 and Z4 locations were deeper (mean = 8 meters; Figure 2a). There was a high percentage of rock substrate at all locations; the Z3 location had more sand cover, and the Z1 location had more coral cover (Figure 2b). The Z3 and Z4 locations had more cavities and higher rugosity (Figure 2c and d). The Z3 location had small-sized rocks, while the Z1 and Z4 locations had larger rocks (Figure 2e). The descriptors of BSC allowed the separation of locations in two groups with different levels of complexity: the shallow Z1 and Z2 stations made up the low BSC group (LBSC), while the Z3 and Z4 stations made up the high BSC group (HBSC) (Figure 2f).

Composition of the Fish Community

A total of 88 monthly sampling trips were conducted in 2005 and 2006. A total of 34,887 fish were counted, belonging to 31 families, 61 genera and 89 species (Table 1). The total richness was 89 species. In the HBSC locations species richness was 89 species with an average of 25 species, while at LBSC locations species richness was 77 species, with average values of 17.36 species at Z1 and 16.36 species at Z2 (Figure 3a).

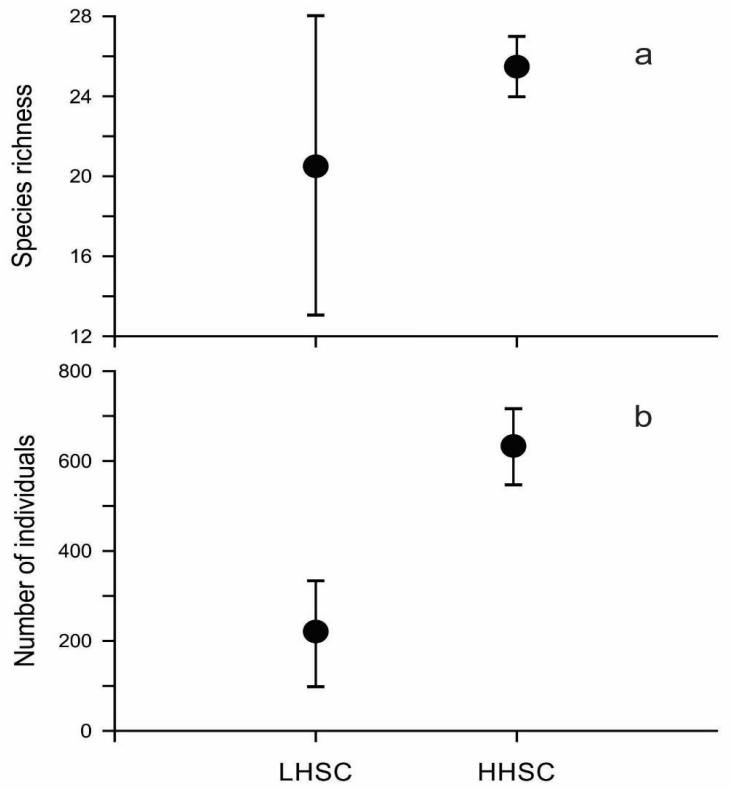

Figure 3. Species richness and total abundance, average and standard deviation (T) at low benthic structural complexity (LBSC) and high benthic structural complexity (HBSC) locations.

Abundance

A total of 34,887 individuals were counted. The highest abundances occurred at the HBSC locations, where 18,284 individuals were recorded, with an average of 415.54 individuals. At LBSC locations 16,603 individuals were counted, with an average of 377.34 individuals (Figure 3b).

Table 1. Systematic list of reef fish species in Los Frailes, including scientific name, abundance, frequency, classification according to abundance and frequency, presence in HBSC or LBSC, warm or cool season. O = species present, X = species absent

Species	relative abundance (n = 34 887)	% occurrence (n = 88)	Classification	HBSC	LBSC	functional trophic group	cold season
CLASS CHONDRICHTHYES							
ORDER MYLIOBATIFORMES							
FAMILY *MYLIOBATIDAE*							
Rhinoptera steindachneri Evermann & Jenkins, 1892	0.02	1.1	Rare	O	X	18	+
CLASS ACTINOPTERYGII							
ORDER ELOPIFORMES							
FAMILY *ELOPIDAE*							
Elops affinis Regan, 1909	0.13	4.5	Common	O	O	1	+
ORDER ANGUILLIFORMES							
FAMILY *MURAENIDAE*							
Gymnothorax castaneus (Jordan & Gilbert, 1883)	0.06	14.8	Rare	O	O	2	+
Muraena lentiginosa Jenyns, 1842	0.01	4.5	Rare	O	X	2	X
ORDER AULOPIFORMES							
FAMILY *SYNODONTIDAE*							
Synodus lacertinus Gilbert, 1890	0.01	3.4	Rare	O	X	3	O
ORDER BERYCIFORMES							
FAMILY *HOLOCENTRIDAE*							
Myripristis leiognathos Valenciennes, 1846	0.05	9.1	Rare	O	O	20	O
Sargocentron suborbitalis (Gill, 1863)	0.18	17.0	Common	O	O	20	O
ORDER GASTEROSTEIFOMES							
FAMILY *FISTULARIIDAE*							
Fistularia commersonii Rüppell, 1838	0.46	35.2	Common	O	O	1	O
ORDER SCORPAENIFORMES							
FAMILY *SCORPAENIDAE*							
Scorpaena mystes Jordan & Starks, 1895	0.07	9.1	Rare	O	O	3	O
FAMILY *SERRANIDAE*							
Alphestes multiguttatus (Günther, 1867)	0.03	5.7	Rare	O	O	12	O

Species	relative abundance (n = 34 887)	% occurrence (n = 88)	Classification	HBSC	LBSC	functional trophic group	cold season
Cephalopholis panamensis (Steindachner, 1877)	0.23	27.3	Common	O	X	2	X
Epinephelus labriformis (Jenyns, 1840)	0.12	29.5	Common	O	O	3	O
Mycteroperca rosacea (Streets, 1877)	0.24	26.1	Common	O	O	3	O
Paranthias colonus (Valenciennes, 1846)	0.45	26.1	Common	O	O	21	O
Rypticus nigripinnis Gill, 1861	0.02	4.5	Rare	O	O	3	O
Serranus psittacinus Valenciennes, 1846	0.44	34.1	Common	O	O	3	O
Serranus sp.	0.00	1.1	Rare	O	O	3	O
FAMILY *APOGONIDAE*							
Apogon retrosella (Gill, 1862)	0.66	15.9	Common	O	O	7	O
FAMILY *NEMATISTIIDAE*							
Nematistius pectoralis Gill, 1862	0.01	1.1	Rare	O	X	1	X
FAMILY *CARANGIDAE*							
Caranx caballus Günther, 1868	0.27	12.5	Common	O	O	1	O
Caranx caninus Günther, 1867	0.50	3.4	Common	O	O	1	X
Caranx melampygus Cuvier, 1833	0.17	10.2	Common	O	O	1	O
Gnathanodon speciosus (Forsskål, 1775)	0.16	9.1	Common	O	O	1	O
Trachinotus rhodopus Gill, 1863	0.31	14.8	Common	O	O	18	O
FAMILY *LUTJANIDAE*							
Hoplopagrus guentherii Gill, 1862	0.06	10.2	Rare	O	O	9	O
Lutjanus viridis (Valenciennes, 1846)	1.20	38.6	Frequent	O	O	3	O
FAMILY *GERREIDAE*							
Gerres simillimus Reagan, 1907	0.01	2.3	Rare	O	O	20	O
FAMILY *HAEMULIDAE*							
Haemulon flaviguttatum Gill, 1862	1.63	6.8	Rare	O	O	20	O
Haemulon maculicauda (Gill, 1862)	0.58	11.4	Common	O	O	7	O
Haemulon sexfasciatum Gill, 1862	0.15	9.1	Common	O	O	21	O
Microlepidotus inornatus Gill, 1862	1.05	17.0	Frequent	O	O	21	O
FAMILY *MULLIDAE*							
Mulloidichthys dentatus (Gill, 1862)	1.26	34.1	Frequent	O	O	21	O
FAMILY *KYPHOSIDAE*							
Kyphosus elegans (Peters, 1869)	0.10	9.1	Rare	O	O	13	O

Table 1. (Continued)

Species	relative abundance (n = 34 887)	% occurrence (n = 88)	Classification	HBSC	LBSC	functional trophic group	cold season
Kyphosus vaigensis (Quoy & Gaimard 1825)	0.01	3.4	Rare	O	O	13	O
FAMILY *CHAETODONTIDAE*							
Chaetodon humeralis Günther, 1860	0.21	19.3	Common	O	O	14	O
Johnrandallia nigrirostris (Gill, 1862)	0.25	27.3	Common	O	O	6	O
FAMILY *POMACANTHIDAE*							
Holacanthus clarionensis Gilbert, 1891	0.30	22.7	Common	O	O	6	O
Holacanthus passer Valenciennes, 1846	1.95	81.8	Frequent	O	O	8	O
FAMILY *CIRRHIRIDAE*							
Cirrhitichthys oxycephalus (Bleeker, 1855)	1.35	58.0	Frequent	O	O	8	O
Cirrhitus rivulatus Valenciennes, 1846	0.04	6.8	Rare	O	O	3	O
FAMILY *POMACENTRIDAE*							
Abudefduf troschelii (Gill, 1862)	3.84	63.6	Frequent	O	O	16	O
Chromis atrilobata Gill, 1862	12.74	70.5	Abundant	O	O	17	O
Chromis limbaughi Greenfield & Woods, 1980	0.73	15.9	Common	O	O	17	O
Microspathodon bairdii (Gill, 1862)	0.02	2.3	Rare	O	O	13	O
Microspathodon dorsalis (Gill, 1862)	2.05	55.7	Frequent	O	O	5	O
Stegastes acapulcoensis (Fowler, 1944)	0.19	10.2	Common	O	O	21	O
Stegastes flavilatus (Gill, 1862)	0.60	48.9	Common	O	O	21	O
Stegastes rectifraenum (Gill, 1862)	3.39	63.6	Frequent	O	O	21	O
FAMILY *LABRIDAE*							
Bodianus diplotaenia (Gill, 1862)	1.63	60.2	Frequent	O	O	8	O
Halichoeres chierchiae di Caporiacco, 1947	0.07	10.2	Rare	O	O	9	O
Halichoeres dispilus (Günther, 1864)	1.49	33.0	Frequent	O	O	8	O
Halichoeres melanotis (Gilbert, 1890)	0.11	4.5	Common	O	O	9	O
Halichoeres nicholsi (Jordan & Gilbert, 1882)	0.17	19.3	Rare	O	O	15	O

Species	relative abundance (n = 34 887)	% occurrence (n = 88)	Classification	HBSC	LBSC	functional trophic group	cold season
Halichoeres notospilus (Günther, 1864)	0.56	12.5	Common	O	O	9	O
Halichoeres semicinctus (Ayres, 1859)	0.70	3.4	Common	O	O	9	O
Novaculichthys taeniourus (Lacepède, 1801)	0.03	4.5	Rare	O	O	19	O
Thalassoma grammaticum Gilbert, 1890	2.98	70.5	Frequent	O	O	21	O
Thalassoma lucasanum (Gill, 1862)	35.36	100.0	Abundant	O	O	21	O
Xyrichtys pavo Valenciennes, 1840	0.04	5.7	Rare	O	O	19	O
FAMILY *SCARIDAE*							
Nicholsina denticulata (Evermann & Radcliffe, 1917)	0.34	11.4	Common	O	O	4	O
Scarus compressus (Osburn & Nichols, 1916)	0.47	25.0	Common	O	O	4	O
Scarus ghobban Forsskål, 1775	0.72	54.5	Common	O	O	4	O
Scarus perrico Jordan & Gilbert, 1882	0.65	52.3	Common	O	O	4	O
Scarus rubroviolaceus Bleeker, 1847	0.32	25.0	Common	O	O	4	O
Scarus sp.	0.10	6.8	Common	O	O	4	O
FAMILY *TRIPTERYGIIDAE*							
Crocodilichthys gracilis Allen & Robertson, 1991	0.01	2.3	Rare	O	O	8	X
FAMILY *BLENNIIDAE*							O
Ophioblennius steindachneri Jordan & Evermann, 1898	0.16	19.3	Common	O	O	5	O
Plagiotremus azaleus (Jordan & Bollman, 1890)	1.08	37.5	Frequent	O	O	8	O
FAMILY *LABRISOMIDAE*							
Labrisomus multiporosus Hubbs, 1953	0.12	6.8	Common	O	O	9	O
Labrisomus xanti Gill, 1860	0.01	3.4	Rare	O	O	12	O
Malacoctenus hubbsi Springer, 1959	0.00	1.1	Rare	O	X	11	X
Malacoctenus margaritae (Fowler, 1944)	0.01	2.3	Rare	O	X	12	X
FAMILY *ZANCLIDAE*							
Zanclus cornutus (Linnaeus, 1758)	0.23	22.7	Common	O	O	6	O

Table 1. (Continued)

Species	relative abundance (n = 34 887)	% occurrence (n = 88)	Classification	HBSC	LBSC	functional trophic group	cold season
FAMILY *ACANTHURIDAE*							
Acanthurus nigricans (Linnaeus, 1758)	0.01	2.3	Rare	O	O	13	O
Acanthurus triostegus (Linnaeus, 1758)	0.88	19.3	Common	O	O	4	O
Acanthurus xanthopterus Valenciennes, 1835	0.57	28.4	Common	O	O	21	O
Prionurus laticlavius (Valenciennes, 1846)	0.40	10.2	Frequent	O	O	4	O
Prionurus punctatus Gill, 1862	7.61	85.2	Common	O	O	4	O
FAMILY *SCOMBRIDAE*							
Euthynnus lineatus Kishinouye, 1920	0.01	2.3	Rare	O	X	1	O
ORDER TETRAODONTIFORMES							
FAMILY *BALISTIDAE*							
Balistes polylepis Steindachner, 1876	0.12	9.1	Common	O	O	9	O
Pseudobalistes naufragium (Jordan & Starks, 1895)	0.30	22.7	Common	O	O	10	O
Sufflamen verres (Gilbert & Starks, 1904)	1.03	68.2	Frequent	O	O	8	O
FAMILY *TETRAODONTIDAE*							
Arothron hispidus (Linnaeus, 1758)	0.04	8.0	Rare	O	O	10	O
Arothron meleagris (Lacèpede, 1798)	1.51	86.4	Frequent	O	O	8	O
Canthigaster punctatissima (Günther, 1870)	1.06	52.3	Frequent	O	O	5	O
Sphoeroides lobatus (Steindachner, 1870)	0.01	3.4	Rare	O	X	15	O
FAMILY *DIODONTIDAE*							
Chilomycterus reticulatus Linnaeus, 1758	0.12	3.4	Common	O	X	19	O
Diodon holocanthus Linnaeus, 1758	0.36	50.0	Common	O	O	19	O
Diodon hystrix Linnaeus, 1758	0.28	13.6	Common	O	X	10	O

Classification of Species According to Their Relative Abundance

Of the 89 recorded species two (*Thalassoma lucasanum* and *Chromis atrilobata)* were categorized as abundant. A total of 16 species were categorized as frequent, including *Prionurus punctatus*, *Abudefduf troschelii*, *Stegastes rectifraenum*, *Arothron meleagris*, and *Plagiotremus azaleus*. A total of 42 species were categorized as common, among which were

included *Acanthurus triostegus*, *Halichoeres semicinta*, *Scarus ghobban* and *Apogon retrosella*. There were a total of 29 rare species, present at one or two locations with a low number of individuals; among these were: *H. chierchiae*, *H. nicholsi*, *Scorpaena plumieri*, *Crocodilichtthys gracilis* and *Synodus lacertinus* (Figure 4). The HBSC locations had a higher number of abundant (4.9%) and rare species (76.5%), as well as higher species richness, while LBSC locations had higher values of frequent (43.7%) and common (76.5%) species (Figure 4).

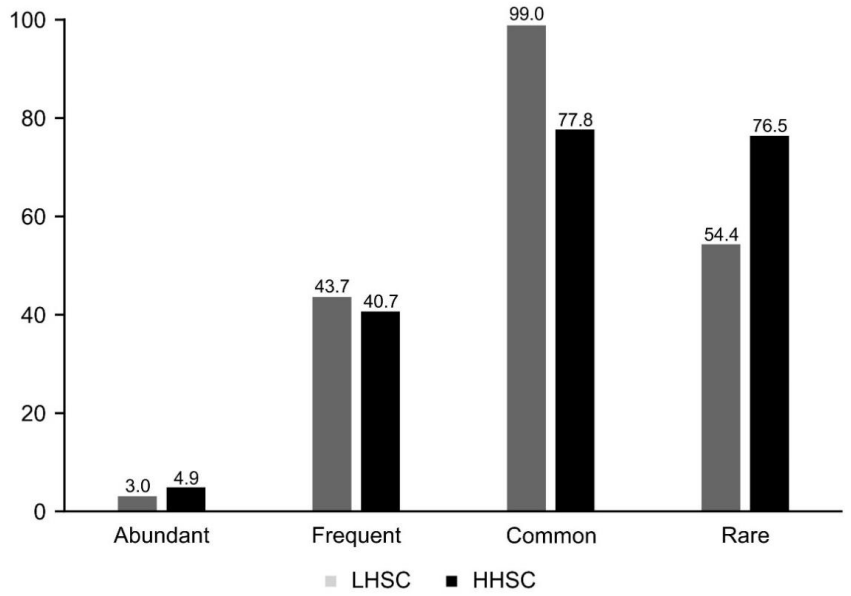

Figure 4. Classification of species according to their frequency and abundance in low benthic structural complexity (black bars) and high benthic structural complexity (grey bars) habitats.

Trophic Organization

Of the 89 species making up the fish fauna of the Los Frailes reef, 32% were at a trophic level between 3 and 3.5; only 21% were level 4 predators, while 12% belonged to low trophic levels (2 – 2.5). A total of 58% of fish species were associated to the bottom, 3% were species associated to the surface, and of the total of recorded species only five were territorial. There was a dominance of small-sized fish (67%), while there were few medium and large fish (25% and 8%, respectively). The type of fin of most species was emarginate (43%), followed by the lunate type (25%) and forked (10%).

Functional Trophic Groups

In HBSC areas a total of 89 species were recorded and a total of 21 functional trophic groups (FTG) were integrated (Figure 5). In the LBSC areas a total of 78 species were recorded, which were grouped into 19 FTG (Figure 6).

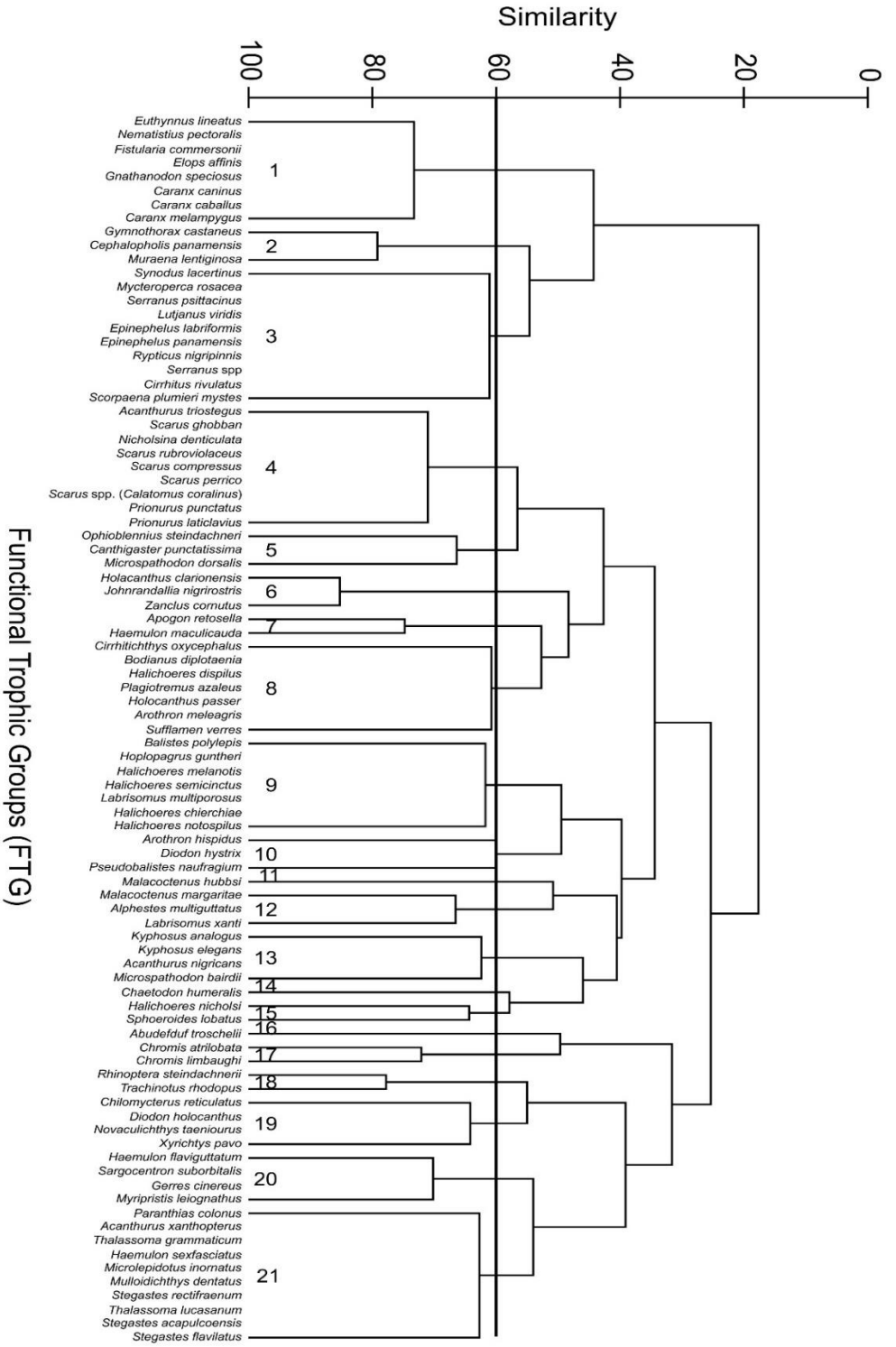

Figure 5. Functional groups in HBSC locations. Numbers represent each functional group and the species that comprise it.

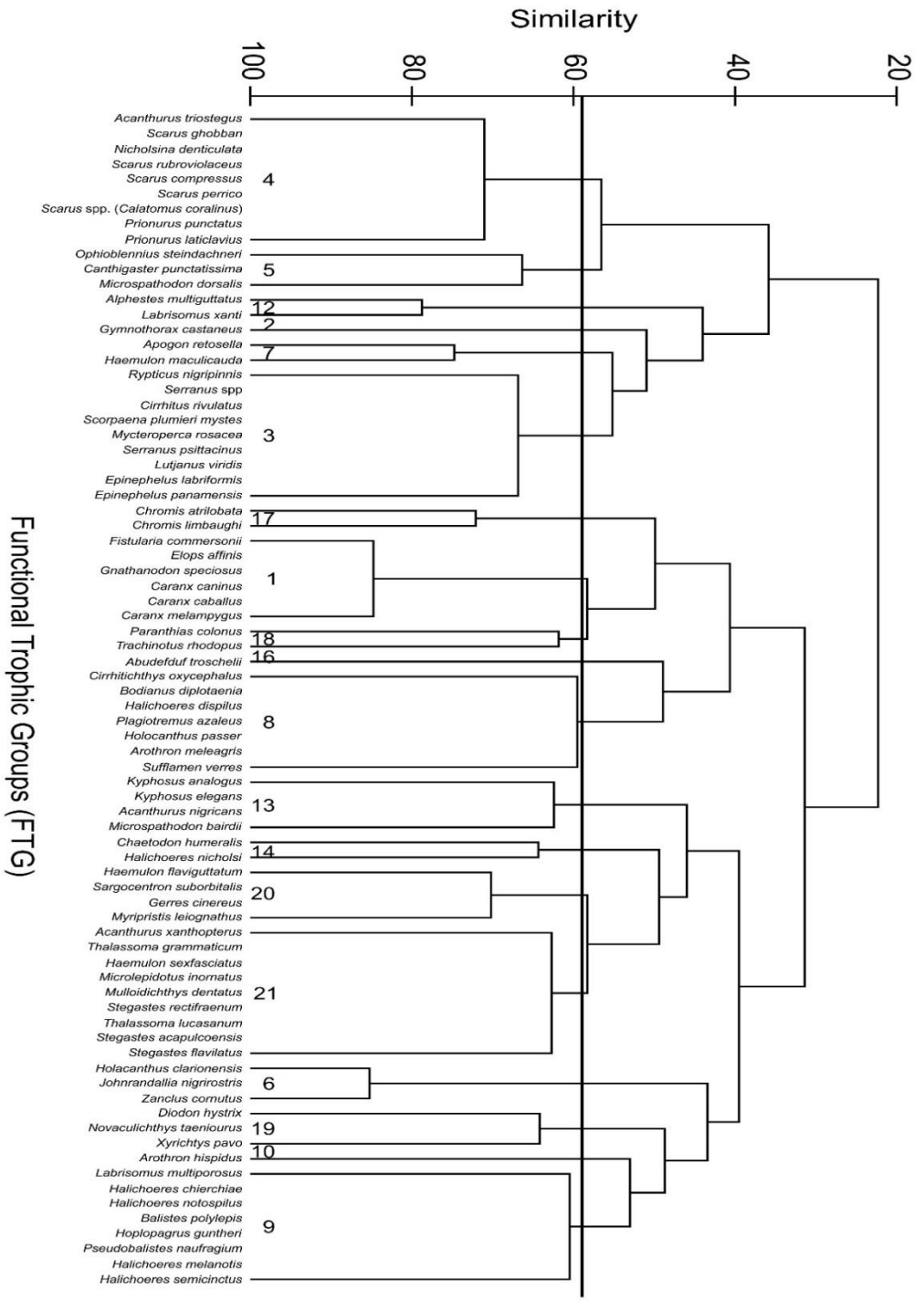

Figure 6. Functional groups in LBSC locations. Numbers represent each functional group and the species that comprise it.

There were differences in the number of species contained in the functional trophic groups between the areas. The FTG in the HSBC locations are denoted as 1, 2, 3, 4…, and FTG in the LSBC locations are denoted as 1ª, 2 ª, 3 ª, 4 ª…

1. This group was made up by mainly piscivorous species, most of which were pelagic and some demerso-pelagic. They were catalogued as common and rare.

1ª. This group comprised the same species as group 1, excepting *E. lineatus* and *N. pectoralis* which did not occur in LBSC areas.

2. This group comprised carnivorous species, which were not abundant and inhabited the reef bottom among rocks and cavities.

2ª. This was a mono-specific group (*Gymnothorax castaneus*).

3. This group was made up by piscivorous species, although some invertebrates were also included (crab, shrimp, polychaetes) in their diet. All these species had a high trophic level (>3.95); they inhabited the bottom and mid-water over the reef, and were common and rare species.

3ª. This group comprised nine species that had the same trophic function in LSBC as in HSBC locations.

4. This group was made up by herbivorous species (70-90% of the diet) and corallivorous species (20-10% of diet). The trophic level of this group was less than 2.5. Species occurred on the reef bottom and in mid-water.

4ª. This group comprised the same nine herbivorous species and had the same trophic relationships in HBSC as in LBSC locations.

5. This group was made up by three herbivorous species (40–60% of diet), although echinoderms, fish remains, sponges and bivalves were also included in their diet. Their trophic level was between 2.75 and 2.85. These were frequent and common species, associated to the bottom and territorial.

5ª. This group included the same species in LBSC locations.

6. In HBSC locations the group was comprised of species that fed on sponges (80 to 85% of diet), algae (5 to 10%), and invertebrates (less than 5% of diet). These were common species associated to the substrate and mid-water, with slow swimming speeds.

6ª. This group of species remained the same in LBSC locations.

7. This group included two species that fed mainly on shrimp and on a lower proportion of crab and other fish. These species were considered common and were associated with the bottom and mid-water.

7ª. This group did not change with the benthic structural complexity; the same members and trophic functions were maintained.

8. This group included species that fed on shrimp, sea urchins, crab, brown algae and sponges. The species were considered common and were found on the bottom and mid-water.

8ª. This group remained the same in LBSC locations.

9. Nine species comprised this group. They fed on echinoderms, pellecipods and gastropods. They were considered common and rare species related to the bottom.

9ª. This group comprised 10 species, also including *Pseudobalistes naufragium*.

10. This group was made up of three species that fed on echinoderms, sipunculids and tunicates. They had low abundance and frequency on the reef and were associated to the bottom.

10ª. This group was made up only by *Arothron hispidus*, since *D. hystrix* was not recorded in LBSC locations and *P. naufragium* was included in group 9 due to its trophic characteristics.

11. This group included only one species that fed mainly on ophiurids (sea stars). It had low frequency and abundance and was associated to the bottom.

11ª. The species that conformed this group in HBSC locations was not recorded in LBSC locations, so that this group did not occur there.

12. This group was made up of three species that fed on crab and complemented their diet with echinoderms and gastropods. They had a trophic level of 3.5 and were considered rare species on the reef.

12ª. This group was made up of only two species: *Alphestes multiguttatus* and *Labrisomus multixanti*, since *Malacoctenus margaritae* was not recorded in LBSC locations.

13. This group was made up of four species that fed on a high percentage of algae (over 90%) and a lower proportion of detritus (10%). In general these organisms had low presence on the reef and were found in mid-water.

13ª. This group did not show changes in the number of members and trophic functions in LBSC locations.

14. The only species in this group was *Chaetodon humeralis*. It fed on copepods, green algae, polychaetes, amphipods and shrimp. It was considered frequent on the reef.

14ª. This group did not occur in LBSC locations.

15. Two species made up this group. They fed on the same organisms (copepods, polychaetes, amphipods, bivalves and gastropods) mainly on the benthos. They had a high trophic level (>3.5) and were considered rare.

15ª. This group did not occur in LBSC locations.

16. The only species in this group was *Abudefduf troschelii*. It fed on anthozoans, copepods, tunicates, polychaetes and fish eggs. Its position was on the reef surface.

16ª. This mono-specific group did not show changes in LBSC locations.

17. This group was made up by two species of the Pomacentridae family, which fed on copepods, fish eggs and shrimp. They were found in the water column and only associated with the substrate to lay eggs.

17ª. This group remained constant in LBSC locations.

18. This group was made up of two species that fed on bivalves and gastropods, and that were considered rare.

18ª. This group was made up by *Trachinotus rhodopus* and *Paranthis colonus*, which consumed bivalves and gastropods. *Rhinoptera steindachneri* was not recorded in LBSC locations.

19. This group was made up by three species (*Diodon holocanthus, Novaculichthys taeniourus* and *Chilomycterus reticulatus*) that fed on gastropods and bivalves. Their trophic level was similar. These species were considered common to rare and were associated to the bottom and mid-water.

19ª. This group was made up by three of the four species, since *Chilomycterus reticulatus* was not recorded in LBSC locations.

20. This group was made up by four species that fed on polychaetes, shrimp, crab, gastropods and other benthic components. They were rare species associated to the benthos and demerso-pelagic.

20[a]. This group did not change as to the number of species or trophic organization in the two types of location.

21. This group was heterogeneous, and included 10 species that fed on ostracods, copepods, polychaetes, chaetognaths, crabs, echinoderms, gastropods, bivalves, sipunculids, algae (brown and green), isopods, fish eggs (associated to the benthos), and sponges. They were considered abundant and common.

21[a]. This group remained constant, excepting *Paranthis colonus*, which was included in group 18. The nine remaining species maintained their trophic functionality in LBSC locations.

Diagram of Biomass Flow in the Reef

In order to estimate the importance of the benthos in the trophic organization of the fish community of the Los Frailes rocky reef, a diagram of biomass flow was created of the different prey species and the fish community, categorizing it according to feeding components as herbivorous, omnivorous or carnivorous.

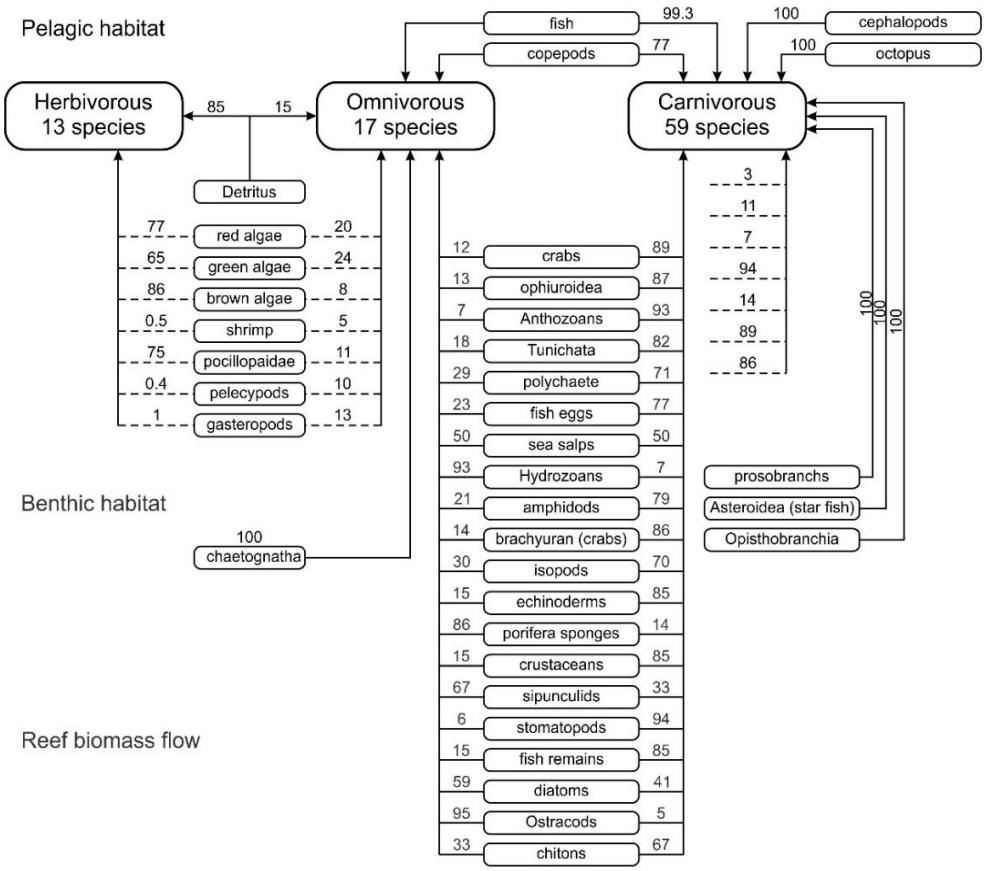

Figure 7. Diagram of biomass flow in Los Frailes reef. Feeding categories (large black rectangles), food components (small black rectangles), connections (arrows), and biomass percentage of each food component.

In the diagram of biomass flow the herbivore category comprised 13 species, which were assigned to trophic guilds 4 and 13. The omnivorous category comprised 17 species assigned to trophic guilds 5, 6, 14 and 21, while carnivores comprised 59 species assigned to the remaining 15 guilds.

Omnivores and carnivores shared 20 food components belonging to the benthos and 2 components from the pelagic zone. The chaetognaths were the only components that were exclusive to omnivores, while cephalopods from the pelagic zone, and prosobranchs, asteroids and opistobranchs from the benthic zone were exclusive to carnivores.

In general, the connections and biomass flow within the reef were varied, but there was great dominance of benthic species, which confirms the importance of invertebrates as a food source for reef fish (Figure 7).

DISCUSSION

Habitat Structural Complexity

The objective of the present chapter was to analyze the effect of benthic structural complexity (BSC) on the trophic organization of the conspicuous ichthyofauna of the Los Frailes reef. The first step was to evaluate some habitat characteristics such as number of cavities, substrate heterogeneity, rugosity, number of rocks, and depth. These attributes were considered ideal to measure the degree of BSC because they are conceptually descriptive, easily measured and allow comparisons at different spatial scales (McCormick, 1994).

According to results of the grouping analysis, zones one (Z1) and two (Z2) were categorized as low benthic structural complexity (LBSC) areas, while zones three (Z3) and four (Z4) were categorized as high benthic structural complexity areas (HBSC). McCormick (1994) and Bartholomew et al. (2000) reported that locations with high values of these attributes are not only more complex from the architectural point of view, but also from the biological point of view, so that it can be inferred that benthic complexity has an effect and is reflected on the trophic organization of the reef (Ángel & Ojeda, 2001).

LBSC locations were found in the shallow zone (between one and three meters depth), while HBSC locations were found at intermediate depths (between eight and nine meters depth). It should be mentioned that the grouping analysis did not take into account depth values because they affect grouping *per se*, and given the shallow depth of the Los Frailes reef (10m) it was considered that the effect of depth on community structure was limited, due to the variability of other factors such as temperature, hydrodynamic processes, water pressure, light intensity, and space available in the water column (Bayle-Sempere et al., 1994; Chabanet & Letourneur, 1995; García-Charton & Pérez-Ruzafa, 2001).

Substrate heterogeneity is an important part of HSC and has a differential effect on the ichthyofauna (Roberts & Ormond, 1987) because depending on its characteristics specific election preferences are created that influence directly the distribution and abundance of fish, and therefore, the community structure (Ruitton et al., 2000).

HBSC locations were characterized by a higher number of cavities than LBSC locations. Hixon & Beets (1989) reported that habitats with these characteristics offer a higher number

of potential refuges, which promotes recruitment by juveniles and increases the number of species (predators and prey) and their density (Shulman, 1984; Kellison & Sedberry, 1998).

HBSC locations were located between the rocky reef and sandy areas of the ocean bottom. This "edge effect" promoted higher abundance and diversity due to the convergence of different species that inhabit both habitat types. In other parts of the world this same pattern has been observed, and the presence of rare species associated only to these transition zones has been detected (Acosta & Robertson, 2002; Friedlander & Parrish, 2002).

Rugosity is defined as the ratio of substrate contour to linear distance (Luckhurst & Luckhurst, 1978), that is, how sinuous the bottom contour is. This characteristic provides refuge and space for new recruits, and settlement areas for algae and invertebrates, in an indirect manner. Martin-Smith (1993) reported that epifaunal settlement is dictated by high substrate rugosity, because substrate is proportional to the contact area of organisms. Rugosity was also different among locations, being lower for LBSC areas.

Each HSC has an effect on the structure of the fish community to a greater or lesser degree and therefore on trophic organization, so that it is important to determine the relationship between the habitat (benthos) and the species, since each modification to HSC will have an effect on the fish community structure.

Fish Community Composition

The list of fish species in the Los Frailes reef during the two year study period comprised 89 species (Table 1). The reef contained approximately 0.8 species m^{-2}, which makes it more diverse than other nearby areas within the Gulf of California. The Los Frailes reef shelters the equivalent of 31% of reef fish species recorded for the entire Gulf of California (281 species; Thomson et al., 2000), and 38% of species reported for the Cabo Pulmo reef (236 species recorded over a 12 year period; Villareal-Cavazos et al., 2000).

It should be mentioned that this chapter focused mainly on conspicuous species, underestimating cryptic and nocturnal species (Sale, 1997; Villareal-Cavazos et al., 2000; Romero-Ponce, 2002). Other contributions of this chapter lay in the systematic sampling and methods used, which, although they did not take into account cryptic species, were relevant for comparative analyses of seasons and habitats with different degrees of structural complexity.

Studies carried out in several areas within the Gulf of California are not easily comparable among each other due to differences in structure, depth, number of refuges and latitude of the reefs, and in some cases, due to the existing variation in the census method used. In other Gulf of California locations fish studies have been carried out using visual censuses. Pérez-España et al. (1996) recorded 75 species in four areas within the Bahía de La Paz; Sánchez-Ortíz et al. (1997) recorded 101 species in 11 locations ranging from north to south from Isla San Dieguito to Isla Cerralvo; Arreola-Robles & Elorduy-Garay (2002) recorded 80 species during censuses carried out in five islets and one wreck in the Espíritu Santo archipelago; Aburto-Oropeza (1999) counted 102 species, of which only 72 were identified using visual censuses (Aburto-Oropeza & Balart, 2001); and Villegas-Sánchez et al. (2009) recorded 84 species at five locations of Isla San José. Species richness in Los Frailes reef (with a lower number of sampling areas) was similar or even higher than the species richness reported in those studies.

Trophic Organization of the Fish Community

The highest abundance of recorded species (89) in the Los Frailes reef corresponded to carnivorous fish, followed by omnivores and a low percentage of herbivores. This type of trophic structure is commonly found in tropical and subtropical areas of the eastern Pacific (Roberson et al., 2004), including Cabo Pulmo (Álvarez-Filip & Reyes-Bonilla, 2006), Bahía de La Paz, Loreto, Bahía de Los Ángeles (Viesca-Lobaton et al., 2007), Bahía de Acapulco (Palacios-Salgado, 2005) and the Clipperton Atoll (Allen & Robertson, 1997).

It should be mentioned that despite there being fewer herbivorous than carnivorous species in these systems, their relative abundance in tropical areas is much higher than in temperate areas, with a gradual decrease from the equator towards the poles. This could be due to fish having more efficient digestive processes in warm waters (Floeter et al., 2004).

Among the most abundant carnivorous species in the Los Frailes reef are *Lutjanus viridis*, *Mycteroperca rosacea*, *Serranus psittacinus* and *Cephalopholis panamensis*. All these species feed mainly on fish, and according to their size and swimming capabilities, can complement their diet with invertebrates from the reef itself or from nearby sandy areas (Hobson, 1968; Thomson et al., 2000). Among low abundance carnivorous species are *Muraena lentiginosa*, *Synodus lacertinus* and *Alphestes multiguttatus*, which are associated to edge areas of the reef; they are ichthyofagous and have a stalking tactic to hunt their prey (personal observation). Carangids such as *Caranx caninus* and *Caranx melampygus* are visual, active predators that spend a great part of their time on the reef searching for prey (Hobson, 1968; Cervigón, 1972).

One of the main herbivorous species on the reef was *Prionorus punctatus*, which is one of the most characteristic fish species in Baja California Sur reefs (Montgomery et al., 1980). It feeds on filamentous and crustose algae, and forms schools that move about the reef stopping briefly to feed (Moreno-Sánchez et al., 2014). Species that feed on coral include the scarids *Scarus ghobban*, *S. compressus*, *S. perrico*, and *S. rubroviolaceus*. These species were abundant probably because of the great quantity of coral present (Álvarez-Filip, 2004).

Plagiotremus azaleas was categorized as a parasitic species, since if fed on the mucus and skin of other fish (Hobson, 1968; Fischer et al., 1995). Omnivores were the second representative group on the reef, of which the species *T. lucasanum*, *S. rectifraenum*, *M. dorsalis*, *H. passer* and *A. meleagris* were most abundant. These species fed on invertebrates and algae, and are considered key in the trophic webs of reef systems (Acero & Rivera, 1992), because detritus enters the food chain of fish mainly through benthic invertebrates that are preyed on by these species (Lieske & Myers, 1996).

Effect of Benthic Structural Complexity on the Trophic Organization of the Ichthyofauna of the Los Frailes Reef

It was determined that the benthic structural complexity had little effect on the trophic organization of the fish community at Los Frailes reef. A total of 21 functional trophic groups (FTG) were defined at LBSC locations, and 19 FTG were identified at LBSC locations. These results contrast with what was reported by Ángel & Ojeda (2001), who compared two macroalgal areas with different HSC levels in the northern Chile coast. These authors found that complex habitats (benthos with different algae species, variation in density, size and

shape of fronds) provided higher spatial heterogeneity for the settlement of a greater quantity and variety of invertebrates and fish. The FTG were more interconnected due to the manner in which prey were shared.

This pattern has also been reported for fish communities in rivers. Pouilly et al. (2006) documented a change in the trophic structure of rivers in Bolivia due to the habitat structural complexity, and recorded changes in conductivity, pH, temperature, slope, speed and width of the river. These factors had a favorable effect on primary productivity.

Willis et al. (2005) detected a positive correlation between benthic structural complexity and functional diversity in the fish associations present in the Cinaruco River in Venezuela. The relationship was based on leaf litter mounds, logs and other substrate providing more area for higher benthic production and resulting in greater density of fish that used these sites for feeding and protection.

The abundance and diversity of resources in this type of habitat allow fish to exploit resources in different ways. For example, fish enter into cavities and crevices of logs to feed and seek protection. Larger omnivores with protractile mandibles can also feed on the organisms found in crevices (Angermeier & Karr, 1984), promoting in this way an increase in the presence of different species and therefore of fish diversity.

Friedlander & Parrish (1998) reported that in several locations in Hawaii the spatial relief of the reef bottom, equivalent to the number and size of cavities, depth, bottom configuration and location of reef patches, was the main promoter of biomass and abundance of the fish community.

The discrepancy between the results presented in this chapter with what was found by the previously-mentioned authors is probably related to the fact that we did not analyze the diets of all species at each location, and that diet was assigned independently of the sampling area. This was decisive in not finding significant differences in the number of FTG. Ángel & Ojeda (2001) stated that richness and abundance of species were not critical in the conformation of the number of FTG. A clear example of this is *Isacia conceptionis*, a species recorded at both location types. In high complexity locations, this species functions as an omnivore feeding on the benthos (on 5 main food components), while in low complexity areas it functions as a pelagic carnivore (with 3 main food components).

It was determined that *Arothron meleagris* was an omnivorous species, since it fed on a great variety of benthic species (sponges, sea urchins *Echinometra vanbrunti*, corals *Pocillopora* spp., *Porites* spp., bryozonas and *Crepidula arenata*). Reyes-Bonilla & Calderón-Aguilera (1999) reported that this same species in a nearby zone of Cabo Pulmo fed on coral, which could be indicative of its trophic plasticity.

The diagram of the fish community biomass flow showed high flow among the different categories. This same pattern was documented in Cape Hatteras, North Carolina, by Garrison & Link (2000), who did not find differences in the trophic structure of 40 species that integrated 14 trophic guilds. In that study a wide spatial scale was covered with 400 sampling stations, a depth range of 8 to 400 m, and diets that were sampled over 25 years. The authors reported that ontogenetic diet changes were the main factors that determined the differences found between trophic guilds, and that these changes were produced by morphological and habitat changes. Although that study was not focused on determining the effect of habitat complexity, the authors mentioned that a more detailed analysis could have found differences related to those factors.

Aburto-Oropeza & Balart (2001) reported that in Bahía de La Paz the structural complexity of the benthos (rugosity, size of cavities, cover of the different substrate types) could have a different effect on the behavior of individuals that chose sites for protection, feeding, reproduction and recruitment. The authors determined that several species were generalists with a wide distribution over different habitats. They also reported that empty spaces can be important for larvae and juveniles, and that survival depends in great part on the characteristics of the location.

Viesca-Lobaton et al. (2007) compared the functional groups formed in Bahía de La Paz, Loreto and Bahía de Los Ángeles. They reported that although the presence of functional groups was maintained in the entire area, there was an ecological substitution of species among regions due to the latitudinal change. This was possibly associated to the different physiological adaptation of species to cold water conditions, and secondarily, to a change in the feeding habits of the species due to resource availability.

It should be pointed out that this same effect occurred in the functional trophic groups (FTG) formed in Los Frailes reef. Although almost the same FTG were maintained, within each FTG some species and their abundance varied slightly depending on the structural characteristics of the habitat. For example, FTG number one was comprised of coastal pelagic species with ichthyofagous habits (*Euthynnus lineatus, Nematistius pectoralis, Fistularia commersoni, Elops affinis, Gnathanodon speciosus, Caranx caninus, C. caballus* and *C. melampygus*) (Hobson, 1968; Sierra et al., 1990; Fischer et al., 1995; Thomson et al., 2000). The first two species did not occur in the LBSC locations because they are mainly oceanic. The presence of FTG one in the reef is related to feeding, because during sampling this FTG was observed feeding on *Harengula thrissina*, an abundant prey species during the summer months, when the presence and frequency of predators was also higher. This same behavior has been reported in several locations of the Gulf of California and Bahía Magdalena (Thomson et al., 2000; Moreno-Sánchez, 2004).

Fistularia commersoni shares few morphological characteristics with the remaining species, but was integrated into FTG one due to its trophic level. It occupies the same space in the water column as the other members of this group, feeding on recently settled fish (mostly in LBSC locations), using a stalking tactic. Hobson (1968) reported that this species feeds on reef fish smaller than 5 cm at any time of day.

The species from the Carangidae family (*Gnathanodon speciosus, Caranx caninus, C. caballus* and *C. melampygus*) feed on fish but also consume components of the benthos to complement their diets. They are active and visual predators that feed mainly during the day (Daneman, 1993; Cruz-Escalona & Abitia-Cárdenas, 2004).

The second trophic functional group included *Gymnothorax castaneus, Muraena lentiginosa* and *Cephalopholis panamensis* at HBSC locations, while *Gymnothorax castaneus* only occurred at LBSC locations. These three species have similar feeding habits, consuming a high percentage of fish and a lower proportion of shrimp and crab (Randall, 1967; Raymundo-Huizar, 2000; Thomson et al., 2000). They are ambush predators associated to rocky bottoms that can be found among cavities and crevices from where they attack their prey; they have no specific feeding time (Hobson, 1968). The HSC could have an important effect on their presence and feeding.

In HBSC areas species usually employ stalking or ambush tactics because there are more places to hide (Coen et al., 1981). In fact, the coexistence of ecologically similar species could be possible due to resource sharing along one or more niche edges (Gladfelter &

Johnson, 1983), which in this case would be the number and size diversity of cavities as well as consumption of prey species, since despite feeding on the same items, the proportion of each species was different. In this scenario *Gymnothorax castaneus* could occur over the whole reef, independently of benthic complexity. This species´ morphology, aggressiveness, voracity and trophic plasticity give it a competitive advantage over the other two species (Werner & Hall, 1988).

The third FTG included species catalogued as roaming species that are distributed near the bottom without depending on it (demersal). They have a wide trophic spectrum comprised of fish, crab, shrimp, and polychaetes (Randall, 1967; Hobson, 1968; Fischer et al., 1995; Raymundo-Huizar, 2000; Thomson et al., 2000).

This FTG included the same members at both locations, except for *Synodus lacertinus*, which was not recorded at LBSC locations because its main prey occur at the edges of rocky reefs and sandy areas. It is a stalking predator, a voracious carnivore that propels itself from the bottom upwards to capture prey, usually small fish (Fischer et al., 1995; Raymundo-Huizar, 2000), and tends therefore to be located in deep areas. The characteristics that could favor this behavior can be observed mainly in HBSC locations. The occurrence of this FTG at both locations suggests efficiency in sharing of feeding resources.

FTG number four was made up by herbivorous species that occurred in both types of benthic complexity, although the abundance and frequency at each location was different. In general these species feed on three types of algae (Chlorophyta, Phaeophyta and Rhodophyta) and coral (Randall, 1967; Montgomery et al, 1980., Thomson et al., 2000). Anaya-Reyna & Riosmena-Rodríguez (1996) reported that 61 species made up the systematic list of macroalgae present in the coral reef of Cabo Pulmo-Los Frailes, highlighting the algal diversity in both areas.

The analysis of the feeding habits of the herbivores *Acanthurus nigricans* and *Prionorus punctatus* indicated that they have different diets (Abitia-Cárdenas et al., 2011; Moreno-Sánchez et al., 2014). *A. nigricans* feeds mainly on *Ulva linza* (50% relative importance) and *P. punctatus* feeds on *Gracilaria* spp., *Jania Mexicana* and *Hypnea musciformis*. Montgomery et al. (1980a, b) compared the diet of two herbivorous species (*Stegastes rectifraenum* and *Microspathodon dorsalis*) that differed considerably in their feeding behavior in this same reef: the first is selective and the second is not. In both cases the sharing of resources among herbivores was manifest.

The presence of species from the Scaridae family (*Scarus ghobban, Nicholsina denticulata, S. rubroviolaceus, S. compressus, S. perrico* and *Calatomus* spp.) could be explained by the nearness of Los Frailes reef to Cabo Pulmo, where they are common, implying that they could move from one reef to the other. These species feed on live coral, which in Los Frailes reef comprises up to 10% of cover. Their presence could also be due to the need to find refuge at night, as these organisms rest among cavities and crevices of rocky areas and segregate a mucus that presumably prevents predators from smelling them (Thomson et al., 2000; Viesca-Lobatón, 2003).

There was temporal segregation between *Acanthurus nigricans* and *Prionurus punctatus* in FTG four. *A. nigricans* occurs in higher abundance during the cold season and in lower abundance during the warm season. *P. punctatus* is common all year long. This FTG was not affected by the HSC, because these species feed on algae with a high turnover rate (Abitia-Cárdenas et al., 2011; Moreno-Sánchez et al., 2014).

FTG five was made up of species that feed mainly on algae and also consume invertebrates (Randall, 1967; Hobson, 1974; Montgomery, 1980; Thomson et al., 2000). These species are morphologically similar; they feed on the same prey and occur in the same position on the reef. Although this functional group was integrated in the two HSC types, the abundances of *O. steindachneri* and *C. punctatissima* were higher at HBSC locations, probably because these species need refuges to get protection from predators (Thomson et al., 2000). However, their dependence on the substrate was markedly higher than that of *M. dorsalis*, which is an herbivorous species that moves near the substrate but only depends on it to lay eggs (Hernández-Olalde, 2008). In general this FTG is little affected by the HSC and occupies both areas.

The sixth FTG occurred at both locations. The species that made up this group fed on algae, invertebrates and a high percentage of sponges (Hobson, 1974). These species had moderate abundance and frequency (common species). The presence of the group at both HSC locations could be related to spacing within the reef because none of these species depends strictly on the substrate (Thomson et al., 2000).

The seventh FTG group was made up by *Apogon retrosella* and *Haemulon maculicauda*, which were present at both HSC locations. Their diet was composed of shrimp, crab and fish. They are considered common species that occupy the space from the bottom to mid-water; they are nocturnal species.

The eighth FTG was one of the most diverse in trophic spectra, feeding behavior and morphology, and could be found at all locations. The diet of the species included this group was made up of algae and invertebrates (Randall, 1967; Hobson, 1968; Hobson, 1974; Pérez-España & Abitia-Cárdenas, 1996; Thomson et al., 2000). The abundances of each species varied among locations. For example, at HBSC locations, *C. oxycephalus*, *H. dispilus* and *P. azaleus* were numerous. These are small-sized species that measure between 5 and 15 cm and need to be close to their refugia to feed and hide (Hobson, 1976). *B. diplotaenia* and *H. passer* were considered roaming species in the reef: *B. diplotaenia* feeds on organisms on the substrate, and *H. passer* is coprofagous, living in the water column, usually under schools of *C. atrilobata* (Reynolds & Reynolds, 1977; Pérez- España & Abitia-Cárdenas, 1996; Aburto-Oropeza et al., 2000; Thomson et al., 2000). *Arothron meleagris* and *Sufflamen verres* were more abundant at LBSC locations. These are generalist species, with great trophic plasticity (Reyes-Bonilla & Calderón-Aguilera 1999; Thomson et al., 2000; Moreno-Sánchez et al., 2009).

The ninth FTG included species that fed on echinoderms, pellecipods and gastropods (Hobson, 1968; Hobson, 1974). These species were not very abundant or frequent, and were affected by the HSC. These labrids are small and live among crevices to avoid being preyed on; they were abundant at HBSC locations (Thomson et al., 2000). Of the four recorded species, two were the most abundant (*H. notospilus* at LBSC locations and *H. semicinctus* at HBSC locations) so that despite this group occurring at both locations, there was spatial segregation.

Hoplopagrus guntheri and *L. multiporosus* are relatively small species that also occupy cavities and crevices for refuge. These are icthyofagous reef-roaming species (Thomson et al, 2000).

Despite this functional group occurring in both areas, the abundance of its members changed according to the HSC. Most members use cavities and crevices to find refuge; therefore the species most dependent on the substrate were most abundant in HBSC areas. In

a similar way to previous cases, there was sharing of resources that facilitates coexistence among species.

The tenth FTG included *Pseudobalistes naufragium, Diodon hystrix* and *Arothron hispidus* in HBSC locations. *A. hispidus* was recorded only in LBSC locations. The three species depend on substrate characteristics to feed and are catalogued as reef-roaming species. The most important food components were sea urchins, which can be consumed due to the characteristics of the mouth apparatus of these species. *Diodon hystrix* and *A. hispidus* have plate-shaped teeth and *P. naufragium* has eight prominent teeth (Fischer et al., 1995; Thomson et al., 2000). These species complement their diet with sipunculids and tunicates (Randall, 1967). This FTG was more abundant at HBSC locations where a greater quantity of echinoderms was recorded.

The eleventh FTG was monospecific, with only *Malacoctenus hubbsi* being recorded. This species requires specific habitat characteristics and is therefore sensitive to benthic complexity. Thomson et al., (2000) reported that this is an aggressive and abundant species that inhabits preferentially depths over 8 m and feeds mainly on sea stars. In the Los Frailes reef it was recorded at HBSC locations, where this type of habitat is commonly found.

The twelfth FTG group included *Alphestes multiguttatus, Labrisomus xanti* and *Malacoctenus margaritae*. These species were recorded at both locations, except *M. margaritae* which was not found at LBSC locations. This is probably related to this being a comparatively small species that also needs small cavities, which are more abundant and frequent at HBSC locations. *A. multiguttatus* and *L. xanti* are larger species that depend on the substrate for feeding but are not territorial. Although the abundances of the members of this group differed depending on the HSC, they could carry out their activities in both areas.

The thirteenth FTG group, which included the herbivorous species *Kyphosus analogus, K. elegans, Acanthurus nigricans* and *Microspathodon bairdii*, was sensitive to the HSC. These species feed on algae, diatoms and detritus (Hobson, 1974; Montgomery, 1980 a and b; Thomson et al., 2000). No effect of benthic characteristics could be identified, since algal production is sufficient for all herbivorous species present in the area, although competition with more territorial or aggressive species could influence the presence of some of the members of this group, such as *S. rectifraenum* (Hobson, 1968, Moreno-Sánchez et al., 2011).

The fourteenth functional group included only *Chaetodon humeralis* at HBSC locations. This fish fed on copepods, green algae, polychaetes, amphipods, and shrimp, among other components (Randall, 1967; Hobson; 1974; Thomson et al., 2000). Due to its morphological characteristics this species could compete with FTG 15. There seems to be spatial segregation since this FTG did not occur at LBSC areas.

Despite *Halichoeres nicholsi* and *Sphoeroides lobatus* being different morphologically (Fischer et al., 1995), they made up the fifteenth functional group because they share food resources (copepods, polychaetes, amphipods, bivalves, gastropods). *H. nicholsi* needs cavities to find refuge from its predators and moves from the bottom to mid-water. *S. lobatus* inhabits preferentially the reef edges (rock-sand interface; Randall, 1967; Thomson et al., 2000). In this system this species was classified as rare. Due to its habitat requirements it was not recorded in LBSC locations. For this FTG the HSC did have an effect on *S. lobatus* due to its habitat requirements.

The sixteenth FTG was monospecific, it only included *Abudefduf troschelii*. This species has characteristics that give it a competitive advantage over other species such as *C. atrilobata*. For example, it has a wide feeding spectrum (including anthozoans, copepods,

tunicates, polychaets and fish eggs) and diverse feeding areas (water column and benthos). The dependence of this species on the substrate occurs during the reproductive period. When it nests its eggs need cavities with specific size and shape, with presence of algae (Hernández-Olalde, 2008). In general the species was well represented in both locations. There are reports of this species interacting with *C. atrilobata* in the pelagic zone and with *S. rectifraenum* on the benthos. *A. troschelli* occurs all year long and practically in all reef environments. It was classified as a frequent species.

The seventeenth FTG included two Pomacentrids, *Chromis atrilobata* and *C. limbaughi*. Both species were recorded in LBSC and HBSC locations, although the highest abundances and frequencies occurred in HBSC locations. At these locations depth is considerable, and in this type of habitat cavities are heterogeneous and are used as refuge areas during the night (Hobson, 1968; Hobson, 1974; Thomson et al., 2000). Strong interspecific competition could be detected in this functional group because both species use benthic resources in a similar way and feed practically on the same prey.

The eighteenth FTG included *Trachinotus rhodopus* and *Rhinoptera stendanchneri* in HBSC locations, while in LBSC locations *R. stendanchneri* was substituted by *Paranthias colonus*. The HSC had an important effect on this FTG, due to the habitat requirements of *R. steindanchneri*. This species needs sandy substrate near the reef edge, where it searches for food (bivalves and gastropods) at depths over 7 meters (Thomson et al., 2000).

Trachinotus rhodopus is a carangid with all the characteristics of a pelagic organism, but it feeds on a high percentage of benthic organisms (bivalves and gastropods; Randall, 1967; Moreno-Sánchez, 2004). *P. colonus* is a mainly planktofagous species, although it also consumes a lower proportion of organisms from the benthos (Thomson et al. 2000). In general this group shares space and food resources, which probably allows them to coexist.

The nineteenth FTG included *Chilomycterus reticulatus, Diodon holocanthus, Novaculichthys taeniourus* and *Xyrichtys pavo*, of which only *C. reticulatus* did not occur in LBSC locations. These species feed on gastropods and bivalves (Randall, 1967; Hobson, 1974; Thomson et al., 2000) and are associated to the bottom and mid-water. Their abundances changed according to the HSC, being higher in HBSC areas, probably due to the greater quantity of invertebrates present at these locations.

The twentieth FTG was made up by *Haemulon flaviguttatum, Sargocentron suborbitales, Gerres cinereus* and *Myripristis leiognathus*, and occurred in both HSC locations. It should be mentioned that all these species are considered rare. They feed on a great variety of benthic organisms such as polychaetes, shrimp, crab and gastropods (Randall, 1967; Hobson, 1968; Thomson et al., 2000), which could explain their presence in both areas.

The twenty-first FTG was one of the most diverse in its feeding habits and was made up by three pomacentrids (*Stegastes rectifraenum, S. acapulcoensis* and *S. flavilatus*), two labrids (*Thalassoma lucasanum* and *T. gramaticum*), two haemulids (*Haemulon sexfasciatum* and *Microlepidotus inornatus*), an acanthurid (*Acanthurus xanthopterus*), a mullid (*Mulloidichthys dentatus*), and a serranid (*Paranthias colonus*).

The feeding requirements of *S. rectifraenum, S. acapulcoensis* and *S. flavilatus* are similar. They occupy a territory of approximately one meter in radius, which they defend constantly to reduce predation pressure (Wellington, 1982; Wellington & Víctor, 1988, Moreno-Sánchez et al., 2011). Pomacentrids occurred at both location types but their abundances were higher in HBSC areas, possibly because there is a greater number of cavities and refuges there. During the two years of sampling it was observed that despite these species

sharing the same areas, there was a succession, with *S. flavilatus* being found first, then *S. rectifraenum*, and lastly *S. acapulcoensis*. It is possible that *S. rectifraenum*, being the dominant species, occupies the best sites.

The labrids *T. lucasanum* and *T. gramaticum* use the benthos in a similar way, with *T. lucasanum* being dominant because its abundance is ten times higher than that of *T. gramaticum*. Its trophic diversity, enormous fecundity, capacity to reproduce twice a year and to tolerate wide variations in temperature confer it a good competitive advantage (Fischer et al., 1995; Thomson et al., 2000; Robertson & Allen, 2002, Hernández-Olalde, 2008).

The serranid *Paranthis colonus* was included in this FTG due to the quantity of copepods that it consumes. This species does not compete with the other group members because it is distributed over the entire water column (Thomson et al., 2000; Robertson & Allen, 2002). *Acanthurus xanthopterus* feeds on benthic invertebrates (Hobson, 1968; Fischer et al., 1995; Thomson
et al., 2000).

The haemulid *Haemulon sexfasciatum* fed on brachiurans, but contrary to other members of this FTG, it fed during the night, roaming around the edges of the reef during the day. It is possible that competition with this species is lower because of the wide heterogeneity of its diet (number and diversity of components). There was no change in this species´ abundance with HSC (Hobson, 1968; Fischer et al., 1995; Thomson et al., 2000).

Microlepidotus inornatus fed mainly on sipunculids and other benthic invertebrates. The highest abundances of this species were recorded at HBSC locations. It is a nocturnal predator and during the day it forms schools that roam the reef (Hobson, 1968; Fischer et al., 1995; Thomson et al., 2000).

Mulloidichthys dentatus feeds on small bivalves and crabs in rocky and sandy areas. It is a nocturnal predator but the smaller individuals feed during the day. This could explain the higher abundances recorded at HBSC locations, where observed individuals were small (10 to 15 cm). Competition for food with the other members of the FTG was low because the total population size is small (Hobson, 1968; Fischer et al., 1995; Thomson et al., 2000).

The benthic structural characteristics did not have a significant effect on the trophic organization of the fish community, because although there were small changes in the number of FTGs and in the species that integrated them, these variations were not significant. This could be indicative of food not being a limiting factor in Los Frailes reef. It is known that upwelling occurs at the mouth of the Gulf of California all year, enriching superficial waters, favoring algal blooms and increasing the general system productivity (Álvarez-Borrego & Lara-Lara. 1991). Results could also be explained by the high redundancy of the functional groups present in the reef (Álvarez-Filip & Reyes-Bonilla, 2006).

CONCLUSION

Benthic heterogeneity, the number and size of cavities, the size of rocks, rugosity, and depth are robust variables that characterize adequately the benthic structural complexity (BSC) of the Los Frailes reef. Ecological indices of the fish community structure (species richness and abundance) were higher at HBSC locations than at LBSC locations. It is possible that this is due to an increase in BSC being related to a higher availability of benthic micro-habitats. The BSC did not have a measurable effect on the trophic organization of the fish

community in Los Frailes reef. It was observed that at both locations there was almost the same number and composition of trophic functional groups (high functional redundancy). Few species differed from this pattern. This is possibly due to the restricted effect that the benthic structural characteristics have on fish feeding habits.

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Chapter 17

MARINE SEAWEEDS OF THE YUCATAN PENINSULA: DIVERSITY, ECONOMIC IMPORTANCE AND CONSERVATION

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ABSTRACT

The Mexican Caribbean and the Atlantic coast of Mexico have 3,294 km of coastline with diverse habitats and rich biota. Coastal ecosystems, unfortunately, are experiencing wide range of pressures due to siltation, eutrophication, coastal development and climate change. Those species that adapt to these pressures will expand their living boundaries while others may fade away. Accordingly, the study of coastal biodiversity is of great concern globally and constitutes an important element of global change research. Peninsula of Yucatan has 1,940 km of coastline, reportedly with rich diversity of seaweeds. Previously published accounts on seaweed biodiversity were mainly in the form of checklists. The present study is a timely publication based wholly on primary data. Data were collected through extensive and systematic field studies conducted by the authors during different seasons over an eleven years period since 2004 to 2014, also we checked herbarium's specimens that are housed in the herbarium ENCB. Analysis of the information showed that Rhodophyta has the greatest diversity with 317 species; Chlorophyta has 180; Phaeophyceae 70 and finally Cyanobacteria with 38 species. One of the groups best represented in the study area is the Order Corallinales with two families and 43 species, these organisms are of major ecological importance in the reefs of the Peninsula. Three species are new records from Mexican Caribbean, *Centroceras*

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hyalacanthum, *Metapeyssonnelia tangerina* and *Sarcodiotheca divaricate*, and sixteen species are new records for the study area The importance of epiphytic algae in the study area is discussed as well as the use of molecular markers in order to solve taxonomic problems of selected genera of algae. The floristic list includes data on distribution, tidal level and habitat. On the other hand Quintana Roo is the state that has the greatest diversity of marine algae in the Gulf of Mexico and Mexican Caribbean's shores with 551 species. Topics on economic importance and extraction of bioactive substances are discussed, as well as suggestions on conservation of seaweeds from Yucatan Peninsula. Finally, several coastal towns in the Yucatan Peninsula are being impacted by natural events such as hurricanes as well as urban; tourism and industrial development, so it is important continue studies that allow us to characterize the changes that populations of seaweeds are undergoing.

INTRODUCTION

Floristic studies on the marine algae growing in Yucatan Peninsula area were made by several workers [1, 2]. Huerta et al. [1] reported on a preliminary survey of shallow water marine algae of coastal and islands area of Yucatan Peninsula, although much pertinent new information has accumulated since its publication. Aguilar-Rosas et al. [3] and Collado-Vides et al. [4] studied the Chlorophyta of the Mexican Caribbean. Callejas-Jiménez et al. [5] reported the seaweeds of several localities of Campeche; Mateo-Cid et al. [6], publishes lists on subtidal Rhodophyta of Cozumel Island, Mendoza-González et al. [7] listed offshore seaweeds of Mujeres Island. Cetz-Navarro et al. [8] reported new records of seaweeds for the Mexican Atlantic coast as well as update data on the floristic richness of the Mexican Caribbean. Mateo-Cid et al. [9] presented the results of the study on benthic marine algae in eight localities from the coast of Campeche; these authors reported 30 new records of seaweeds for Campeche. Until now have been recorded 635 species of seaweed for the Yucatan Peninsula, in this sense, it is noted that the algal flora of this region is still not well known. On the other hand is obvious the lack of knowledge on ecology and conservation of marine algae in this region, in addition oil spills and pollution are a persistent danger for the significant risk involved for human lives and the health of shallow and deep marine habitats. Furthermore, it is remarkable the numbers of hurricanes that have affected the Yucatan Peninsula in the last ten years, the risks associated with hurricanes are: storm surge, high winds, heavy rains, landslides and flooding. It is expected that under these arguments is important to know the changes in the short and medium terms of marine algae in this region, which possible to assess the risks to which these organisms as well as their management and sustainable use.

The present chapter was undertaken with a view to know the composition of seaweed and its distribution in the study area, as well some effects of pollution and human. The data achieved for a period of ten years from July 2004 to June 2014 are presented in this chapter and would be very much useful to the seaweed industries about the occurrence of economically important agar and algin yielding seaweeds for commercial exploitation and for conservation.

Study Area

The Yucatan Peninsula is a biogeographical area comprising the entire territory of the Mexican states Campeche, Yucatan and Quintana Roo, adjacent small portions of Tabasco and Chiapas, The El Petén department in Guatemala, and the northern half of Belize (Figure 1). The coastal environment of the Yucatan Peninsula includes a wide variety of ecosystems ranging from mangroves to coral reefs, resulting in a heterogeneous landscape. Specifically, the marine system is characterized by environmental differences which respond to regional and local forcing functions such as marine currents and groundwater discharges. Yucatan Peninsula has 1,940 km of coastline, its coast has been characterized as low lying coastal area, where 57% is represented by coastal lagoons with barrier islands and 43% is ocean front, 85% of this ocean front is sandy coast. The tidal regime is mixed with a diurnal dominance, with a tidal variety of 0.1 m for neap tides and 0.8 m for spring tides. Reported grain size values are only available for the Progreso beach area ranging between 0.2 mm (at 0.5 m depth) to 0.5 mm (swash zone) with poorly sorted grains. Coastal ecosystems of the Yucatan Peninsula experience three well-defined seasons: dry (March to May), rainy (June to October), and "nortes" (November to February), which is dominated by cold fronts. Additionally, the hurricane season (from August to September) has a strong influence on coastal lagoon stability and disturbance regime. The Yucatan Peninsula has unique hydrogeological characteristics, including low relief, lack of rivers, highly permeable karst-derived soils, and substantial submarine groundwater discharge [10].

The Yucatan Peninsula is affected by extreme wind waves generated by two meteorological systems: (i) mid latitude anticyclonic meteorological systems which generate northerly cold fronts known as "nortes"; and (ii) cyclonic systems known as Tropical Cyclones including tropical depressions and hurricanes [11]. Along the Caribbean coast of the Yucatan Peninsula; brackish ground water (mixed fresh water and sea water) is channeled through upper Pleistocene limestone via fracture-controlled caverns. In caves, cenotes, and caletas at Xcaret, Yalku, and Tancah, this open-flow coastal mixing zone comprises three major layers: (1) an upper dilute zone of gradually increasing salinity with depth (slow mixing), (2) a thin intermediate zone of rapidly increasing salinity with depth (rapid mixing), and (3) a lower saline zone of gradually increasing salinity with depth. The intermediate layer occurs at different absolute salinities at different localities, and it generally corresponds to the level of a notch in the wall rock of the caverns [12].

Collections were made using SCUBA for subtidal; while, seaweeds in the intertidal zone were collected during low tide periods by hand picking in different habits such rocky and sandy. Collections at 40 locations were made during twenty five visits since 2004 to 2014. Algae were preserved in formalin/sea water at a 1:19 ratio. Semi-permanent slides were prepared using corn syrup/water 1:1 with a trace of phenol added to prevent fungal growth. Identification was made with specialized literature [13, 14, 15, 16, 17]. Voucher slides and specimens are deposited at the herbarium of the Escuela Nacional de Ciencias Biológicas (ENCB) at the Instituto Politécnico Nacional in Mexico City, D.F. Mexico. The taxa were identified with from the collections are listed following the order proposed by Wynne [18] and Guiry and Guiry [19]. Each species is presented with data regarding distribution in the study area, tidal level, habitat and observations (Table 1).

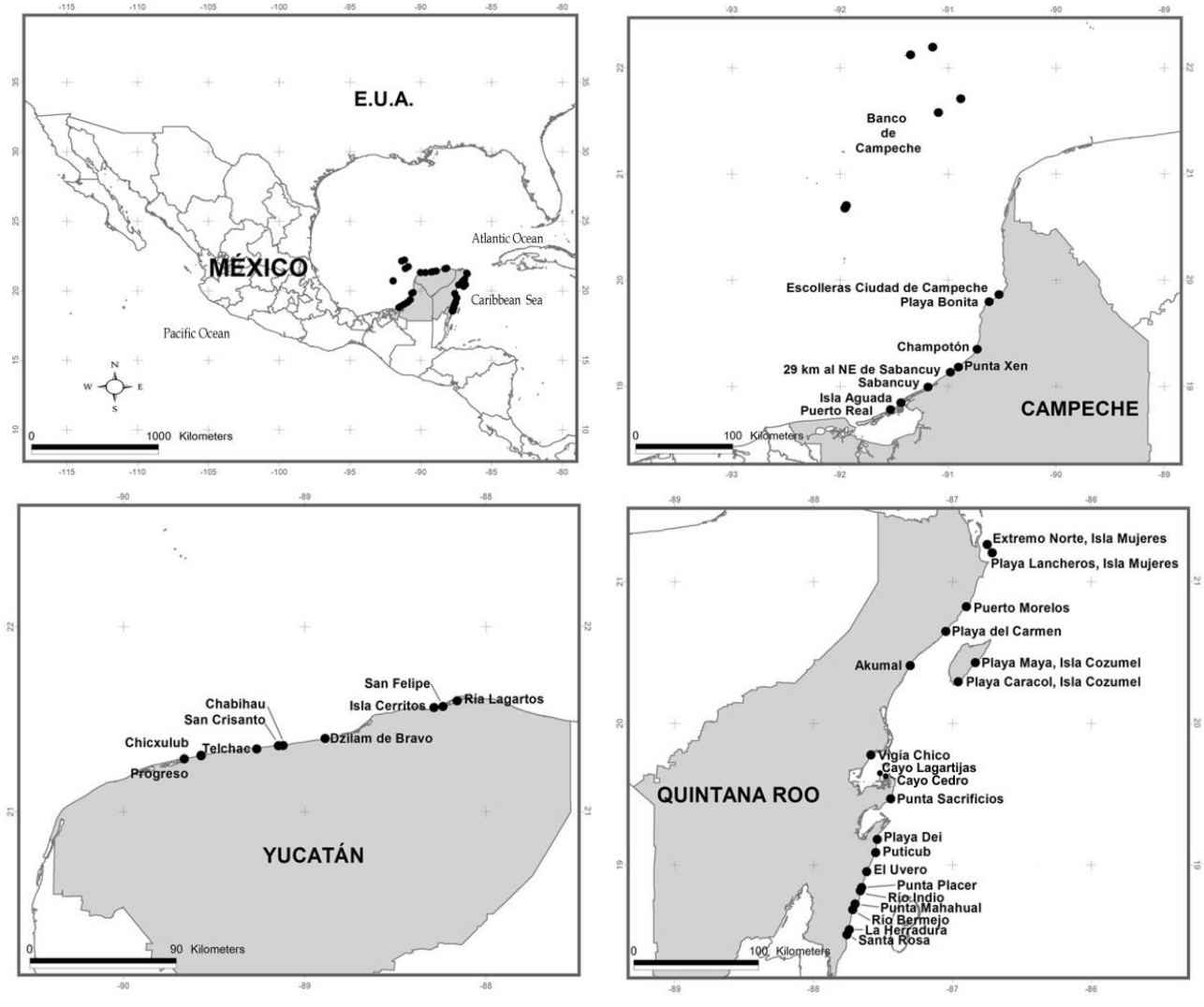

Figure 1. Study area and sample localities.

Table 1. Marine algae of Yucatan Peninsula

SYMBOLOGY
STATES
CAMP= Campeche; YUC= Yucatan; QROO= Quintana Roo
LEVEL/HABITAT
I= Intertidal; S= Subtidal 1 to 50 m Deep
R= Rocky; A=Sandy; Ep= Epiphityc; Ro=Maërl; M= Mangrove Roots; En= Endophyte; Par= Parasite
OBS
NRM= New record Mexico; NRP= New record Peninsula

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
CYANOBACTERIA **Cyanophyceae** **Oscillatoriales** **Oscillatoriaceae** 1. *Blennothrix lyngbyacea* (Kützing ex Gomont) Anagnostidis & Komárek	X	X	X	I, S /R, Ep	
2. *Lyngbya confervoides* C. Agardh ex Gomont	X	X		I /R	
3. *L. lutea* Gomont ex Gomont			X	I/Ep	
4. *L. majuscula* (Dillwyn) Harvey ex Gomont	X		X	I/Ep	
Phormidiaceae 5. *Phormidium limosum* (Dillwyn) P. C. Silva	X			I/Ep	
6. *P. retzii* Kützing ex Gomont			X	I/Ep	
7. *P. submembranaceum* Gomont			X	I/Ep	
8. *Porphyrosiphon notarisii* Kützing ex Gomont			X	I/R, Ep	
Pseudanabaenales **Schizotrichaceae** 9. *Schizothrix arenaria* Gomont			X	I/R	
10. *S. calcicola* Gomont		X	X	I/Ep	
11. *S. mexicana* Gomont		X	X	I/R	
12. *Trichocoleus tenerrimus* (Gomont) Anagnostidis	X			I/Ep	NRP
Pseudanabaenaceae 13. *Leptolyngbya marina* (Gardner) Anagnostidis	X	X		I/Ep	
14. *Pseudanabaena catenata* Lauterborn			X	S/Ep	
Symphyonemataceae 15. *Brachytrichia quoyi* (C. Agardh) Bornet & Flahault	X		X	I/R, Ep	
Microcoleaceae 16. *Microcoleus vaginatus* Gomont ex Gomont		X	X	I, S/ Ep	
17. *Symplocastrum purpurascens* Gomont Anagnostidis			X	I, S/Ep	NRP
Nostocales **Nostocaceae** 18. *Anabaena oscillarioides* Bory de Saint-Vincent ex Bornet & Flahault		X	X	I, S/Ep	
19. *Nodularia spumigena* Mertens			X	I, S/R, Ep	

Table 1. (Continued)

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
Rivulariaceae 20. *Rivularia atra* Roth ex Bornet & Flahault		X		S/R	
21. *Scytonematopsis crustacea* (Thuret ex Bornet & Flahault) Kovál ik & Komárek	X	X	X	I, S/R, Ep	
Scytonemataceae 22. *Scytonema hofmannii* C. Agardh ex Bornet & Flahault			X	I/R	
Chroococcales **Microcystaceae** 23. *Chroococcus turgidus* (Kützing) Nägeli			X	S/Ep	
Hydroccocaceae 24. *Radaisia epiphytica* Setchell & N. L. Gardner		X		S/Ep	NRP
25. *Microcystis zanardinii* (Hauck) P. C Silva	X		X	S/Ep	
Dermocarpellaceae 26. *Dermocarpa acervata* (Setchell & Gardner) Pham-Hoàng Hô	X			S/Ep	NRP
27. *D. willei* (Gardner) P. C. Silva	X			S/Ep	
Xenococcaceae 28. *Xenococcus gilkeyae* Setchell & N. L. Gardner	X			S/Ep	
29. *X. pyriformis* Setchell & N. L. Gardner	X	X		I, S/Ep	
Entophysalidaceae 30. *Chlorogloea endophytica* M. A. Howe			X	S/En	NRP
31. *Entophysalis conferta* (Kützing) F. E. Drouet & W. A. Daily	X	X	X	I, S/Ep	
32. *E. deusta* F. E. Drouet & Daily			X	S/Ep	
33. *Placoma violacea* Setchell & N. L. Gardner		X		I/R	NRP
Cyanobacteriaceae 34. *Aphanotece microscopica* Nageli		X		S/Ep	NRP
35. *A. stagnina* (Sprengel) A. Braun			X	I, S/Ep	
Gomphosphaeriaceae 36. *Gomphosphaeria aponina* Kützing		X	X	S/Ep	
Spirulinaceae 37. *Spirulina subsalsa* Oersted ex Gomont			X	S/Ep	
Synechococcales **Merismopediaceae** 38. *Aphanocapsma marina* Hansgirg			X	I, S/Ep	
RHODOPHYTA **Stylonematophyceae** **Stylonematales** **Stylonemataceae** 39. *Chroodactylon ornatum* (C. Agardh) Basson	X	X	X	I, S/Ep	
40. *Stylonema alsidii* (Zanardini) K.M. Drew	X	X	X	I, S/Ep.	

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
Compsopogonophyceae **Erythropeltidales** **Erythrotrichiaceae** 41. *Erythrotrichia carnea* (Dillwyn) J. Agardh	X	X	X	I, S/Ep	
42. *E. vexillaris* (Montagne) G. Hamel	X			S/Ep	
43. *Sahlingia subintegra* (Rosenvinge) P. Kornmann	X	X	X	I, S/Ep	
Rhodochaetales **Rhodochaetaceae** 44. *Rhodochaete pulchella* Thuret ex Bornet			X	S/Ep	
Bangiophyceae **Bangiales** **Bangiaceae** 45. *Pyropia leucosticta* (Thuret) Neefus & J. Brodie	X			I/R	
Florideophyceae **Acrochaetiales** **Acrochaetiaceae** 46. *Acrochaetium flexuosum* Vickers		X	X	I, S/Ep	
47. *A. globosum* Borgesen			X	I, S/Ep	
48. *A. microscopicum* (Nägeli ex Kützing) Nägeli		X	X	I, S/Ep	
49. *A. savianum* (Meneghini) Nägeli		X	X	I, S/R, Ep	
Colaconematales **Colaconemataceae** 50. *Colaconema daviesii* (Dillwyn) Stegenga	X	X	X	I, S/Ep	
51. *C. hallandicum* (Kylin) Afonso-Carrillo, Sansón, Sagil & Díaz-Villa	X		X	I, S/Ep	
52. *C. hypneae* (Børgesen) A. A. Santos & C. W. N. Moura		X	X	I, S/Ep	
Corallinales **Hapalidiaceae** 53. *Lithothamnion occidentale* (Foslie) Foslie	X	X	X	S/Ro	
54. *L. sejunctum* Foslie			X	I/R	
55. *Melobesia membranacea* (Esper) J. V. Lamouroux	X	X	X	I, S/Ep	
56. *Mesophyllum mesomorphum* (Foslie) W. H. Adey	X		X	S/Ep	
Corallinaceae 57. *Amphiroa beauvoisii* J. V. Lamouroux			X	I, S/R	
58. *A. fragilissima* (Linnaeus) J. V. Lamouroux	X	X	X	I, S/R	
59. *A. rigida* J. V. Lamouroux	X	X	X	I, S/R	
60. *A. tribulus* J. V. Lamouroux		X	X	I, S/R	
61. *A. valonioides* Yendo			X	I, S/R	
62. *A. vanbossea* Me. Lemoine			X	I, S/R	
63. Goniolithon decutescens (Heydrich) Foslie ex M. A. Howe			X	I/R	
64. Hydrolithon boergesenii (Foslie) Foslie	X		X	I/R	
65. H. farinosum (J. V. Lamouroux) D. Penrose & Y. M. Chamberlain	X	X	X	I, S/Ep	

Table 1. (Continued)

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
66. *H. improcerum* (Foslie & M. Howe) Foslie			X	S/R	
67. *H. onkodes* (Foslie) D. Penrose & W.H. Woelkerling	X		X	S/R	
68. *H. pachydermum* (Foslie) J. C. Bailey, J. E. Gabel & D. W. Freshwater			X	S/R	
69. *H. reinboldii* (Weber-van Bosse & Foslie) Foslie			X	S/Ro	
70. *Jania adhaerens* J. V. Lamouroux	X	X	X	I, S /R, Ep	
71. *J. capillacea* Harvey	X	X	X	I, S /R, Ep	
72. *J. cubensis* Montagne ex Kützing	X	X	X	I, S /R, Ep	
73. *J. rubens* (Linnaeus) J.V. Lamouroux	X	X	X	I, S/R	
74. *J. subulata* (J. Ellis & Solander) H. W. Johansen		X	X	I, S/R	
75. *Lithoporella atlantica* (Foslie) Foslie			X	I, S /Ep	
76. *L. bermudensis* (Foslie) W. H. Adey		X	X	I, S /R	
77. *Lithophyllum incrustans* Philippi			X	S/R	NRP
78. *L. stictaeforme* (Areschoug) Hauck			X	S/R	
79. *Neogoniolthon accretum* (Foslie M. A. Howe) Setchell & L. R. Mason			X	I, S/R	
80. *N. acropetum* (Foslie & M. Howe) H. W. Adey		X	X	I, S/R	
81. *N. affine* (Foslie & M. Howe) Setchell & Mason			X	I, S/R	
82. *N. erosum* (Foslie) W. H. Adey			X	I, S /R, Ep	
83. *N. fosliei* (Heydrich) Setchell & L. R. Mason		X	X	I, S/R	
84. *N. mamillare* (Harvey) Setchell & L. R. Mason			X	I, S/R	
85. *N. propinquum* (Foslie) Me. Lemoine			X	I, S/R	
86. *N. rhizophorae* (Foslie) Setchell & L. R. Mason		X	X	I, S/R	
87. *N. siankanensis* Mateo-Cid, Mendoza-González & P. W. Gabrielson			X	I, S/R	
88. *N. spectabile* (Foslie) Setchell & L. R. Mason	X	X	X	S/Ro	
89. *N. strictum* (Foslie) Setchell & L. R. Mason		X	X	S/Ro	
90. *N. trichotomum* (Heydrich) Setchell & L. R. Mason			X	S/R	
91. *Pneophyllum confervicola* (Kützing) Y.M. Chamberlain	X	X	X	I,S/Ep	
92. *P. fragile* Kützing	X	X	X	I,S/Ep	
93. *Spongites yendoi* (Foslie) Y.M. Chamberlain	X			I, S/R	
94. *Titanoderma pustulatum* (J.V. Lamouroux) Nägeli	X		X	I, S /R, Ep	
Nemaliales		X	X	S/R	
Liagoraceae					
95. *Ganonema farinosum* (J.V. Lamouroux) K.C. Fan & Yung C. Wang	X				
96. *G. megagynum* (Børgesen) Huisman			X	S/R	
97. *G. pinnatum* (Harvey) Huisman			X	S/R	
98. *Gloiocallis dendroidea* (P.L. Crouan & H.M. Crouan) S.M. Lin, Huisman & D.L. Ballantine			X	S/R	
99. *Helminthocladia calvadosii* (J.V. Lamouroux ex Duby) Setchell			X	I/R	
100. *Liagora albicans* J.V. Lamouroux			X	S/R	
101. *L. ceranoides* J.V. Lamouroux			X	I, S/R	

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
102. *L. tsengii* Huisman & M. J. Wynne			X	S/R	
103. *Trichogloeopsis pedicellata* (M. A. Howe) I.A. Abbott & Doty			X	I, S/R	
104. *Titanophycus validus* (Harvey) Huisman, G. W. Saunders & A. R. Sherwood		X	X	I, S/R	
Galaxauraceae 105. *Dichotomaria marginata* (J. Ellis & Solander) Lamarck	X		X	S/R	
106. *D. obtusata* (J. Ellis & Solander) Lamarck	X			S/R	
107. *Galaxaura comans* Kjellman			X	S/R	
108. *G. rugosa* (J. Ellis & Solander) J.V. Lamouroux			X	S/R	
109. *Tricleocarpa cylindrica* (J. Ellis & Solander) Huisman & Borowitzka			X	S/R	
110. *T. fragilis* (Linnaeus) Huisman & R. A. Townsend			X	S/R	
Scinaiaceae 111. *Scinaia complanata* (F. S. Collins) A. D. Cotton	X		X	S/R	
Bonnemaisoniales **Bonnemaisoniaceae** 112. *Asparagopsis taxiformis* (Delile) Trevisan de Saint-Léon	X	X	X	I, S/Ep	
Naccariaceae 113. *Naccaria antillana* W. R. Taylor			X	S/Ep	
Ceramiales **Callithamniaceae** 114. *Aglaothamnion boergesenii* (Aponte & D. L. Ballantine) L'Hardy-Halos & T. Rueness			X	I, S/Ep	
115. *A. cordatum* (Børgesen) Feldmann-Mazoyer			X	I, S/R, Ep, M	
116. *A. felliponei* (M. A. Howe) D. L. Ballantine, N. E. Aponte & J. N. Norris			X	I, S /R, Ep	
117. *A. herveyi* (M. A. Howe) N. E. Aponte, D. L. Ballantine & J. N. Norris	X	X		I, S/R, Ep	
118. *Callithamnion corymbosum* (Smith) Lyngbye			X	I, S/M, Ep	
119. *Crouania attenuata* (C. Agardh) J. Agardh			X	I, S /Ep	
120. *C. mayae* Mateo-Cid, Mendoza-González & Searles		X	X	S/R, Ep	
121. *C. pleonospora* W. R. Taylor			X	S/R, Ep	
122. *Crouanophycus latiaxis* (I. A. Abbott) A. Athanasiadis			X	S/Ep	
123. *Seirospora occidentalis* Børgesen			X	S/Ep	
Ceramiaceae 124. *Antithamnion cruciatum* (C. Agardh) Nägeli			X	S/Ep	
125. *A. decipiens* (J. Agardh) Athanasiadis			X	S/Ep	
126. *Antithamnionella boergesenii* (Cormaci & G. Furnari) Athanasiadis			X	S/Ep	
127. *A. elegans* (Berthold) J. H. Price & D. M. John	X		X	S/Ep	
128. *Callithamniella tingitana* (Schousboe ex Bornet) Feldmann-Mazoyer	X		X	I,S/Ep	
129. *Centrocerocolax ubatubensis* A. B. Joly			X	S/Par	
130. *Centroceras hyalacanthum* Kützing	X	X	X	I, S /R, Ep	**NRM**
131. *C. micracanthum* Kützing	X	X	X	I, S /R, Ep	
132. *Ceramium brevizonatum* var. *caraibicum* H.E. Petersen & Børgesen	X	X	X	I, S/R, Ep	
133. *C. cimbricum* H. E. Petersen		X	X	I, S/R, Ep	
134. *C. codii* (H. Richards) Mazoyer	X	X		I, S/R, Ep	

Table 1. (Continued)

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
135. *C. corniculatum* Montagne	X	X	X	I, S/R, Ep	
136. *C. cruciatum* F. S. Collins & Hervey	X			I, S/R, Ep	
137. *C. deslongchampii* Chauvin ex Duby			X	S/Ep	
138. *C. floridanum* J. Agardh			X	S/R	
139. *C. leptozonum* M. A. Howe	X	X	X	S/Ep	
140. *C. leutzelburgii* Schmidt	X	X	X	I, S/R, Ep	
141. *C. nitens* (C. Agardh) J. Agardh			X	I, S/R, Ep	
142. *C. subtile* J. Agardh			X	I, S/R, Ep	
143. *C. tenerrinum* (G. Martens) Okamura			X	I, S/R, Ep	
144. *C. virgatum* Roth			X	S/R	
145. *Corallophila verongiae* (D. L. Ballantine & M. J. Wynne) R. E. Norris			X	S/R	
146. *Gayliella flaccida* (Harvey ex Kützing) T.O. Cho & L. J. McIvor	X	X	X	I, S/R, Ep	
147. *G. mazoyerae* T. O. Cho, Fredericq & Hommersand	X	X	X	I, S/R, Ep	NRP
148. *G. transversalis* (F. S. Collins & Hervey) T.O. Cho & Fredericq			X	I, S/R, Ep	
149. *Dohrniella antillana* (W. R. Taylor) Feldmann-Mazoyer			X	S/Ep	
Spyridiaceae 150. *Spyridia filamentosa* (Wulfen) Harvey	X	X	X	I, S/R	
151. *S. hypnoides* (Bory de Saint-Vincent) Papenfuss	X	X	X	I, S/R, Ep	
Wrangeliaceae 152. *Anotrichium tenue* (C. Agardh) Nägeli	X	X	X	I, S /Ep	
153. *Griffithsia globulifera* Harvey ex Kützing			X	S /Ep	
154. *G. heteromorpha* Kützing			X	S /Ep	
155. *G. radicans* Kützing			X	I, S /Ep	
156. *G. schousbei* Montagne			X	S /Ep	
157. *Gymnothamnion elegans* (Schosboe) J. Agardh			X	S /Ep	
158. *Grallatoria reptans* M. A. Howe			X	S /Ep	
159. *Haloplegma duperreyi* Montagne			X	S /Ep	
160. *Ptilothamnion speluncarum* (F. S. Collins & Hervey) D. L. Ballantine & M. J. Wynne			X	S/R	
161. *Spermothamnion gymnocapum* M. A. Howe			X	I, S/Ep	
162. *S. investiens* (P. Crouan & H. Crouan) Vickers			X	I, S/Ep	
163. *S. macromeres* Collins & Harvey			X	I, S/Ep	
164. *S. repens* (Dillwyn) Rosenvinge			X	I, S/Ep	
165. *Tiffaniella gorgonae* (Montagne) Doty & Meñez	X	X	X	I, S/Ep	
166. *Wrangelia argus* (Montagne) Montagne	X		X	I, S/R, Ep	
167. *W. bicuspidata* Borgesen			X	I, S/R, Ep	
168. *W. penicillata* (C. Agardh) C. Agardh			X	S/R	

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
Delesseriaceae 169. *Caloglossa leprieurii* (Montagne) G. Martens	X	X	X	I/M	
170. *Hypoglossum hypoglossoides* (Stackhouse) Collins & Harvey			X	S/Ep	
171. *H. involvens* (Harvey) J. Agardh			X	S/Ep	
172. *H. simulans* M. J. Wynne, I. R. Price & D. L. Ballantine			X	S/Ep	
173. *H. subsimplex* M. J. Wynne			X	S/Ep	
174. *H. tenuifolium* (Harvey) J. Agardh			X	S/Ep	
175. *Martensia fragilis* Harvey			X	I, S/R, Ep	
176. *Nitophyllum adhaerens* M. J. Wynne			X	S/Ep	
177. *Taenioma nanum* (Kützing) Papenfuss			X	I, S/Ep, M	
Dasyaceae 178. *Dasya baillouviana* (S. G. Gmelin) Montagne	X	X	X	I, S/R	
179. *D. caraibica* Børgesen	X		X		
180. *D. collinsiana* M. A. Howe	X	X	X	I, S/R, Ep	
181. *D. corymbifera* J. Agardh	X	X	X	I, S/R, Ep	
182. *D. crouaniana* J. Agardh	X	X	X	S/R	
183. *D. haitiana* S. Fredericq & J. N. Norris			X	S/R	
184. *D. hutchinsiae* Harvey			X	S/R	
185. *D. mollis* Harvey			X	S/R	
186. *D. ocellata* (Grateloup) Harvey	X	X	X	I, S /R, Ep	
187. *D. ramosissima* Harvey			X	S/R	
188. *D. rigidula* (Kützing) Ardissone	X	X	X	I, S /R, Ep	
189. *Dictyurus occidentalis* J. Agardh			X	S/R	
190. *Halydictyon mirabile* Zanardini	X	X	X	S/R	
191. *Heterosiphonia crispella* (C. Agardh) J. M. Wynne	X	X	X	I, S /Ep	
192. *H. gibbesii* (Harvey) Falkenberg			X	I, S /R	
193. *Thuretia bornetii* Vickers			X	I,S/R	
Rhodomelaceae 194. *Acanthophora muscoides* (Linnaeus) Bory de Saint-Vincent	X	X	X	I,S/R	
195. *A. spicifera* (M. Vahl) Børgesen	X	X	X	I,S/R	
196. *Amansia multifida* J. V. Lamouroux		X	X	I,S/R	
197. *Bostrychia montagnei* Harvey			X	I/M	
198. *B. moritziana* (Sonder ex Kützing) J. Agardh			X	I/M	NRP
200. *B. radicans* (Montagne) Montagne	X	X	X	I,S/R, M	
201. *B. tenella* (J.V. Lamouroux) J. Agardh	X	X	X	I,S/M	
202. *Bryothamnion seaforthii* (Turner) Kützing	X	X	X	I,S/R	
203. *B. triquetrum* (S. G. Gmelin) M. A. Howe	X	X	X	I,S/R	
204. *Chondria baileyana* (Montagne) Harvey	X	X	X	I,S/R	
205. *Ch. capillaris* (Hudson) M. J. Wynne			X	S/R	
206. *Ch. cnicophylla* (Melvill) De Toni	X	X	X	S/Ep	
207. *Ch. collinsiana* M. A. Howe	X	X	X	I,S/R, Ep	

Table 1. (Continued)

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
208. *Ch. curvilineata* F. S. Collins & Hervey	X		X	I,S/R	
209. *Ch. dasyphylla* (Woodward) C. Agardh	X	X	X	I,S/R	
210. *Ch. floridiana* (F. S. Collins) M. A. Howe			X	S/R	
211. *Ch. leptacremon* (Melvill ex G. Murray) De Toni	X	X	X	I,S/R	
212. *Ch. littoralis* Harvey	X		X	I,S/R	
213. *Ch. platyramea* A. B. Joly & Ugadim	X		X	S/Ep	
214. *Ch. polyrhiza* F.S. Collins & Hervey	X	X	X	I,S/Ep	
215. *Digenea simplex* (Wulfen) C. Agardh	X	X	X	I,S/R	
216. *Dipterosiphonia dendritica* (C. Agardh) F. Schmitz			X	I/Ep	
217. *D. rigens* (Shousboe ex C. Agardh) Falkenberg			X	I/Ep	
218. *Herposiphonia bipinnata* M. A. Howe			X	I, S/R, Ep	
219. *H. pecten-veneris* (Harvey) Falkenberg	X	X	X	I, S/R, Ep	
220. *H. secunda* f. *tenella* (C. Agardh) M. J. Wynne	X	X	X	I, S/R, Ep	
221. *Heterodasya mucronata* (Harvey) M. J. Wynne		X	X	S/R	
222. *Laurencia brongniartii* J. Agardh			X	S/R	
223. *L. caraibica* P. C. Silva			X	I, S/R, Ep	
224. *L. filiformis* (C. Agardh) Montagne	X	X	X	S/R	
225. *L. intricata* J. V. Lamouroux	X	X	X	I, S/R, Ep	
226. *L. laurahuertana* Mateo-Cid, Mendoza-González, Senties & Díaz-Larrea			X	I/Ep	
227. *L. microcladia* Kützing	X		X	I, S/R, Ep	
228. *L. obtusa* (Hudson) J. V. Lamouroux	X		X	I, S/R, Ep	
229. *L. venusta* Yamada	X		X	S/R	
230. *Lophosiphonia cristata* Falkenberg	X		X	I, S/R, Ep	
231. *L. obscura* (C. Agardh) Falkenberg	X		X	I, S/R, Ep	
232. *Lophocladia trichoclados* (C. Agardh) F. Schmitz			X	I, S/R, Ep	
233. *Meridiocolax polysiphoniae* (E. C. Oliveira & Ugadim) J. Morrill			X	I/Par	
234. *Murrayella periclados* (C. Agardh) F. Schmitz	X	X	X	I, S/R, Ep	
235. *Neosiphonia echinata* (Harvey) N. Mamoozadeh & D. W. Freshwater		X	X	I, S/R, Ep	
236. *N. ferulacea* (Suhr ex J. Agardh) S.M. Guimarães & M. T. Fujii		X	X	I, S/R, Ep	
237. *N. gorgoniae* (Harvey) S. M. Guimarães & M. T. Fujii		X	X	I,S/Ep	
238. *N. harveyi* (J. Bailey) M.-S. Kim, H.-G. Choi, Guiry & G. W. Saunders			X	I, S/R, Ep	
239. *N. hawaiiensis* (Hollenberg) M.-S.Kim & I. A. Abbott	X	X	X	I, S/R, Ep	
240. *N. howei* (Hollenberg) Skelton & G.R. South		X	X	I, S/R, Ep	
241. *N. sertularioides* (Grateloup) K. W. Nam & P. J. Kan		X	X	I, S/R, Ep	
242. *N. sphaerocarpa* (Børgesen) M. S. Kim & I. K. Lee	X	X	X	I, S/R, Ep	
243. *Osmundaria obtusiloba* (C. Agardh) R. E. Norris			X	I/R	
244. *Palisada corallopsis* (Montagne) Sentíes, Fujii & Díaz	X		X	S/R	
245. *P. flagellifera* (J. Agardh) K. W. Nam			X	S/R	

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
246. *P. perforata* (Bory de Saint-Vincent) K. W. Nam	**X**	**X**	X	I, S/R, Ep	
247. *Polysiphonia atlantica* Kapraun & J. N. Norris	X	X	X	I, S/R, Ep	
248. *P. binneyi* Harvey	X	X	X	I, S/R, Ep	
249. *P. denudata* (Dillwyn) Greville ex Harvey	X			I, S/R, Ep	
250. *P. exilis* Harvey			X	S/R	
251. *P. pseudovillum* Hollenberg			X	I, S/R, Ep	NRP
252. *P. scopolorum* var. *villum* (J. Agardh) Hollenberg			X	S/Ep	
253. *P. subtilissima* Montagne	X		X	I, S/R, Ep	
254. *P. ramentacea* Harvey			X	S/R	
255. *Yuzurua poiteaui* (J.V. Lamouroux) Martin-Lescanne	X		X	S/R	
256. *Y. poiteaui* var. *gemmifera* (Harvey) M.J. Wynne	X			S/R	
257. *Wrightiella tumanowiczii* (Gatty ex Harvey) F. Schmitz			X	S/R	
Sarcomeniaceae 258. *Cottoniella sanguinea* M. A. Howe			X	S/R	
Gelidiales **Gelidiaceae** 259. *Gelidium americanum* (W. R. Taylor) Santelices	X	X	X	I, S/R, Ep	
260. *G. corneum* (Hudson) J. V. Lamouroux			X	I, S/R	
261. *G. crinale* (Hare ex Turner) Gaillon	X	X	X	I,S/R	
262. *G. pusillum* (Stackhouse) Le Jolis	X	X	X	I, S/R, Ep	
263. *G. spinosum* (S. G. Gmelin) P. C. Silva			X	S/R	
Gelidiellaceae 264. *Gelidiella acerosa* (Forsskål) Feldmann & G. Hamel	X		X	I,S/R	
265. *G. lubrica* (Kützing) Feldmann & G. Hamel			X	I,S/Ep	
266. *Parviphycus pannosus* (Feldmann) G.XFurnari			X	I,S/R, Ep	
267. *P. setaceus* (Feldmann) J. Afonso-Carrillo, M. Sanson, C. Sangil & T. Díaz-Villa	X	X		I,S/R	
268. *P. trinitatensis* (W. R. Taylor) M. J. Wynne			X	S/R	
Pterocladiaceae 269. *Pterocladiella capillacea* (S. G. Gmelin) Santelices & Hommersand	X		X	S/R	
270. *P. sanctarum* (Feldmann & G. Hamel) Santelices	X	X		S/R	
271. *P. taylorii* (Joly) Santelices	X			I,S/R	
Gigartinales **Gigartinaceae** 272. *Chondracanthus elegans* (Greville) Guiry			X	S/R	
273. *C. teedei* (Mertens ex Roth) Kützing			X	S/R	
Caulacanthaceae 274. *Catenella caespitosa* (Withering) L. M. Irvine			X	I/Ep	
275. *C. impudica* (Montagne) J. Agardh	X	X	X	I/Ep	
Cystocloniaceae 276. *Hypnea musciformis* (Wulfen) J. V. Lamouroux	X	X	X	I,S/R, Ep	
277. *H. spinella* (C. Agardh) Kützing	X	X	X	I,S/R, Ep	
278. *H. valentiae* (Turner) Montagne			X	I,S/R, Ep	NRP

Table 1. (Continued)

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
279. *Hypneocolax stellaris* Børgesen	X			I/Par	
Sebdeniaceae 280. *Sebdenia flabellata* (J. Agardh) P. G. Parkinson	X		X	S/R	
Kallymeniaceae 281. *Kallymenia westii* Ganesan			X	S/R	
Solieriaceae 282. *Agardhiella ramossisima* (Harvey) Kylin	X		X	S/R	
283. *A. subulata* (C. Agardh) Kraft & M. J. Wynne	X		X	S/R	
284. *Eucheuma isiforme* (C. Agardh) J. Agardh	X		X	S/R	
285. *Meristotheca gelidium* (J. Agardh) E. J. Faye & M. Masuda	X			S/R	
286. *Solieria filiformis* (Kützing) P. W. Gabrielson	X			S/R	
287. *Flahaultia tagetiformans* W. R. Taylor			X	S/R	
288. *Sarcodiotheca divaricata* W. R. Taylor	X			S/R	**NRM**
289.*Wurdemannia miniata* (Sprengel) Feldmann & G. Hamel			X	I,S/R, Ep	
Dumontiaceae 290. *Dudresnaya crassa* M. A. Howe			X	S/R, Ep	
Nemastomatales **Nemastomataceae** 291. *Predaea feldmanii* Børgesen	X		X	S/R	
Acrosymphytales **Acrosymphytaceae** 292. *Acrosymphyton caribaeum* (J. Agardh) Sjöstedt			X	S/R	
Phyllophoraceae 293. *Gymnogongrus griffithsiae* (Turner) Martius			X	I,S/R	
294. *G. tenuis* J. Agardh			X	I,S/R	
Rhizophyllidaceae 295. *Ochtodes secundiramea* (Montagne) M. A. Howe			X	I,S/R	
Gracilariales **Gracilariaceae** 296. *Gracilaria blodgetii* Harvey	X	X	X	I,S/R	
297. *G. bursa-pastoris* (S. G. Gmelin) P. C. Silva	X		X	I,S/R	
298. *G. cervicornis* (Turner) J. Agardh	X		X	I,S/R	
299. *G. cuneata* Areschoug	X			I,S/R	
300. *G. cylindrica* Børgesen	X		X	I,S/R	
301. *G. damaecornis* J. Agardh	X		X	I,S/R	
302. *G. debilis* (Forsskål) Børgesen	X		X	I,S/R	
303. *G. dominguensis* (Kützing) Sonder ex Dickie			X	I,S/R	
304. *G. lacinulata* (M. Vahl) M. A. Howe			X	I,S/R	
305. *G. isabellana* Gurgel, Fredericq & J. N. Norris	X	X	X	I,S/R	
306. *G. mammillaris* (Montagne) M.A. Howe	X		X	I,S/R	

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
307. *G. multipartita* (Clemente) Harvey			X	I,S/R	
308. *G. ornata* Areschoug			X	I,S/R	
309. *G. tikvahiae* McLachlan	X		X	I,S/R	
310. *Gracilariopsis andersonii* (Grunow) E. Y. Dawson	X		X	I,S/R	NRP
311. *G. lamaneiformis* (Bory de Saint-Vincent) E. Y. Dawson, Acleto & Foldvik	X		X	I,S/R	
312. *G. longissima* (S. G. Gmelin) M. Steentoft, L. M. Irvine & W. F. Farnham			X	I,S/R	
313. *Hydropuntia caudata* (J. Agardh) Gurgel & Fredericq			X	I,S/R	
314. *H. cornea* (J. Agardh) M. J. Wynne	X		X	I,S/R	
315. *H. crassissima* (J. Agardh) M. J. Wynne			X	I,S/R	
Halymeniales **Halymeniaceae** 316.*Dermocorynus dichotomus* (J. Agardh) Gargiulo, M. Morabito & Manghisi			X	S/Ep	
317. *Halymenia echinophysa* F. S. Collins & M. A. Howe	X			S/R	
318. *Halymenia floresii* (Clemente) C. Agardh	X	X	X	S/R	
319. *H. floridiana* J. Agardh	X		X	S/R	
320. *H. pseudofloresia* F. S. Collin & M. A. Howe			X	S/R	
321. *H. rosea* M. A. Howe & W. R. Taylor			X	S/R	
322. *Cryptonemia crenulata* (J. Agardh) J. Agardh	X		X	S/R	
323. *Grateloupia filicina* (J. V. Lamouroux) C. Agardh	X			I,S/R	
324. *G. gibbesi* Harvey			X	S/R	
Rhodymeniales **Champiaceae** 325. *Champia minuscula* A. B. Joly & Ugadim	X	X	X	I,S/R, Ep	
326. *C. parvula* (C. Agardh) Harvey	X	X	X	I,S/R, Ep	
327. *C. parvula* var. *postrata* L. G. Williams			X	I,S/R, Ep	
328. *C. salicornioides* Harvey			X	I,S/R	
329. *Coelothrix irregularis* (Harvey) Børgesen			X	I,S/R	
330. *Gastroclonium parvum* (Hollenberg) C. F. Chang & B. M. Xia			X	I,S/R	
Lomentariaceae 331. *Ceratodictyon intricatum* (C. Agradh). R. E. Norris	X	X	X	I,S/R	
332. *C. planicaule* (W. R. Taylor) M. J. Wynne	X	X	X	I,S/R	
333. C. variabile (Greville ex J. Agardh) R. E. Norris	X		X	I,S/R	
334. Lomentaria divaricata (Durant) M. J. Wynne			X	S/Ep	
335. L. rawitsheri A. B. Joly	X		X	S/Ep	
Rhodymeniaceae 336. Asteromenia peltata (W. R. Taylor) Huisman & A. J. Millar			X	S/R	
337. Botryocladia occidentalis (Børgesen) Kylin	X	X	X	S/R	
338. B. pyriformis (Børgesen) Kylin			X	I,S/R	
339. B. spinulifera W. R. Taylor & I. A. Abbott			X	I,S/R	
340. Chrysymenia enteromorpha Harvey			X	S/R	
341. C. halymenioides Harvey			X	S/R	

Table 1. (Continued)

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
342. Coelarthrum cliftonii (Harvey) Kylin			X	S/R	
343. Cordylecladia peasiae F.S.Collins			X	S/R	
344. Rhodymenia pseudopalmata (J. V. Lamouroux) P. C. Silva			X	S/R	
Faucheaceae 345. *Gloiocladia atlantica* (Searles) R. E. Norris			X	S/Ep	
Peyssonneliales **Peyssonneliaceae** 346. *Metapeyssonnelia tangerina* D. L. Ballantine & Ruiz			X	S/R	**NRM**
347. *Peyssonnellia armorica* (P. Crouan & H, Crouan) Weber- van Bosse in Børgesen			X	S/R	
348. *P. boergesenii* Weber-van Bosse			X	S/R	
349. *P. boudouresquei* Yoneshigue			X	S/R	
350. *P. conchicola* Piccone & Grunow			X	S/R	
351. *P. inamoena* Pilger			X	S/R	
352. *P. nordstedtii* Weber-van Bosse			X	S/R	
353. *P. rubra* (Greville) J. Agardh			X	S/R, Ep	
354. *P. simulans* Weber-van Bosse			X	S/R, Ep	
355. *P. stoechas* Boudouresque & Denizot			X	S/R	
HETEROKONTOPHYTA **Phaeophyceae** **Dictyotales** **Dictyotaceae** 356. *Canistrocarpus cervicornis* (Kützing) De Paula & De Clerck	X	X	X	I, S/R, Ep	
357. *C. cervicornis* f. *pseudohamatus* (Cribb) M. J. Wynne			X	S/R	
358. *Dictyopteris delicatula* J. V. Lamouroux	X	X	X	I, S/R, Ep	
359. *D. jamaicensis* W. R. Taylor			X	I, S/R, Ep	
360. *D. jolyana* E. C. Oliveira & R. P. Furtado			X	S/R	
361. *D. justii* J. V. Lamouroux	X		X	S/R	
362. *D. polypodioides* (A. P. De Candolle) J. V. Lamouroux	X		X	S/R	
363. *Dictyota bartayresiana* J. V. Lamouroux	X		X	I, S/R, Ep	
364. *D. caribaea* Hörnig & Schnetter Montagne			X	I, S/R, Ep	
365. *D. ciliolata* Kützing	X	X		I, S/R	
366. *D. dichotoma* (Hudson) J. V. Lamouroux	X		X	I, S/R, Ep	
367. *D. hamifera* Setchell			X	S/Ep	
368. *D. guineënsis* (Kützing) P. L. Crouan & H. M. Crouan			X	I, S/R, Ep	
369. *D. jamaicensis* W.R. Taylor	X	X	X	S/R	
370. *D. menstrualis* (Hoyt) Schnetter, Hörning & Weber-Peukert	X		X	I, S/R, Ep	
371. *D. mertensii* (Martius) Kützing			X	I, S/R	
372. *D. pinnatifida* Kützing	X	X	X	I, S/R	
373. *D. pulchella* Hörnig & Schnetter	X	X	X	I, S/R, Ep	

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
374. *Lobophora variegata* (J. V. Lamouroux) Womersley ex E. C. Oliveira	X	X	X	I, S/R, Ep	
375. *Padina boergesenii* Allender & Kraft	X	X	X	I, S/R	
376. *P. gymnospora* (Kützing) Sonder	X	X	X	I, S/R	
377. *P. haitiensis* Thivy	X		X	I, S/R	
378. *P. pavonica* (Linnaeus) Thivy	X	X	X	I, S/R	
379. *P. sanctae-crucis* Børgesen	X	X	X	I, S/R	
380. *Spatoglossum schroederi* (C. Agardh) Kützing	X	X	X	I, S/R	
381. *Stypopodium zonale* (J. V. Lamouroux) Papenfuss			X	I, S/R	
382. *Zonaria tournefortii* (J. V. Lamouroux)		X	X	S/R	
Ectocarpales **Acinetosporaceae** 383. *Hincksia rallsiae* (Vickers) P. C. Silva	X	X		I, S/Ep	
384. *H. onslowensis* (Amsler & Kapraun) P. C. Silva			X	I, S/Ep	
Asteronemataceae 385. *Asteronema breviarticulatum* (J. Agardh) Ouriques & Bouzon			X	I, S/R, Ep	
386. *A. rhodochortonoides* (Børgesen) D. G. Müller & E. R. Parodi			X	S/Ep	
387. *Feldmannia duchassaingiana* (Grunow) Aisha & Shameel		X	X	I, S/Ep	
388. *F. mitchelliae* (Harvey) H. S. Kim		X	X	I, S/R, Ep	
389. *Herponema tortugense* (W. R. Taylor) W. R. Taylor			X	S/Ep	
Chordariaceae 390. *Myrionema strangulans* Montagne			X	S/Ep	
391. *Cladosiphon zosterae* (J. Agardh) Kylin	X			S/Ep	
392. *Levringia brasiliensis* (Montagne) A. B. Joly		X		I/R	
393. *Streblonema invisibile* Hoyt	X			S/Ep	
Ectocarpaceae 394. *Kuetzingiella elachistaeformis* (Heydrich) M. Balakrishnan & Kinkar	X		X	I,S/Ep	
395. *Ectocarpus confervoides* Harvey in J.D. Hooker		X		I, S/R, Ep	
396. *E. siliculosus* (Dillwyn) Lyngbye			X	I, S/R, Ep	
397. *E. siliculosus* var. *dasycarpus* (Kuckuck) Gallardo	X	X		I, S/R, Ep	
398. *E. variabilis* Vickers			X	I,S/Ep	
Scytosiphonaceae 399. *Chnoospora minima* (Hering) Papenfuss			X	I, S/R	
400. *Colpomenia sinuosa* (Roth) Derbes & Solier	X		X	S/R	
401. *Hydroclathrus clathratus* (C. Agardh) M. A. Howe			X	S/R	
402. *Rosenvingea intricata* (J. Agardh) Borgesen			X	S/R	
403. *R. sanctae-crucis* Børgesen		X		S/R	
Onslowiales **Onslowiaceae** 404. *Onslowia bahamaensis* E.C. Henry			X	S/Ep	
405. *O. endophytica* Searles	X		X	S/En	
406. *Verosphacela ebrachia* E.C. Henry			X	S/Ep	

Table 1. (Continued)

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
Sphacelariales **Sphacelariaceae** 407. *Sphacelaria brachygonia* Montagne			X	I, S/Ep	
408. *S. novae-hollandiae* Sonder		X	X	I, S/Ep	
409. *S. rigidula* Kützing		X	X	I, S/Ep	
410. *S. tribuloides* Meneghini			X	I, S/R	
Ralfsiales **Neoralfsiaceae** 411. *Neoralfsia expansa* (J. Agardh) P. E. Lim & H. Kawai ex Cormaci & G. Furnari	X	X		I/R	
Fucales **Sargassaceae** 412. *Sargassum buxifolium* (Chauvin) M. J. Wynne			X	S/R	
413. *S. cymosum* C. Agardh	X		X	I,S/R	
414. *S. filipendula* C. Agardh	X	X	X	I,S/R	
415. *S. fluitans* (Børgesen) Børgesen	X	X	X	I,S/F	
416. *S. furcatum* Kützing	X		X	I,S/R	
417. *S. natans* (Linnaeus) Gaillon		X	X	I,S/F	
418. *S. platycarpum* Montagne			X	I,S/R	
419. *S. polyceratium* Montagne			X	I,S/R	
420. *S. polyceratium* var. *ovatum* (F.S. Collins) W. R. Taylor			X	I,S/R	
421. *S. ramifolium* Kützing			X	I,S/R	
422. *S. vulgare* C. Agardh		X	X	I,S/R	
423. *Turbinaria tricostata* E. S. Barton			X	I,S/R	
424. *T. turbinata* (Linnaeus) Kuntze			X	I,S/R	
Sporochnaceae 425. *Sporochnus pedunculatus* (Hudson) C. Agardh	X			S/R	
CHLOROPHYTA **Ulvophyceae** **Phaeophilales** **Phaeophilaceae** 426. *Phaeophila dendroides* (P. L. Crouan & H. M. Crouan) Batters	X	X	X	I, S/En	
Ulvales **Ulvellaceae** 427. *Epicladia ventriculosa* (Børgesen) M. J. Wynne		X	X	I, S/Ep	
428. *Ulvella lens* P. L. Crouan & H. M. Crouan		X	X	I, S/Ep	
429. *U. viridis* (Reinke) R. Nielsen, C. J. O¨Kelly & B. Wysor		X	X	I, S/Ep	
Ulvaceae 430. *Ulva compressa* Linnaeus	X		X	I, S/R	
431. *U. chaetomorphoides* (Børgesen) Hayden, Blomster, Maggs, P. C. Silva, M. J Stanhope & J. R. Waaland			X	I, S/R, Ep	

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
432. *U. flexuosa* Wulfen	X	X	X	I, S/R	
433. *U. lactuca* Linnaeus	X		X	I, S/R	
434. *U. intestinalis* Linnaeus	X	X	X	I, S/R	
435. *U. linza* Linnaeus	X	X	X	I, S/R	
436. *U. rigida* C. Agardh			X	I, S/R, Ep	
Kornmanniaceae 437. *Pseudendoclonium marinum* (Reinke) Aleem & E. Schulz			X	S/Ep	
Cladophorales **Anadyomenaceae** 438. *Anadyomene howei* D. S. Littler & M. M. Littler			X	S/R	NRP
439. *A. menziesii* Harvey	X	X	X	S/R	
440. *A. pavonina* (J. Agardh) Wille	X			S/R	NRP
441. *A. saldanhae* A.B. Joly & E.C. Oliveira	X		X	S/R	
442. *A. stellata* (Wulfen) C. Agardh			X	I,S/R, Ep	
443. *Microdictyon marinum* (Bory de Saint Vincent) P.C. Silva	X		X	S/R	
Boodleaceae 444. *Cladophoropsis fasciculata* (Kjellman) Wille			X	I,S/R	
445. *C. membranacea* (Hofman Bang ex C. Agardh) Børgesen	X	X	X	I,S/R	
446. *C. macromeres* W.R. Taylor			X	I,S/R	
447. *Phyllodictyon anastomosans* (Harvey) Kraft & M. J. Wynne		X	X	I/R	
448. *P. pulcherrimum* J. E. Gray		X		I/R	
Phitophoraceae 449. *Dictyosphaeria cavernosa* (Forsskål) Børgesen			X	I, S/R	
450. *D. ocellata* (M. A. Howe) Olsen-Stojkovich			X	I, S/R, Ep	
Siphonocladaceae 451. *Chamaedoris peniculum* (J. Ellis & Solander) Kuntze			X	S/R, Ep	
452. *Ernodesmis verticillata* (Kützing) Børgesen	X		X	I,S/R	
453. *Siphonocladus rigidus* M.A. Howe			X	I, S/R, Ep	
454. *S. tropicus* (P. L. Crouan & H. M. Crouan) J. Agardh			X	I,S/R, Ep	
Valoniaceae 455. *Valonia aegagropila* C. Agardh			X	I, S/R, Ep	
456. *V. macrophysa* Kützing			X	I, S/R, Ep	
457. *V. utricularis* (Roth) C. Agardh			X	I, S/R, Ep	
458. *V. ventricosa* J. Agardh			X	I, S/R, Ep	
Chaetosiphonaceae 459. *Blastophysa rhizopus* Reinke			X	S/Ep	
Cladophoraceae 460. *Chaetomorpha aerea* (Dillwyn) Kützing	X	X	X	I, S/R, Ep	
461. *Ch. antennina* (Bory) Kützing	X	X	X	I, S/R	
462. *Ch. bracygona* Harvey			X	I, S/Ep	

Table 1. (Continued)

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
463. *Ch. clavata* Kützing			X	I, S/Ep	
464. *Ch. crassa* (C. Agardh) Kützing			X	I, S/Ep	
465. *Ch. geniculata* Montagne			X	I, S/Ep	
466. *Ch. gracilis* Kützing	X	X	X	I, S/Ep	
467. *Ch. linum* (O. F. Müller) Kützing	X	X	X	I, S/Ep	
468. *Ch. minima* F. S: Collins & Hervey			X	I, S/Ep	
469. *Cladophora albida* (Nees) Kützing	X	X	X	I, S/R, Ep	
470. *Cl. catenata* (Linnaeus) Kützing		X	X	I, S/R, Ep	
471. *Cl. coelothrix* (Hudson) Kützing	X		X	I, S/R, Ep	
472. *Cl. constricta* F. S. Collins			X	I, S/Ep	
473. *Cl. corallicola* Børgesen			X	I, S/Ep	
474. *Cl. crispula* Vickers			X	I, S/R, Ep	
475. *Cl. dalmatica* Kützing			X	I, S/Ep	
476. *Cl. fracta* (O.F. Müller ex Vahl) Kützing	X			I, S/R, Ep	
477. *Cl. glomerata* var. *crassior* (C. Agardh) Hoek			X	I, S/R, Ep	
478. *Cl. intertexta* F. S. Collins	X		X	I, S/R, Ep	
479. *Cl. laetevirens* (Dillwyn) Kützing	X			I, S/Ep	
480. *Cl. lehmanniana* (Lindenberg) Kützing	X	X		I,S/Ep	
481. *Cl. liebetruthii* Grunow	X	X		I, S/Ep	NRP
482. *Cl. liniformis* Kützing	X	X		I, S/Ep	NRP
483. *Cl. montagneana* Kützing	X	X	X	I, S/Ep	
484. *Cl. pellucida* (Hudson) Kützing			X	I, S/Ep	
485. *Cl. sericea* (Hudson) Kützing	X		X	I, S/R, Ep	
486. *Cl. vadorum* (Areschoug) Kützing	X	X	X	I, S/Ep	
487. *Cl. vagabunda* (Linnaeus) van den Hoek	X		X	I, S/R, Ep	
488. *Rhizoclonium africanum* Kützing	X	X	X	I, S/Ep	
489. *Rh. crassipellitum* West & G. S. West		X	X	I, S/Ep	
490. *Rh. riparium* (Roth) Harvey	X	X	X	I, S/Ep	
491. *Rh. tortuosum* (Dillwyn) Kützing	X			S/Ep	
Bryopsidales **Bryopsidaceae** 492. *Bryopsis hypnoides* J. V. Lamouroux	X	X	X	I,S/R, Ep	
493. *B. pennata* J. V. Lamouroux	X	X	X	I,S/R	
494. *B. pennata* var. *leprieuri* (Kützing) Collins et Hervey	X			I,S/R, Ep	
495. *B. plumosa* (Hudson) C. Agardh	X		X	I,S/R	
496. *B. ramulosa* (Montagne)			X	I,S/R	
497. *Pseudobryopsis blomquistii* Díaz-Piferrer			X	S/R	
498. *Trichosolen duchassaingii* (J. Agardh) W. R. Taylor	X		X	I,S/R	

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
Derbesiaceae 499. *Derbesia fastigiata* W. R. Taylor			X	S/R	
500. *D. marina* (Lyngbye) Solier			X	I,S/R, Ep	
501. *D. osterhoutii* (L. R. Blinks & A. C. H. Blinks) J. Z. Page		X	X	S/Ep	
502. *D. vaucheriaeformis* (Harvey) J. Agardh		X	X	I,S/R	
503. *Pedobesia simplex* (Meneghini ex Kützing) M. J. Wynne & Leliaert		X		I/R	NRP
Codiaceae					
504. *Codium decorticatum* (Woodward) M. A. Howe	X		X	I,S/R	
505. *C. intertextum* Collins & Hervey		X	X	I/R	
506. *C. isthmocladum* Vickers	X		X	S/R	
507. *C. taylorii* P. C. Silva			X	I/R	
Caulerpaceae 508. *Caulerpa ashmeadii* Harvey	X	X	X	S /A	
509. *C. brachypus* Harvey			X	S/A	
510. *C. chemnitzia* (Esper) J. V. Lamouroux	X	X	X	S/A	
511. *C. cupressoides* (H. West) C. Agardh	X	X	X	S/A	
512. *C. cupressoides* var. *lycopodium* Weber-van Bosse			X	S/A	
513. *C. cupressoides* var. *turneri* Weber-van Bosse	X	X		S/A	
514. *C. cupressoides* var. *flabellata* Børgesen	X	X	X	S/A	
515. *C. fastigiata* Montagne	X	X	X	S/A	
516. *C. mexicana* Sonder ex Kützing		X	X	S/A, R	
517. *C. mexicana* f. *laxior* (Weber-van Bosse) W. R. Taylor			X	S/A	
518. *C. mexicana* var. *pectinata* (Kützing) W. R. Taylor			X	S/A	
519. *C. microphysa* (Weber-van Bosse) Feldmann	X	X	X	S/A	
520. *C. paspaloides* (Bory de Saint Vincent) Greville			X	S/A, R	
521. *C. paspaloides* f. *phleoides* (Bory de Saint-Vincent) Weber-van Bosse	X			S/A	
522. *C. paspaloides* var. *laxa* Weber-van Bosse	X			S/A, R	
523. *C. paspaloides* var. *wurdemannii* Weber-van Bosse	X	X	X	S/A	
524. *C. lamourouxii* (Turner) C. Agardh	X	X	X	S/A	
525. *C. prolifera* (Forsskål) J. V. Lamouroux		X	X	S/A	
526. *C. prolifera* f. *obovata* J. Agardh			X	S/A	
527. *C. prolifera* f. *zosterifolia* Børgesen	X	X	X	S/A	
528. *C. racemosa* (Forsskål) J. Agardh	X	X	X	S/R	
529. *C. racemosa* var. *macrophysa* (Kützing) W. Taylor			X	S/R	
530. *C. racemosa* var. *occidentalis* (J. Agardh) Børgesen			X	S/A	
531. *C. serrulata* (Forsskål) J. Agardh			X	I, S/A	
532. *C. sertularioides* (S. Gmelin) M. Howe	X	X	X	I, S/A, R	
533. *C. sertularioides* f. *brevipes* (J. Agardh) Svedelius			X	I, S/A	
534. *C. sertularioides* f. *farlowii* (Weber-van Bosse) Børgesen	X			I; S/A, R	
535. *C. sertularioides* f. *longiseta* (Bory de Saint-Vincent) Svedelius	X	X	X	S/A, R	
536. *C. verticillata* C. Agardh	X	X	X	I, S /A, M	
537. *C. webbiana* Montagne			X	S/A	

Table 1. (Continued)

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
538. *Caulerpella ambigua* (Okamura) Prud'homme van Reine et Lokhor			X	S/A, Ep	
Halimedaceae 539. *Halimeda copiosa* Goreau & E. A. Graham	X	X	X	S/A	
540. *H. discoidea* Decaisne	X		X	S/A	
541. *H. favulosa* M. A. Howe			X	S/A	
542. *H. goreauii* W. R. Taylor	X		X	S/A	
543. *H. gracilis* Harvey ex J. Agardh	X	X	X	S/A	
544. *H. incrassata* (J. Ellis) J. V. Lamouroux	X	X	X	S/A	
545. *H. lacrimosa* M.A. Howe		X	X	S/A	
546. *H. monile* (J. Ellis & Solander) J.V. Lamouroux	X	X	X	S, I/A, R	
547. *H. opuntia* (Linnaeus) J. V. Lamouroux	X		X	S/R	
548. *H. scabra* M. A. Howe	X		X	S/A	
549. *H. simulans* M. A. Howe			X	S/A	
550. *H. tuna* (J. Ellis & Solander) J.V. Lamouroux		X	X	S/A	
Dichotomosiphonaceae 551. *Avrainvillea asarifolia* Børgesen			X	S/A	
552. *A. digitata* D. S. Littler & M. M. Littler			X	I, S/R	
553. *A. ellioti* A. Gepp & E. S. Gepp	X	X	X	S/A	
554. *A. levis* M. A. Howe			X	S/A	
555. *A. longicaulis* (Kützing) G. Murray & Boodle			X	S/A, R	
556. *A. mazei* G. Murray & Boodle			X	S/A	
557. *A. nigricans* Decaisne			X	S/A, R	
558. *A. rawsonii* (Dickie) M. A. Howe			X	S/A	
559. *Cladocephalus luteofuscus* (P. L. Crouan & H. M. Crouan) Børgesen			X	S/A	
560. *C. scoparius* M. A. Howe			X	S/A	
Pseudocodiaceae 561. *Pseudocodium floridanum* Dawes & Mathieson	X			S/A	NRP
Rhipiliaceae 562. *Rhipilia tomentosa* Kützing			X	I, S /A	
563. *Rhipiliopsis profunda* (Eiseman & S. Earle) J. N. Norris & S. Blair			X	S/Ep	
564. *R. stri* (Earle & J. R. Young) Farghaly & Denizot			X	S/A	
Udoteaceae 565. Boodleopsis pusilla (F. S. Collins) W. R. Taylor, A. B. Joly & Bernatowicz	X	X	X	I,S/R, A	
566. Penicillus capitatus Lamarck	X	X	X	S/A, R	
567. P. capitatus f. laxus Børgesen	X	X	X	S/A	
568. P. dumetosus (J.V. Lamouroux) Blainville			X	S/R	
569. P. dumetosus f. expansus Børgesen	X	X	X	S/A	
570. P. lamourouxii Decaisne	X	X	X	S/A, R	
571. P. pyriformis A. Gepp & E. Gepp			X	S/A, R	

Species	States			Level/ Habitat	OBS
	CAMP	YUC	QROO		
572. *Rhipidosiphon floridensis* D. S. Littler & M. M. Littler			X	S/A	
573. *Rhipocephalus oblongus* (Decaisne) Kützing			X	I,S/A	
574. *R. phoenix* (J. Ellis & Solander) Kützing		X	X	S/A, R	
575. *R. phoenix* f. *brevifolius* A. Gepp & E. S. Gepp	X	X	X	S/A, R	
576. *Rhipocephalus phoenix* f. *longifolius* A. Gepp & E. Gepp	X		X	S/A, R	
577. *Udotea caribaea* D. S. Littler & M. M. Littler			X	S/A	
578. *U. conglutinata* (J. Ellis & Solander) J.V. Lamouroux	X		X	S/A	
579. *U. cyathiformis* Decaisne			X	S/A	
580. *U. cyathiformis* f. *sublittoralis* (W. R. Taylor) D. S. Littler & M. M. Littler	X		X	S/A	
581. *U. dixonii* D. S. Littler & M. M. Littler	X	X	X	S/A	
582. *U. flabellum* (J. Ellis & Solander) M. A. Howe	X		X	I, S/A	
583. *U. looensis* D. S. Littler & M. M. Littler	X		X	S/A	
584. *U. luna* D. S. Littler & M. M. Littler			X	S/A	
585. *U. occidentalis* A. Gepp & E. Gepp	X		X	S/A	
586. *U. spinulosa* M. Howe			X	I, S/A	
587. *U. unistratea* D. S. Littler & M. M. Littler			X	I, S/A	
588. *U. wilsonii* A. Gepp, E. S. Gepp & M. A. Howe			X	S/A	
Dasycladales **Dasycladaceae** 589. *Batophora oerstedii* J. Agardh	X	X	X	I, S/R, Ep	
590. *B. occidentalis* (Harvey) S. Berger & Kaever ex. N. J. Wynne			X	I, S/R	
591. *B. occidentalis* var. *largoensis* (J. S. Prince & S. Baker) S. Berger & Keaever ex M. J. Wynne			X	I, S/R, M	
592. *Cymopolia barbata* (Linnaeus) J. V. Lamouroux			X	S/R	
593. *Dasycladus vermicularis* (Scopoli) Krasser	X		X	S/R	
594. *Neomeris annulata* Dickie			X	S/R	
595. *N. cokeri* M. A. Howe			X	S/R	
596. *N. dumetosa* J. V. Lamouroux			X	S/R	
597. *N. mucosa* M. A. Howe			X	S/R	
Polyphysaceae 598. *Acetabularia caliculus* J. V. Lamouroux	X	X	X	I, S/R	
599. *A. crenulata* J.V. Lamouroux	X	X	X	I, S/R	
600. *A. farlowii* Solms-Laubach		X	X	I, S/R	
601. *A. schenckii* K. Möbius	X		X	I, S/R	
602. *Chalmasia antillana* Solms-Laubach			X	S/R	
603. *Parvocaulis polyphysoides* (P. L.Crouan & H. M.Crouan) S. Berger, U. Fettweiss, S. Gleissberg, L. B. Liddle, U. Richter, H. Sawitzky & G. C. Zuccarello		X	X	S/R	
604. *P. pusillus* (M.A. Howe) S. Berger, U. Fettweiss, S. Gleissberg, L. B. Lidlle, U. Rithcer, H. Sawitzky & G. C. Zuccarello			X	S/R	
Orden Ulotrichales **Ulotrichaceae** 605. *Ulothrix flacca* (Dillwyn) Thuret	X		X	I, S/Ep	

RESULTS

A total of 605 species of benthic marine algae were found in Yucatan Peninsula (Table l), of the total of marine algae identified, 38 species correspond to Cyanobacteria, 317 to Rhodophyta, 70 to Phaeophyceae and 180 to Chlorophyta (Figure 2). The percentage of species for each group is: Cyanobacteria 6.2%; Rhodophyta, 52.3%; Phaeophyceae, 11.6%; Chlorophyta, 29.9%, of them three species are new records from Mexican Caribbean, *Centroceras hyalacanthum*, *Metapeyssonnelia tangerina* and *Sarcodiotheca divaricata.* In addition sixteen species are new records for the study area; seven corresponds to Cyanobacteria, three to Rhodophyta and six to Chlorophyta (Table 1, column of observations). The family with highest species richness of the division Rhodophyta was Rhodomelaceae with 64 species, eleven of the genus Chondria and eight of *Laurencia*, *Neosiphonia* and *Polysiphonia.* It is followed in importance by the family *Corallinaceae*, with 39 taxa of which twelve belong to the genus *Neogoniolithon* and seven to the genus *Amphiroa.* Ceramiaceae has 26 species, of which 13 are of the genus *Ceramium.*

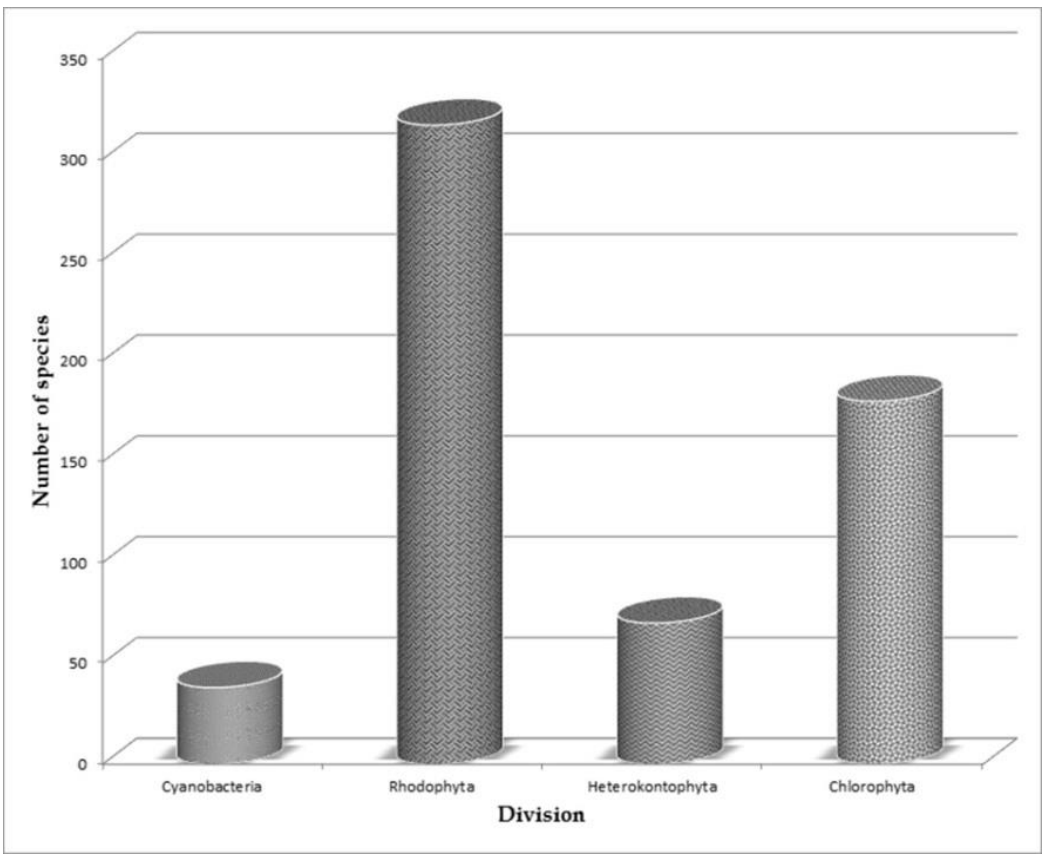

Figure 2. Total number of species for divisions in Yucatan Peninsula.

Regarding the class Phaeophyceae, the family Dictyotaceae was represented by twenty seven species, eleven of the genus *Dictyota* and five of the genus *Padina.* Concerning Chlorophyta the families Caulerpaceae and Cladophoraceae were the best represented with 31

taxa each one, 30 of genus *Caulerpa* and 19 of *Cladophora*. Udoteaceae was represented by 24 species of which twelve belong to the genus *Udotea*. The group with the lowest number of species was the division Cyanobacteria; the families Oscillatoriaceae, Phormidiaceae, Schizotrichaceae and Entophysalidaceae were represented by four species each one (Figure 3). The number of species found each state is: Campeche 271; Yucatan 214 and finally Quintana Roo with 551 (Figure 4), these data shows that the last state has the greatest diversity of marine algae in the Gulf of Mexico and Mexican Caribbean's shores [1, 2, 5, 8].

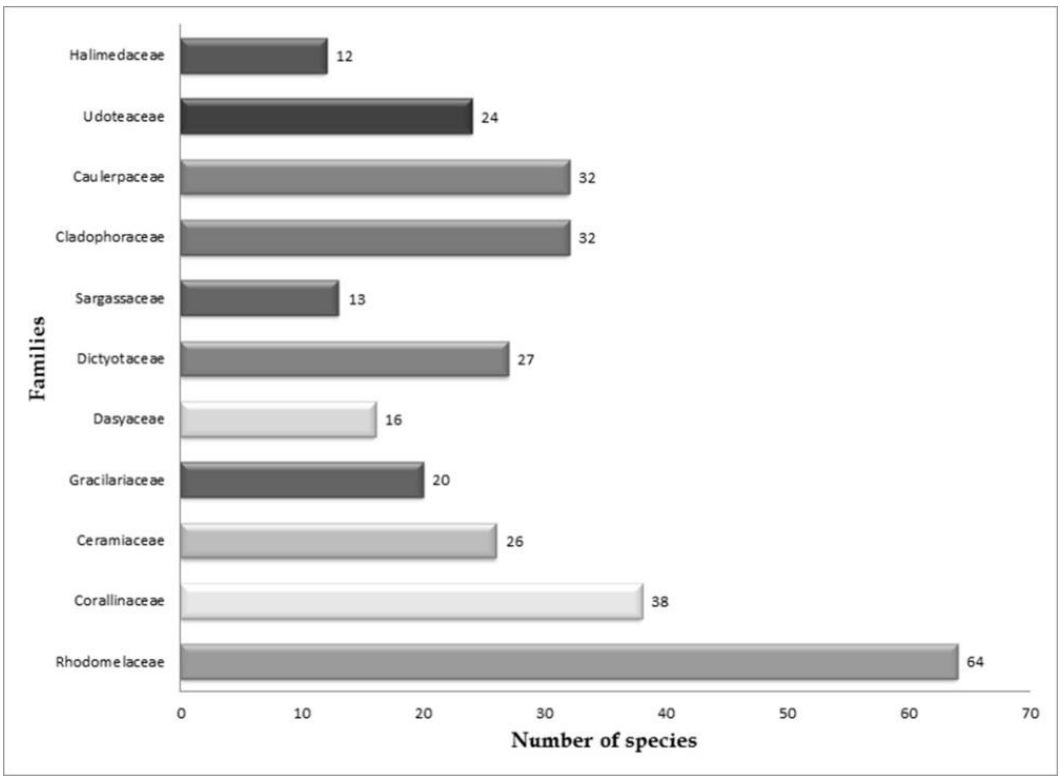

Figure 3. Number of species of best represented families in Yucatan Peninsula.

Most marine algae are indifferent to the chemical nature of their substrate; epiphytic algae can develop on other algae. Epiphytes that occur on a certain species of alga are closely related to the texture of the host, whose life span should be long enough to allow the epiphyte to complete its life cycle on it. In some cases, the occurrence of an epiphyte on a host is fortuitous, whereas in others, there is a high degree of specificity. The presence of epiphytic marine algae on other larger algae is a well-known phenomenon. Most epiphytes use the host as a support structure [20]. A significant number of epiphytes were found in this chapter, in total 257; of which 240 occurred on different algae, seagrasses and mangrove roots, with no preference for any one of them (Figure 5). The other 17 did show specificity, such as *Catenella impudica*, *Bostrychia scorpioides, B. montagnei* and *Laurencia laurahuertana***,** among others. In fact, it is known that a large number of species of algae are obligate epiphytes and in many cases form permanent associations with some species of algae, marine phanerogams and mangroves.

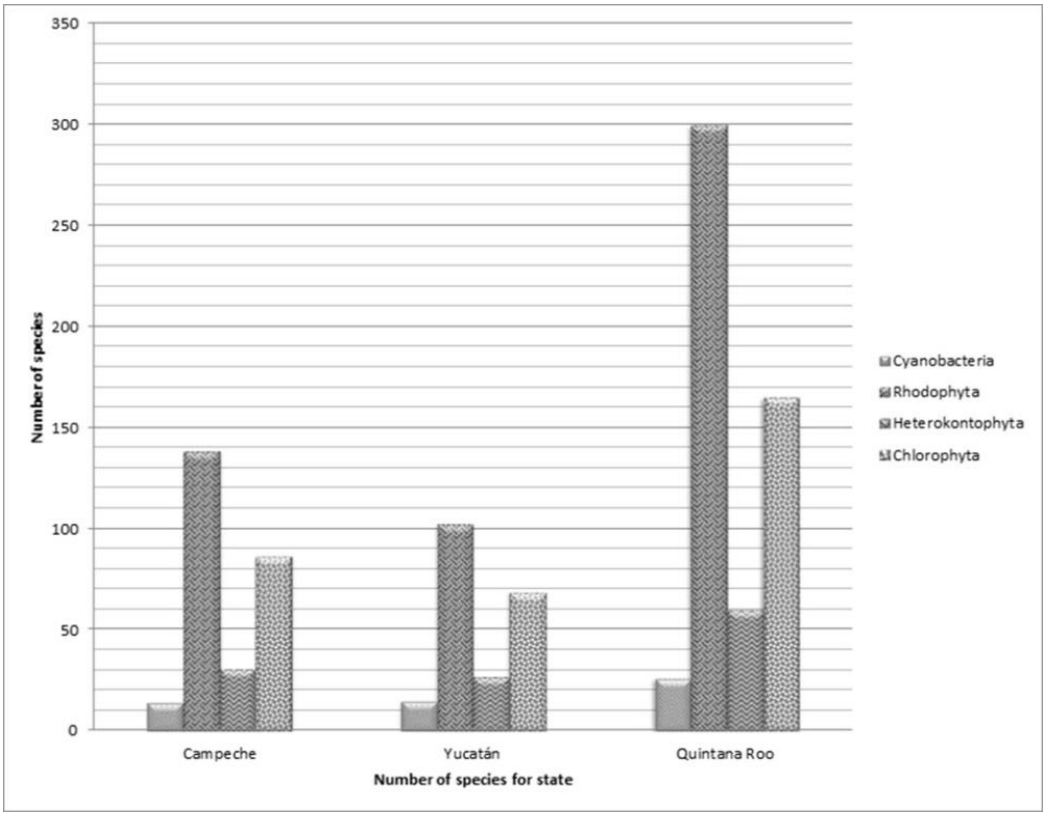

Figure 4. Number of species for states in Yucatan Peninsula.

On the other hand, there are genera as *Amphiroa, Hydrolithon, Palisada, Neogoniolithon, Ceramium, Chondria, Udotea* and *Caulerpa* which represent a taxonomic defiance, because of their high morphological plasticity and that there are probably cryptic species. The use of DNA sequence data has profoundly altered our understanding of the phylogenetic relationships among seaweeds and new Order, families, genera and species have been stablished into Rhodophyta, Chlorophyta and Phaeophyceae, among others, based on DNA sequence data and supported by morpho-anatomical characters [21, 22, 23]. In this sense, it is necessary to use molecular methods to resolve the taxonomic problems that arise in the above-mentioned genera.

Species of *Padina, Acetabularia, Liagora* and *Halimeda* were observed in Yucatan and Quintana Roo but they are not well distributed. In Quintana Roo *Sargassum* and *Turbinaria* species dominated in rocky shore environment. *Neogoniolithon, Udotea, Caulerpa* and *Halimeda* showed dominance in rocky with sandy areas. Coralline algae like *Amphiroa, Jania, Hydrolithon* and *Neogoniolithon* dominated in the reef areas of Peninsula, these organisms are of major ecological importance in the coral reefs of Campeche, Yucatan and Quintana Roo. Commercially important seaweeds like *Gracilaria cervicornis, G. damaecornis, G. debilis, Gracilariopsis andersonii, Hydropuntia crassissima* and *Eucheuma isiforme* were found on rocky coast of several localities of Campeche and Quintana Roo. *Sargassum cymosum, S. vulgare* and *Turbinaria turbinata* were found in large quantities in Quintana Roo coast.

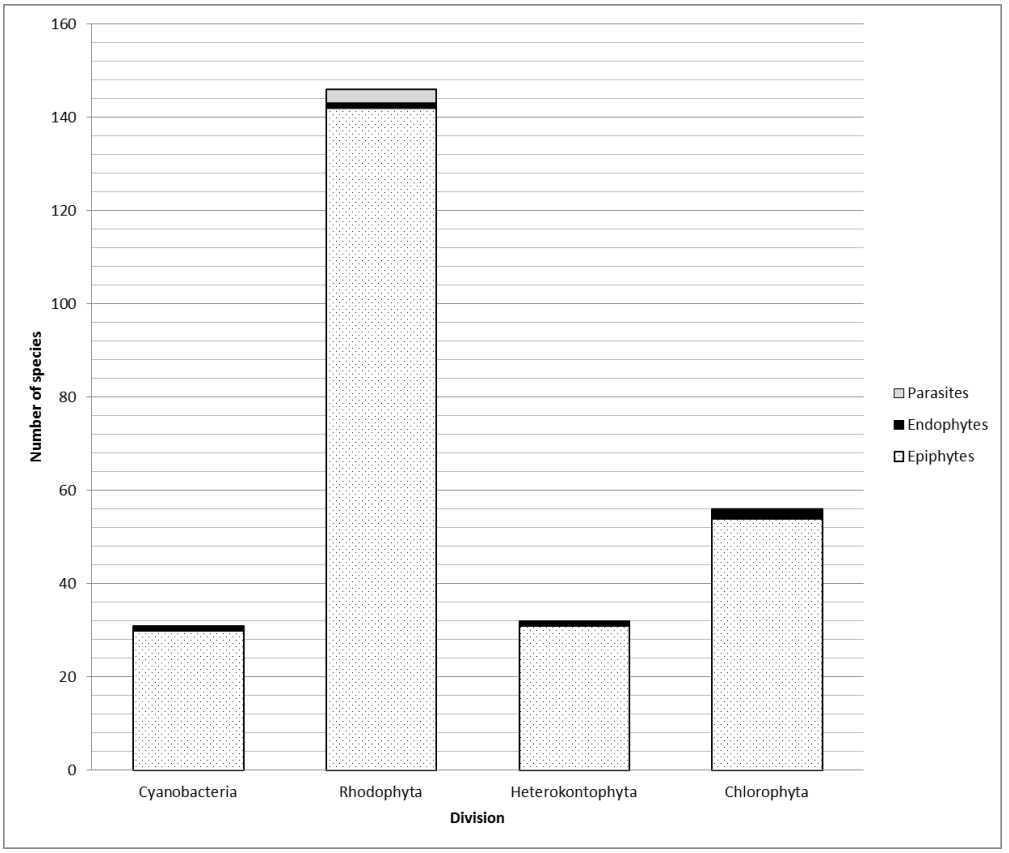

Figure 5. Number of species epiphytes, endophytes and parasites in Yucatan Peninsula.

Seaweeds are potential renewable resource in the marine environment. Most of the compounds of marine algae show anti-bacterial activities. Many metabolites isolated from marine algae possess bioactive principles. Compounds with cytostatic, antiviral, anthelmintic, antifungal and antibacterial activities have been detected in green, brown and red algae. For example, *Ulva fasciata* have showed antimicrobial activities against *Staphylococcus aureus*. Several extractable compounds such as cyclic polysulfides and halogenated compounds are toxic to microorganisms and they are responsible for the antibiotic activity of seaweeds [24]. Seasonal and geographical variation also contributes in the antimicrobial activity levels of marine algae. Species found in Yucatan Peninsula as *Ulva compressa*, *U. lactuca*, *Caulerpa lamourouxii*, *C. mexicana*, *C. sertularioides* and *C. paspaloides* are potential species for use as a source of bioactive substances.

CONSERVATION

The biological diversity, high productivity, and ecosystem services provided by tropical coastal systems are well recognized; indeed, these same characteristics may promote human settlement and urban development. Approximately 60% of the total human population lives in coastal areas and human impacts on coastal systems are increasing [10]. While losses of coral

reefs and mangrove habitats are probably the most significant in terms of losses of biodiversity it should not be forgotten that other critical coastal habitats are also disappearing. Wetland areas, estuaries and seagrass beds are known to be key nursery areas for coastal fisheries and yet are being destroyed rapidly without there being full ecological and economical appraisal of the consequences even in developed countries, also the eutrophication caused by excess of nutrients and sewage discharged into coastal waters is an expanding problem and incidents are known from almost for every coastal countries. The initial effects are of altered species composition both in the water columns and in benthic communities [25]. Furthermore, examples of infrastructure damage and coastal use alteration produced by coastal storms are numerous; for instance, one of the most recent events of this kind was hurricane Sandy which impacted the mid-Atlantic region on Oct. 29 and 30, 2012 with economic damages estimated in 30 - 50 billion US dollars. These hazards are becoming more common, as can be stated by the increasing trend found in the number and intensity of storms in the last decade [11]. Yucatan Peninsula has been affected by several hurricanes in the last decade. In 2005, "Emily" made landfall in Playa del Carmen as a category IV, affecting the zones of Xel-Ha at Riviera Maya, about 145 miles south of Cancun. In the same year "Wilma" struck the Yucatan and Quintana Roo, as a category IV, with sustained winds of 250 km/h. In 2007, Hurricane "Dean" hit southern Quintana Roo, hurting mainly 2 types of vegetation: mangrove and evergreen tropical forest. Hitting the coast in category V, just north of the Majahual, with maximum winds of 280 km/h. In 2009, the most intense hurricane was "Bill" category IV, with maximum sustained winds of 215 km/h, with gusts of 260 km/h. In 2010, three hurricanes hit the area: the most intense was "Igor" with category IV, and maximum winds of 240 km/h and gusts of 295 km/h. The hurricane Alex made landfall on the southwestern tip of the state of Quintana Roo, as a tropical storm, 90 miles southwest of Chetumal, Quintana Roo, with maximum sustained winds of 95 km/h and gusts of 110 km/h. "Karl," was another cyclone which affected Mexican territory, impacting as a tropical storm, 15 miles south-southwest of Puerto Bravo, Quintana Roo, with maximum sustained winds of 100 km/h and gusts of 120 km/h. In early August 2011, "Ernesto" made landfall as a Category I in southern Quintana Roo. Under these arguments it is evident that the Yucatan Peninsula is exposed to natural disasters and the ever-increasing human influence. In addition, the total diversity of the Yucatan peninsula flora, including previous records and the results of this investigation are 739 species; however, of these total species 134 of them were not located in the present study (Table 2) probably due to the effects of hurricanes, anthropogenic influence, especially for the modification on the coast for both of these influences which brings diverse habitats disappear with its species, the climatic conditions present and the negative effect of the expansion of the national oil industry in offshore waters, because the unfortunately accidental blowout of the Ixtoc-I in June 1979 caused the first- world massive oil spill in tropical environment. More than 3.4 million of barrels of crude oil were liberated in an ecosystem, which produced two effects one for the oil and other for the long-term environmental consequences derived from the residual hydrocarbon compounds accumulated in coastal of the Southern Gulf of Mexico [26]. In this chapter, is found a great marine algae biodiversity in Coral Reefs rather than the coast of Yucatan and Campeche. The best way to conserve marine diversity is to protect habitat and landscape diversity in the coastal area. Marine protected areas are only a part of the conservation strategy needed. It is suggested that a framework for coastal conservation is integrated coastal area management where one of the primary objectives is sustainable use of insular and coastal biodiversity.

Table 2.

Species	Nomenclatural Status	Reference
CYANOBACTERIA 1.*Lyngbya aestuarii* Liebman ex Gomont		[2]
2.*L. major* Hansgirg	*Oscillatoria major* Vaucher ex Forti	[2]
3.*L. mucicola* Lemmermann	*Leptolyngbya mucicola* (Lemmermann) Anagnostidis & Komárek	[2]
4.*L. penicillata* (Gomont) Hoffmann		[2]
5.*L. perelegans* Lemmermann	*Leptolyngbya perelegans* (Lemmermann) Anagnostidis & Komárek	[2]
6.*Oscillatoria laetevirens* Hofman-Bang ex Forti		[2]
7.*O. margaritifera* Kützing ex Gomont		[2]
8.*Phormidium corallinae* (Gomont ex Gomont) Anagnostidis & Komárek	*Oscillatoria corallinae* Gomont ex Gomont	[2]
9.*Phormidium persicinum* Gomont	*Pseudanabaena persicina* (Reinke ex Gomont) Anagnostidis	[2]
10.*Porphyrosiphon miniatus* (Gomont) F. E. Drouet	*Arthrospira miniata* Gomont	[2]
11.*Heteroleibleinia infixa* (Frémy) Anagnostidis & Komárek		[2]
12.*Plectonema nostocorum* Bornet ex Gomont	*Leptolyngbya nostocorum* (Bornet ex Gomont) Anagnostidis & Komárek	[2]
13.*Hydrocoleum comoides* Gomont	*Blennothrix comoides* (Gomont) Anagnostidis & Komárek	[2]
14.*H. glutinosum* Gomont ex Gomont	*Blennothrix glutinosa* (Gomont ex Gomont) Anagnostidis & Komárek	[2]
15.*Sirocoleum guyanense* Kützing ex Gomont		[2]
16.*Calothrix aeruginea* Thuret ex Bornet & Flahault		[2]
17.*C. juliana* Bornet & Flahault ex Gomont	*Homoeothrix juliana* (Bornet & Flahault ex Gomont) Kirchner	[2]
18.*Dichothrix fucicola* Bornet & Flahault		[2]
19.*D. penicillata* Zanardini ex Bornet & Flahault		[2]
20.*D. rupicola* F. S.Collins		[2]
21.*Symploca hydnoides* Kützing ex Gomont		[2]
22.*Trichodesmium thiebautii* Gomont ex Gomont		[2]
23.*Arthrospira major* (Kützing ex Gomont) W. B.Crow	*Spirulina major* Kützing ex Gomont	[2]
24.*Spirulina meneghiniana* Zanardini ex Gomont		[2]
25.*S. princeps* West & G. S.West		[2]
26.*S. subtilissima* Kützing ex Gomont		[2]
27.*S. tenerrima* Kützing ex Gomont		[2]
28.*S. versicolor* Cohn ex Gomont		[2]
29.*Leibleinia epiphytica* (Hieronymus) Compère		[2]
30.*L. gracilis* (Rabenhorst ex Gomont) Anagnostidis & Komárek		[2]
31.*Microchaete vitiensis* Askenasy ex Bornet & Flahault		[2]
32.*Anabaena propinqua* Setchell & N. L.Gardner	*Trichormus propinquus* (Setchell & N. L.Gardner) Komárek & Anagnostidis	[2]
33.*Nodularia harveyana* Thuret ex Bornet & Flahault		[2]
34.*Isactis plana* Thuret ex Bornet & Flahault		[2]
35.*I. centrifuga* Bornet		[2]
36.*Rivularia polyotis* Roth ex Bornet & Flahault		[2]
37.*Mastigocoleus testarum* Lagerheim ex Bornet & Flahault		[2]
38.*Entophysalis granulosa* Kützing		[2]
39.*Solentia stratosa* Ercegovic		[2]

Table 2. (Continued)

Species	Nomenclatural Status	Reference
40.*Aphanothece elabens* (Brébisson ex Meneghini) Elenkin		[2]
41.*Agmenellum termale* (Kützing) Drouet & Daily	*Merismopedia thermalis* Kützing	[2]
42.*Coelosphaerium dubium* Grunow		[2]
43.*Dermocarpa acervata* (Setchell & Gardner) Pham-Hoàng Hô		[2]
RHODOPHYTA 44.*Bangiopsis dumontoides* (P. L.Crouan & H. M.Crouan) V. Krishnmurthy		[2]
45.*Erythrocladia pinata* W. R.Taylor		[2]
46.*Acrochaetium antillarum* W. R. Taylor		[2]
47.*A. bisporum* (Børgesen) Børgesen	*Colaconema bisporum* (Børgesen) I.-K. Hwang & H.-S.Kim	[2]
48.*A. robustum* Børgesen		[2]
49.*A. unipes* Børgesen		[2]
50.*Lithophyllum daedaleum* Foslie & M. Howe	*Lithophyllum congestum* (Foslie) Foslie	[2]
51.*L. prototypum* (Foslie) Foslie		[2]
52.*Amphiroa brasiliana* Decaisne		[2]
53.*Acrothamnion butlerae* (F.S. Collins) Kylin		[8]
54.*Aglaothamnion uruguayense* (W. R. Taylor) N. E. Aponte, D. L. Ballantine & J. N. Norris		[2]
55.*Anthithamnion antillanum* Børgesen		[2]
56.*A. cruciatum* (C. Agardh) Nägeli		[2]
57.*A. decipiens* (J. Agardh) Athanasiadis		[2]
58.*A. ternatum* A. B. Joly & Cordeiro	*Antithamnionella breviramosa* (E.Y. Dawson) Wollaston	[2]
59.*Ceramium brasiliense* A. B. Joly		[2]
60.*C. dawsonii* A. B. Joly		[2]
61.*C. evermanii* Setchell & N. L.Gardner		[2]
62.*C. tenerrimum* (G. Martens) Okamura		[2]
63.*C. uruguayense* W.R. Taylor		[2]
64.*Grallatoria reptans* M.A. Howe		[2]
65.*Griffithsia caribaea* G. Feldmann		[2]
66.*G. scchousboei* Montagne		[2]
67.*Gymnothamnion elegans* (Schousboe ex C. Agardh) J.Agardh		[2]
68.*Platysiphonia caribaea* D.L. Ballantine & M.J. Wynne		[2]
69.*Dasya antillarum* (M. A. Howe) A. J. K. Millar		[2]
70.*D. arbuscula* (Dillwyn) C. Agardh	*Aglaothamnion sepositum* (Gunnerus) Maggs & Hommersand	[2]
71.*Bryocladia cuspidata* (J. Agardh) De Toni		[2]
72.*Chondria atropurpurea* Harvey		[2]
73.*C. sedifolia* Harvey		[2]
74.*Herposiphonia secunda* (C. Agardh) Ambronn		[2]
75.*Herposiphonia tenella* (C. Agardh) Ambronn		[2]
76.*Laurencia chondrioides* Børgesen		[2]
77.*Laurencia corymbosa* J. Agardh		[2]
78.*Micropeuce sarcocaulon* (Harvey) Kylin ex P.C. Silva		[8]
79. *Neosiphonia tongatensis* Harvey ex Kützing) M.-S. Kim & I.K. Lee		[2]

Species	Nomenclatural Status	Reference
80.*Ophidocladus simpliciusculus* (P. L. Crouan & H. M. Crouan) Falkenberg		[2]
81. *Polysiphonia denudata* (Dillwyn) Greville ex Harvey		[2]
82. *Polysiphonia foetidissima* Cocks ex Bornet		[2]
83. *Polysiphonia hapalacantha* Harvey		[2]
84.*Polysiphonia havanensis* Montagne		[2]
85. *Polysiphonia opaca* (C. Agardh) Moris & De Notaris		[2]
86.*Wrightiella blodgettii* (Harvey) F. Schmitz		[2]
87. *Pterocladiella caloglossoides* (Kützing) Santelices & Hommersand		[2]
88. *Pterocladiella caerulescens* (M. A. Howe) Santelices		[2]
89. *Gracilaria armata* (C. Agardh) Greville		[2]
90. *Gracilaria compressa* (C. Agardh) Greville		[2]
91. *Gracilaria curtissiae* J. Agardh		[2]
92. *Gracilaria gracilis* (Stackhouse) M. Steentoft, L.M. Irvine & W.F. Farnham		[2]
93. *Gracilaria usneoides* (C. Agardh) J. Agardh	*Hydropuntia usneoides* (C. Agardh) Gurgel & Fredericq	[2]
94. *Gracilaria venezuelensis* W. R. Taylor		[2]
95. *Grateloupia dichotoma* J. Agardh	*Dermocorynus dichotomus* (J. Agardh) Gargiulo, M. Morabito & Manghisi	[2]
96. *Halymenia duchassaingii* (J. Agardh) Kylin		[2]
97. *Eucheuma acanthocladum* (Harvey) J. Agardh		[2]
98. *Eucheuma isiforme var. denudatum* D. P. Cheney		[2]
99. *Kappaphycus inermis* (F. Schmitz) Doty ex H. D. Nguyen & Q. N. Huynh		[2]
100. *Solieria tenera* (J. Agardh) M. J. Wynne & W. R. Taylor		[2]
101. *Hypnea cornuta* (Kützing) J. Agardh		[2]
102. *Wurdemannia miniata* (Sprengel) Feldmann & G. Hamel		[2]
103. *Contarinia magdae* Weber-van Bosse		[2]
104.*Chrysymenia planifrons* (Melvill) J. Agardh		[2]
PHAEOPHYTA 105.*Dictyota linearis* (C. Agardh) Greville	*Dictyota implexa* (Desfontaines) J.V. Lamouroux	[2]
106.*Acinetospora crinita* (Carmichael) Sauvageau		[2]
107.*Feldmannia indica* (Sonder) Womersley & A. Bailey		[2]
108.*Sphacelaria fusca* (Hudson) S. F. Gray		[2]
109.*Hummia onusta* (Kützing) J. Fiore		[2]
110.*Sargassum hystrix* J. Agardh		[2]
111.*Sargassum vulgare var. foliosissimum* (J. V. Lamouroux) C. Agardh		[2]
CHLOROPHYTA 112.*Gomontia polyrhiza* (Lagerheim) Bornet & Flahault		[2]
113.*Blidingia marginata* (J. Agardh) P. J. L. Dangeard		[2]
114.*B. minima* (Nägeli ex Kützing) Kylin		[2]
115.*E. prolifera* (O. F. Müller) J. Agardh	*Ulva prolifera* O.F. Müller	[2]
116.*Dictyosphaeria versluysii* Weber-van Bosse		[2]
117.*Valoniopsis pachynema* (G. Martens) Børgesen		[2]

Table 2. (Continued)

Species	Nomenclatural Status	Reference
118.*Cladophora brasiliana* G. Martens		[2]
119.*Cladophora conferta* P. L. Crouan & H. M. Crouan		[2]
120.*Cladophora constricta* F. S. Collins		[2]
121.*Cladophora jongiorum* Hoek		[2]
122.*Cladophora patentiramea* (Montagne) Kützing		[2]
123.*Cladophora pellucidoidea* Hoek		[2]
124.*Rhizoclonium crassipellitum var. robustum* G. S. West		[2]
125.*Bryopsis halliae* W. R. Taylor		[2]
126.*Codium carolinianum* Searles		[2]
127.*Codium spongiosum* Harvey		[2]
128.*Caulerpa cupressoides* var. *serrata* (Kützing) Weber-van Bosse		[2]
129.*Caulerpa lanuginosa* J. Agardh		[2]
130. *Caulerpa paspaloides* var. *compressa* (Weber-van Bosse) M. A. Howe		[2]
131.*Caulerpa taxifolia* (M. Vahl) C. Agardh		[2]
132.*Rhipilia tomentosa* Kützing f. *zonata* A. Gepp & E.S. Gepp		[2]
133.*Udotea verticillosa* A. Gepp & E. S. Gepp		[2]
134.*Ostreobium quekettii* Bornet & Flahault		[2]

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Chapter 18

BENTHIC ASSEMBLAGES IN SOUTH AMERICAN INTERTIDAL ROCKY SHORES: BIODIVERSITY, SERVICES, AND THREATS

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ABSTRACT

Rocky shores are areas of high diversity and productivity providing goods and services. Since humans are altering nature at an unprecedented rate, producing shifts in important parameters for life such as temperature, habitat availability, water quality, among others, it is expected that species will respond by changing their natural distributions and/or abundances. To understand how species will respond to such changes, it is necessary to learn the processes that determine these patterns. The South American Research Group on Coastal Ecosystems was established to assess marine diversity and biomass along both coasts of South America through an international collaboration. The main goals of SARCE are to: (1) Test hypotheses about latitudinal gradients and patterns of local and regional biodiversity, (2) Identify the relationship between biodiversity and ecosystem functioning, (3) Assess the effect of environmental gradients and anthropogenic stressors, (4) Carry out capacity building and training activities aimed to solve environmental problems for the benefit of society. The SARCE network has sampled the coasts of nine countries around South America with a standardized protocol in more than 150 sites (2010-2014), ranging from 11º North to 55º South. This chapter provides a description of the biodiversity of the sites sampled by SARCE, along with a review of the uses and services that these ecosystems provide to human populations and the main threats and impacts these uses have caused.

INTRODUCTION

Biodiversity has been a subject of interest for many decades by scientists and conservationists. More recently, other groups such as managers, government agencies and industries have also been involved in establishing its ecological and economical value, as well

as the consequences of its loss. Up to date, an important number of papers have attempted to identify the importance of biodiversity for ecosystem functioning (Loreau et al. 2001, Pachepsky et al. 2001, Cardinale et al. 2002, Pfisterer & Schmidt 2002, Gessner et al. 2004) and the processes by which any diversity loss will be negatively reflected in the number and quality of services that a particular system might provide (Balvanera et al, 2006; Cardinal et al, 2006, O´Connor et al., 2006).

Figure 1. Map of South America showing the localities sampled by the SARCE network (South American Research Group in Coastal Ecosystems) in the Caribbean Sea, and in the Atlantic and Pacific oceans.

Coastal marine ecosystems have a very high biodiversity (Ray 1996). Within these, the macroalgal habitats rank among the highest along with coral reefs and seagrass communities, due to the fact that they are dominated by bio-engineering organisms that build three-

dimensional structures, providing substrate, food and habitat complexity, which ultimately increases species richness (van Oppen et al. 1996, Phillips 1997, Walker & Kendrick 1998, Wysor et al. 2000, Duarte 2000, Engelhardt & Ritchie 2001, Duffy et al. 2001, Sommerfield et al. 2002, Bulleri et al. 2002). On the other hand, due to their particular location (i.e. land-sea interface) these coastal areas are also severely impacted by human activities such as fisheries overexploitation, alteration of the physical environment, pollution, introduction of alien or invasive species and recreational activities, all of which have inevitably impoverished marine biodiversity (Beatley 1991, Norse 1993, Gray et al., 1997, Walker & Kendrick 1998, Cury 1999, Bax et al. 2001, Tilman & Lehman 2001, Piazzi et al. 2001, Barnes 2002).

In this sense, the study of biodiversity is crucial for the sustainable use of coastal resources (Gray 1997), especially in Marine Protected Areas (Ray 1985, Olsen 1999, Ward et al. 1999). Biodiversity has been measured at many different levels and scales and by different methods (France & Rigg 1998). This has made comparisons difficult, so a unified approach to study biodiversity at a global scale was much needed (Rabb & Sullivan 1995, Valero et al. 1998, Mikkelsen & Cracraft 2001). As a response to this need, the NaGISA project (Natural Geography in Shore Areas: www.nagisa.coml.org) of the Census of Marine Life program (CoML: www.coml.org) provided the necessary framework to study biodiversity in rocky shores at a global scale. The NaGISA project was a collaborative effort aimed at inventorying and monitoring habitat specific biodiversity with a standard protocol in coastal marine areas at a global scale (Konar et al. 2010). Thanks to NaGISA (2003-2010), the first global baseline of nearshore biodiversity was initiated [see: Diversity in the Nearshore: The NaGISA Collection (2010) PLoS Collections: http://dx.doi.org/10.1371/issue.pcol.v01.i06], and in South America, it has continued through the South American Research Group on Coastal Ecosystems network (SARCE). This network was established to assess marine diversity and biomass along the Pacific and Atlantic (including the Caribbean) coasts of South America through an international collaboration. The main goals of SARCE are to: (1) Test hypotheses about latitudinal gradients and patterns of local and regional biodiversity, (2) Identify the relationship between biodiversity and ecosystem functioning, (3) Assess the effect of environmental gradients and anthropogenic stressors, (4) Carry out capacity building and training activities aimed to solve environmental problems for societal benefit. The SARCE network includes more than 30 researchers from 9 South American coastal countries and has sampled with a standardized protocol in more than 150 sites around the continent (Figure 1). In this chapter we provide a description of the biodiversity of the sites sampled with the SARCE protocol (http://sarce.cbm.usb.ve/for-scientists/), along with a review of the uses and services that these ecosystems provide to human populations and the main threats and impacts these uses have caused.

THE INTERTIDAL ROCKY SHORES IN SOUTH AMERICA: MAIN FEATURES AND ASSOCIATED BIODIVERSITY

The Caribbean

Colombia

The Caribbean coastline of Colombia has an extension of 1760 km, of which 25% are rocky shores (Posada & Henao 2008), mainly composed by unstable shores highly affected by

wave action and coastal erosion. Most of these shores have steep slopes and in the areas where the platform occurs can harbor rich and abundant macro-algal communities (Garcia & Diaz-Pulido 2006). The tide range is 0.5 m due to this is common to find in small areas a mixture of organisms that belongs to different intertidal levels (high, mid and low tide), factors as wave action, substrate type and slope, determine the community composition (Lopez-Victoria et al. 2004). Due to the small tidal range only high and low tide levels can be easily differentiated. According to Lopez-Victoria et al. (2004) the rocky shore can be divided into two types of rocks, cohesive or non-cohesive, each having a particular associated community (algae and macroinvertebrates). The first type of rocks is stable, hard and with low erosion rates, with a high rate of colonization and a well developed community with advanced succession stages. The second type of rocks is unstable, the shores are highly affected by wave action, and therefore, species diversity and richness is lower, and the community cannot reach advanced succession stages. The climate presents two main periods: dry and rainy seasons with a transition season in between. The sea surface temperature and salinity during the dry and rainy seasons vary between 25.5-27.5ºC / 35.6-37 ppt and 27-29.5ºC / 34.5-36.5 ppt respectively. The waves are higher during the dry season (1.5 to 2.5 m) in relation to the rainy season (0.5 to 1.4 m) (Posada & Henao 2008). An upwelling system is present from December to March in the north coast and the sea surface temperature can drop below 20ºC.

In the Colombian Caribbean, the SARCE project sampled in 15 sites within two localities: Santa Marta and Darien. In Santa Marta (Figure 2A), the rocky shore is dominated by cliffs (metamorphic schist rocks), with different size boulders at the base that gives complexity to the shore and offers a variety of habitats that can be exploited by intertidal organisms. The cliffs are part of the Sierra Nevada de Santa Marta system that branches down to the sea. Due to the upwelling, there is a major change in algae composition, species of *Sargassum* can reach a meter in length and other algae species grow and cover most of the rocky substrate. Along the different sites of the Santa Marta bay, the geology varies from cliffs that continue as rocky shores with boulders in the north (Punta Verde) which are highly exposed to wave action, to rocky platforms between 5 to 10 m wide in the south (Playaca). The most abundant invertebrate species in the high intertidal in the north are *Nerita versicolor* and *Littorina* sp, while the low intertidal is dominated by coralline algae, by *Palisada perforata*, *Zoanthus pulchellus* and *Hypnea musciformis*, and the invertebrates *Echinometra lucunter* and *Isognomon bicolor*. In the south, the high intertidal is mostly rock; with some areas with filamentous algae. The most abundant macroinvertebrate species are *Plicopurpura patula*, *I. bicolor* and *Dendropoma* sp. In the low intertidal, dominant species are coralline and filamentous algae along with *Laurencia obtuse, E. lucunter*, *I. bicolor*, *Spirobranchus giganteus* and *Balanus* sp. The low intertidal is also covered by the canopy of large *Sargassum.* The east side of the bay (Playa Grande) is characterized by a rocky platform covered by a thin layer of vermetids. Here, the high intertidal is mostly rock with some areas covered by filamentous algae, the dominant macroinvertebrate species being *N. tesellata* and *Brachidontes domingensis*. In the low intertidal, dominant species are the macroalgae *Acanthophora spicifera*, *Dictyopteris deliculata* and *Neoralfsia expansa* and the invertebrates *Dendropoma* sp. and *I. bicolor*. Other localities sampled in the Santa Marta region were Inka Inka, Puerto Luz, and Aeropuerto. The first two were rocky platforms, while the third was an exposed sandy beach with flat rocks that may be periodically covered by sand due to wave action. In these, the high intertidal was dominated by *N. tesellata*, *P. patula*, *Echinolittorina*

ziczac (Inka Inka); *E. ziczac*, *E. angustior* and *P. patula* (Puerto Luz); and *E. interrupta*, *E. angustior* and *Chthamalus sp*. (Aeropuerto). The low intertidal was dominated by coralline algae, vermetids, filamentous algae, *E. lucunter*, *I. bicolor*, *Mitrella ocellata*, *Sargassum* (Inka Inka), *A. spicifera*, *L. obtuse*, filamentous algae, *Dendropoma* sp., *E. lucunter*, *I. bicolor* (Puerto Luz), and *A. spicifera*, *Lyngbya* sp., coralline algae, *Centroceras* sp., *B. domingensis*, *Fissurella nimbosa*, *Stramonita haemastoma* (Aeropuerto).

The locality of Darien is located west of the Uraba Gulf, where mangroves and soft bottoms dominate the landscape; the sediments and nutrients are brought by the Atrato River, one of the largest rivers in the Atlantic basin of Colombia. Tropical rainy forest is the most common type of vegetation; however, grasses for cattle growth have replaced large extensions of this forest. In this region, the volcanic rock shore is located north of the delta of River Atrato, followed by large sandy beaches and abrasion platforms of coralline origin towards the Panama border. This area has little urban development and human settlements are small, there are no roads and the main way of transportation is by boats and most of the settlements are located on the coast. The Darien was sampled in the Trigana area which is located north to the Atrato River delta, with a rocky shore of volcanic origin, interrupted by sandy beaches, and with several small islands in front of the coast. The intertidal in this area is affected by freshwater runoff. The sites ampled were Isla Napú, an islet with a narrow rocky platform that falls to the sea reaching a maximum of 2 m depth in the surrounding areas; Trigana, a platform 10 m wide; Titumate, an islet with a soft slope and shallow sandy bottom (< 1 m) and sea grasses; Capurgana, located in the northwest part of the Uraba Gulf with a rocky shore composed by abrasion platforms of coralline origin; Sapzurro, a bay close to the Panama border, with a soft slope shore and an abrasion platform of coralline origin; and Isla Narza, an islet of volcanic origin in front of Capurgana village, the shore consisting of a cliff in the exposed side and boulders in the sheltered side. In these sites, the high intertidal was dominated by E. angustior, *E. ziczac* and *E. interrupta* (Napu), *E. angustior* and *S. rustica* (Trigana), *E. angustior*, *B. domingensis* and *N. tesellata* (Titumate), and *E. angustior* (Sapzurro and Isla Narza). The low intertidal is dominated by *L. obtusa*, *Pterocladiella capillacea*, *Lyngbia* sp., *Gracilaria domingensis*, *Centroceras* sp., the barnacles *Chthamalus* sp. and *Balanus* sp. which form a complex with vermetids (Napu); filamentous and coralline algae, *S. serratum*, *Centroceras* sp., *L. obtuse*, *G. domingensis*, *Chthamalus sp*., *S. rustica* (Trigana); *S. serratum*, *L. obtuse*, *Centroceras* sp., *Chthamalus* sp., *S. rustica* (Titumate); *L. obtuse*, coralline algae, *E. lucunter*, *Cittarium pica* (Sapzurro), and coralline and filamentous algae, *E. lucunter*, *Ceratozona squalida*, and *Chiton squamosus* (Isla Narza).

Venezuela

Rocky shores along the 3964 km of the Venezuelan coastline are very heterogeneous in terms of their geological composition and structure (Miloslavich et al., 2005). Due to small tidal ranges in the southern Caribbean (20-30 centimeters) (Torres and Tsimplis, 2012) Venezuelan rocky shores have been described considering only two levels or strata: high intertidal and low intertidal. The low intertidal is constantly under wave action whereas, the high intertidal is washed rarely by waves, only receiving sea's spray. Consequently, in some Venezuelan rocky shores, gaps openings and/or depressions in the substrate of only few centimeters of depth can generate zones that remain submerged most of the year. These habitats, henceforth called substrates depressions, are located between the high and the low intertidal on platform rocky shores and their species composition is completely different to

that found in the high and low intertidal. Given their large extensions, "substrates depressions" are different to rock pools or tide pools.

Characteristics of rocky shores are very heterogeneous and respond to the geomorphology of the different regions of the Venezuelan coast. In the Western coast, from the Paraguana Peninsula to Patanemo, rocky shores are emerging platforms and are composed of limestone rocks (Figure 2B). The Central coast, from Ocumare to Chirimena (Figure 2C), is characterized by narrow rocky stripes formed by sandstones and conglomerates. Sites sampled on the mainland of the Eastern coast, from Santa Fe to La Pared (Figure 2D), had a very steep slope, whereas those sampled in the insular region were emerging horizontal rocky platforms. Nevertheless, all rocky shores in the East are formed by limestone's rocks. Most of the platforms are narrow (3-10 meters), except for San Juan de los Cayos platforms that are between 60 to 120 meters wide. The length of platforms is highly variable. In the western region, they can reach few kilometers, whereas in the central coasts they do not surpass hundreds or tens of meters (Kennedy et al., 2014; Ellenberg, 2010).

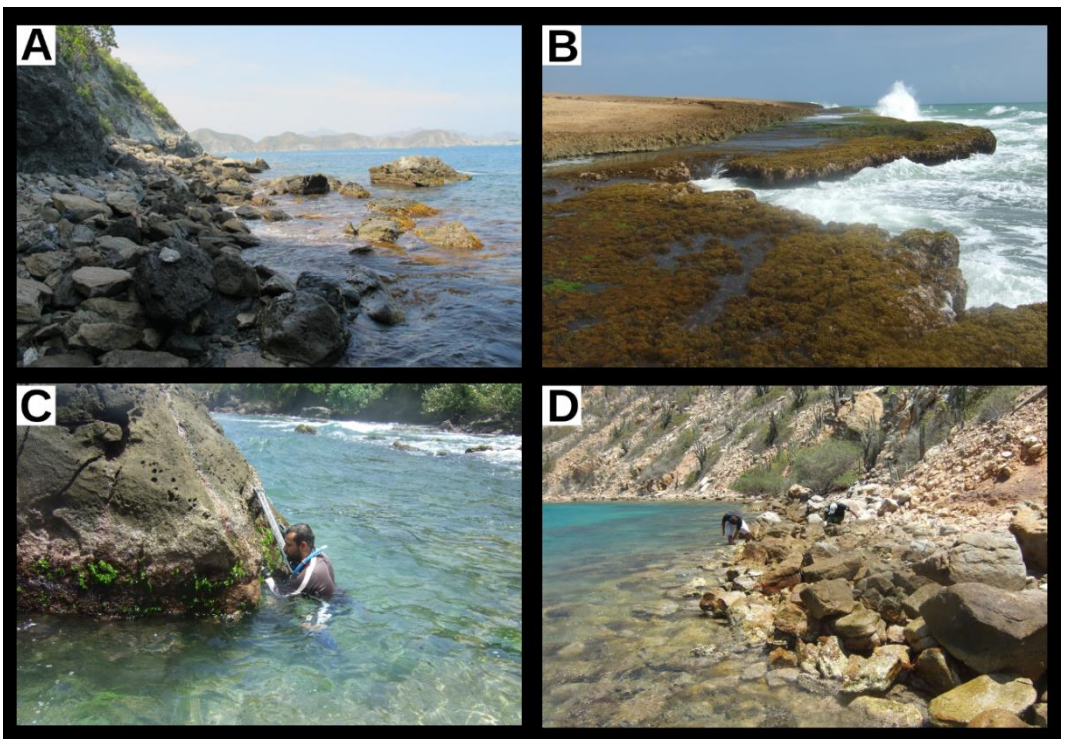

Figure 2. Sampling sites in the Caribbean. A. Colombia – Taganaga, Santa Marta. B. Venezuela - Cabo San Román, West Coast. C. Venezuela – Chuspa, Central Coast. D. Venezuela – Mochima, East Coast.

Estimates of rugosity were moderately high for both strata: 0.809 ± 0.002 and 0.745 ± 0.003 for low and high intertidal, respectively. The rock's irregularities form microhabitats that are used by some organisms like whelks, crabs, limpets and snails that protect them from desiccation and high temperatures during low tides, which in turns contribute to the high diversity reported for the Southern Caribbean. Crevices, scars and holes over the rocky substrate are due to erosion of wind and wave, as well as to the activity of some organism such as the sea urchin *Echinometra* sp. (Bak, 1994).

Despite a small tidal amplitude (Torres and Tsimplis, 2012), desiccation levels can vary significantly due to the effect of wind. Northern Trade winds blow on Venezuelan coasts intensively between December and June, increasing intensity and height wave, and consequently decreasing exposition levels. Besides, trade winds are responsible of annual upwelling, between January and May, in different zones of Venezuelan coast (Castellanos et al., 2002; Muller-Karger, 2004); which has been reported to enhance algae growth and increase the production of herbivores in rocky shores (Wieters, 2005; Bosman et al., 1987). This has not been tested for the Venezuelan Coast, however, the only study done (western region, Peninsula de Paraguna) found no correlation between Sea Surface temperature Changes generated by upwelling and changes in the structure of assemblages associated with rocky shores (Herrera unpublished data).

Like many rocky shores in the continent and the world, assemblages living in Venezuelan rocky shores are mainly composed of seaweeds and small mollusks. The most common seaweeds belong to phylum Rhodophyta, being genera such as *Laurencia* and *Polysiphonia* the most abundant. Also, algae of phylum Chlorophyta (mainly *Ulva* spp.) and Ochrophyta (mainly *Sargassum* spp.) can be widely found. The species complex made of crustose coralline algae (commonly named CCA) was present in almost all sampled sites. It is very likely that the species composition of these complexes change among zones, sites, regions and strata; but identification of species of crustose coralline algae in the field is not possible. This complex was present as primary cover usually below corticated, articulated and foliose algae, as well as secondary cover on top of sessile and mobile animals. The most common mollusks living in Venezuelan rocky shores were snails, limpets, whelks, key-hole limpets and chitons.

The dominant functional groups in Venezuela were primary producers (macroalgae), small herbivores (gastropods and chitons), large herbivores (sea urchins and crabs), filter feeders (bivalves and barnacles) and predators (mainly the gastropods *Plicopurpura patula*, *Stramonita* and *Vasula* species; and octopuses). In the southern Caribbean, unlike other rocky shores, the dominant echinoderm was not a sea star but the sea urchin *Echinometra lucunter*. It has been proposed (but not tested) that *E. lucunter* plays a key role in structuring these assemblages due to its high densities, high herbivory rates and bio-erosive activity. Another peculiarity of Venezuelan rocky shores is the low density of barnacles in the intertidal. In Venezuela, they are only present in the central and western coast, usually associated to rock-walls, cliffs and artificial structures.

Continuous monitoring of assemblages associated with rocky shores in Venezuela has shown that they vary importantly at different temporal and spatial scales, and between strata. For example, during the rainy season, conspicuous changes occur due to massive algae cover decrease, and the composition is dominated by opportunistic species such as *Ulva*, *Dictyota*, *Padina* and *Chaetomorpha*. Also, from a spatial point of view and despite large variation at small spatial scales (10s to 100s meters), important differences can be detected among regions (e.g. presence of barnacles only in central and western coast).

Intertidal rocky shores of Venezuela are part of the highly diverse coastal ecosystems of the Southern Caribbean. Using SARCE's protocol, 31 sites across the Venezuelan coast were sampled, detecting a total of 217 species in total: 85 marine algae (40 Rodophyta, 20 Chlorophyta, 18 Ochrophyta, 5 Cyanobacteria, 2 seaweeds not identified), 89 molluscs (66 Gastropoda, 17 Bivalvia, 6 Polyplacophora), 21 cnidarians (17 Anthozoa, 4 Hydrozoa), 8 arthropods (5 Malacostraca, 3 Maxillopoda), 5 echinoderms (2 Echinoidea, 2 Ophiuroidea, 1

Holothuroidea), 5 marine sponges (Demospongie), 3 ascidia (Ascidiacea) and 1 seagrass (Tracheophyta).

In the high intertidal of Venezuelan rocky shores, algae presence was uncommon; and when they were, these usually were crustose calcified algae CCA and *Pseudolithoderma extensum*, or filamentous algae such as *Lyngbya* spp. and *Bostrychia tenella*. Mobile species were represented mostly by small herbivores mollusks such as *Nerita versicolor*, *Nerita* peloronta, *Nerita tessellata, Echinolittorina ziczac, Echinolittorina interrupta, Acanthopleura granulata, Echinolittorina angustior*, *Cechritis muricatus*, *Tectarius antonii* and *Acmaea* and *Siphonaria* species. Carnivorous mollusks (i.e. *Plicopurpura patula*) were also present but were not as abundant as herbivores species. Assemblages in the high intertidal were highly dominated by few species, especially by Littorinids that commonly had abundances between thousands and tens of thousands of individuals per square meter. Neritidae species were not as abundant, but densities could reach hundreds per square meter. In crevices and gaps, bivalves belonging to genus *Brachidontes* and *Isognomon*, were found in low densities. Sessile mollusks of the family Vermetidae were also occasionally found in very dense patches with abundances ranging between the tens and hundreds of individuals per square meter.

Assemblages in depressions or mid platforms shallow lagoons were dominated by corticated foliose algae such as *Dictyota* and *Padina*, articulated calcareous algae such as *Halimeda opuntia*, foliose calcareous algae such as *Udotea sp* and *Penicillus sp*, and the foliose algae *Ulva* spp. Because these environments are constantly covered by water, cnidarians belonging to genus *Zoanthus* and *Palythoa* were commonly found. Principal mobile organisms in these microhabitats were small fishes belonging to Gobiidae family and hermits crabs. These two groups, however, were not considered in this study. These habitats are constantly submerged by water that is constantly being replaced, but due to their shallow characteristics, temperature is usually few degrees above normal Sea Surface temperature. Consequently, substrate tends to be dominated by one or two of the species mentioned above.

The low intertidal of Venezuelan rocky shores was dominated by macroalgae, mollusks and sea urchins; whereas some cnidarians and other echinoderms (sea cucumbers and brittle stars) were found occasionally. The most abundant and commonly found algae in all sampled sites, were the crustose calcified algae comples CCA and *P. extensum*, the corticated corticated algae *Laurencia obtusa*, *Laurencia papillosa* and *Gelidiella acerosa*, the filamentous algae *Polysiphonia atlantica*, the leathery macrophyte *Sargassum* spp, and filamentous microalgae *Lyngbya* spp. Few species, as the opportunistic green foliose algae *Ulva* spp, the green filamentous algae *Chaetomorpha* spp, and cnidarians *Palythoa* and *Zoanthus* were not commonly seen in all sites; but when they were present, they occupied an important proportion of the primary and secondary substrata. The most abundant mobile species in the low intertidal were herbivores *E. lucunter*, *Chiton squamosus* and *Fissurella* spp, as well as carnivores *Stramonita rustica*, *P. patula* and *Vasula deltoidea*. The carnivore gastropod *P. patula* has its highest densities in the high intertidal (very close to the transition between the high and the mid), however it is present in the low intertidal as well. The sea urchin *E. lucunter* was commonly found in almost all the sites sampled, reaching densities of up to 72 ind/m^2. It is likely that *E. lucunter* plays a key role as the principal herbivorous on Venzuelan rocky shores, but this conceptual model has not yet been tested. Other large herbivores such as the gastropods *Cittarium pica* and *Astraea* spp, are found in low densities and small sizes, very likely due to the pressure of artisanal fishermen.

Trinidad and Tobago

Trinidad and Tobago is a twin-island state located on the continental shelf of north eastern South America. Trinidad is approximately 12 kilometers from the mainland while Tobago is 30 kilometers North East of Trinidad. Trinidad is the most southerly of the Caribbean islands. The continental origin of the islands is reflected in the similarity of terrestrial fauna and flora. The coastal areas of Trinidad and Tobago are largely comprised of sedimentary rocks. The north coast however is comprised of non-sedimentary rock with low grade metamorphic and small areas of volcanic rock (Georges, 1983). The geomorphology is generally of gently sloping beaches and cliffs. Current flow around Trinidad and Tobago is driven by the South Equatorial current coming north from South America. The current splits with movement to the west entering the Gulf of Paria and to the east moving and merging with the Atlantic Ocean.

Prior to the NAGISA project (2005), the intertidal rocky shores of Trinidad and Tobago had not been studied in any detail. The sites sampled with the SARCE project are on the north-east coast (Saybia, Toco) and the north coast (Maracas Bay). In the north-east coast location (Saybia, Toco there is a fringing reef which offers some protection although there is a strong westerly longshore current. In the north coast (Maracas Bay) area there are generally strong offshore winds and a strong longshore current, although some protection is offered by the headlands. The mean tidal range in Trinidad and Tobago is around 1.2m and is semi-diurnal with a high and low every 12hrs.

The biodiversity associated with the intertidal sites typically included the common groups: macroalgae (green, red, and brown), bivalves (*Isognomon* sp. the most common but only found at Salybia and Toco Bay), gastropods (*Littorina* sp. the most common), polyplacophorans, crustaceans (barnacles found at all sites except Las Cuevas), and tunicates. The red algae, mostly *Heterosiphonia* were the most dominant species, while brown algae were least dominant, and *Chaetomorpha* sp. was the most common green algae (found at Maracas and Blanchisseuse). Coralline algae were well represented at all sites except for Salybia. There were greater numbers of species of soft corals (22) as opposed to soft corals (2), all of which were found in Salybia and Toco Bay, sites nearby to coral reef systems.

The Atlantic

Brazil

The Northeast: Ceara

The Ceará state coastline is dominated by long sand beaches, interrupted occasionally by beachrock reefs (Aquasis, 2003). The beachrocks have a more recent origin, and are composed by sand, shell fragments and pebbles cemented by calcium carbonate and iron oxide (Smith and Morais, 1984). These reefs are generally tabular, of variable extension and sloping gently towards the sea. The coastline is more E-W oriented, along typically equatorial latitudes. The climate is typically semi-arid, ruled by the intertropical convergence zone, with 2 seasons characterized by the pluviometry: a rainy season from January to June, and a dry season from July to December (IPECE, 2013). The coast is washed by the North Brazil current, running from E to W, following the strong trade winds that are characteristic for the region (Aquasis, 2003). The North Brazil current water mass is considered oligotrophic, with

temperatures varying little around 26ºC and salinity around 36 (NOAA, 2014). The constant trade wind regime blowing from E-SE with 6.4 m.s-1 on average, reaching more than 10 m.s-1 during the dry season (Jimenez et al., 1999). Wind waves are permanently splashing over the reefs, sometimes combined with swell waves, ranging from 1.8 to 3.6 m in height (Aquasis, 2003). The tidal regime is typically semidiurnal, with a mean spring tidal range of 3.3 m, and mean neap tidal range of 1.2 m.

The beachrock reefs of Ceara host a very diverse community which is still poorly studied. The rocky intertidal shows a typical biodiversity zonation from the supralittoral to the subtidal zones. The supralittoral fringe is barely colonized, and the dominant species is *Echinolittorina lineolata*, a small mobile gastropod that fits into minute crevices avoiding extreme desiccation. The same species is also abundant in the high intertidal, but the space is occupied now by barnacles, especially *Chthamalus proteus* and patches of the green algae *Ulva fasciata*. The association of these two species characterizes the whole upper littoral zone, which is considerably poor in terms of species richness. The mid littoral zone is more diverse and the dominant species may vary at different locations. In Caucaia (Figure 3A), near urban capital Fortaleza, there is a belt of the bivalve *Brachidontes exustus* at the higher portion of the upper littoral zone, and a continuum of large colonies of the polychaete *Phragmatopoma caudate* is also present. The dominant algae species are *Chondracanthus acicularis*, *Gelidiella acerosa*, and *Hypnea musciformis*. Other dominant species are *C. proteus* and *U. fasciata*, which are still abundant in the lower littoral zone. In Trairi, there is not a conspicuous band of *B. exustus*, and the mid littoral zone is then dominated by *U. fasciata*, *Pterocladiella caerulescens* and *Laurencia papillosa*. Large colonies of *P. caudata* are present, and the zoanthid *Palythoa grandiflora* also covers large areas of this zone. The mobile fauna present in the mid littoral zone is mainly composed of crabs, especially *Pachygrapsus transversus*, hermit crabs, such as *Clibanarius antillensis* and *Calcinus tibicen* (Herbst, 1791). The lower littoral zone is the most diverse, and fully dominated by algae. The most abundant species vary by location and site. In Caucaia, the most common species are *Centroceras clavulatum*, *C. acicularis*, and *U. fasciata*, while in Trairi the dominant species are *P. caerulescens*, *Gracillaria* spp., *Amansia multifida*, and crustose coralline algae. The mobile fauna at the lower littoral zone is also more diverse and includes the species mentioned for the mid littoral zone plus the gastropods *Stramonita haemastoma* and *Tegula viridula* (Gmelin, 1791). Recent surveys of the whole littoral zone detected a total of 110 species for Caucaia and 103 for Trairi. Most species are shared by the two locations, but Trairi is considerably more diverse in terms of sessile organisms (both animals and algae), while Caucaia has more motile animals. In terms of composition, the most specious taxa, in decreasing order, were: Rodophyta (45 spp.), Chlorophyta (18 spp.), Mollusca (18 spp.), Ochrophyta (11 spp.), Crustacea (8 spp.), Porifera (7 spp.), Ascidiacea (7 spp.), Cnidaria (5 spp.), Polychaeta (3 spp.) and Echinodermata (2 spp.).

The data collected using the SARCE protocol for large and conspicuous organisms in the intertidal shows that there is a considerable biodiversity along the beachrock reefs in Ceará. There are about 130 species in total, the vast majority of them of algae, especially red and green algae. There is also a tenable difference among locations and sites along this stretch of coast, which highlights also a degree of beta diversity. It is worth mentioning that the protocol favored the algae component of the intertidal community by accounting only for organisms "over" the substrate. Previous surveys aimed at producing inventories had come out with different numbers. For instance, results form the PROBIO initiative in Ceará indicated for the

same area 109 species of algae, 53 species of Crustacea, 44 species of Mollusca, 28 species of Ascidiacea, 24 species of Polychaeta, 22 species of Cnidaria, and 9 species of Echinodermata (Matthews-Cascon and Lotufo, 2006). Even these numbers are underestimating the total diversity, because the effort was still punctual.

The Northeast: Sergipe

Sergipe is the smallest Brazilian State, and the third shortest coast in extension (160 km). Located in the northeastern, it limits with the State of Alagoas in the north, and the State of Bahia in the south. Aracaju, the main city, comprises 25% of the state's population and has two harbor complexes. Sergipe has no real rocky shores, but rather beaches with boulders and rocky outcrops, and few areas with biolithic substrates (*Pragmatopoma caudata*). Beaches are composed by fine sand, and the linear coast is fringed by esturies and mangroves, associated with the rivers Real, Vaza-Barris, Sergipe and São Francisco.

The State has 47.3% of its territory inside of the "Drought Polygon" polígono das secas (FAO). The climate is tropical, with the highest humidity by the coast and in semi-arid backlands. The highest rainfall occurs between January and March.

In Sergipe, the SARCE project sampled, from south to north in three beaches: Praia do Saco, Coroa do Meio, and Praia do Jatobá. A total of 106 taxa were found represented by 34 species of invertebrates, 66 of macroalgae, and 6 species of filamentous cyanobacteria. The supralittoral zone is characterized mostly by bare bolders, which may be colonized by periwinkle gastropods of the complex *Echinolittorina ziczac*, and filamentous cyanobacterias. The high/medium intertidal, not always well zonated, contains a belt of the barnacle *Chthamalus bisinuatus* and the mussel *Brachidontes solisianus*, usually fouled by the red algae *Bostrychia* spp. and other filamentous red and green macroalgae. The low intertidal was the most diverse characterized mainly by barnacles (*Tetraclita stelifera*), oysters (*Crassostrea rhizophorae*), crab (*Aratus pisonii*), *Lottia* (*Collisela*) *subrugosa*, and mussels (*Perna perna*). Regarding macroalgal assemblages, the biomass was dominated in general by filamentous, leathery and terete functional groups. A total of 66 taxa were found: 41 *Rhodophyta*, 16 *Chlorophyta* and 9 *Phaeophyceae*, dominated by *Rhodomelaceae* (9 species), *Ceramiaceae* (5 species.), *Corallinaceae*, *Cystocloniaceae*, *Cladophoraceae* and *Ulvaceae* (4 species). The most conspicuous species were *Gayralia brasiliensis*, *Rhizoclonium riparium*, *Ulva flexuosa*, *Sargassum platycarpum*, *Centroceras clavulatum*, *Gracilaria cervicornis*, *Jania adhaerens*, *Hypnea valentiae*, *Solieria filiformis* and *Pterocladiella capillacea*.

The Northeast: Bahia

In Bahia, rocky substrates are mainly biogenic and sandstone, but some granitic substrate is also found. The SARCE project sampled five locations: Itacaré (Arruda, Corais, Figure 3B, 3C, 3D), Ilhéus (Backdoor, Praia do Sul), Itaparica (Penha, Mar Grande), Salvador (Stella Maris), and Litoral Norte (Guarajuba, Itacimirim), Litoral Norte and Itacaré located within a State marine protected area. Among these, Corais, Penha and Stella Maris are the only sampling sites that are characterized by granitic substrate.

The total number of species found at these sites varied between 29 (Mar Grande) and 58 (Arruda). Macroalgal diversity at all sites was very high and dominated the assemblage. The total number of macroalgal species varied between 18 (Mar Grande) to 46 (Arruda), with red algae being the most speciose group, followed by green and brown algae. In the high intertidal, the most abundant species were *Brachidontes*, *Chthamalus*, *Lyngbya* and *Ulva*

flexuosa. The mid intertidal was dominated by *Ulva lactuca, Palisada perforata* and *Gelidiela acerosa*, but in general, *P. perforata* and *G. acerosa* were also abundant species in this zone. The low intertidal was dominated by *Sargassum*, but *Bryothamnion triquetrum* and *Amphiroa anastomosans* were also dominant species at Penha and Praia do Sul respectively.

The Southeast: Espírito Santo

Espirito Santo State is bordered by Minas Gerais, Bahia and Rio de Janeiro. Climate is coastal humid tropical. Rainfall is highest during summer (1.000 mm and 1.500 mm/year), and mean air temperatures are around 22°C and 24°C. The south coast is rocky, with sandstone cliffs, and in the central coast, biolithic and granite formations can be found. The south-central coast is very indented with coves and bays sheltered by rocky outcrops. The coast is more indented in the center-south, and open sea to the north. The State comprises the higher seaweed diversity and biomass in Brazil, been influenced by the South Atlantic Central Water upwelling. The SARCE project sampled sites at the localities of Paraty, Ubu (Figure 3E), and Manguinhos.

A total of 183 taxa were found comprised by 48 invertebrate species and 131 macroalgae conspicuos taxa. The supralittoral zone is mostly bare but periwinkle gastropods of the complex *Echinolittorina ziczac*, and the green filamentous algae *Rhizoclonium* may be found. The high/mid intertidal, not always well zonated, contains a belt of the barnacle *Chthamalus* sp., oysters (*Crassostrea rhizophorae*), the mussel *Brachidontes solisianus*, and a complex of the red algae *Bostrychietum*. The low intertidal was more diverse and characterized by the barnacles *Tetraclita stelifera*, and *Lottia (Collisela) subrugosa*, muricid gastropods, and *Palithoa caribeorum*. Regarding macroalgal assemblages, the biomass was dominated in general by foliose, terete and calcareous (crustose and articulated) functional groups. A total of 131 taxa were found: 69 Rhodophyta, 37 Chlorophyta and 25 Phaeophyceae. The most conspicuous species were *Anadyomene stellata*, *Caulerpa* spp. (maily *C. racemosa* and *C. cupressoides*), *Codium* (mainly *C. intertextum* and *C. isthmocladum*), *Valonia aegragopila*, *Canistrocarpus cervicornis*, *Colpomenia sinuosa*, *Dictyopteris delicatula*, *Dictyota menstrualis*, *Neoralfsia expansa*, *Padina gymnospora*, *Sargassum cymosum*, *Arthrocardia flabellata*, *Dichotomaria marginata*, *Gelidium* spp., *Hypnea spinella*, *H. valentiae*, *Jania adhaerens*, *Lithotamnium/Lithophylum* complex, *Ochtodes secundiramea*, and *Palisada flagellifera*.

The Southeast: São Paulo

Nine rocky shores were sampled along 150 km of the coast of São Paulo State, Brazil, within three localities: Baixada Santista (Guaiúba/Guarujá, Ilha Porchat/São Vicente, Itaquitanduva/Praia Grande), São Sebastião (Baleeiro/São Sebastião, Feiticeira/São Sebastião, Itassucê/São Sebastião), and Ubatuba (Enseada/Ubatuba, Itaguá/Ubatuba, Praia Grande/Ubatuba). This coastline faces south - southeast, with variable complexity, from long linear stretches in Bertioga (Figure 3F) and southern São Sebastião, where long sandy beaches prevail, to very intricate coasts in Ubatuba, where small sandy beaches (tens to a few hundred meters) and mangrove forests are interspersed along a general rocky shoreline, forming several small bays and coves (Tessler et al. 2006). This general feature is an outcome of major tectonic dynamics leading to a gradual emergence towards the southwest, leading to the formation of coastal plains and long sandy shorelines, and to submersion towards the northeast, where a sinking mountain range, part of the Serra do Mar system, makes up most

of a remarkably convoluted shoreline and coastal islands (Martin & Suguio 1975, Almeida 1976). The rocky intertidal is usually steep, never forming large platforms, and often broken into very large boulders. As in most of the Serra do Mar, rocks are mostly constituted by gneiss and granite (Almeida & Carneiro 1998). The climate regime varies from tropical do humid subtropical (Sant'Anna Neto 1990) within this area. In Ubatuba, where historical climate data are available and have been extensively modeled (e.g. Valentim et al. 2013), temperature is maximum during February [27.8ºC air temperature (AT), 28.6ºC sea surface temperature (SST)] and minimum during July (21.1ºC AT, 21.9 ºC SST). Continuous measurements during the austral summer of 2011, taken at Baleeiro, São Sebastião, showed that temperature at the rock surface in the mid intertidal averaged 28.8 ºC and occasionally exceeded 40ºC (Kasten & Flores 2013).

The sampled shores varied from sheltered (Enseada, Itaguá), moderately exposed (Guaiuba, Ilha Porchat, Itaquitanduva, Baleeiro, Feiticeira, Itassucê) and exposed (Praia Grande), within an area where wave height frequencies from 0.5 to 2.0 m sum up 90%, with a wave height interval between 1.0 to 1.5 m making up half the observations (Bomtempo 1991). Wave exposure is apparently related to the midshore height (MH), from the upper limit of the coralline algal turf to the upper limit of the chthamalid barnacle cover. Roughly, the MH is lower than 0.5 m at sheltered shores, betwen 0.5 and 1.0 m at moderately exposed shores, and higher than 1.0 m at exposed shores. In this area, coastal primary production is comparatively low when compared to temperate areas prone to intensive seasonal upwelling (Gianesella et al. 2008). Estimates of nitrate concentration based on SST time series taken in the São Sebastião Channel indicated variation from only 0.2 to 0.7 μM (Flores unpublished data). Local upwelling of South-Atlantic Central Waters (SACW) may take place sporadically, during summer months, but more frequent inputs to the coastal zone take place via remote forcing, mostly during the passage of cold fronts during winter (Ciotti et al. 2010). The tidal regime at this coastline is a semidiurnal one, with the tidal range at spring tides ranging from 1.1 to 1.5 m. There is often a clear intertidal zonation, with the barnacle *Chthamalus bisinuatus* dominating the upper midlittoral (level 1), the mussel *Brachidontes solisianus*, and the volcano barnacle *Tetraclita stalactifera* prevailing in the lower midlittoral zone (level 2), and a coralline algal turf, associated to a very diverse assemblage of other macroalgae, making the most of the infralittoral fringe (level 3). These three levels were the targets of sampling protocols attempting a complete report of species presence and abundance.

Intertidal biological assemblages at the study sites - The upper levels sampled in this survey (levels 1 and 2) showed little variation among localities, but species turnover was very high in the lowest level (level 3), rendering almost shore-specific assemblages. At this lowest level, diversity was very high due to the presence of a large number of small macroalgal species. The most common species at all sites in level 1 in terms of cover were *Chthamalus bisinuatus* and *Brachidontes solisianus*, while *Collisella subrugosa* was one of the most abundant reaching densities of more than 70 ind/m^2. Level 2 was dominated by *Phragmatopoma caudate, Brachidontes solisianus, Tetraclita stalactifera,* and *Chthamalus bisinuatus*. Level 3 showed differences in among the different sites, with *Collisella subrugosa, Fissurella clenchi, Phragmatopoma caudate, Ulva lactuca, Stramonita haemastoma,* and *Caulerpa fastigiata* as some of the most abundant and conspicuous species.

The South: Paraná and Santa Catarina

The rocky coasts along south Brazil are formed by granitic or basaltic rock, resulting from the erosion of the border of the Serra do Mar mountain chain, which lies parallel to the coastline. Biolithic formations are also observed as an important coastal substrate, mainly at Paraná and Santa Catarina States. It is not a continuous ecosystem, but forms more or less extended outcroppings between sandy beaches and around numerous coastal islands. In some beaches there is a large rocky wall with different inclinations, where the intertidal zone is 5 – 6 m wide, but in most cases boulders of different sizes accumulate in front of these walls and the intertidal community covers a band of just 1.5 – 2 m high in each boulder. Usually the tide ranges from -0.2 to 1.8 m and water temperatures range from 17 – 23 ºC but surface water temperatures can reach peaks of 26 – 28ºC. The Paraná comprises the shortest coast in extension in the South and the second shortest in Brazil (98 Km). The area is between two Estuarine Complexes (Paranaguá and Guaratuba Bays), resulting in low transparency and high concentration of dissolved organic matter.

The monitoring of rocky coasts in the Brazilian southern region covered the following beaches from north to south: Morro do Farol (Figure 3G) and Praia Grande (Mel Island), Farol Island (Matinhos), Ferry Boat and Morro do Cristo (Guaratuba), in state of Paraná; First outcrop and Third outcrop (Itapema do Norte, Itapoá), Papagaio Point and Praia de Cima (Palhoça), in state of Santa Catarina; and Praia da Cal and Guarita Park (Torres) in state of Rio Grande do Sul. The northern seven sites listed are close to large estuarine systems (Paranaguá Bay, Guaratuba Bay, Babitonga Bay) and consequently exposed to low salinities (33-34) and high loads of sediment (turbid waters) and high concentration of dissolved organic matter.

A total of 160 species were found, around 60 invertebrates and 100 conspicuous macroalgae distributed along the three states, but known species richness in the southern Brazilian coast can reach 220 taxa at Santa Catarina (ca.), 131 taxa in Paraná (Pellizzari et al. 2014) and 85 taxa in Rio Grande do Sul (ca.). Rhodophytes dominated over Chlorophytes and Phaeophycean. The supralittoral zone mostly comprises bare space used by the periwinkle gastropods *Echinolittorina lineolata* (d'Orbigny, 1840), which are the most common and characteristic organisms at the lower part of this zone. Abundances can be as high as 150 individuals per 100 cm2. The high intertidal contains a dense belt of the barnacle *Chthamalus bisinuatus* Pilsbry, 1916, where many *Echilittorina* are still present. The mid intertidal is dominated by the mussel *Brachidontes solisianus*, usually fouled by the algae *Pyropia* (formerly known as *Porphyra*) *suborbiculata*, *Bostrychia* spp., and *Gelidium pusillum* during the winter time. In this zone sand accumulates among the bivalves, sometimes almost covering all the shells, in which a community of vagile invertebrates such as polychaetes and nematodes is found. The community of the low intertidal is more variable among sites. In some beaches, barnacles (*Tetraclita stalactifera*) are very common (Morro do Farol, Morro do Cristo, Ponta do Papagaio), while in others, the mussel *Perna perna* is the dominating species (Ferryboat, Praia de Cima, Praia da Cal, Guarita Park). Below the barnacle and mussels zone, high densities of the sabelariid polychaete *Phragmatopoma caudata* are found forming sand reefs that can extend 50-70 cm away from the substrate. Not all rocks are covered by the sand reefs and macroalgae are also very abundant in this zone. Regarding seaweed assemblages, filamentous, foliose and terete functional groups dominated the biomass. Species with higher coverage in the low intertidal were *Acantophora spicifera, Centroceras clavulatum, Gelidium spp., Gymnogongrus griffthsiae, Hydropuntia caudata,*

Hypnea musciformis, Pyropia acantophora, Laurencia spp., *Bryopsis pennata, Cladophora* spp., *Codium taylorii, Gayralia brasiliensis, Ulva lactuca, U. flexuosa, Bachelotia antillarum, Colpomenia sinuosa, Dictyota* sp., *Padina gymnospora and Sargassum* spp. The most important herbivores in the area are sea-urchins and turtles. In some beaches, hydroids are also common in the low intertidal such as *Obelia dichotoma, Orthopyxis sargassicola,* and *Acharadria crocea*. The sea anemone *Bunodosoma caissarum* as well as the sponge *Hymeniacidon heliophila* were also frequent. When the tide is very low and the sublittoral fringe gets exposed, a few ascidian species can be found (*Polysyncraton aff. amethysteum, Didemnum galacteum, Botryllus planus,* and the introduced *Eudistoma carolinense*). Among the grazers, there were five mollusk species and one sea-urchin, while among the predators, *Stramonita braziliensis* was the only ubiquitous and abundant invertebrate species. No sea-stars were found, however, 20 years ago, *Asterina stellifera* was common in the intertidal zone of this region.

Along the Brazilian coast in general, macrofauna does not show significant differences in composition, however seaweed assemblages are strongly marked by latitudinal differences on their composition and biomass. The highest diversity of macroalgae is found between the coasts of Espírito Santo and Bahia States, while the highest biomass is found in the Brazilian Northeastern and also Santa Catarina, in the South, associated probably to the influence of the South Atlantic Central Water (ACAS), distance to large estuaries (affect water transparency), and finally to the availability of hard substrates.

Uruguay

The Uruguayan marine and estuarine coastlines (ca. 500 km, between 34º and 35ºS) include sandy beaches interrupted by streams and coastal lagoons and rocky (mainly metamorphic and igneous) outcrops forming capes or peninsulae. Since the Uruguayan coast is under the influence of the the Río de la Plata estuary, one of the largest estuaries of South America, a salinity gradient roughly oriented east–west can be identified. Based on salinity, three main regions can be identified: a west region influenced by freshwater (<1ppt), a central region that is influenced by water of variable salinity (1–30 ppt) and an east region open to ocean waters (>30 ppt) (Brazeiro, Borthagaray, and Giménez, 2006; Defeo et al., 2009; Giménez et al., 2010). Diluted waters (i. e. salinity <33.2) dominate shallow coastal area (i.e. depths <50m) and can reach the offshore producing a buoyant fresh water layer during extreme continental discharge that determine variations in coastal water input (Ortega and Martínez, 2007). Upper waters temperature could exceed 20ºC (e.g. Tropical Water) (Thomsen, 1962) at surface. In the boundary between the estuarine and oceanic zone (Punta del Este), water temperature can fluctuate between 10.7ºC in winter and 24.6ºC in summer, (Burone and Bayseé, 1985; Milstein and Juanicó, 1985). The coast experiences a semidiurnal tide (range < 0.5 m) with the water level influenced mainly by wind conditions (direction and speed). Winds blow south-west during winter and north-east during summer. The rocky platforms have variable slopes and are exposed to different degrees of wave action according to their orientation. SARCE sampling sites are located in the east region (Figure 3H).

Across the Uruguayan coast, intertidal species richness of both macroalgae and invertebrates, increased from west to east; this was most notable for sessile fauna and macroalgae (Giménez et al., 2010). In the east region, two to three zones can be identified,

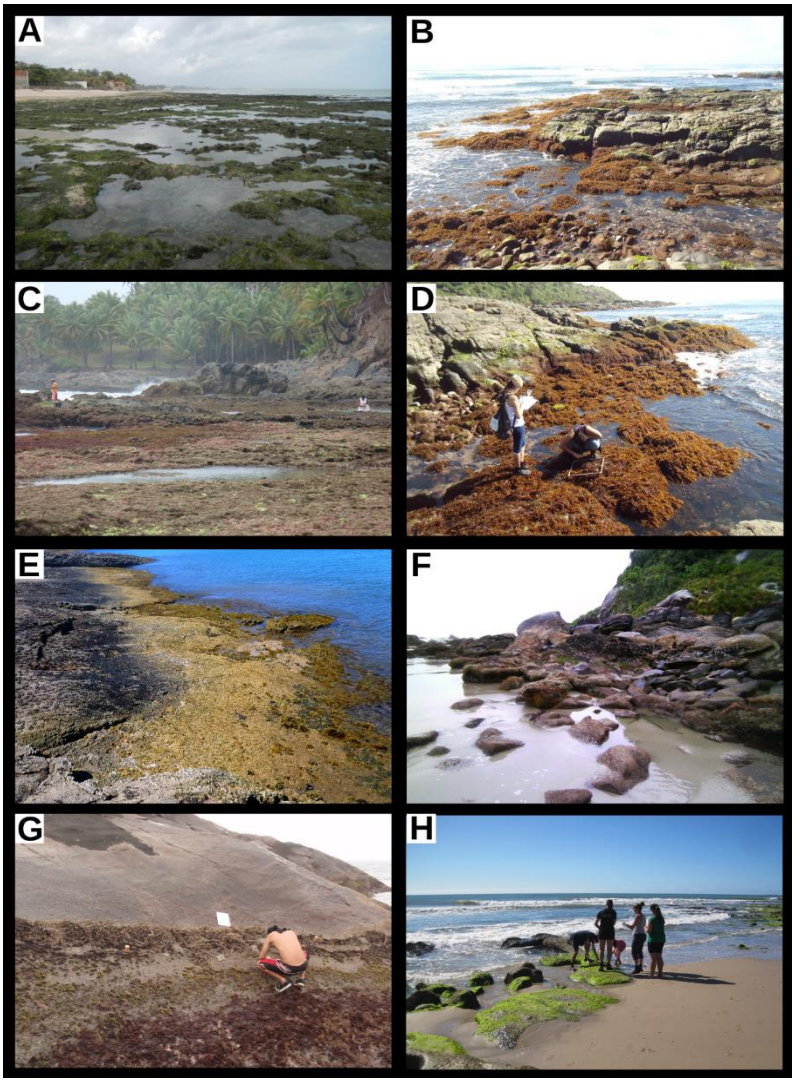

Figure 3. Sampling sites in the Brazilian and Uruguayan Atlantic. A. Northeast Brazil – Caucaia. B. Northeast Brazil – Bahia/Itacaré. C. Northeast Brazil – Bahia/Arruda, octopus fishing. D. Northeast Brazil – Bahia/Itacaré. E. Southeast Brazil – Costao de Ubu/Espirito Santo. F. Southeast Brazil – Sao Lourenco. G. South Brazil – Morro de Farol/Parana. H. Uruguay – Punta del Este.

following classical zonation schemes: a high intertidal zone dominated by a cyanobacterial film, a middle intertidal zone dominated by barnacles and a low intertidal and shallow subtidal zone characterized by a dense cover of mussels and/or macroalgae. Intertidal mussel beds are thus a conspicuous feature of Uruguayan rocky shores, providing important economic and ecological services (Borthagaray and Carranza, 2007; Riestra and Defeo, 1994; Riestra and Defeo, 2000). Along this gradient, the intertidal mussel beds changes in species composition and structure. Currently, the western region is characterized by the invasive mussel *Limnoperna fortunei*. *Brachidontes darwinianus* and *Mytella charruana* occupy consolidated substrata along the central region (Maytía (Maytia and Scarabino, 1979; Neirotti, 1981; Scarabino et al., 2006), overlapping with *Brachidontes rodriguezii* from the

eastern half of the central region and being replaced by this species in the eastern region (e. g. Amaro (Amaro, 1965; Maytia and Scarabino, 1979; Scarabino et al., 2006). *Mytilus edulis*, in turn, is distributed from the eastern half of the central region, being the dominant mussel species in this zone. *Brachidontes rodriguezii* and *Mytilus edulis* originate dense banks in the more saline part of the Río de la Plata, the first being characteristic of the intertidal (also occurring in the Atlantic shores), the second mostly subtidally although some intertidal exposed zones have been observed to be dominated by this species (Borthagaray and Carranza, 2007). The brown mussel *Perna perna* originates large banks in the subtidal of the eastern coast (Rocha) but its presence (in very low abundances) reaches Punta del Este. The Uruguayan coast was massively colonized by this species in the late ´50ths. Since this first colonization, *P. perna* almost disappeared in the late 70s to 1997, when a new process of colonization occurred in the area (Carranza and Borthagaray, 2008; Orensanz et al., 2002). The annotated list of macroalgal species of the Uruguayan coast given by Coll and Oliveira (1999) reported the presence of 69 species sampled from 27 sites located along the central and east sectors of the Uruguayan coast. Conversely, a single site (Cerro Verde) can yield more than 40 invertebrate taxa, including the small mobile (e.g. amphipods, polychaetes), and encrusting (e.g., briozoa, hydrozoa) fauna. Under the SARCE sampling protocol, two sites located in the eastern region showed a combined richness of 20 species, including 9 metazoans (4 Gastropoda, 2 Bivalvia, 2 Cnidaria Anthozoa and 1 Cirripedia), 4 Chlorophyta and 7 Rhodophyta bortha (Borthagaray and Carranza, 2007).

Argentina

The Argentinean marine coast extends along more than 4700 km and twenty degrees of latitude. It comprises the Argentinean and Magellanic biogeographical provinces, which are delimited by the Valdés Peninsula. The Argentinean Biogeographic Province extends from 36 to 43º S, including the provinces of Buenos Aires and Río Negro, and the north of Chubut province. The Magellanic Biogeographic Province, extends from 43ºS to 56ºS along southern Chubut province as well as Santa Cruz and Tierra del Fuego provinces (the latter includes the Malvinas/Falklands and South Atlantic Islands) (López Gappa et al., 2006, Balech and Ehrlich, 2008). Intertidal rocky platforms in Argentina increase in frequency and extend from North to South. In the northernmost coastal province (Buenos Aires), rocky coastal stretches are markedly discontinuous. They rarely exceed 1 km length and are frequently associated to urban areas. Most intertidal platforms in this province are formed by consolidated sediments (e.g., limestones, sandstones, calcretes) that support both epilithic and endolitic biota (Bagur et al. 2013, 2014). The only exceptions are a handful of metamorphic rock (ortoquartzite) platforms adjoining the city of Mar del Plata (38° S).

SARCE sampled two sites were sampled in the province of Buenos Aires: Playa Chica, an orthoquartzite platform located in the urban zone of Mar del Plata (Figure 4A) and at a calcrete platform located immediately north of the port of Quequén. Both localities are exposed to waves and face the open sea. The tidal regime at these sites is semidiurnal and microtidal (mean and maximum amplitude are 0.83 and 1.65 m respectively). Water temperature varies from a media of 5º C in winter to a media of 18 º C in summer (Servicio Meteorologico Nacional-Argentina, http://www.smn.gov.ar). The next province to the south, Rio Negro, presents intertidal platforms of varying substrate, including sedimentary (e.g., sandstones, limestones) and igneous rock types (e.g., granite, ignimbrites) (Kokot et al. 2004). Tidal regimes are semidiurnal and macrotidal through the whole coastal range. The sampling

sites in this province were: El Espigón, La Lobería (Figure 4B), Playa Los Suecos, and Punta Colorada. El Espigón and La Lobería face the open ocean and are characterized by sedimentary rock substrates and maximal tidal amplitude of 4.32 m. Playa Los Suecos and Punta Colorada are located within the San Matías Gulf and show igneous rock substrates and maximal tidal amplitude of 8.72 m (Kokot et al. 2004). Southwards, the rocky shores of Chubut province are exposed to unusually harsh physical conditions, particularly with regard to desiccation (see Bertness et al. 2006). The region is characterized by persistent and intense winds (up to 90 km/h, annual average 16.6 km/h) and low precipitation (mean 235.9 mm/yr (Paruelo et al., 1998, Labraga & De Davies, updated 2013). The tidal regime is semidiurnal and macrotidal. Consolidated sediments are the dominant substrate type across these rocky shores. Sampling sites in Chubut were Puerto Lobos, Punta Este (Figure 4C), and Camarones. Puerto Lobos is located by the Southern end of the San Matías Gulf and its maximal tidal amplitude is about 6 m. Punta Este is located within the Nuevo Gulf, 8 km south from the city of Puerto Madryn. Mean tidal amplitude at this site is 3.8 m (Maximal: 5.7 m). The town of Camarones is located within the homonymous bay. This site is characterized by monthly-averaged wind speeds ranging from 13 to 31 km/h and an average tidal amplitude of 4 m, platforms are characterized by sedimentary rocks although some igneous rock are present. The southernmost continental sampling site was Puerto Deseado (Figure 4D) in Santa Cruz Province. This site is located in the Deseado Massif geological province, and characterized by igneous rock substrates (rhyolites; see Pankhurst and Rapela, 1995; Pankhurst et al., 1998). Climate is also dry and windy with a mean annual precipitation around 200 mm and an average annual air temperature of 8.2 °C (Servicio Meteorologico Nacional-Argentina, http://www.smn.gov.ar). The tidal regime is mesomacrotidal (Isla and Bujalesky, 2008), with amplitudes ranging between 2.5 and 5.5 m. Two additional sites were sampled in Tierra del Fuego Island: Estancia Viamonte (Figure 4E) and Playa Larga (Figure 4F). Estancia Viamonte is located 40 km south of the city of Río Grande and characterized by an extensive limestone abrasion platform (the low tide level is ca. 2 km distant from the high tide line) that faces the open ocean. The tidal regime is macrotidal, with amplitudes ranging between 2.2 and 8.4 m (Bujalevsky 1997, 2007). The area shows a dry and windy climate (340 mm/yr precipitation) with the mean annual temperatures of 5-6 °C and low between-month variations (Bujalevsky 1997, 2007). Playa Larga is located 3.5 km east of the city of Ushuaia in the Beagle Channel. The rocky shore at this site is characterized by methamorphic rocks and a sharp slope. The tidal regime is microtidal (1.1 m mean amplitude). Mean annual temperature and precipitation are 6°C and 500 mm, respectively. This shore faces the dominant SW-W winds and, thus, is exposed to considerable wave splash. The subtidal all along the Beagle channel is characterized by dense forests of *Macrocystis pyrifera* (Figure 4G) down to the channel's mouth to the Atlantic at Estancia Moat, a planned sampling site (Figure 4H).

The main feature of this extensive coast is the low biodiversity of its rocky intertidal shores, which at the same time involve low biomass (Wieters et al. 2012). From North to South, the two localities in Buenos Aires Province have different geological substrates, and are 120 km apart. Even if both assemblages have the same species composition; the structure and relative abundance of species between localities were different. In Mar del Plata, the locality with quarzitic substrate, the bivalve *Brachidontes rodriguezii*, the limpet *Siphonaria lessoni* and the introduced barnacle *Balanus glandula* are the more abundant species of the high intertidal. In the mid intertidal level, several algae species were added to the

assemblages, being *Hildenbrandtia* sp. *Polysiphonia fucoides*, *Ulva* sp. and two non-indigenous red algae *Anfeltiopsis devoniensis* and *Schyzimenia dubyi* the most abundant. In the low intertidal level, the most representative species are *Polysiphonia fucoides* and *Siphonaria lessoni* in Mar del Plata and *Corallina officinalis* and *Balanus glandula* in Quequén. The presence of non-indigenous algae species is limited to Schyzimenia *dubyi*, and in very low coverage.

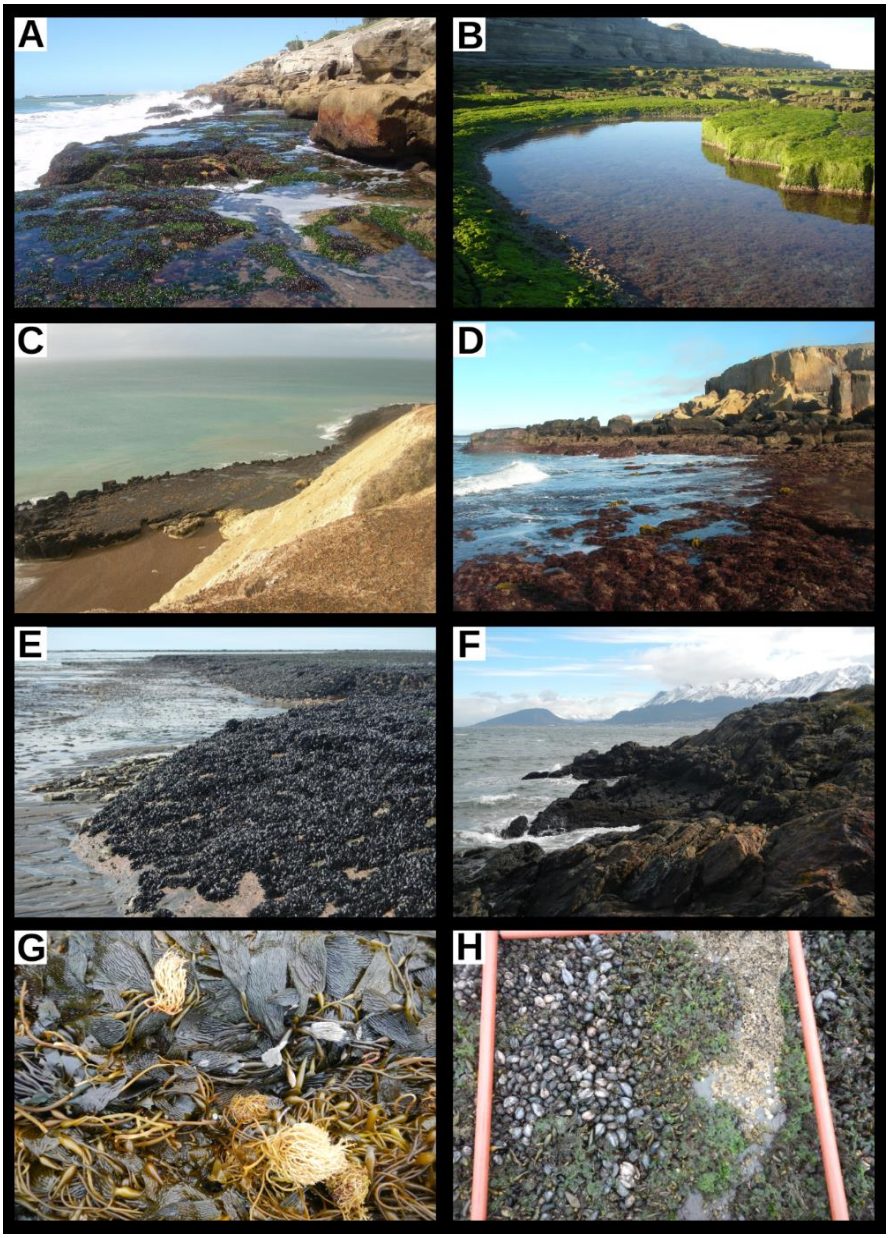

Figure 4. Sampling sites in Argentina. A. Mar del Plata. B. La Lobería. C. Punta Este. D. Puerto Deseado. E. Río Grande. F. Playa Grande. G. Estancia Moat – *Macrocystis pyrifera*. H. Estancia Moat.

The Rio Negro Province comprises two localities and also an area of ecotone among two biogeographic regions, the Argentinean and Magellanian provinces. The differences among localities involve not only changes in the species composition but abundance of the ones that are present in both regions. In El Espigón and Loberia, a great variability was found between sites, the high intertidal is inhabited mainly by *Mytilus platensis* in one site and by *Ralfsia expansa* and *Enteromorpha linza* in the other. The mid intertidal level could have up to 98 % coverage of *Brachidontes rodriguezii* in one site and 0 % in the other. *Siphonaria lessoni* is the second more abundant species in this level. In the low intertidal the areas are patchly covered by *E. linza* and *Corallina officinalis* in one site or almost exclusively covered by *C. officinalis* in the other. In Playas Doradas, the most abundant species that inhabits the high intertidal are *Siphonaria lessoni* and *Brachidontes rodriguezii*, but in a very low percentage cover (approximately 12 %). In the mid intertidal, *Brachidontes pupuratus* replaces *B. rodriguezii* and it is the most abundant species followed by *Ralfsia expansa* and *S. lessoni*. For the low intertidal, *C. officinalis* is the most abundant followed by a complex of *Aulacomya actra* and *Mytilus platensis* forming mussel beds. The Chubut Province is characterized by sites with no human settlements. In the north of the province, the biodiversity pattern is similar to the localities in the province of Rio Negro, with *B. purpuratus* being the most abundant species in the high and mid intertidal and *C. officinalis* in the low intertidal. In Puerto Madryn, a site with a local population of 80,000 residents, the pattern is similar but with a greater proportion of the green algae *Ulva* sp. in the mid intertidal, depending on the season. High and mid intertidal are dominated by the mytilid complex of *Brachidontes rodriguezii* and *Brachidontes purpuratus* that produce an heteregenous habitat that facilitates settlement for several species. Also the non-indigenous barnacle *Balanus glandula* is present in high and mid intertidal levels, while the gastropod *Trophon geversianus* is a carnivore specialized on mytilid bivalves. The low intertidal is dominated by the alga *Corallina officinalis* and the herbivore gastropod *Tegula patagonica*. In Camarones, the zonation is similar to Puerto Madryn, presenting zones with 100% of coverage of *Brachidontes purpuratus* dominating the mid intertidal, and the invasive species *Balanus glandula* in the mid and high intertidals. The low interidal is dominated by the calcareous algae *Corallina officinalis*, while *Aulacomya atra* and *Siphonaria lessoni* are abundant in some localities. The gastopod *Trophon geversianus* is less common in this zone, probably due to the harsh physical stress.

Santa Cruz is the last province of the continent and the one with less population, with nearly 10,000 residents on the coast, but with an important port in the sampling locality Puerto Deseado. Here, even if biodiversity increased slightly, patterns of the most abundant species were kept. The high and mid intertidal were dominated by *Brachidontes purpuratus* and the algae *Bostrichia*, while the in the low tide *Corallina officinalis* and *Chondria* were the most abundant. In the low intertidal level the presence of the non-indigenous red algae *Anotrichium furcellatum* was detected as one of the most abundant. In Tierra del Fuego Island, assemblages are different according to their degree of exposure (open ocean vs Beagle channel). In the open ocean locality, Estancia Viamonte, near Ushuaia (56,000 residents), mussel beds are composed mainly by *Mytilus edulis platensis* and covered from 50 to 87% of the mid and high intertidal. In the low intertidal, the incrusting algae *Corallina* sp. is the more abundant sessile species. The biodiversity of mollusks increased in this site, being *Nacella magellanica*, *Kerguelenella lateralis* and *Trophon geversianus* the most abundant. In the locality in the Beagle Channel, Playa Larga, macroalgal biodiversity increased, being the

most conspicuous group in the low and mid intertidal along with *M. edulis platensis*. The limpet *Notochthamalus scabrosus* is the more abundant species in the high intertidal. As observed in the open ocean site, the biodiversity of mobile mollusks increased with *S. lessoni*, *K. lateralis*, *N. magellanica* and *T. gerversianus* the most abundant species in the three intertidal levels.

The Pacific

Colombia

In general terms, two main regions/features can be recognized on the littoral of the Pacific coast of Colombia: mangrove swamps and muddy flats to the south and rocky shores to the north; with a hybrid zone almost in the middle of the coast: Málaga and Buenaventura Bays. The shoreline is very broken, interrupted by numerous small rivers and creeks characteristic of one of the rainiest and most bio-diverse areas in the World: The Tumbes-Chocó-Magdalena region. Despite this condition or perhaps due to it, most of the coast is unpopulated, with only two relatively large cities: Buenaventura (the most important commercial port in Colombia), located almost in the middle of the coast and Tumaco, a smaller city located at the south, near the border with Ecuador. Although these two are the main cities, there are numerous small towns, such as Guapi and Bahía Solano, among others. The transportation system in the region is precarious. The underdeveloped roads infrastructure is limited to Buenaventura and Tumaco, and all other settlements can only be reached by boat or in few cases by plane, a fact that makes it difficult and expensive to undertake research projects in the Pacific coast of Colombia.

The Pacific coastline has an extension of nearly 1544 km, of which 636 km are exposed rocky shores (Londoño-Cruz et al., 2008, Londoño-Cruz et al., 2014). Despite this exposure, wave action is moderate most of the year round, reaching, on average, wave heights of up to 1 m in most locations (INVEMAR, 2003). Tidal range is relatively large (ca. 4.5 – 5.0) and has a semidiurnal frequency (ca. 6:15 hrs. between high and low tide). Currents, on the other hand, respond to prevalent winds and to the movement of the Intertropical Convergence Zone (ITZC). The most important surface currents are the North Equatorial Current, the North Equatorial Counter-current, the Panama Gulf Current, and the Colombia Current. Although there are hypothesis regarding the existence of upwellings in the Pacific coast of Colombia, these have not been unambiguously confirmed; if they do occur, they happen during the first months of the year and at the northernmost of the Colombian Pacific (Vides and Sierra-Correa 2003). Rocks forming the large extension of rocky shores on the Colombian Pacific are composed by volcanic rocks from the Secondary or Tertiary periods and by sedimentary rocks from the Quaternary. The volcanic rock characterizes the rocky shores of the northern regions and the Gorgona and Malpelo Islands, while the sedimentary rocks characterize the rocky shores of central (Málaga Bay, Pichidó Isthmus, and Tortuga Gulf) and southern (Gallo Island) regions (INVEMAR, 2004). Due to the relatively large tidal range, the rocky intertidal can be easily divided into three levels, which vary in dimension depending on the slope of the shore. It is very common to find cliffs along the coast, with scattered abrasion platforms and rocky/boulder beaches. Zonation in the intertidal zone is very typical, with periwinkles and *Nerita* spp. occupying the upper intertidal; barnacles, limpets, other snails and bivalves in the middle and a richer arrange of species in the lower. In these rocky shores, the algal coverage

is very low as compared to shores in higher latitudes or the Colombian Caribbean; so although species richness in general is high, species abundances are relatively low. It is also important to note that space seems not to be a limiting factor, since there is plenty of free space in almost every rocky shore along the coast. One might hypothesize that the reason for this low abundance is low algal coverage and long exposure periods during low tide, which may bring very high temperatures or very low salinities (during high rainfall).

The localities sampled by SARCE (from North to South) include El Choco (Punta Ardita, Cabo Marzo) (Figure 5A), Málaga Bay (Los Negritos, Isla Palma), and Gorgona Island (La Ventana, La Camaronera, Piedra Redonda) (Figure 5B). Punta Ardita at El Choco is the northernmost locality, near to the border with Panamá, practically undisturbed by human presence. The volcanic rocky shores in this locality are edged by large sandy beaches. The high intertidal is mostly bare rock with *Cladophoropsis* sp., *Nerita scabricosta*, *Echinolittorina conspera*, *Chthamalus panamensis*, *Lottia mesoleuca*, and *Acanthina brevidentata*. The mid intertidal is dominated by *Cladophoropsis* sp., *Acanthina brevidentata*, *Fissurella microtrema*, *Phragmatopoma* sp., *Fissurella microtrema*, and *Lottia mesoleuca*. The most common species in the low intertidal are *Cladophoropsis* sp., *Echinometra vanbrunti*, *Telmatactis* sp., bryozoa, and *Balanus* sp. Cabo Marzo is an isolated locality with no human settlements nearby and practically undisturbed. Rocks, as in the previous locality, are volcanic. There is some coralline formation in this place, waters are very transparent. Wave conditions are relatively rough. Dominant species of Cabo Marzo are *Chthamalus panamensis*, *Echinolittorina conspera*, and *Nerita scabricosta* in the high intertidal, Corallinales, *Fissurella virescens*, and *Siphonaria maura* in the mid intertidal, and Corallinales, *Chiton stokesii*, *Siphonaria maura*, *Nucella melones*, and *Chama frondosa* in the low intertidal.

At Málaga Bay the rocks are sedimentary. This bay is part of a National Natural Park and there is a relatively large human settlement (Juanchaco) at the mouth of the bay, as well as a Navy Base along with several other scattered minor settlements. Seasonal tourism is, perhaps, the main economic income for the inhabitants. The first site, Isla Palma, is an island (uninhabited), while the second site, Los Negritos, is an intertidal rocky reef with both volcanic and sedimentary rocks. Dominant species at Isla Palma were *Chthamalus panamensis*, *Balanus* sp., *Lottia mesoleuca*, *Echinolittorina paytensis*, *Echinolittorina dubiosa*, and *Echinolittorina apicina* in the high intertidal; *Verrucaria* sp., *Lithophyllum* sp., *Nerita funiculata*, *Lottia mesoleuca*, and *Balanus* sp. in the mid intertidal; and *Cladophoropsis* sp., *Bostrychia* sp., *Lithophyllum* sp., *Echinometra vanbrunti*, *Nucella melones*, and *Brachidontes* sp. in the low intertidal.

Gorgona Island is also a National Natural Park. Most of the island's shores are rocky and cliffy. Rocks are volcanic with few exceptions. Basically all sort of rocky shore types can be found in the island. This locality is perhaps, the most sampled area in the entire Pacific coast of Colombia. The main rocky ecosystems sampled (La Ventana, La Camaronera, and Piedra Redonda) are located at the south and western sides of the Island. At La Ventana, the dominant species were *Nerita scabricosta*, *Cladophoropsis* sp., *Echinolittorina conspera*, and *Siphonaria gigas* in the high intertidal, *Nerita funiculate* and *Cladophoropsis* sp. in the mid intertidal, and *Nucella melones* and *Nerita funiculata* in the low intertidal. At La Camaronera, high intertidal is dominated by *Echinolittorina conspera* and *Nerita scabricosta*, the mid intertidal by *Nerita funiculata*, and the low intertidal by *Cladophoropsis* sp., *Nerita funiculata*, and *Tegula pellisserpentis.* At Piedra Redonda, the high intertidal is dominated by

Nerita scabricosta, the mid intertidal by *Cladophoropsis* sp., *Nerita funiculata*, and *Fissurella virescens*, and the low intertidal by *Cladophoropsis* sp. and *Nucella melons*.

Ecuador

The coast of Ecuador extends for 4,403 km from north to south and includes several isles, islets and estuaries. The continental platform exceeds 100 km in amplitude mainly at the Gulf of Guayaquil (Sonnenholzner et al, 2013) and a depth of 200 m from the coastline (Mora et al, 2010). About a third of the coast is covered by mangroves, mostly in the north and in the south. The central and part of the north coasts are characterized by large sandy beaches interrupted by a few rocky areas, cliffs, lagoons and rocky reefs (Sonnenholzner et al, 2013; Miloslavich et al, 2011). The rocky shore intertidal has mid to steep slopes formed by stratified rocks within cliffs entering the sea and forming platforms within the sandy beaches. At the base of some of the cliffs, an eroded narrow terrace can be observed, or the beach may be narrow with boulders. Despite the importance of rocky shores, they have been poorly studied in Ecuador, and knowledge on its biodiversity is limited to some taxonomic studies on specific groups such as molluscs (Bonilla 1967; Cruz 1977, 1983, 1992a, b, 1996, 2007 y 2009; Mora and Reinoso 1981; Mora 1989, 1990; Arias 2012), poliychaetes (Villamar 1983, 1986, 1989), echinoderms (Avilés 1984, Sonnenholzner et al. 2013), and macroinvertebrates (Massay et al. 1993; Arroyo and Calderón 2000; Mair et al. 2002; Cruz et al. 2003; Ayala 2010; Mora et al. 2010).

SARCE sampled at two localities at the central and south-west of the coast of Ecuador: Caráquez Bay (Punta Bellaca and Punta Gorda) and at the Santa Elena Peninsula, the localities of Puntilla de Santa Elena (Base Naval and La Lobería) (Figure 5C) and Ballenita (El Barco and El Faro) (Figure 5D) which are located at the provinces of Santa Elena and Manabí. The sites with more human impact were Punta Bellaca and Ballenita, while Punta Gorda and Puntilla de Santa Elena were less impacted.

Punta Bellaca and Punta Gorda are characterized by almost vertical, high cliffs of up to 100 m interrupted by deep valleys with steep slopes (Ochoa et al, 1987). These cliffs are unstable and landslides are common. The beach is narrow and in some places, eroded rock terraces can be found inserted within sandy beaches (Boothroyd et al, 1994). Weather in this area is tropical dry with an average temperature of 25°C. Rainfall is not uniform due to the complexity of the Oceanic Front, and varies between 200-800 mm (Ochoa et al, 1987). A total of 16 species were found (10 at Punta Bellaca and 4 at Punta Gorda), mostly represented by molluscs (50%), macroalgae (25%), and crustaceans (25%). The most diverse group was the mollusks represented by 7 species of gastropod and one bivalve species. The most abundant species were the gastropods *Nodilittorina aspera*, *Nodilittorina paytensis* and the macroalgae *Enteromorpha* sp. and *Bachelotia* sp..

The high intertidal was mostly represented by bare rock and patches of sand, but some rocks were colonized by the *Brachidontes semilaevis*/*Balanus amphitrite* complex. The most common species in the mid intertidal were *Balanus amphitrite* and *Enteromorpha* sp. and *Bachelotia* sp. in the low intertidal.

At the Santa Elena Peninsula, Ballenita is a public watering place with hotels all along the border of the coast. The weather is tropical with a mean average temperature of 24°C. The intertidal is characterized by short, vertical, and unstable cliffs. The rocky platforms are inserted within the sandy beaches, and have a soft slope (Ochoa et al, 1987). Boothroyd et al. (1994) proposed that the low cliff originated from a system of barrier/littoral plain formed by

sand poorly cemented to the carbonates and clay. Wave energy is highest during the rainy season (Brito, 2014). The rainy season occurs in January-April followed by the dry season which extends to November-December (Ochoa et al, 1987). Precipitation varies from 62.5 to 125 mm (Ochoa et al, 1987).

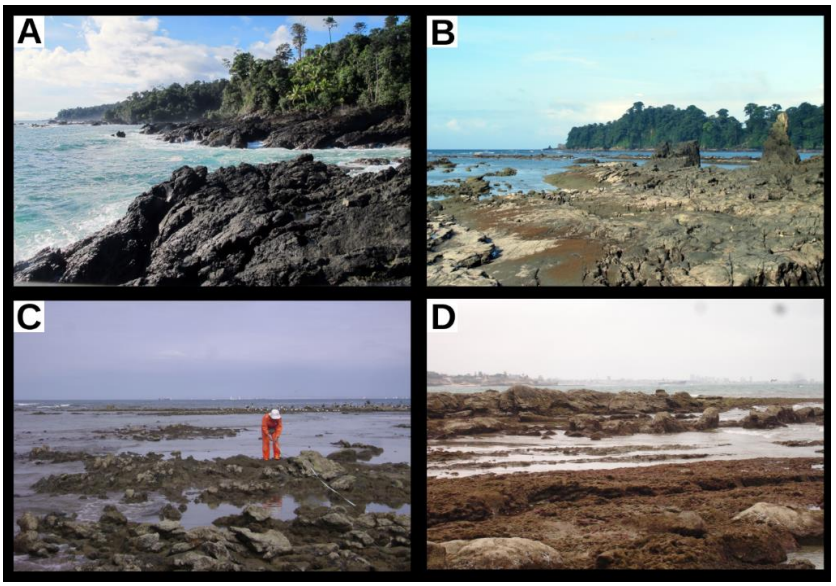

Figure 5. Sampling sites in the Colombian and Ecuadorian Pacific. A. Colombia – Ñuqui/Choco. B. Colombia – La Ventana/Gorgona. C. Ecuador – Puntilla de Santa Elena. D. Ecuador – Ballenita.

The Puntilla de Santa Elena is within a marine protected area known as "Reserva de Producción de Fauna Marina Costera Puntilla de Santa Elena" or REMACOPSE. The intertidal here is irregular, with low vertical, unstable cliffs that continue at sea, emerged, for a few hundred meters. Between these formations, sandy beaches with coarse sand and steep slopes are found. The base of the cliffs are continuously eroded by wave action (Boothroyd et al, 1994; Soledispa, 2008). From the geological point of view, the most outstanding feature at La Lobería is the Cayo Formation, represented by sandstone, chert and silicified clays (Soledispa, 2008). Wave action is stronger than in the previous site and also highest during the rainy season (Brito, 2014). At the Navy Base, the Beach is characterized by medium size rocks covering a great extension that goes into the sea. According to Soledispa (2008), these lay directly on top of the Cayo Formation where deposits from the quaternary are found and composed by calcarean sandstone and conglomerates with abundant fossils. When the tide is low, numerous intertidal pools can be observed among the sand. Weather in this area is arid, with a mean annual temperature of 24°C, and precipitation, determined by the Humboldt Current, varies between 62.5 and 125 mm (Ochoa et al, 1987).

At these localities, a total of 66 species were found. These were mostly represented by molluscs (39%), macroalgae (24%), crustaceans (14%), cnidarians (9%), bryozoans (5%), echinoderms (5%), sponges (3%), and tunicates (1%). Ballenita had a higher number of species (30 species) in comparison to Puntilla de Santa Elena (19 species). The most diverse group were the mollusks (29 gastropod species plus 2 bivalve species) followed by macroalgae (9 species of rodophytes, 3 of chlorophytes, 3 of phaeophytes plus an unidentified

species), and barnacles. The most abundant species were *Balanus amphitrite*, *Brachidontes semilaevis*, *Nodilittorina aspera*, *Nodilittorina paytensis* and one bryozoan. The dominant species in the high intertidal were *Nodilittorina aspera*, *Nodilittorina paytensis*, and the complex *Balanus amphitrite/Brachidontes semilaevis*, while the mid intertidal was dominated by *Pachygrapsus transversus*, *Echninometra vanbrunt*, and *Nerita funiculate;* and the low intertidal by *Nodilittorina aspera*, *Thais brevidentata*, *Padina* sp., and a bryozoan species. The vertical zonation was more evident in the high and mid intertidal which are dominated by littorrinid gastropods, barnacles and mussels (*Nodilittorina aspera*, *Nodilittorina paytensis* and the complex *Balanus Amphitrite*/*Brachidontes semilaevis*) as observed by Cruz (2009) and Brito (2014) at the Santa Elena peninsula. This pattern is dependant on the tide and time of exposure to air (Sibaja-Cordero and Vargas-Zamora, 2006).

Peru

The coastline of Peru extends for 2414 km along the Peruvian Biogeographic Province and is greatly under the influence of the Humboldt Current System, one of the major upwelling systems of the world (Miloslavich et al., 2011). SARCE sampled at seven localities, from north to south: Paita, Huarmey (Figure 6A), Ancón, Paracas (Figure 6B, 6C), Marcona, La Meca, and Punta Colorada (Figure 6D). The geological and physical conditions of these localities are very variable according to their origin and latitude, which ranges from a tropical warm province in the north, to a cold, sub-Antarctic province in the south. These changes are reflected in the composition of the intertidal flora and fauna.

In the north, Paita, located near the border with Ecuador, at the Panamic Province of the Tropical West Pacific is one of the most intense upwelling zones in the coast of Peru and characterized by high productivity (Fahrbach 1981, Huyer 1987, Grados 2002, Graco 2007). It is considered suptropical with very dry weather. Coastal diversity is high at these warm conditions (Ramírez et al., 2003), however, it is temporally affected by ENSO events (Paredes et al. 1998, Paredes et al. 2004), that produce the migration of some vertebrate species and the arrival of larvae or propagules of more tropical species. Sampled areas are relatively small beaches (~100 m in extension), exposed to wave action and located south of the bay of Paita. In this bay, 5 to 9 km from the sampling sites, functions the second largest port of Peru, dedicated mainly to fishing activities and transportation of agriculture products. The coast is characterized by black metamorphic rocks, over which Cretacic rocks can be found, mainly from the quaternary forming sandstones and cliffs of up to 50 m (Palacios Moncayo 1994). Tides are semidiurnal that may reach 1.73 m (HIDRONAV, 2012), and SST varies between 15-29°C (INEI, 2014), with the highest values in summer (February-March) and the lowest in spring (September-October). Paita is also under the influence of of the Peruvian Coastal Current which is characterized by cold Waters but also of tropical ecuatorial surface warm waters (Zuta & Guillen 1970, Cabrera et al. 2005). Very little is known about the biodiversity of these coasts. An abundant species is the barnacle *Pollicipes elegans* which is commercially exploited for exportation (Villena 1995, Oliva 1995, Pinilla 1996). Population density of this barnacle is very high during ENSO events, but after the event, population decreases significantly and is replaced by other invertebrates such as *Semimytilus algosus*, *Austromegabalanus psittacus* and *Balanus* spp (Kameya and Zeballos 1998). As for macroalgae, a total of 35 species were identified in the intertidal zone, of which 22% are

considered to be endemic. This diversity is seasonal, decreasing during the winter or during other cold events such as La Niña (Benavente 1994).

In central Peru, the sites sampled at the Bay of Huarmey are exposed to wave action and located at 6.5-11.7 km north of the Port of Huarmey, important for mineral transportation and fishing activities. The rocky shores are part of the Casma Formation, from the late Cretacic, mainly volcanic spills of weathered andesite and inserted sediments. The weather is considered dry, subtropical desert, and tidal amplitude reaches 1.16 m (HIDRONAV, 2012). SST varies between 18-22 °C (Puerto de Chimbote, INEI 2014). Upwelling events are not frequent, but when they occur, SST may decrease to 15 °C (Berru Paz et al 2007). The most common species found in the rocky shores are *Fissurella* spp, *Polyplacophora*, *Pyropia* spp., *Chondrocanthus chamissoi*, which are also commercial species and monitored by IMARPE as artisanal fishing resources (Tam et al. 2007). The sites sampled at Ancón are within the area known as Volcánico de Ancón, which is characterized by pyroclastic rocks and volcanic andesites, typically metamorphic with plagioclases. Also a desert area, it was declared a natural protected area in 2011 along with the islands in front of the coast. The main port of Peru, El Callao, is located 33 km south of Ancón. Sampled sites are protected from wave exposure, tides reach 1.16 m, and SST varies between 14-22°C (Tarazona, unpublished). Upwelling events are frequent and generate hypoxia and even anoxia by the effect of bubbling from the bottom of sulphur compounds and other gases (Tarazona 1984, Tarazona et al. 1988, Tarazona et al. 1996). Biodiversity studies have been carried out since the 1970s (Paredes, 1974), and report 127 invertebrate species. The high intertidal is characterized by barnacles (*Jehlius cirratus* and *Notochthamalus scabrosus*) and littorinid gastropods (*Nodilitorina peruviana* and *Austrolittorina araucana*), the mid intertidal by a zone of mytilids in two bands, the upper band of *Perumytilus purpuratus* and the lower band of *Semimytilus algosus*, among macroalgae, and the low intertidal is characterized by *Austromegabalus psittacus* (Paredes & Tarazona 1980). Other spcies found in the intertidal are *Fissurella* spp., Polyplacophora, *Chondracanthus chamisoii*, and *Patallus mollis.* The locality of Paracas is located within the protected area known as Reserva Nacional de Paracas, the first marine reserve in Peru. The landscape is a coastal cordillera that reaches the sea forming cliffs of 50 to 400 m in height (Palacios et al. 1995). One of the sampling sites is located in the Ambo Formation from the Carboniferous, and the rocks are characterized by carbon sheets, which were formerly exploited. The other site is located on the Paracas Formation from the early Tertiary, characterized by phosphate sandstone and bentonites (Fernandez Dávila 1993). Both sampling sites are protected from wave exposure, tidal range is 1.12 m (HIDRONAV, 2012), and SST varies between 13 a 17 °C, however, at spatial scales of 1 to 2 kilometros, increases of up to 7°C may be observed due to water circulation and wave exposure in the bay (Romero 2000; Quispe et al. 2010, Moron et al. 1998). Intertidal fauna is represented by *Concholepas concholepas*, *Fissurella* spp., *Pyropia* spp., Polyplacophora, *Lessonia nigrecens*, among other species.

In the south of Peru, Marcona is an important area of mineral extraction, but also an area for the conservation of sea lions and pinguins (Punta Marcona). Rocky shores of the sampling sites are characterized by granitic formations from the Coastal Basal Complex, on which sedimentary rocks of the San Juan and Pisco formations have deposited sandstone of calcareous origin (Caldas Vidal 1978). These shores are exposed to wave action, however this is mitigated by a surrounding rocky reef. Tidal amplitude reaches 1.23 m (HIDRONAV, 2012) and SST varies between 12-24°C, with a marked seasonal pattern (Apaza & Figari

1999), and predominance of cold coastal waters. Upwelling events are common in this area (Rojas de Mendiola 1981). The most common species in these shores are the macroalgae *Lessonia* spp., *Macrocystis pyrifera* and *Pyropia* spp., and the invertebrates *Loxoechinus albus* and *Concholepas concholepas* (Galindo et al 1999). La Meca is located near the Wetlands of Ite, which were once heavily polluted by heavy metals from mining. Sampled sites are continuos rocky shores of sedimentary origin with sandstone and some volcanic outcrops from the Chocolate Formation of the coastal cordillera (Acosta Pereira et al. 2012). The sites are exposed to wave action but this is mitigated by rocky reefstidal range reaches 1.38 m (HIDRONAV, 2012) and SST is around 14 °C. This area is visited by artisanal fiherman extracting crabs (*Leptograpsus variegatus*), *Concholepas concholepas*, and macroalgae. The most southern locality is Punta Colorada, also characterized by outcrops of the coastal cordillera and some volcanic Rocks. The shore is exponed but some rocky reefs are also present. Between thes two localities, there are several artisanal fishing ports (Estrella Arellano et al. 1998).

Chile

The coastline of continental Chile extends over 4200 km (from ca. 18°S to 56°S) encompassing from subtropical to sub-Antarctic waters (Santelices, 2001). The regular and relatively straight coast changes south of Chiloé Island (41°29'S) which is replaced by many gulfs, islands, channels and fjords. The rocky shores in northern and central Chile are mostly exposed to strong wave action (Thiel et al., 2007). Substratum is composed of rock of volcanic, granitic or sedimentary origin. Most of the coastal range consists of Jurassic and Cretaceous volcanic rocks (Fariña et al., 2008). Most of the coastline is influenced by the flowing Humboldt Current System, coastal upwelling and periodic occurrence of El Niño-Southern Oscillation (ENSO) (Thiel et al., 2007).

Northern Chile: Iquique, Antofagasta and Copiapó

This area represents one of the driest regions of the world, annual precipitation is extreme low (1 mm to 80 mm) with occasional rainfall episodes during austral summer, but no large differences between winter and summer exist (Schulz et al. 2011) (Figure 6E, 6F).

Species richness at Iquique, Antofagasta and Copiapó intertidal rocky sites was 41, 37 and 52 respectively. The high intertidal communities are dominated by the chthamalid barnacle *Jehlius cirratus* and the periwinkle *Echinolittorina peruviana*, occasionally small limpets *Siphonaria lessoni* and *Scurria variabilis* can be also found. Few macroalgae are present, mostly *Porphyra* sp. and *Pyropia* sp. The middle intertidal is dominated by the anemones *Phymactis papillosa* and *Anemonia alicemartinae*, the purple mussel *Perumytilus purpuratus* and macroalgae such as the ephemeral green alga *Ulva* spp. and the fleshy crustose brown *Ralfsia* sp., particularly in Antofagasta sites the middle and low intertidal are dominated by a dense turf of the red alga *Caulacanthus ustulatus* but in Antofagasta Bay extensive aggregations of the barrel-shaped tunicate *Pyura preaputialis* dominated the middle and low intertidal fringe, this is a non-indigenous species that affect the presence of native organisms (Caro et al. 2011). The low intertidal is dominated by a conspicuous belt of the kelp *Lessonia berteroana*, this brown alga is an ecosystem bioengineers and its holdfast provides habitat for high variety of small invertebrates (Vásquez & Santelices 1984), patches of the red algae *C. ustulatus* and *Corallina officinalis* var. *chilensis* are also present. At several sites the calcareous crusts of *Mesophyllum* sp. and *Lithophyllum* sp. dominates the

substrata, over the crusts several individuals of the snail *Tegula atra*, the black sea urchin *Tetrapygus niger*, the edible barnacle *Austromegabalanus psittacus* and the large mollusc *Enoplochiton niger* can be found. At shadow protected places, the sea cucumber *Patallus mollis* and the anemone *Phymactis papillosa* (mostly the blue morph) are quite abundant. Several filamentous algae can be also found at middle and low intertidal, such as *Centroceras clavulatum*, *Polysiphonia* sp. and *Ceramium* spp. In some places the brown algae *Colpomenia sinuosa*, *C. tuberculosa* forms patches of several individuals.

Central-Northern Chile: Coquimbo, Los Vilos and San Antonio

In central Chile, Mediterranean climate is predominant characterized by a winter rainy season and a dry period in summer. The ocean proximity moderates temperatures, averages between 10°C in winter and 17°C in summer can be found. Presence of snow and frost are rare, day-night oscillation is also lower. Species richness at Coquimbo, Los Vilos and San Antonio intertidal rocky sites was 58, 69 and 70, respectively. The chthamaloid barnacle *Jehlius cirratus* dominates the sessile communities at high intertidal. The mobile communities are dominated for *Echinolittorina peruviana* and the small limpet *Siphonaria lessoni*. Mostly fleshy crustose macroalgae are present, such as *Hildenbrandia lecanellieri*, *Ralfsia* spp. and patches of the lichen *Thelidium chilensis*. The middle intertidal is dominated mostly by beds of the purple mussel *Perumytilus purpuratus*, and red algae *Mazzaella laminarioides*, *Hildenbrandia lecanellieri* and the turf-forming alga *Gelidium chilensis*. The mobile organisms in the middle zone are dominated by small limpets such as *Scurria araucana*, *Scurria variabilis* and the pulmonate gastropod *Siphonaria lessoni*. The low intertidal zone is dominated in rocky exposed shores mainly by crustose algae such as *Lithothamnium* spp., *Hildenbrandia lecanellieri* and the articulate calcareous coralline *Corallina officinalis* var. *chilensis*. In lower proportion the mussel *Semimytilus algosus* can be found and a conspicuous belt of the *Lessonia spicata*. The most common mobile organisms in the low intertidal zone were *Scurria araucana*, *S. scurria* and the key hole-limpets *Fissurella crassa* and *F. costata*. Several species such as *Perumytilus purpuratus*, *Gelidium chilense* and *Corallina officinallis* var. *chilensis* are considered ecosystem bioengineers and it loss can have significant changes in the community structure (Kelaher et al. 2007). On the other hand, is important to mention that in Central Chile a meso-scale eddy activity has been described (around 30ºS) (Hormazábal et al. 2004). Therefore expected differences in the structure of populations across this region can be found (Narváez et al. 2006).

Central-Southern Chile: Concepción and Valdivia

This area represents one of the rainiest regions in Chile, where the annual precipitation is very high, reaching up to 1250 mm (average last 10 years = 847 mm) at Concepción and 2400 mm (average last 10 years = 1800 mm) at Valdivia, with constant rainfall episodes during austral winter (June-September), and also occasionally rainfall during spring, and even during austral summer (December-February) (Figure 6G).

Species richness at Concepción and Valdivia intertidal rocky sites was 48 and 52 respectively, the communities at the high intertidal are dominated principally by the chthamalid barnacle *Jehlius cirratus* and the littorinid snails *Austrolittorina araucana*, occasionally the small limpets *Siphonaria lessoni*, *Scurria scurra* can be also found.

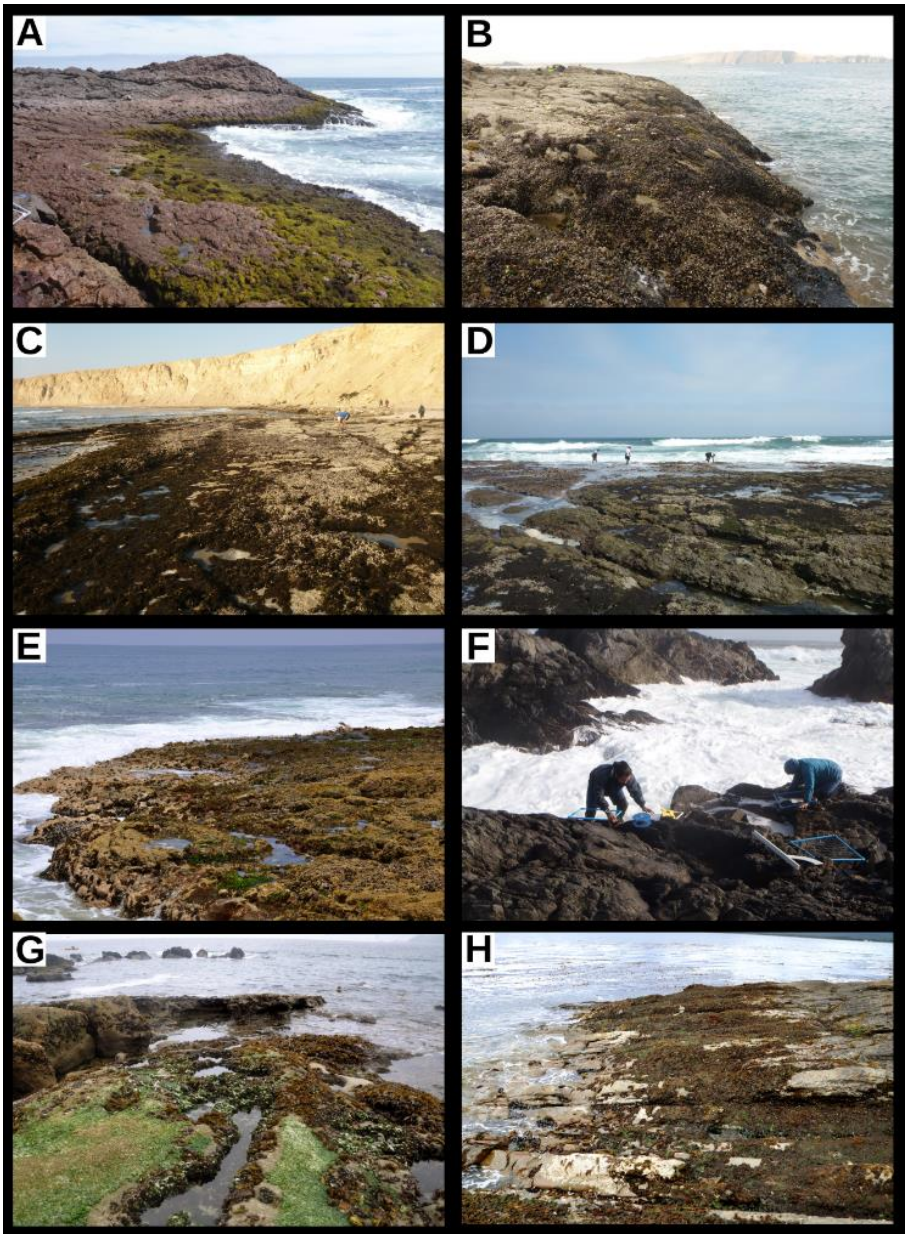

Figure 6. Sampling sites in the Peruvian and Chilean Pacific. A. Peru – Huarmey. B. Peru – Paracas. C. Peru – Paracas. D. Peru – Tacna/Punta Colorada. E. Northern Chile - Huayquique. F. Northern Chile - Copiapo. G. Central-southern Chile – Cocholgue. H. Southern Chile – Fuerte Bulnes.

The middle intertidal is dominated by dense beds of mussels *Perumytilus purpuratus* and *Semimytilus algosus* covered by the red macroalgae *Mazzaella laminarioides*, and in some sites, the red macroalgae *Mastocarpus latissimus* and *Gelidium pseudointrincatum*. Among mobile species, the limpets *Scurria scurra* and *S. variabilis*, and the snail *Tegula atra* are common inhabitant in the middle zone. The low intertidal is dominated by the red macroalgae *Ahnfeltiopsis furcellata* and patches of *Corallina officinallis* var. *chilensis*. At several sites, the foliose green macroalgae *Ulva* sp. is also found. Over and into primary substrate

individuals of the snails *Tegula atra* (in some places assorted with *Prisogaster niger*) and *Acanthina monodon* are very abundant. At shadow protected places, the anemone *Phymactis papillosa* is quite abundant. Others species as the polychaete *Phragmatopoma moerchi*, the solitary ascidian *Pyura chilensis*, and the kelp *Lessonia spicata*, although less important in density, are considered important component on these latitudes because are considered ecosystem bioengineers, which may change significantly the surrounding community structure (Cancino & Santelices 1984, Sepúlveda et al. 2003a, 2003b).

Southern Chile: Punta Arenas

This area represents one of the most austral regions of the world, where annual precipitation is high, reaching up to 640 mm at Punta Arenas, with constant rainfall, snow and hail episodes during all year (Figure 6H). Winds are frequent and often exceed 100 km/h. The minimum temperature during winter can drop up to -2°C approximately (Butorovic 2013). Species richness at Punta Arenas intertidal rocky sites was 48. The communities at high intertidal are dominated by the barnacle *Jehlius cirratus* and the red macroalgae *Porphyra* spp. and *Pyropia* spp., and over the primary substrate the small limpets *Siphonaria lessoni* can be found in medium densities. In the middle intertidal patches of several macroalgae can be found, such as the coarsely branched *Nothogenia fastigiata*, *Mazzaella laminariodes*, the brown alga *Adenocystis utricularis*, *Ulva* sp. and filamentous of several Ceramiales species (e.g. the introduced species *Polysiphonia morrowii*), in this zone *S. lessoni*, and patches of *Perumytilus purpuratus* are also found. The low intertidal is dominated by the brown algae *Caepidium antarcticum* and *Lithophyllum rugosum*, however, the most conspicuous component are the gastropods *Nacella magellanica* and *N. deaurata*, which are found in high densities.

USES AND THREATS TO THE INTERTIDAL ROCKY SHORES IN SOUTH AMERICA

The intertidal rocky shores around South America represent a valuable resource for local populations in many aspects. The main uses given to these shores along with the threats that such uses produce is summarized in Table 1. In general, localities with dense human settlements face the problems associated to urbanization and sewage discharges along with unregulated tourism, while in less densely populated areas, the uses are basically associated to the extraction of invertebrates and macroalgae for food. Industrialization (e.g. oil and gas extraction, mining) is another issue affecting the services that these ecosystems may provide.

The Caribbean

In the Colombian Caribbean, besides populated human settlements, the main economic activity is tourism, so the marine environment is under pressure constantly due to these related activities and waste disposal. Exploitation of some resources also occurs, for example the snail locally known as Burgao or Cigua (*Cittarium pica*), lobsters (*Panulirus* spp.), the Caribbean king crab (*Damithrax spinosissimus*), various fishes (snappers, groupers),

octopuses and chitons (Lopez-Victoria et al. 2003). Due to the exploitation, sometimes exceeding sustainable population limits, several of these species have been allocated in different risk categories of red lists (Ardila et al. 2002, Mejia et al. 2002). In less densely populated areas such as Taganaga, next to the Tayrona Natural National Park, small fishermen villages (ca. 4000 people) have precarious sanitary services polluting the coast with untreated sewage.

Table 1. Summary of main uses, threats and impacts at intertidal rocky shores in South America

Country/Locality	Uses	Threats/Impacts
CARIBBEAN		
Colombia	Food (snails, lobster, crab, fish, octopus, chitons) Urbanization, tourism	Decline in species abundance (some under threat categories by IUCN) Pollution, sedimentation
Venezuela	Food (snails, bivalves, sea urchins Tourism, urbanization	Decline in species densities Freshwater runoff, sedimentation, pollution (sewage), oil spills, solid waste
Trinidad and Tobago	Food (fish), tourism, urbanization	Pollution, freshwater runoff, overfishing
ATLANTIC		
Northeast Brazil	Food (artisanal and tramp fisheries of shrimp, crab), tourism, urbanization	Pollution (sewage, petrochemical), freshwater (urban, agriculture) runoff, solid waste, erosion
Southeast Brazil	Tourism, food, urbanization, marinas	Pollution (ore mining, petrochemical, heavy metals, sewage), maritime traffic, sedimentation (dredging navigation channels)
South Brazil	Food (octopus, crabs, oyster, mussel), tourism, urbanization	Pollution (sewage), invasive species, destruction of sand dunes, freshwater runoff, port activities
Uruguay	Food (macroalgae, mussels), tourism,	Invasive species (*Rapana*)
Argentina	Urbanization, food, tourism	Invasive species, pollution (sewage)
PACIFIC		
Colombia	Food (lobsters, fish, snails, shrimp)	Rock removal
Ecuador	Tourism, urbanization, food	Pollution (sewage), sedimentation, gasoline spills
Peru	Food (crabs, snails, macroalgae, chitons, sea urchins) Mining	Pollution (heavy metals from mining)
Chile	Food (macroalgae, snails)	Pollution (sewage), decline in macroalgal density

In Venezuela, some of the threats to Venezuelan rocky shore's biodiversity are related to freshwater runoff, sedimentation, pollution, tourist pressure, oil spills and urbanization (Miloslavich et al., 2003; Paz-Villaraga et al., 2015). In particular, two of the most important sources of freshwater on the Venezuelan coast are the Tocuyo River, and the Tuy-Carenero Rivers system. The Tocuyo River discharges near Morrocoy National Park (Bastidas et al.,

1999), and the sediment plume of Tuy-Carenero system can be large enough to affect the rocky shores of Cabo Codera and Chirimena (Cedeño, 2009). Both rivers are highly polluted, they receive untreated water from a wide array of agricultural and industrial sources, and the discharge from drain sewage from urbanization and rural areas; including untreated waters from Caracas, the capital city of Venezuela. However, no correlations were found between the structure of assemblages associated with rocky shore and their relative distances to these rivers. It is very likely that other factors such as the selective collection, for human consumption, of gastropods, bivalves, and urchins could be affecting these communities. Some populations of invertebrates have shown an alarming decrease of their densities, which might be related to fishermen activities. Some of the gastropods in this situation are *Cittarium pica* and *Astraea tecta*, whose juveniles can be found on the low intertidal (Díaz-Ferguson et al., 2010). *C. pica* is the second gastropod most heavily fished in the Caribbean (Gómez-Gaspar, 1999; Miloslavich and Huck, 2009; Schmidt et al., 2002); however, there are no statistics available for this fishery (Robertson 2003) in the Venezuelan coast. When *C. pica* was present (7 of 31 sites), the diameter sizes ranged between 25mm and 40mm, which represented a clear diminution when compared to values reported in the Archipelago Los Roques National Park on 1987, where maximum size was 115 mm (Castell, 1987; Osorno et al., 2009). Also, this size is classified as small for *C. pica* in the Colombian Caribbean coast, Virgin Island and Puerto Rico (Schmidt et al., 2002; Robertson, 2003; Osorno et al., 2009). Minimum and maximum densities of *C. pica* were 0.4 ind/m^2 and 1.2 ind/m^2, respectively; which is lower than densities reported by Castell in 1987 (5.6 ind/m^2). Finally, despite intertidal rocky shores in Venezuela are not the principal touristic attraction on the coast, some of them (e.g. Peninsula of Paraguaná, Morrocoy and Mochima Nationals Parks, Patanemo, La Sabana and Chirimena) are visited by an important number of tourists, where random collection of shells and invertebrates is a common practice.

In Trinidad & Tobago, the rocky shore areas are very important for shoreline protection and they provide habitats for various species of fish, crustaceans, molluscs and macroalgae. Some rocky areas are very popular for recreational fishing and ecotourism. The main threats to biodiversity and the marine ecosystem in T&T is from land-based activities. These include expanding industrialization and urbanization (e.g., land clearing for housing etc.), and accompanying pollution and contamination (solid, liquid and gaseous wastes). As with the rest of the region, overfishing and unmanaged coastal development and agricultural practices also exacerbate these problems. More recently, extreme weather conditions reflected in increased rainfall during the wet season and extremely dry seasons (attributed to climate change) continue to result in increased flooding, freshwater runoff and sedimentation. Trinidad and Tobago is a highly industrialized country with 2 very large industrial estates involved in a range of activities dominated by the petrochemical sector. As the largest oil and natural gas producer in the Caribbean, Trinidad and Tobago's also houses one of the largest natural gas processing facilities in the Western Hemisphere. With 11 ammonia plants and seven methanol plants, Trinidad and Tobago is the world's largest exporter of ammonia and the second largest exporter of methanol, according to IHS Global Insight (2013). Trinidad and Tobago's coastal areas contain rich biodiversity reserves including productive and critical habitats- coral reefs, sea grass beds, estuaries, mangrove forests and coastal swamps, beaches and bays. These coastal areas account for approximately 90% of annual fish production. Fishing occurs throughout the marine environment around both islands, in estuaries, nearshore coastal waters and deep oceans. Today, the local fishing industry is largely

artisanal, based on resources occurring in the coastal and territorial waters, and is characterized by multi-species, multi-gear and multi-fleet operations (Fisheries Division, 2002). In 2005, the marine fisheries sector contributed $63 million to the Gross Domestic Product (GDP). Other coastal and marine resources include crustaceans (shrimps, lobsters, crabs), cephalopods (squid), cetaceans (marine mammals including whales, dolphins, and porpoises) and sea turtles. Historically, T&T has not been a recognized tourist destination and as recent as 2010 T&T's contribution to the overall Caribbean was only 10.9%. It was estimated that on an annual basis approximately 33% of visitors to Trinidad and Tobago use the coastal resources (Tourism Development Company, 2010). Several beaches in Trinidad and Tobago (Pigeon Point, Maracas, Mayaro etc.) are very popular for recreation and tourism but it is the Buccoo Reef in Tobago which generates the greater tourism income (both local and foreign). The reefs provide livelihoods for a large portion of the local population through both fisheries and tourism (Burke, 2008). In 2006, the value of the reefs to recreation and tourism was estimated to be between US$100 and $130 million, or approximately 45% of Tobago's GDP for that year. At the same time, the value of the reef fisheries was approximately 0.8 to 1 million USD. The coral reef shoreline protection value was calculated at between US$18 and US$33 million in 2006 (WRI 2008). These ecosystem services (coral reef and rocky shores) are important to the island, at the same time they are most vulnerable to erosion and storm damage.

The Atlantic

In the region of Ceará in Brazil, the intertidal reefs are vital for many human populations established along the shore. Most of the small villages depend on the artisanal fisheries and tourism for subsistence, but the aquaculture has also gained importance in the last decades. The reefs play an essential role as nursery habitat for many fish species and also for the spiny lobster, the most important economic resource of the region (Igarashi 2010; Godinho and Lotufo 2010; Cunha et al. 2008). Tourism is a relevant source of income for the state's economy and is present in more or less intensity along the whole coast. In the last three decades, the development of tourism has followed what happened throughout the world, with an increasing number of tourists visiting especially the coastal zone, attracted by the warm water and constantly sunny days of the region (SETUR 2009; Aquasis 2003). The beachrock reefs in Ceará have been strongly impacted by human activities along the shore. The reefs closer to large urban areas, such as Caucaia, receive a large amount of pollutants carried by the rivers that run through farmlands, industrial districts and densely populated cities (Nilin et al. 2007). Locations outside the influence of urban areas are also under stress, because of the exploitation of algae for industrial use, mainly *Gracillaria birdiae* (Plastino and Oliveira 2002). Although not properly evaluated, the impact of algae exploitation is easily noticed when the areas are visited. As an alternative, the cultivation of the algae has been stimulated in different coastal communities, with variable degrees of success. Also, fishermen looking for young lobsters, crabs and especially octopuses, constantly visit these reefs. The strategy used by fishermen for capturing octopus is dislodging the animal with the use of chlorine or large amounts of salt thrown directly on the burrows or tide pools. Aquasis (2003) has presented and discussed extensively the importance of the state's coastal zone, pointing out the conflicts and problems with the management (or absence of management) at both local

and regional scales. The main problem is that rocky shores are one of the least studied areas of the Brazilian coast, and the northeast region as a whole is going through an accelerated process of development and increasing pressure over these coastal ecosystems.

At Sergipe, the coastal zone is a contrasting area, in which there are many activities, interests and conflicts, in a scenario that consists of urbanized areas, agricultural (sugar cane, orange and coconut), extractive, industrial and port activities, besides tourism and sale of properties. Moreover, this area is permeated by low density of occupation and occurrence of ecosystems of high environmental significance, but which have been subject of accelerated occupation, a tourism subproduct. This issue generates environmental degradation that hinder the practice of many activities, including tourism. Regarding the disorderly occupation, it noteworthy that 25% of the territory is coastal zone. Sergipe's coastal zone has extensive areas of mangroves associated with estuaries. However, mangroves have been the target of multiple human impacts, mainly shrimp farming and crab catching. In addition, mangroves and dunes are turning into garbage dumps, without any legal or environmental criteria in the area. Petroleum, natural gas, limestone and potassium (largest mine in the Southern Hemisphere) are the main products from mineral extraction, Sergipe is the 6th Brazilian state in oil production, following Rio de Janeiro, Rio Grande do Norte, Amazonas, Bahia and Espirito Santo. Pirambu is a new port area in the State comprising a large off-shore terminal operating primarily with petro and chlorochemical, besides being a vector to expand the economy and the tourism, it is also a potential environmental problem. The most important stressing factors on Sergipe rocky shores are the harbor presence, the trampling effects of tourists and shrimp and crab catching. Other stressing factors are the influence of the petro and chlorochemical industries, sewage pollution, and coastal erosion.

In Bahia, the main use observed at the rocky shores is trampling, especially for fisheries of octopus, sea urchin and fish. However, there is no sign of overfishing in these areas, as there were low numbers of fishermen and most was subsistence fishing by local communities. Even though all sampling sites are considered important touristic areas, there is no evidence of strong pressure in most sampling sites. Itaparica and Litoral Norte are the closest locations to Bahia state capital, Salvador, and thus a popular touristic destination. Tourism in Litoral Norte increased during the past 15 years with the construction of luxurious hotel complexes and the improvement of a state highway. During the holiday season it is common to see people walking on the rock shores. However, considering the number of visitors in that area, it is likely that the rocky shores are affected by trampling and fishing, but this needs further experimental tests. This is especially important in Litoral Norte as it provides feeding and resting habitats for adult and sub-adult green turtles (Jardim et al., 2014). Natural sedimentation is also common in Litoral Norte. Mar Grande (Itaparica location) and Praia do Sul (Ilhéus) are the most threatened sampling sites regarding pollution. Both are located close to urban areas and are subjected to urban runoff, solid waste and domestic sewage discharge. Even though Stella Maris is located in Salvador, it is not a high populated area when compared to the other parts of Salvador and not highly exposed to urban runoff and pollution. Ilhéus is the second largest city, among all sampling sites, with around 220 thousand inhabitants. Itacaré sites are inside an ecological touristic area with a relative low number of visitors. Thus, touristic pressure and water pollution and solid wastes, here, are not a threat.

Espirito Santo, in contrast to the Paraná State where ca. 68% of the territory is preserved by specially protected areas (APA), has only 2% of APA. Furthermore the coastal area of Espirito santo is out of the Conservation Units (UC). The most important stressing factors on

Espírito Santo rocky shores, directly or indirectly, are tourism, sewage pollution, industrial complex presence (trading coffee, chocolate, edible oils, citric juices and cellulose), and a harbour complex that handles petrochemical (mainly oil and natural gas, being the second petroleum province in the country). Espirito Santo also encompasses the second largest ore mining dock in the world managed by Vale do Rio Doce Company.

In Sao Paulo, coastal land use and human impacts - The Baixada Santista, at the Southern end of the sampled coastline, is a major economic zone within São Paulo State and heavily urbanized area, which includes the cities of Praia Grande, São Vicente, Santos and Guarujá. Together, these cities sum up 1.6 million habitants, imposing severe impacts on the coastal environment. Particularly problematic is the Santos Harbour, the largest in South-America. Apart from a very intense traffic, the channel needs to be frequently dredged to allow the passage of large cargo vessels, further impacting the seafloor and the water column due to excessive siltation and suspended materials, including heavy metals and other major pollutants. Further north, socio-economical activities are leaded by tourism, although there is increasing pressure for the expansion of the São Sebastião Harbour. At the northernmost locality, Ubatuba, the coastal impact is relatively small, although the population has been gradually increasing. Today, the population in Ubatuba is around 85.000 habitants, and domestic sewage is already a major pollution source.

In Parana, the most important stresses on rocky shores communities are the trampling effects of tourists and fisherman, the selective collecting for food, oyster and crabs catching, and mussel seeds for cultures, sewage pollution and bio-invasions. All the sites studied receive a large amount of tourists every summer (from the end of December to the beginning of February) and weekends. Coastal environmental problems in the South also include unplanned occupation nearby the shoreline. Besides the destruction of the frontal dunes, the occupation invades the beach altering the balance of the whole coastal system. Trampling effects on the community have been studied only in one rocky coast at the southeastern region and a negative effect on *Chthamalus bisinuatus* was observed suggesting that the cumulative effect over years is significant (Ferreira & Rosso, 2009). Furthermore, the small towns at the coast double or triple their population during summer months and domestic sewage pollution and consequent eutrophication of coastal areas is certainly occurring, but the consequences to rocky shore communities are not known. The brown mussel (*Perna perna*) is the main item harvested both to be used directly for food, for commercialization or as seeds for cultures. The Brazilian government started a regulation since 2006 forbidding the extraction of this species from natural stocks from September to December each year and also regulating its extraction during the rest of the year. Although the brown mussel was supposedly introduced in Brazil from Africa (Sousa et al. 2004), it is well established in the intertidal community along the southeast and south Brazilian coasts. More recently the brown mussel belt has been invaded by another mussel, *Isognomon bicolor* (Adams, 1845), which forms dense aggregations in some sites in southeastern Brazil displacing *P. perna*, but that have not been so abundant in the Paraná and Santa Catarina coasts. We only found *I. bicolor* in one beach (Praia de Cima) during 2010 surveys and none in 2013 surveys. Also in the south of Brazil, the Paranaguá Harbor is the largest cereal port in Latin America, exporting mainly soybean, and the 3rd largest port of containers from Brazil, following Itajaí, a strategic zone to monitor alien or invasive species. The port area also presents serious problems of waste disposal. The presence of large ports along the south coast (Paranaguá, São Francisco, Itajaí and Imbituba) in addition to the extensive area of bivalve culture in Santa Catarina poses a constant threat of

species invasion in this region. During the surveys we found introduced barnacles at most sites visited, being *Megabalanus coccopoma* (Darwin, 1854) the most frequent, but also *Amphibalanus amphitrite* (Darwin, 1854) and *A. reticulatus* (Utinomi, 1967) (Kloh et al. 2013). In the Paranaguá Bay, *Ulva australis* (formerly known as *U. pertusa*) was detected as a free floating thallus. Monitoring is being carried out to confirm the alien species in the area.

In Uruguay, human uses of rocky shores are mainly recreational, although some species e.g. mussels) may be harvested by tourists or by a subsistence, small scale hand-gathering fishery and/or to be sold in local markets (Carranza et al., 2009; Scarabino, 2004). Extraction of the algae *Ulva* spp. may also occur associated to uses in local gastronomy, especially during the summer. Sport fishing (generally unregulated) are frequently observed in these sites, although some rocky shores are included in the National Protected Area System (SNAP). Commercial fisheries are restricted to subtidal mussel beds located at Isla Gorriti (34º57'S 54º58'W) and Isla de Lobos (35º0'S 54º53'W), targeting the blue mussel *Mytilus edulis* (Defeo, 1991; Niggenmayer and Masello, 1992; Riestra and Defeo, 1994). To date, threats to rocky shores biodiversity have not been evaluated at a national level. However, although not occurring in the intertidal, the invasive rapa whelk *Rapana venosa* is a matter of concern since this species preys mainly over mussels in the estuarine-oceanic interphase (Carranza et al., 2010; Carranza, Delgado, and Martinez, 2013). Other exotic species such as *Isognomon bicolor* (Breves et al., 2014) has been detected, but does not seem to have established so far.

In Argentina, the Buenos Aires province coastline is highly urbanized and harbors important ports. Playa Chica is located ca. 1.2 km of Mar del Plata Harbour (the most important fishing port in Argentina) and Quequén is located ca. 2 km m from Quequén Harbour. Both sites are subject to intense recreational use by summer visitors and pollutants associated to maritime traffic and urban runoff, such as policyclic aromatic hydrocarbons (PAHs), trace metals, and Tributyltin (TBT), all of which have been detected in their nearby ports (Marcovecchio et al. 1998, Bigatti et al. 2009, Albano et al. 2013). The Quequén site may also be impacted by a nearby (ca. 5 km) sewage effluent (López-Gappa et al. 1990). Río Negro is not very populated (El Espigón, Punta Colorada) and human settlements are mostly limited to small summer vacationing villages (La Lobería, Playa Los Suecos). Yet, the shipping of iron pellets from a loading dock in Punta Colorada might have contributed with pollutants to this area in spite that the dock in question was intermittently operational over the past two decades. In Chubut Province, Puerto Lobos is an artisanal fishermen place, with no evident contamination, and no ports are present. In Puerto Madryn, the local population is about 80,000 residents but in the summer season this number may duplicate due to the tourism. In this city, the 2nd port in importance regarding fisheries landings in Argentina is settled, as well as different industries in nearby the port. Pollutants such as (PAHs), organochlorinated compounds, trace metals and TBT are present in areas with intense maritime activity in Golfo Nuevo coasts (Commendatore et al., 2000; Esteves et al., 2006; Gil et al., 2006; Commendatore and Esteves, 2007; Massara Paletto et al., 2008; Bigatti et al., 2009; Commendatore et al., 2012). Imposex (masculinization of female gastropods) has been detected in the port zone but at a low frequency in the sampling sites. These are used mainly for tourism at Punta Este during the summer season. In Camarones, the population is around 1300 habitants and the port is used by fishing boats. Near the port, imposex and TBT contamination were detected (Bigatti et al., 2009), while the sampling sites far from this place are imposex free. The sampling site "Algueros" is a place of algae collection for industrial

purposes, and the other places are used by tourist for recreation and sport fishing. At Puerto Deseado, near the port, where the maritime traffic is high, TBT contamination has been detected (Bigatti et al., 2009), however, TBT was not detected in the sampling sites. Recollection of the limpet *Nacella magellanica* is common within the local people. At Tierra del Fuego Island, Playa Larga is likely the most impacted by human activities due to its proximity to Ushuaia. This city is situated in the coast of the Beagle Channel and hosts the southern port of South America, characterized by intense maritime traffic. Contamination by TBT (Bigatti et al., 2009), PAHs (Esteves et al., 2006), metals (Giarratano et al., 2010) and sewage were detected at this area, and up to 100% incidence of imposex was observed in female gastropods.

The Pacific

In the rocky shores of the Colombian Pacific, the main resources exploited are lobsters (*Panulirus gracilis*) and fishes (snappers, groupers), however, some other species such as oysters (families Ostreidae and Pteriidae), and snails (families Littorinidae and Muricidae) are locally exploited. Another source of perturbation is the removal of rocks in the search for shrimps (*Upogebia* spp.) that are used as bait in fishing activities (Lopez-Victoria et al. 2003). The population density on the Pacific coast is very low in comparison to the Caribbean, with most of the shore basically uninhabited. In this way, human disturbance is localized and/or of low impact.

The rocky shores of Ecuador are used for urbanization, fishing, tourism, recreation, and marinas. Punta Gorda is relatively far away from human settlements and therefore, less impacted, but Punta Bellaca, more accessible was once a fishing ground for the lobster *Panulirus gracilis* and it is used sporadically at present by tourism. The main threats detected in the coast of Ecuador are sedimentation, pollution with sewage waters, diesel spills at the marinas, development of vacational complexes and unplanned human settlements.

In Peru, fisheries are a key component of the countries economy. Such fisheries are mostly pelagic resources, however, several species of the coastal zone are also fishing targets. At Paita, the barnacle *Pollicipes elegans* is commercially exploited and exported (Villena 1995, Oliva 1995, Pinilla 1996). Along the coast, there are several benthic species subject to artisanal fisheries in the intertidal including invertebrates and macroalgae: *Fissurella* spp, Polyplacophora, *Pyropia* spp., *Chondrocanthus chamissoi*, *Patallus mollis*, *Concholepas concholepas*, *Lessonia nigrecens*, *Lessonia* spp., *Macrocystis pyrifera*, *Pyropia* spp., and *Loxoechinus albus* among others (INRENA, 2002). At Paracas, subtidal artisanal fisheries also occurs (Mendo & Wolff 2003). Mining has been very intensive in Peru and some areas show pollution associated to mineral exploitation including heavy metals (Jacinto et al. 2001, Jacinto et al. 2003, Jacinto et al. 2008).

In Chile, the impact of human activities on the rocky intertidal has been studied extensively (see Fernández et al. 2000 and references therein). Several types of human impacts clearly affecting nearshore ecosystems can be identified along the coast of Chile, although the intensity, extent, and persistence of these sources vary geographically (Fernández et al. 2000). One of the most important human impacts along the Chilean coast, in terms geographical extent and persistence, are sewage discharges and the harvesting of invertebrates and algae in rocky shores (Gross & Hayek 1998). The removal of several

important ecologically species during low tides have a dramatic effect on the structure of the intertidal communities. Historically, intertidal populations were exploited as a food resource but in modern times, flora and fauna are also collected for fish bait, for research, as souvenirs, and for home aquaria uses. A keystone muricid gastropod *Concholepas concholepas*, locally known as "loco" is intensively targeted by food gatherers affecting the functioning of food webs at intertidal rocky shores (Castilla, 1999). The increasing international demand for brown large macroalgae and local requirement as food for abalone aquaculture has caused deterioration of natural kelp populations along the rocky shore in Chile, especially between 18° and 42°S (Vega et al. 2014). Impact of non-indigenous species have been poorly documented, some species have displayed an expansion of their geographical range and increasing of their abundance, such as the anemone *Anemonia alicemartinae* (Hausermann & Forsterra 2001) and the red alga *Mastocarpus* sp. (Macaya et al. 2013). Together with exploitation of marine species, sewage discharges are important in geographical extension and persistence having an impact along the Chilean coast (Fernández et al. 2000).

GAPS IN OUR KNOWLEDGE AND FUTURE PROSPECTS

Knowledge of the intertidal rocky shore ecosystem is very variable among the different South American countries, and also between different areas within each of the countries. While some countries seem to have a long tradition of research in these ecosystems (e.g. Chile), other are just beginning to study them (e.g. Venezuela). In the Southern Caribbean, rocky shores remain virtually unexplored, despite the high diversity that these ecosystems support (Miloslavich et al., 2010). For example, in Venezuela, most of the research articles on these ecosystems are non-published descriptions or informal inventories about the fauna and algae that inhabit them. Few studies have considered quantitative description of patterns of temporal and across different scales, which would allow proposing underlying mechanisms that drive assemblages associated with intertidal rocky shores (Cruz-Motta, 2007). Furthermore, the total number of scientific publications for this ecosystem is the lowest for coastal and marine ecosystems of Venezuela (Miloslavich et al., 2003). Consequently, describing patterns and determining processes affecting assemblages associated with Caribbean intertidal rocky shores is still a prevailing necessity. Caribbean rocky shores harbor an important biodiversity, however their economic benefits and ecosystem services are still poorly studied and understood. Threats to biodiversity in this ecosystem, and other coastal ecosystems, are imminent; therein lies the importance not only of knowing their biodiversity, but also of monitoring the ecosystem to be able to understand their patterns and processes, their connectivity to other coastal ecosystems, the population dynamics of key species, and finally to have the necessary knowledge to transfer to policy makers so they can take scientifically based informed decisions.

There are several limitations to achieve these goals. The first of them is the funding required to go to the field on a yearly basis, and the commitment to do so, especially at such large geographical scale like the one presented in this chapter. Initiating a long term time series is not an easy task. The second is related to human capacity. The team required to produce quality information is quite complex, with background in marine ecology, biology, taxonomy, genetics, fisheries, and oceanography to mention a few. This expertise is rarely

found altogether within a same country, therefore, the importance of establishing and collaborating within the umbrella of an international network. Initiatives like SARCE, and previously NaGISA of the Census of Marine Life, have contributed significantly in the region to increase our understanding of these important but traditionally neglected ecosystems.

Finally, for all the South American region, the use and abuse of the rocky intertidal resources has been intensive during the last years, needing a full re-evaluation of the human impacts along the South American coastline, in order to demonstrate the need for conservation of these ecosystems. The implementation of marine reserves and laws aimed to halt the collection of organisms has proved to be successful in some areas but ultimately depends on the enforcement of the laws and compliance by the public (e.g. Chile). Even, if collecting is stopped through enforcement, other impacts of human use, ranging from local and specific activities such as trampling or overturning of rocks to large scale impact such as pollution and climate change, still persists. Therefore, effective protection of rocky intertidal communities will require an approach that may need to go beyond the singular focus on collecting to reduce the full suite of impacts (Smith et al. 2008).

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Chapter 19

EVALUATION OF BIOMASS AND REPRODUCTIVE ASPECTS OF INVASIVE ALGAE *ACANTHOPHORA SPICIFERA* IN PUNTA ROCA CAIMANCITO B.C.S. MEXICO

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ABSTRACT

Acanthophora spicifera is a red alga, native to the Caribbean, was introduced to the Hawaiian Islands in the early 1950's, where he Invasive catalog. His success as an invader is attributed to having the ability to reproduce both sexually and vegetatively by fragmentation or by spores. Their growth is accelerated and invades Pacific coral reefs. Prevents nutrients and sunlight to penetrate these ecosystems producing that reduce populations of resident seaweeds and corals. In Punta Roca Caimancito, BCS, a blanket of *A. spicifera*, which formerly dominated the brown algae of the genus Sargassum found. The temperature at which develops in Punta Roca Caimancito was 20-29. It can be found growing on a variety of substrates epilithic hard rock, dead coral or shells, even as an epiphyte on other species of macroalgae or epizoica invertebrates. They have a simpodial and apical growth, presents secondary branches, has a rough or rough texture, has a disk-shaped, in its main axis no thorns. It is characterized by a highly branched and bushy, leafy thallus. Apical meristem, spines of 3 mm, which may vary morphologically.

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In cross-section was observed that pericentral cells are dense. Commonly axial cells in the center. In the study area this invasive alga reproduces asexually (fragmentation). The extension of the mantle in Punta Roca Caimancito of has continued to increase since 2006 in which you registered for the first time. The current extension (2009) is 31536.93m^2 or 3.16 Hectares. The maximum average biomass dry weight was 8.12 t/ha and 3.16 minimum 1.18 Ton/3.16 h. The color varies from brown to red. The average length of the thalli was 163 mm and 108 mm maximum minimum in the period October 2008 to October 2009. The annual average was 154 mm. The ANOVA showed differences between different months being October others to present longer that season. The maximum number of branches value was 7 and at least 5 branches per thallus. ANOVA performed for the numbers of branches not show significant differences. The average monthly number of spines has a maximum value of 5.4 mm^2 and a minimum of 3.9 mm^2. The ANOVA shows differences per month, October being the one with the highest number of spines. The biomass is higher in the autumn season. And in winter drastically reduces the population. The average of the highest biomass is 0.26 kg/m^2 and was presented in November; the minimum dry weight biomass was presented in February 0.0375 kg/m^2. The average annual biomass dry weight is 0.29 kg/m^2. The flora and fauna that was associated with A. spicifera goes from epiphytic algae molluscs, echinoderms, sponges to fish. The maximum monthly average was 0.20 kg / m^2 and a minimum of 0.031 kg/m^2. The result of the correlation indicates no relationship between biomass and plant-fauna (r = -0.1892.). We conclude that *A. spicifera* its development at temperatures ranging from 20-29°C. Morphological characters posing *A. spicifera* of Punta Roca Caimancito are similar to those reported by other authors. The main mode of reproduction of *A. spicifera* is asexually by fragmentation and occurs all year round (perennial). Sexual reproduction was limited in spring-summer, only empty conceptáculos were observed. Presents greater lengths during autumn and lower in winter. The number of spines is not related to the length of the branch. The maximum number of branches was 7 in autumn and winter and minimum of 4 branches per talo in summer. The maximum number of thorns thorns was 5.4 mm-2 and the minimum was 3.9 mm^{-2} spines. The percentage of wet weight biomass of A. spicifera is greater in autumn with 3.50 kg/m2 and the lowest was in winter with 0.59 kg/m^2. The percentage of biomass dry weight of *A. spicifera* is greater in autumn with 0.86 kg/m^2 and the lowest was in winter with 0.0375 kg/m^2. The impact of *A. spicifera* in Punta Roca Caimancito is negative for coral reefs because high densities of this alga can affect the uptake of light and nutrients and thus affect their growth.

INTRODUCTION

Invasive species play an important role in marine and terrestrial ecosystems, and damaging to native species and move. Within this theme different definitions that must be understood are handled, the first is naturalized, it relates to non-native species that were established in the region. The second definition is the invasion that has been used for the species has become naturalized and has spread widely in the region. And last and most important invasive species refers to any species that occupy a region in which it was not historically present (Sax et al. 2005).

Invasive species have always been a controversy around the world, because as the name says, invading regions and affect native organisms. This occurs both on land and in the marine environment. Within the marine environment have been studied invasive species in

much of the world's oceans (Carlton et al. 1990; Shiganova 1998; Godwin 2003; Work et al. 2003).

Ecological and Economic Impact of Marine Invasive Species

Alterations of ecosystems caused by global climate change with introduced species are the result of biotic homogenization; this is a process by which ecosystems dominated dominant species and opportunists. This pattern has always been observed in localities affected by environmental degradation and invasive species (FAO 2010).

The ecological impact of non-residential marine groups has been observed in some ecosystems in the Bay of San Francisco on the Asian clam *Potamocorbula amurensis*, Schrenck 1861, which was discovered in 1980 in North America, is characterized by be an invasive species that has colonized freshwater bodies in many countries in Europe and America. This produces major environmental and economic problems as it has a great ability to reproduce. Studies have been done in Portugal where they say this clam sequesters carbon and alters the channel and ecosystem functioning, also presents risks of mass mortality from sudden changes in temperature (Carlton et al. 1990; Godwin 2003).

Another invasive species such as jellyfish *Mnemiopsis leidyl*, A. Agassiz 1865 in the Black Sea, feeding on eggs and larvae of fish and has been a fall in stocks of economic importance (Shiganova 1998).

Among vertebrates, the fish Lutjanus Kasmira, Forsskål 1775, is an invasive fish from the 1950 in the Marquesas Islands belonging to the French Polynesian. Its introduction was intentional and harmed native reef fish (Work et al. 2003; Godwin 2003).

Finally the starfish Asterias amurensi, Djakonov 1950, was discovered in the year 1980 in Tasmania, Victoria and South Australia. A. amurensis has the potential to create large populations in new areas. It is a threat and is considered a pest because it caused the decline of fish belonging to Hand Brachionichthyidae Family. Also in mariculture has mainly played havoc in cultured mussels, oysters and salmon (Godwin 2003; Dommisse et al. 2004).

Impact on Coral Reef

Coral reefs are an important ecosystem as it forms part of the habitat of many organisms and is one of the most productive in the world. These ecosystems can be composed of reef zones, barriers, patches or atolls. Coral reefs are a potential attraction for tourists and are important because they form a natural protection and prevent the erosion of the waves on the coast.

The economic value of reefs is considerable. For example, in Indonesia the annual contribution of these ecosystems for tourism is 191 million dollars, 221 million dollars fishing health and $ 4.8 million dollars, making an annual total of 327 million dollars. Also in Hawaii fisheries in these ecosystems generates 2.5 million annually, tourism $ 304 million, and the value of biodiversity is around $ 17 million. Therefore, coral ecosystems in this region generate an annual total of 363.5 million dollars (Cesar et al. 2004).

Currently, coral reefs are being depleted rapidly in many parts of the world due to factors such as illegal fishing, coral mining, marine pollution and sedimentation. Another important

aspect that affects the reefs are invasive species such as the red alga *Acanthophora spicifera* (M.Vahl) Børgesen 1910, this like the others mentioned aspects contribute to the decline of coral reef ecosystems where it has invaded.

One effect of A. spicifera on coral reefs is competing for light and nutrients associated with corals and algae. On the other hand, industrial pollution (chemicals and industrial wastes) is an anthropogenic factor that contributes to the deterioration of coral reefs (Cesar 2002).

Economic Importance

It has now been recognized that biodiversity and marine resources of the world are threatened by human activities influence. In particular, overfishing and climate change have produced an alteration and destruction of habitat. Invasive algae are increasingly popular because they often have dramatic effects on ecosystem structure and function (Schaffelke et al. 2007).

Globally about 221 species of algae of commercial importance, of which 145 are used for food, for production of phycocolloids 110 (agar, carrageenan, acids, alginic) are known. Some algae are only used for direct food source and you are *Ulva* Linnaeus, 1753, *Caulerpa*, J.V Lamouroux, 1809, *Codium* Stackhouse, 1797, *Pyropia* J.Agardh, 1899 *Laminaria* Lamouroux, 1813 and *Undaria* Suringar, 1873 (Khan et al. 2003).

In countries like Japan, China, Chile, Indonesia, USA have also been used for pharmaceutical or nutritional studies. In this case, the chemical substances extracted from seaweed are used to treat diseases. While nutrition, are used as a food supplement and as part of the diet of many organisms.

Food additives are also extracted as carrageenan, agar and alginates (phycocolloids) these are the most traditional uses of algae. The red seaweed *Digenea* C. Agardh, 1822, produces an acid derived from glutamic acid, which is used as a central nervous system stimulant, the prototype of amino neuroexcitation used in experimental animals. Other red algae as *Ptilota* C. Agardh, 1817 produce a protein called lecithin. Asparagopsis Montagne in Barrer-Webb and Berthelot, 1840 *Sarconema* Zanardini, 1858, are used to control goiter is iodine deficiency (Khan et al. 2003).

The seaweed production has increased in the past twenty years and has reflected an overall increase in trade and economic activities. This production is currently given in 35 countries among which we can find North Korea, South Korea, Japan, China, Chile, Indonesia, United States and India. These countries account for 95% of world production of algae and 90% of production comes from crops. Annual earnings for algae production globally is estimated between $ 5.5 and $ 6 billion. The total of the products consumed by humans is $ 5 trillion. 7.5 An annual production of 8 million tons of wet weight, either algae extracted from wild and cultivated is obtained (Pickering, et al. 2007; Schaffelke et al. 2007). The brown algae are the most widely grown with over 5 million tons, while red algae approximately 1 million tons are produced annually and finally green algae with approximately 33,700 tons per year. The algae is cultivated kelp *Laminaria japonica* J.E Areschoug, 1851, production of this alga constitutes 60% of the total, the rest is for *Pyropia* and *Kappaphycus* Doty, 1988, *Undaria* Suringar, 1873, *Eucheuma* J. Agardh, 1847 and *Gracilaria,* Greville 1830. *Pyropia* is one of the most grown in China and its production

generates $ 1.8 billion annually (Mumford et al. 1988). Philippines contribute about 80% of the production of *Eucheuma* Weber-van Bosse cottoniicon, 1913 which equates to 130,000 tons per year (Khan et al. 2003). Khan et al., 2003 mentions that between 1981 and 2000 the production of aquatic plants increased from 3.2 million tons to 10.1 million tons of wet weight. By 2000 world trade in seaweed left an economic impact of $ 6 billion, while in 1990 it was only $ 250 million (Khan et al. 2003; Valentine et al. 2003).

Algae Introduced

The assessment of the ecological impacts of invasive species has been recognized as a research priority in recent years (Johnson et al., 2007). The impact of invasive algae has been identified as one of the major stressors in coastal ecosystems. A recent study in Hawaii confirmed that the economic impact of invasive species is higher than $ 30 million dollars per year (Schaffelke 2007). However, relatively few studies on the impact of invasive algae in relation to marine animals (Johnson et al. 2007).

Currently there have been over 260 species of introduced algae. Current key impacts of invasive algae have spread to other taxa. It is believed that invasive algae are potentially destructive and can cause a potentially serious impact, which can alter ecosystem structure monopolize space. Particularly worrying is its rapid spread is the effective spread, along with significant environmental and economic consequences (Schaffelke 2007).

Documented impacts of invasive algae are really few. As is the case of *Caulerpa taxifolia* (M. Vahl) C. Agardh, 1817, this alga was documented as invasive in the Mediterranean since 1980. *C. taxifolia* was introduced into the Mediterranean through the wastewater from the Oceanographic Museum in Monaco where he was planted aquarium purposes. Currently covers more than 13,000 hectares of seabed in this region and directly affects all the other vegetation and algae, especially Posidonia. But go slowly eliminating native vegetation indirectly so does the wildlife that feed or live in that environment (fish, sponges, sea urchins, and other less mobile) (Anderson 2005)

Codium fragile Suringar, 1867 is kind is known worldwide and has had an easy reproduction (asexual) and may displace other native algae and crustaceans, polychaetes and tunicates (Trowbridge 1999).

Sargassum muticum (Yendo) Fensholt 1955, is an invasive species native to the coasts of China-Japan and the Pacific Northwest. Its primary means of dispersal have been boat and floating pieces of sticks, has a rate of growth and high fertility. It also produces the displacement of other native species (Harries et al. 2007).

Undaria pinnatifida (Harvey) Suringa1873 r is native to Asia and now invades the coastal zone in many countries around the world. In 1971 he was found in the Mediterranean later in the Atlantic Ocean in Britain and subsequently settled in UK, Spain, New Zealand, Australia, Argentina, and the North Pacific Ocean (Martin et al. 2008; Russell et al. 2008). The shipping traffic is perhaps the most important vector of accidental dispersal of the species, which can be introduced into new geographic areas. This can be carried attached to the hulls of ships as well as spores in ballast water. In summary, invasive algae have been great and dramatic changes in marine ecosystems, as you move through geographical or ecological barriers and displace residents algae (Kilar et al. 1986; Martin et al. 2008 ; Valentine et al. 2003).

Acanthophora spicifera an Invasive Seaweed

Acanthophora spicifera was introduced to the Hawaiian Islands in the early 1950's, and was classified as invasive. Today it is one of the most common in this archipelago. Successful Invasive Acanthophora is attributed to having the ability to reproduce both sexually and vegetatively by fragmentation.

The advantages that may have reproductive capacity are: (1) the extent of distribution of the species, (2) increase in the abundance of organisms and individual biomass and (3) colonization of areas where the sexual propagules have a high mortality rate. Plus it plays throughout the year. Pacific reef is in constant competition with the resident marine algae such as Laurencia spp. and Hypnea cervicornis. It is characterized by epifitar different taxa including invertebrates and other algae such as *Laurencia* (J.V Lamouroux 1813) *Gracilaria* (C. Agardh) Greville 1830), *Padina* ((Linnaeus) Thivy in WR Taylor 1960), *Caulerpa* ((Forsskål) JV Lamouroux 1809), *Halimenia*, and seagrasses, *Halodule* Den Hartog. Has a wide adaptability to environmental conditions.

In the last three decades have presented two important negative alterations are invading seaweed 1) threat in the coral reef habitat by preventing the growth of corals and other photosynthetic organisms in the absence of light and given its high abundance also affects recruitment, biodiversity and sustainability of the structure of reef ecosystems, 2) at the periphery of mangroves creates a barrier that directly affects water quality and flows, ie, decreases the wave propagation and this affects the entire biota (Smith et al. 2002; Walters et al. 2002; O'Doherty et al. 2007; Schaffelke et al. 2007; Tsuda et al. 2008; Weijerman 2008).

Another important aspect is that *A. spicifera* not only cause negative effects where it has been introduced, as has also been found to be a source of food for the green turtle, *Chelonia mydas* (Linnaeus 1758), herbivorous fish and sea urchins crustaceans (Kilar et al. 1986; Russell 2003).

In an experimental study in Kaloko Honokohau, Hawaii in 2005, he tried to control *Acanthophora* using herbivorous fish. The obtained results showed that the introduction of herbivorous fish can reduce the biomass itself *Acanthophora* but for better control, biological suggested combining this method with a regular manual removal (Weijerman 2008).

The main objective of this work was to identify and investigate the phenology, biomass and reproductive aspects of *A. spicifera* Punta Roca beach Caimancito Bay of La Paz, BCS providing critical information to their taxonomy, ecology and reproduction in a new ecosystem is invasive algae.

Description of Genus

Genus of Acanthophora (J.V. Lamouroux 1813) is within the Phylum Rhodophyta in *Chondrieae* tribe. This is one of the oldest groups of eukaryotic plants.

Within this class we find about 4000 species (Guiry et al. 2010; Lee 2008). Genre *Acanthopho*ra described 27 species of which only 6 have been taxonomically recognized as: *Acanthophora aokii* Okamura 1934 *Acanthophora dendroides* Harvey1855, *Acanthophora nayadiformis* (Delile) Papenfuss 1968 formerly *Fucus navadiformis* (Delile 1813), *Acanthophora pacific* (Setchell) Kraft 1979 *Acanthophora ramulosa* Lindenberg ex Kützing 1843, and *Acanthophora spicifera* (M.Vahl) Børgesen 1910 (Cecere 2002).

Description of the Species

One of the unique features of *Acanthophora spicifera* is that the main shaft has no thorns. They are upright plants that grow up to 250 mm, with solid cylindrical branches 2-3 mm wide. (Oliveira et al. 2005) Some branches are short and have irregular shapes with numerous spines that are arranged radially. The apical growth is present, grows attached to a hard substrate with a long and irregular bra which arise many erect spines. In deep water, where there is less water movement, they are smaller and can measure 40-100 mm long, are rather compact and very dense thallus. Compared with specimens from shallow water, where the petals are 100 to 250 mm and more open branches. The coloration of this alga can vary, it is usually red but can see in the following colors: purple, the Yellow, orange and brown. The greater the depth the algae will be darker. In shallow areas the color is lighter or sandy color. *A. spicifera* is abundant in shallow calm waters, reefs, intermediate and low intertidal rocky areas. Usually lives embedded in hard substrates such as rocks, mollusks and corals. Can also be found fragments of alga are floating freely in the water column. As for structural characteristics, must be the apices are pyramidal with trichoblasts. Pericentral cells are dense and Corticadas, axial cells are commonly in the middle. *A. spicifera* commonly associated with other algae that are more tolerant to exposure of the tide and are able to retain water when exposed to air (Kilar et al. 1986; Perrone et al. 2006).

Geographical Distribution

Acanthophora members of the genus are found in all latitudes from Ecuador to the coldest seas. The mean sizes of algae are varied depending on the geography of the region. The more fleshy species usually occur in temperate areas, while in tropical areas can find smaller and filamentous (De Reviers 2003; Lee 2008).

Floristic Records of Algae

Since the last century there have been collections of marine algae in the Gulf of California (Dawson 1944). Although no taxonomic studies of macroalgae made almost over 260 years in the Gulf of California, in any of them referring to the presence of *Acanthophora spicifera* in the Gulf of California is made. (Howe 1911; Setchell and Gardner 1924; Dawson 1953; Dawson 1966; Norris 1972; Norris 1975; Mendoza-Gonzalez and Mateo-Cid 1986; Martinez-Lozano et al. 1991; Mateo-Cid et al. 1993; Serviere-Zaragoza et al. 1993; Mateo Cid et al. 1994; Casas-Valdez et al. 1997; Serviere-Zaragoza et al. 1998; Rodriguez-Morales and Siqueiros-Beltrones 1999; Aguilar-Rosas et al. 2000; Paul Chavez and Riosmena-Rodríguez 2000 and Aguilar-Rosas et al. 2002). For this reason, it is necessary to perform extensive sampling of this species in the Gulf of California, in order to have further evidence of its distribution in the Gulf of California. In 2006 *A. spicifera* it began observing in the Gulf of California and has been dispersed in shallow areas. *A. spicifera* could harm local flora decreasing coverage and biomass of these.

In the Bay of La Paz *A. spicifera* associated with rocky reefs where we can observe that the forests of *Sargassum* area residents, which exhibit rapid growth, may be observed

displaced. *Acanthophora* has been established at points where could financially affect residents because fishermen associated commercial fish are extracted *sargassum* forests. So it could also be endangered coral reefs because they can take to prevent sunlight necessary for their development. This event directly affect the fish that live there, also disturbs the attraction also directly harm the regional economy (Riosmena et al. 2009).

Acanthophora spicifera alga is a native of the Caribbean and now has spread throughout many tropical and subtropical marine ecosystems. In the Hawaiian archipelago this alga was introduced by ships since the 50`sy is considered an invasive species that has caused severe impacts on the ecosystem.

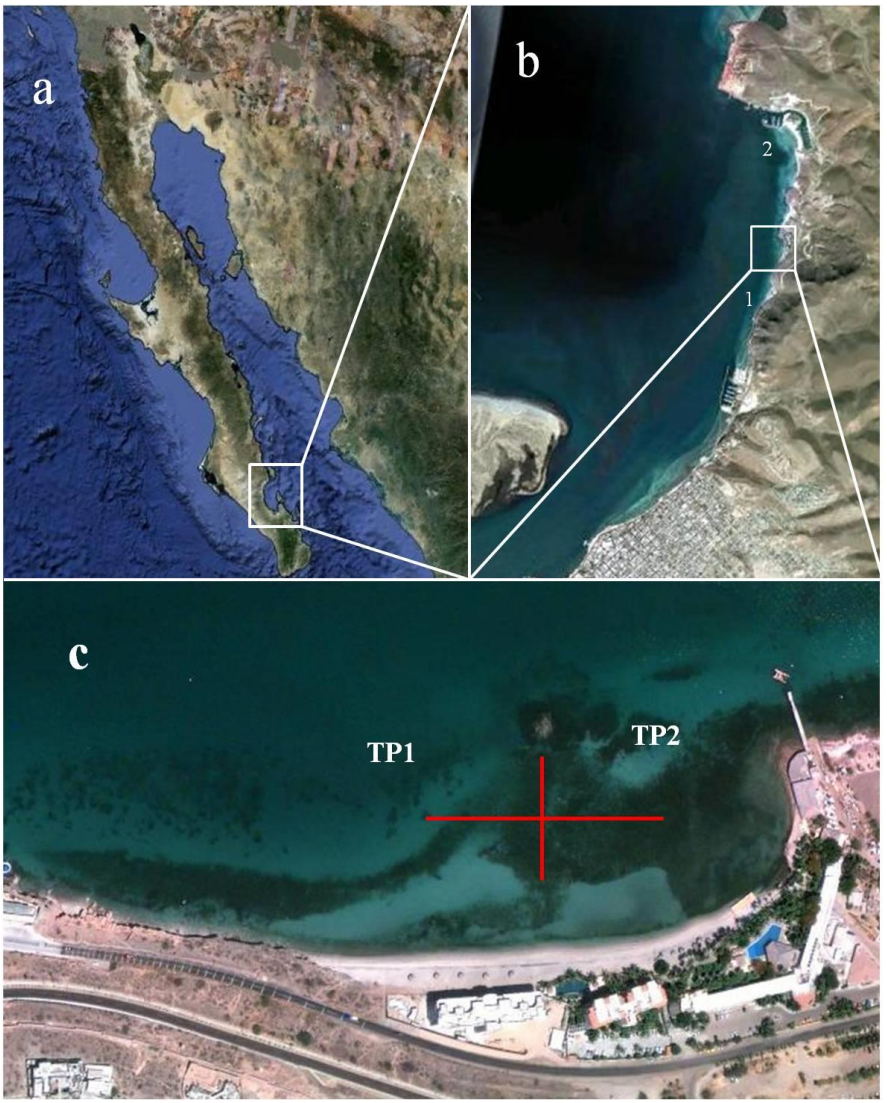


Figure 1. a) Image of the Baja Peninsula where the Bay of La Paz, Baja California Sur, Mexico b) Caimancito Punta Roca beach that lies between the Coromuel (1) and Costa Baja beach (2), c) location is located study area, Beach Punta Roca Caimancito (TP1) transect parallel to the coast line (TP2) transect perpendicular to the shore line.

However, recent studies indicate that this alga has the Mexican Pacific coast come (Riosmena-Rodriguez et al. 2009) no research has been about their adaptive capacities and the effects it has on the ecosystem. This alga is found in parts of the Bay of La Paz (southwestern Gulf of California). Therefore it is essential to study some aspects of its life cycle as their type of reproduction (sexual and asexual), biomass, distribution and behavior of the same in the marine environment as it can be harmful to many organisms. An example is the coral reef as was mentioned harms the ecosystem along with all the associated species.

The aim ofthe work was establish the phenology reproductive, estimate the variation in the biomass an annual cycle, and other aspects of *A. spicifera* in the Bay of La Paz, BCS, Mexico.

Study Area

The Bay of La Paz (Figure 1a) is a semi-protected body of water that is located on the south eastern side of the peninsula of Baja California. It covers approximately 2635 km^2, has an oval shape with a width of 35 km and 80 km long and is separated from the Gulf by a narrow peninsula. Its depth can vary from 10 m to 450 m.

It also presents a system of mixed type semidiurnal tide with a higher level at 1 m during spring tides (Obeso-Fog et al. 2008). Caimancito Beach Punta Roca (Figure 1b) is in the extreme southwest of the Bay (24º12'07.36 "N and 110º18'03.10" W), between the beach and the Coromuel Costa Baja (figure 1c). It is characterized by a shallow area of sandy bottom with patches of rock. On this beach you pass a deep channel through which water enters and leaves the Ensenada de La Paz. This beach has waves of very low intensity and algal community is located at shallow depths (<2 m) (Figure 2) (Ceseña-Arce 2003).

MATERIALS AND METHODS

In the study area where Caimancito Punta Roca A. spicifera distributed randomly placed two quadrants PVC 25x25 cm (0.0625 m^2) at a distance of 20 m from each other at a depth of 1.5 m. Within each quadrant all thalli of *A. spicifer*a present, and associated organisms (flora and fauna) were collected. The material was placed in plastic bags previously labeled.

Then, in the laboratory of Marine Botany UABCS partner agencies separated this invasive macroalgae and weight (g) of each taxonomic group with an analytical balance (Chyo JL180) was obtained. Subsequently, *A. spicifera* samples were dried in an oven (VWR-1350F) at 60°C to obtain the dry weight (g). With these data, the biomass (g. dry weight m^{-2}) *Acanthophora* and partner agencies estimated. Furthermore, in the study area were collected randomly from 20 thalli *A. spicifera* with which the average maximum length (mm) was determined as the average number of branches (number of branches per thallus) and the number of spines per branch, the average of each side branch with thorns (number of spines per linear mm of secondary branches) was taken. The average number of spines in three heights of the plant (apical, middle and bottom) was also compared. These measurements were made in order to determine whether the structural complexity of the plant varies over thallus and also temporarily.

Figure 2. Mantle *Acanthophora spicifera* at Beach Punta Roca Caimancito, BCS.

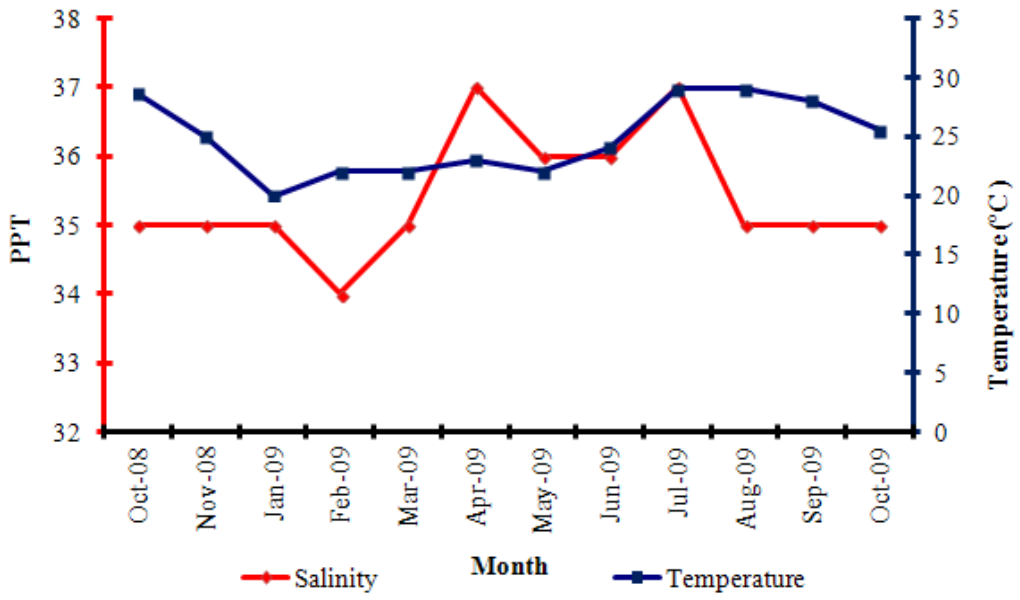


Figure 3. Seasonal variation of temperature (ºC) and salinity at Punta Roca Caimancito.

This allows us to pursue a relationship with the diversity and abundance of associated organisms. It was also noted if the thalli containing reproductive structures, for this three transects along the talus was performed and a sample of each side ie top, middle and base

took part. The cuts were applied aniline blue staining in order to have a better resolution of the reproductive structures. Observations were made under a light microscope (Olympus CX31, CX40) with a resolution of 10, 20 and 40 X, and pictures of the different structures were taken with a (Olympus, Model C-4040ZOOM) digital camera.

The frequency of these samples was monthly within the study area, October 2008 to October 2009. Only in December 2008 did not sample.

RESULTS

Environmental Conditions

A maximum temperature of 29°C in the months of October 2008 and July and August 2009 and a low of 20°C in the month of January 2009 (Figure 3) was recorded. While the higher salinity (37) was recorded in April and July 2009 and the lowest (34) in February (Figure 3).

General Features and Morphological of Punta Roca *Acanthophora spicifera* in Punta Roca Caimancito

In Punta Roca Caimancito, an extension of the mantle (Figure 4) of Acanthophora has continued to increase since 2006, when it was first recorded in this area. The current extension (2009) is 31536.93 m^2 or 3.16 hectares.

Figure 4. a) Mantle *A. spicifera* Punta Roca Caimancito over time. In b) shows the area before the onset of *A. spicifera* in 2004 and 2005, one can observe a lower density of various algae. c) In 2006, first observed at Punta Roca *A. spicifera* Caimancito d) shows how the mantle has increased in the last year (2009) sampling Caimancito Punta Roca.

Table 1. Flora and fauna associated with *A. spicifera* in Punta Roca Caimancito

Flora associated a *A. spicifera*	Fauna associated a *A. spicifera*
*Halimeda (*Lamouroux,1812)	Cnidaria (Hatschek, 1888)
*Caulerpa (*Lamouroux,1809)	Sponges (Grant, 1836)
Codium (Stackhouse, 1797)	Crustacea (Brunnich, 1772)
Ulva (Linnaeus,1753)	Pisces (Bateson,1885)
*Laurencia (*Lamouroux, 1813)	Molliska (Linnaeus, 1758)
Ceramium (Roth, 1797)	
Spyridia (Harvey in Hooker,1833)	
Gracilaria (Greville, 1830)	
Hypnea (Lamouroux, 1813)	
Jania (Lamouroux, 1812)	
Amphiroa (Lamouroux, 1812)	
Lithophyllum (Philippi, 1837)	
Gelidiella (Feldmann y Hamel, 1934)	
Champia (Desvaux, 1809)	
Gigartina (Stackhouse, 1809)	
Padina (Adanson, 1763)	
Dictyota (J.V Lamouroux, 1809)	
Sargassum (C.Agardh, 1820)	
Hydroclathrus (Bory de Saint-Vincent, 1825)	
Rosenvingea (Børgesen, 1914)	

The estimated maximum biomass entire mantle was 8.12 ton DW in November, and the lowest was 1.18 ton in February DW (Figure 5). The Wet weight was 110.71-ton maximum, and the minimum was 18.71 ton WW (Table 6 and 7).

A. spicifera in this area can be found growing on a variety of hard substrates, including epifitando other species of seaweeds or invertebrates (Table. 1) (cnidarians, corals, sponges, mollusks) (Figure 6). However, a major substrate adheres is rock, coral and shells died (Figure 6), this is referred to as epizoic and epilithic, that is, which are subject to animals and rocks respectively. The coloration of *A. spicifera* going from brown to red (Figure 7a and b). In mature thalli coloration goes from light to dark (Figure 7a) brown.

Present an apical sympodial growth and presents secondary branches, has a roughened or rough texture, has a discoidal holder irregularly, in the main axis no spines. It is characterized by a highly branched and bushy, leafy (Figure 7a) talo. Thalli that were found were usually mature and very few were juveniles. We can also see runners with rhizoidal form at the bottom of the algae, they also serve to hold onto the substrate and give rise to a new thallus (Figure 7c).

A. spicifera presents an apical growth meristemal (Figure 8a). It has thorns with an average length of 4.5 mm, which can vary morphologically. These are pointy and there is a space between them, that is, are not on each other (Figure 8b). In cross-section was observed that the pericentral cells are dense Figure 9b). Axial cells are in the center of the talus and give support (Figure 9c).

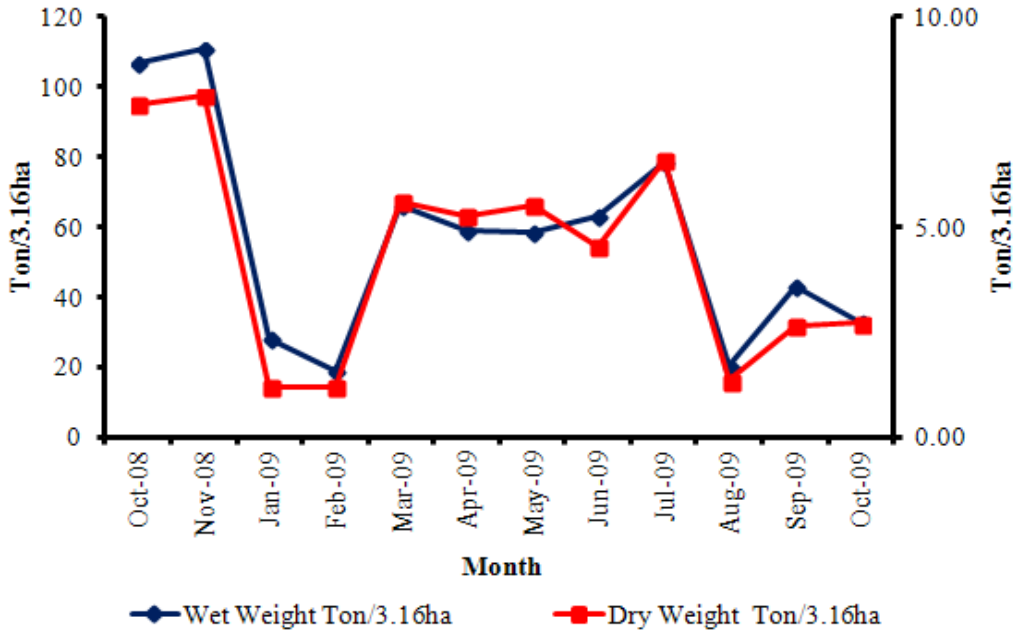


Figure 5. Total biomass mantle *A. spicifera* in Punta Roca Caimancito wet weight (blue) and dry weight (Red).

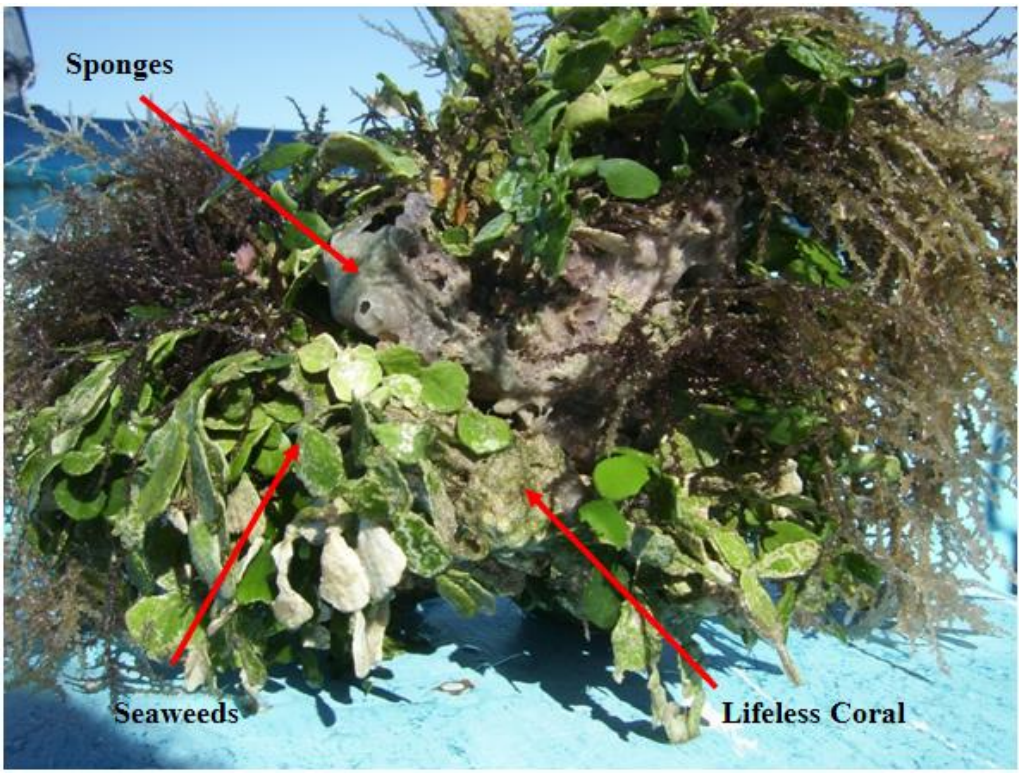


Figure 6. *A. spicifera* specimens growing on different substrate types (coral, algae and spong).

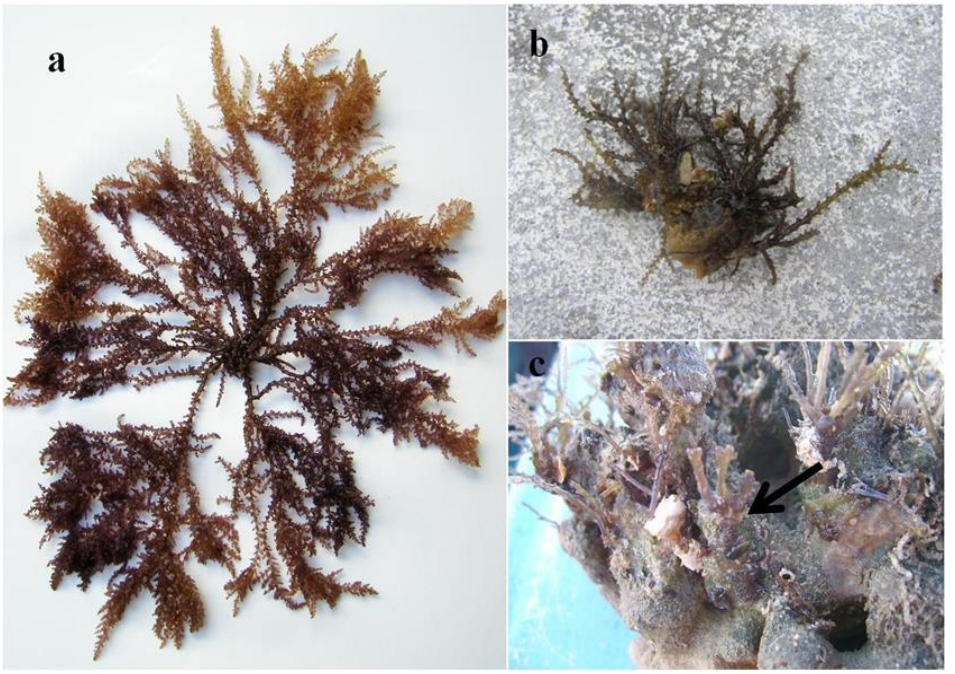


Figure 7. Anatomical characteristics of the thallus of *A. spicifera* Punta Roca Caimancito BCS, Different shades of color are distinguished; **a)** red specimen, **b)** brown specimen. **c)** proliferation of youth fronds in the bottom of the runners (black arrow).

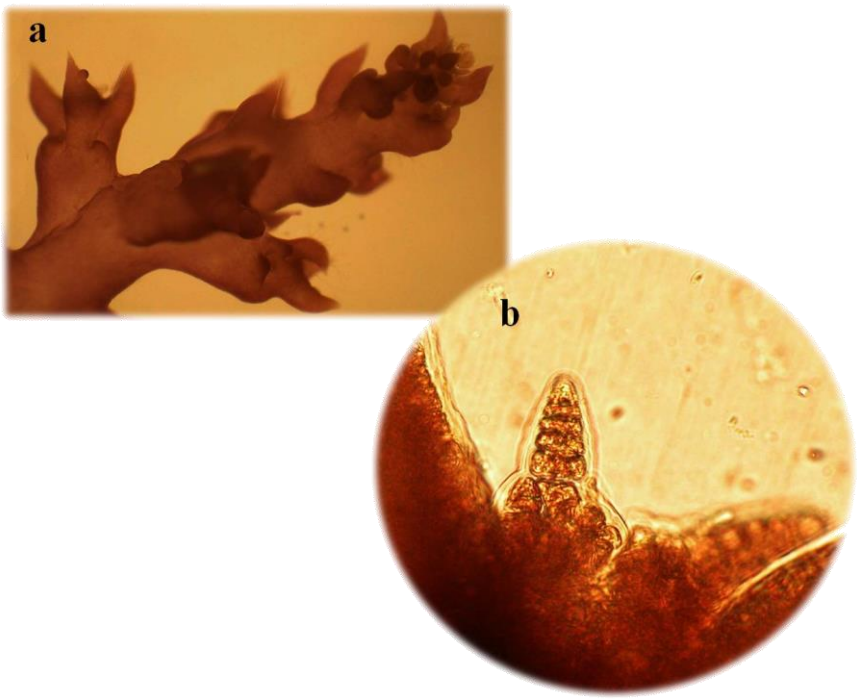


Figure 8. **a)** Morphology of spines. **b)** Meristemal cells in the apical growth zone *A. spicifera*.

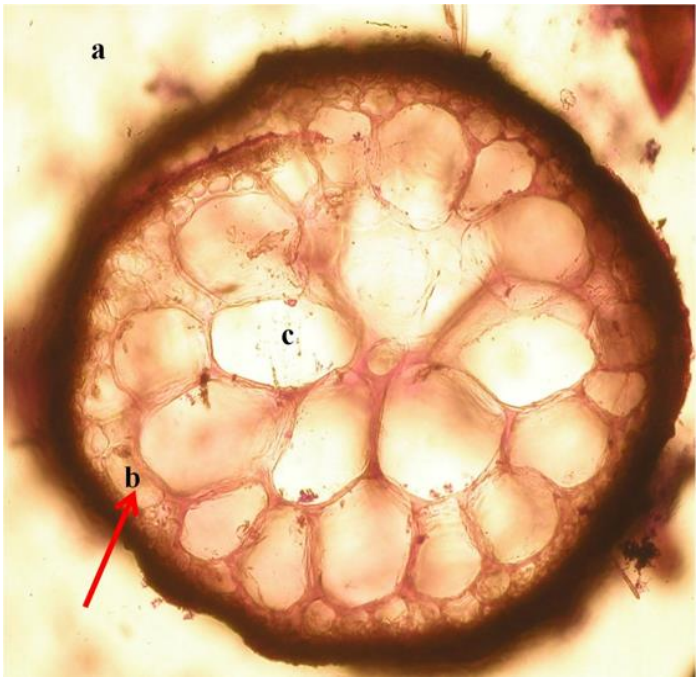


Figure 9. a**)** Cross section where marrow cells that support the thallus of A. spicifera, **b)** pericentral cells (red arrow) **c)** axial support or cells are observed.

Figure 10. *A. spicifera* pericentral cell in the apical part of the thallus.

In this cross-section (Figure 10) we can see that the pericentral cells are less dense by being in the apical region where they do not act as supporting cells. Marrow cells alone have these characteristics, as they are found in the basal part of the stalk (Figure 10).

In the study area this invasive alga reproduces mainly asexually (fragmentation). However, indications were observed also reproduced sexually. Female gametophytes were observed in the apical part (Figure 11a) where the bones are, and only cystocarp spina found. It was observed that was ripe cystocarps (Figure 11b). Male gametophyte, called spermatangia (Figure 11c) have long, thin filaments called compact trichoblasts found in the apical part of the male gametophyte (Figure 11d).

The average maximum length of the thallus of A. spicifera was 163 mm in the fall of 2008 (October) and the minimum length was 108 mm in summer (August) (Figure 12).

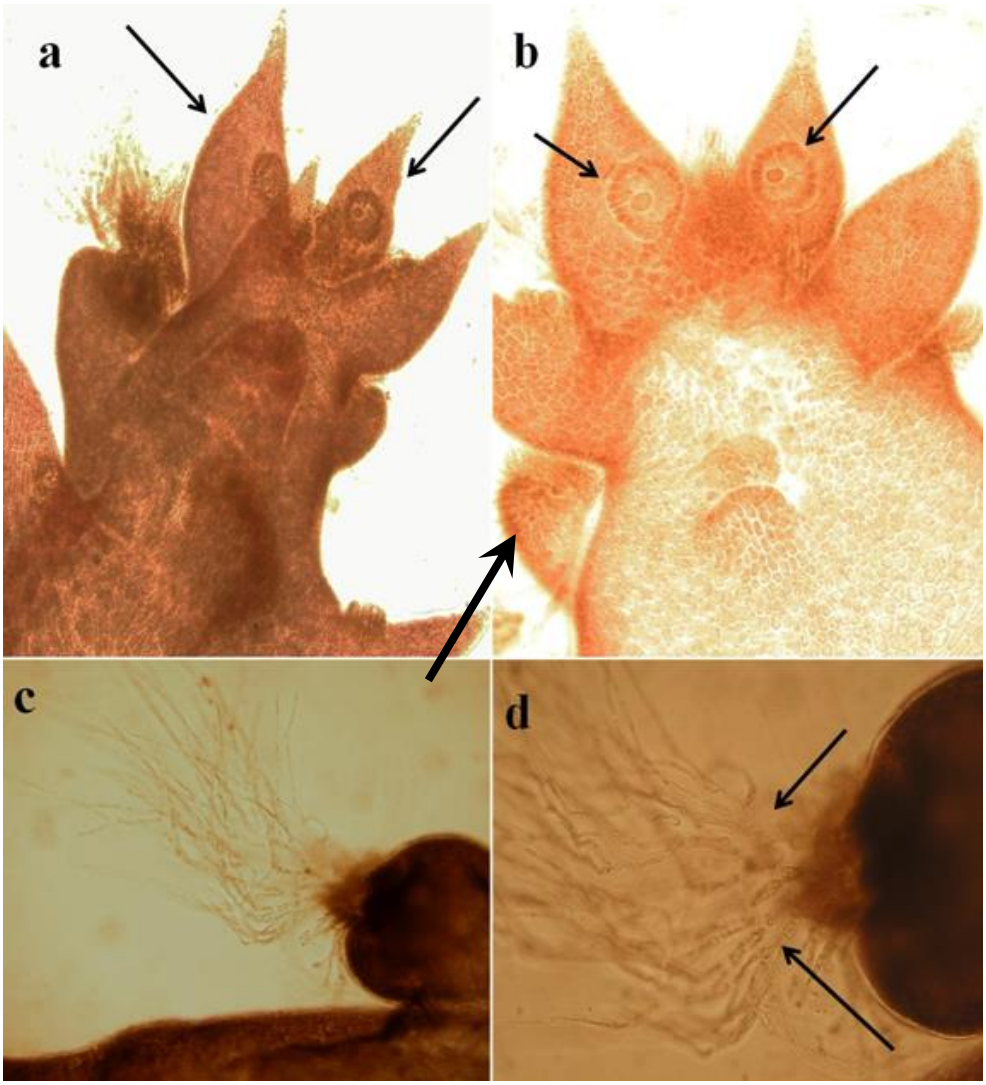


Figure 11. a) The arrows show the area sympodial growth of the female gametophyte. b) Mature cystocarp. c) Structure of the male gametophyte. d) Approach espermatangio where trichoblasts, the thallus of *A. spicifera* (black arrows).

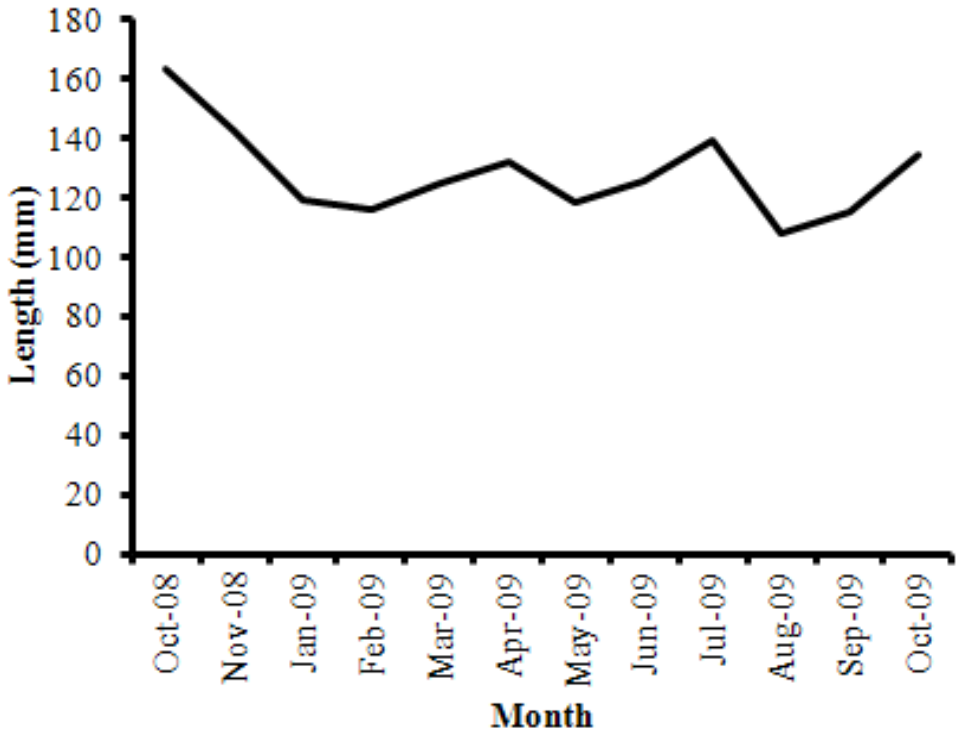


Figure 12. Average monthly length thalli *A. spicifera* in Punta Roca Caimancito.

The annual average was 154 mm. Before analyzing ANOVA data was obtained that are normal and are not homogeneous ($p < 0.05$). The result of ANOVA by ranks (Kruskal-Wallis) (Appendix, Table 3) shows that the length of the thallus varied significantly over time ($p < 0.001$). Thallus length was significantly higher in the month of October 2008. Branch number per talli did not change significantly during the study period. The monthly average was 7 branches maximum and a minimum of 4 branches per thallus (Figure 13).

The annual average was 5 branches. An analysis of variance of a channel, where a priori tests indicated that the data are normal ($p = 0.579$) and varianzias are homogeneous ($P = 0.670$) was performed. The resulting ANOVA (Table 4) showed no statistical difference in the number of branches between months ($p > 0.05$).

Number of Spines. The number of spines by 4.5 mm long linear branchessecondary in the thallus of A. spicifera not vary significantly between areas (apical, medium, low) of the plant (ANOVA, $p > 0.05$). The maximum monthly average was 5.4 linear 4.5 mm thorns and spines minimum 3.9 mm by 4.5 (Figure 14). The annual average was 4.5 spines 4.5 mm length of the side branch. However there were significant differences (ANOVA, $p < 0.05$) were recorded throughout the year (Table 5). Tukey's test indicated that the density of spines in November (2008) was significantly higher than in the other months.

Percentage of Wet and Dry Weight

The average wet weight biomass (WW) was higher in November (2008) WW 3.5 kg/m^2 and lowest in February with WW 0.59 kg/m^2 (Table 6).

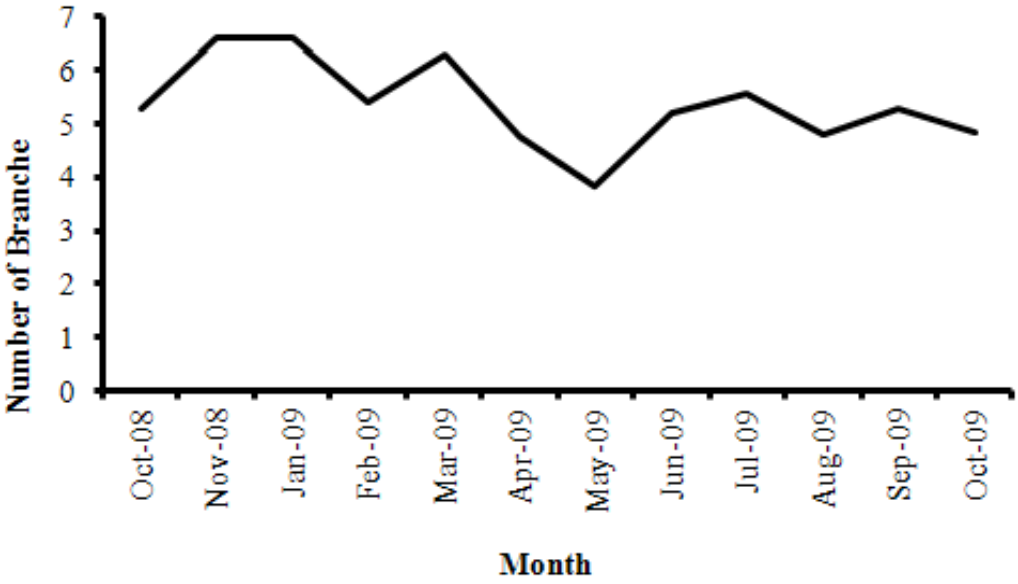


Figure 13. Seasonal variability in the average number of branches per thallus *A. spicifera* in Punta Roca Caimancito.

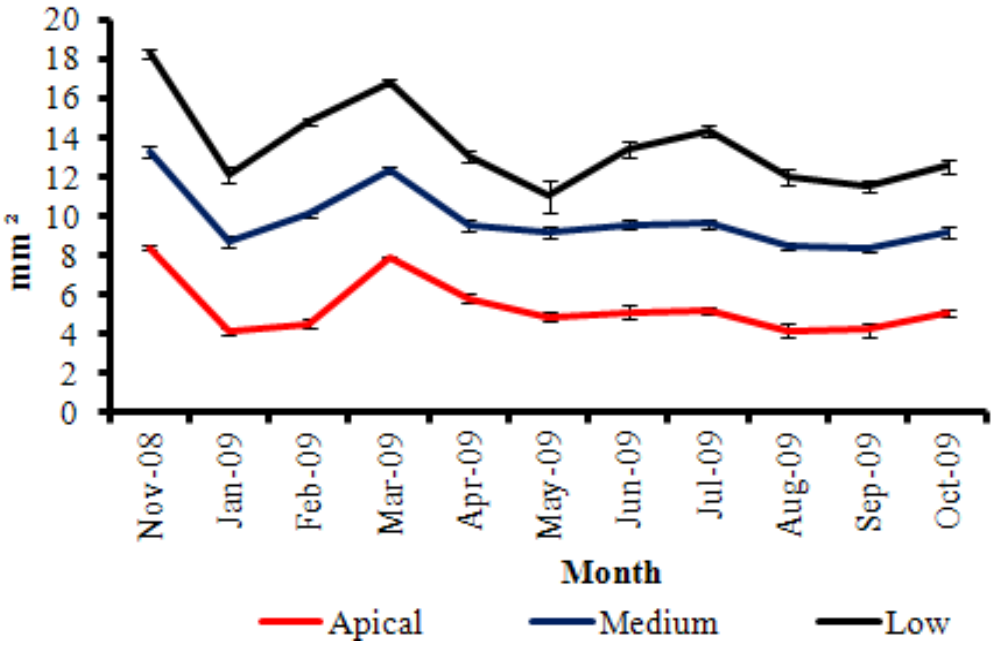


Figure 14. Number of thorn in 4.5 mm linear length of the secondary branches of the thalli of *A. spicifera* in Punta Roca Caimancito.

The annual mean biomass was 1.63 kg WW/m^2 (Figure 15). The variation of biomass dry weight (DW) showed a similar trend, the highest monthly average was recorded in October 2009 and was 0.86 kg DW/m^2 and the minimum in February (2009) is 0.0375 kg DW/m^2 (Table 7) (Figure 16). The annual average biomass dry weight was 0.29 kg DW / m^2 (Table

7). Priori testing resulted not exhibit normal ($p < 0.050$) nor homogeneity of variance ($p < 0.050$). So the ANOVA (Table 8) rank (Kruskal-Wallis) showed us that there are significant differences in biomass throughout the year ($P < 0.001$).

Flora and Fauna of *Acanthophora spicifera*

The flora and fauna associated with *A. spicifera* was represented by several groups, of which the most common were molluscs, echinoderms, epiphytic algae from different divisions, seagrasses, sponges and fish (Figure17). The maximum of these organisms monthly biomass was 0.20 kg/m^2 (6.27 ton for the entire mantle) in September and the minimum of 0.03 kg/m^2 (1.01 ton) in November. The annual average biomass associated with this cloak of *A. spicifera* organisms was 0.071 kg/m^2 (Table 9).

The result of the correlation indicated no relationship between dry weight biomass and biomass *Acanthophora* flora-fauna in dry weight ($r = -0.1892$).

DISCUSSION

Acanthophora spicifera is native Caribbean but now has been documented in many tropical and subtropical coastal zones around the world, which has been considered as an invasive species (Collado-Vides, et al. 1995; O'Doherty et al. 2007; Quiros-Rodríguez et al. 2010;. Tsuda et al. 2008 Serviere-Zaragoza et al. 1992).

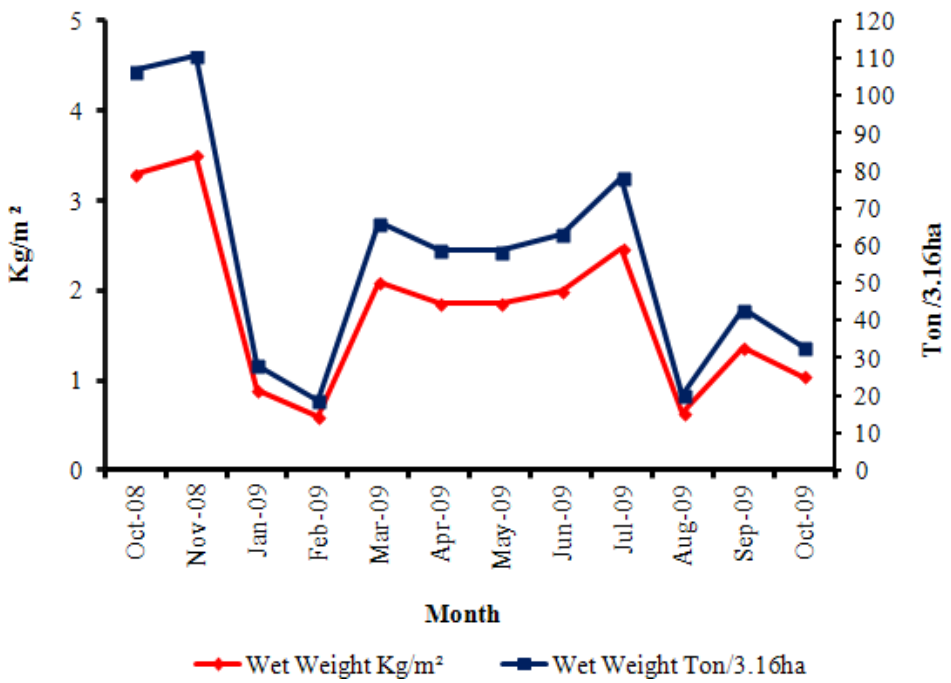


Figure 15. Seasonal variability in the wet biomass *Acanthophora spicifera* per square meter per hectare in Punta Roca Caimancito.

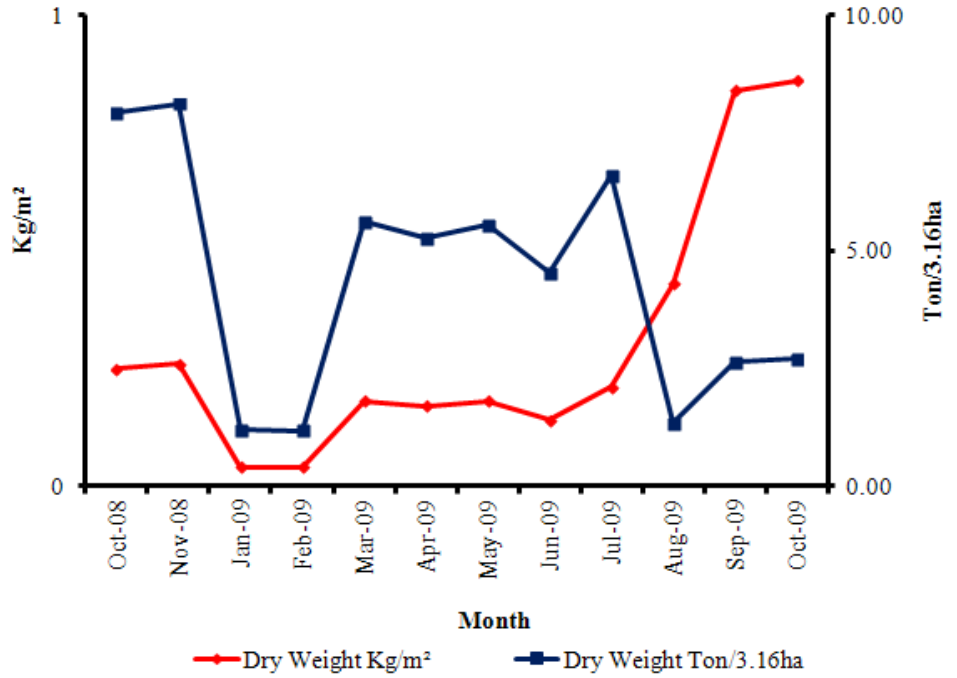


Figure 16. Seasonal variation of biomass dry weight and *Acanthophora spicifera* per square meter per hectare Total in Punta Roca Caimancito.

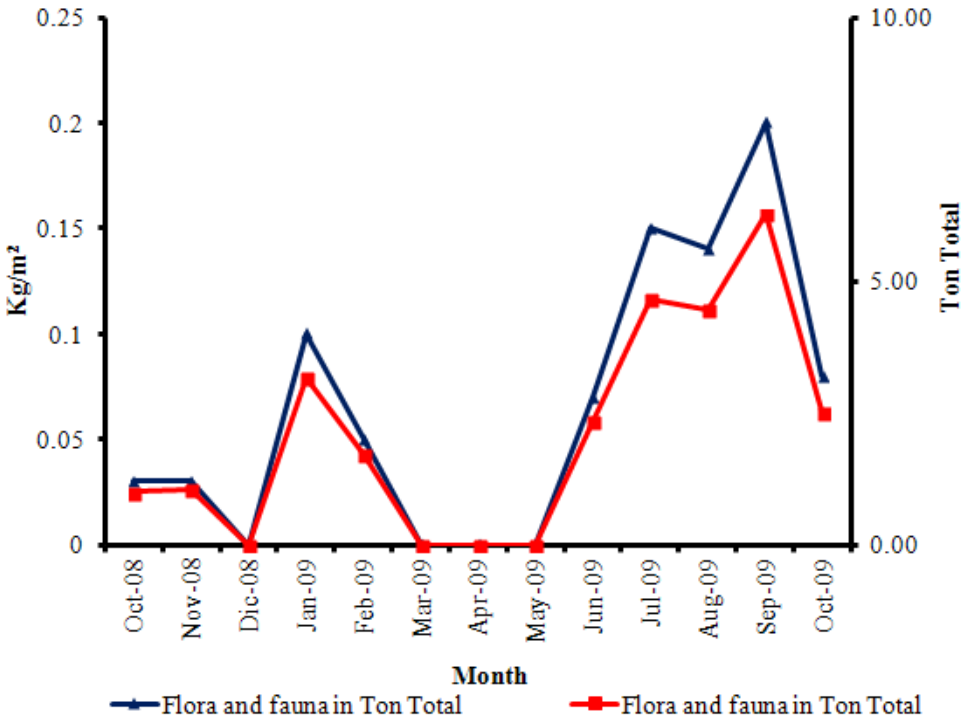


Figure 17. Monthly Average biomass of all the flora and fauna associated with the mantle of *A. spicifera* Caimancito Punta Roca.

Caribbean of Panama for example, it has been reported that the optimum temperature for this species is 25°C. Also on Pearl Harbor, O'ahu, Hawaii, we can find it adapted to a temperature range of 23-29°C (Coles 2006). In our study area (Punta Roca Caimancito) temperature conditions ranged from 20-29ºC. And considering that tolerates a wide temperature range, this species could in turn spread through all the bays and shallow waters of the Gulf of California, and that within this system water temperature varies between 16-29ºC (White-Betancourt et al. 2004) similar to those in our study area (Naim 1993) values. In the Caribbean, this species can be found from the intertidal zone to a depth of 8-22 m (Kilar et al. 1986; Littler et al. 2000). In Pearl Harbor, O'ahu, Hawaii dwells at an average depth of 9.2 m (Coles 2006). At sites where it is distributed within the bay of La Paz (Mexico), this algae can be found from 50 cm to 5 m depth in the subtidal zone in Punta Roca Caimancito this depth distribution could be associated with the availability hard substrate (rock, coral, shell, etc.).

The length of the thalli of *A. spicifera* in Punta Roca Caimancito ranged from 99-171 mm and maximum sizes were recorded in October (autumn 2008) and the minimum in August (summer 2009). In Tanzania length posing A. spicifera is 200 mm (Olivera et al. 2005). In the Caribbean there have been *A. spicifera* lengths over 250 mm (Littler et al. 2000). Making a comparison with the thalli Bay Peace these are much smaller than those in the Caribbean and the Indian Ocean.

In Punta Roca Caimancito a clear seasonal variation was detected in the length of this species in other regions. This macroalgae showed larger sizes in autumn and minimum in summer. Hawaii for example, a peak in summer is recorded and subsequently a decrease in the length in the winter. In fact, it has been observed that during the winter the thallus showing signs of necrosis with minimal possibility of adhering to a substrate and persist (Tsuda et al. 2008). In the eastern Arabian Sea A. spicifera reaches greater lengths during spring (March) (Desai et al. 2003). *A. spicifera* is one of the most dominant algae in the subtidal zone of Punta Roca Caimancito. The biomass of this alga was higher in October 2008 with 15.70 kg/m^{-2} and the lowest was in February 2009 with 1.33 kg/m^{-2}. The decrease in biomass detected in winter possibly because the availability of nutrients in the summer is higher than in winter (Reyes-Salinas et al. 2003). Other species of the same genus as *A. nayadiformis* reaches higher biomass values in summer and autumn with a strong decrease in winter. Therefore in the Mediterranean in those stations *A. nayadiformis* is the most dominant species among the community of subtidal algae with a rate approximately 40% (Cecere et al. 2000).

In this study we observed that *A. spicifera* had pericentral and central cells. The spines are dense and are oval in major axis extremities separated from each other. The length of the spines is 0.5 to 6 mm. For records of the species in the Caribbean measuring up to 0.55 mm in length (Littler et al. 2000). In this sense, some authors who have studied species of this genus show that if the spine is very swollen means there may be spores within it (Cecere et al. 2002). In our study this phase was observed, this might be because he had expelled the gametes. In the cross sections was observed that the spines containing an empty cavity may also be that under these environmental conditions sexual reproduction is not very common.

A. nayadiformis in the number of thorns varies depending on the length of the branch, that is, the longer the greater the number of spines (Cecere et al. 2002). The same author also mentions that the spines are made up of cells that build tissues and present of 3-5 spines on the apical side. *A. nayadiformis* plants develop from primordium spines are more robust and abundant monopodial growth (Cecere et al. 2002). The most common is that the thalli can

become vegetative propagules. Branches can be regenerated from anywhere in the talo and form new thalli without sexual reproduction (Cecere et al. 2002).

A. spicifera is an abundant algae in the subtidal zone of Punta Roca Caimancito where it forms part of a small reef where various organisms (flora and fauna) benefit to get shelter and available substrate (Table 1 and 2). Within associated algae we can find species phyllum Chlorophyta (green), Rhodophyta (red) and Heterokontophyta (brown). A lot of animals living among *A. spicifera* such as fish, sponges, cnidarians, molluscs, arthropods were also found.

A. spicifera not only found in coral reefs but it also can be found associated complexes mangrove ecosystems (Barrios et al. 2003). However, in some Hawaiian coral ecosystems invaded by *A. spicifera*, has detected a negative impact because it prevents nutrients and sunlight reaching the coral community, which may attract some problems for communities of animals and plants who live there (Martinez-Danaras 2006).

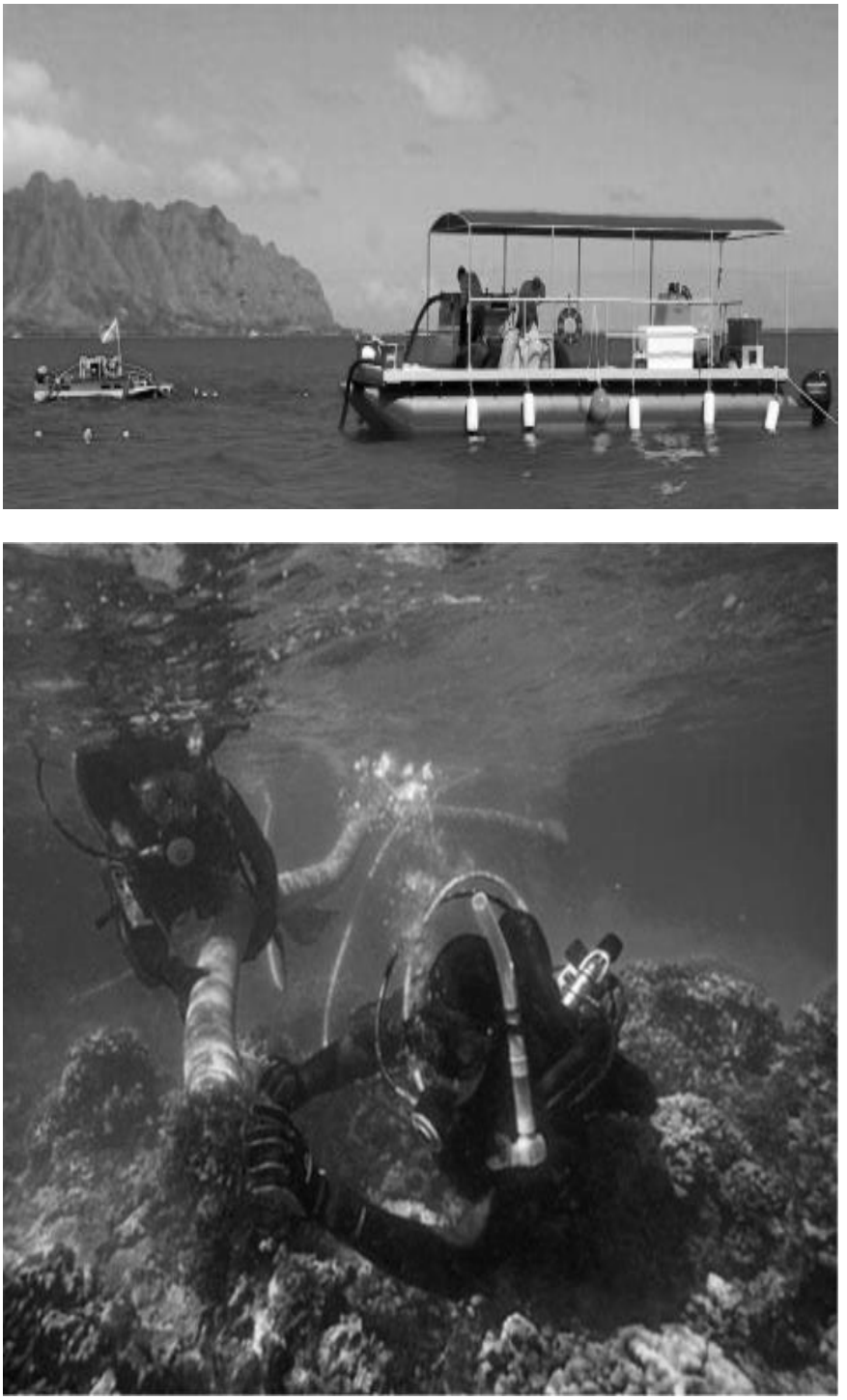
In Punta Roca Caimancito thalli *A. spicifera* easily fragmented (even without waves or strong currents) generating clones that are dispersed by currents as in other regions (Kilar et al. 1986). This feature has favored the spread of this invasive alga by different sites within the Bay of La Paz. O'Doherty *et al*., 2007 mention *A. spicifera* that is a species capable of even flourish under range of environmental conditions for this species and do not need a special environment to reproduce asexually. Unlike sexual reproduction that does require specific environmental conditions that stimulate the production of Tetrasporangia (structures within which tetraspores occur) and also involves a lot of energy expenditure. In populations of *A. spicifera* present in the Hawaiian Islands has been suggested that this truncated life story and that have not been observed gametofitos O'Doherty *et al*., (2007). In Punta Roca Caimancito the tetrasporophytes were observed. Ie carposporangia but empty or perhaps initiating development were observed but not viable.

This alga has spread rapidly through the Bay of La Paz and is present throughout the year (perennial species). It is currently distributed to over 20 km in different parts of the east coast of the Bay of La Paz. Contrary to what happens with native algae like Sargassum is annual, we only see their fronds (higher density and size) during summer and winter only survive fasteners and lots of stipes (Riosmena-Rodríguez et al. 2009) or Porphyra that only occurs during the autumn-winter period (Smith 2002).

CONCLUSION

Acanthophora spicifera is a perennial species that is present throughout the year in Punta Roca Caimancito and tolerates a wide temperature range of 20-29°C. Morphological characters posing *A. spicifera* Punta Roca Caimancito are similar to those reported by other authors for other regions of the world.

The main mode of reproduction of *A. spicifera* is asexually through fragmentation and occurs throughout the year. Sexual reproduction was evidenced by the presence of carposporangia between spring and summer, however, these were never observed containing carpospores. Presents *Acanthophora spicifera* presents larger during fall and lowest during the summer. The number of branches in the thallus of *A. spicifera* was significantly higher in the period of autumn-winter than in the summer.



Ecofriend, 2011; Schrope, 2008.

Figure 18. "Super Sucker" vacuum invasive algae in the coral reefs of Hawaii.

The maximum number of spines was 5.4 4.5 mm linear spines on secondary branch and the minimum was 3.9mm for linear secondary branch. The percentage of biomass by wet weight of *A. spicifera* is higher in autumn and winter less. The percent dry weight of biomass is greater *A. spicifera* fall and lower in winter. *A. spicifera* impact in the study area can be positive for some species of wildlife that benefit from this alga to obtain shelter and food, but for the case of coral communities, the impact appears to be negative as the high density of the algae can affect light and nutrient uptake and thereby affect its growth.

RECOMMENDATION

As already mentioned *A. spicifera* is an invasive algae that has invaded marine ecosystems in the Pacific, and has been shown to be a highly competitive body (light and nutrients) with native corals and algae. It has also been noted that competes for space with a great diversity of sessile organisms. Here, in Hawaii has developed a technology to remove the algae from the invaded area. It is a vacuum machine called "Super Sucker", which has been successful in controlling the algae as it can draw up to 800 kg per hour. Subsequently, these invasive algae are packed in bags and delivered to farmers for use as fertilizer. Therefore, it could recommend the use of these machines to control the high biomass of A. spicifera in places where it is distributed in the Bay of La Paz. Although it is also recommended to first assess the impact of the cleaner for other native organisms associated (National Geographyc 1996-2011; Ocean Power Magazine 2011).

ANNEX

Table 2. ANOVA monthly average length of thalli of *A. spicifera*

Source Variables	gl	H cal	H cri	P
Length per Month	11	102.589	21.03	<0,001

Table 3. ANOVA the average number of branches per thallus of *A. spicifera*

Source Variables	gl	SC	CM	F	P
Among Months	1	0,0817	0,0817	0,0695	0,795
Residual	22	25,847	1,175		
Total	23	25,928			

Table 4. Monthly wet weight in g / cm^2 *A. spicifera* data extrapolates the area covered by the mantle (3.16 ha)

Wet weight	Oct 2008	Nov 2008	Jan 2009	Feb 2009	Mar 2009	Apr 2009	May 2009	Jun 2009	Jul 2009	Aug 2009	Sep 2009	Oct 2009
g/cm^2	211.26	215.39	55.88	38.12	130.72	116.39	116.05	124.91	154.93	39.87	52.92	65.04
kg/m^2	3.30	3.50	0.89	0.59	2.09	1.86	1.85	1.99	2.47	0.64	1.36	1.04
kg/ha	33801.44	35037.57	8940.72	5923.40	20914.65	18622.94	18567.20	19985.26	24788.19	6378.99	13606.26	10406.96
Ton/ha	33.80	35.03	8.94	5.92	20.91	18.62	18.56	19.98	24.78	6.37	13.60	10.40
Ton Total	106.81	110.71	28.25	18.71	66.09	58.84	58.67	63.15	78.33	20.15	42.99	32.88

Table 5. Monthly dry weight in g / cm 2 *A. spicifera* data extrapolates the area covered by the mantle (3.16 ha)

Dry weight	Oct 2008	Nov 2008	Jan 2009	Feb 2009	Mar 2009	Apr 2009	May 2009	Jun 2009	Jul 2009	Aug 2009	Sep 2009	Oct 2009
g/cm^2	12.47	15.70	2.38	1.33	11.10	10.43	10.94	8.98	13.41	2.66	5.23	5.38
kg/m^2	0.25	0.26	0.04	0.04	0.18	0.17	0.18	0.14	0.21	0.43	0.84	0.86
kg/ha	2512.31	2570.67	380.14	375.21	1775.59	1668.85	1750.54	1437.43	2085.68	425.28	837.15	860.75
Ton/ha	2.51	2.57	0.38	0.37	1.77	1.66	1.75	1.43	2.08	0.42	0.83	0.86
Ton total	7.93	8.12	1.20	1.18	5.61	5.27	5.53	4.54	6.59	1.34	2.64	2.71

Table 6. ANOVA dry weight monthly *A. spicifera*

Source Variables	gl	H cal	H cri	P
Month	11	64,820	21.03	<0,001

Table 7. The dry weight of the flora and fauna in monthly g / cm 2 *A. spicifera* data extrapolates the area covered by the mantle (3.16 ha)

Flora and Fauna	Oct 2008	Nov 2008	Jan 2009	Feb 2009	Mar 2009	Apr 2009	May 2009	Jun 2009	Jul 2009	Aug 2009	Sep 2009	Oct 2009
g/cm^2	2.00	2.07	6.30	6.30	0.00	0.00	0.00	4.63	9.20	8.80	12.40	5.00
kg/m^2	0.03	0.03	0.10	0.05	0.00	0.00	0.00	0.07	0.15	0.14	0.20	0.08
kg/ha	318.40	331.20	1006.40	544.00	0.00	0.00	0.00	739.20	1472.00	1408.00	1984.00	800.00
Ton/ha	0.32	0.33	1.01	0.54	0.00	0.00	0.00	0.74	1.47	1.40	1.98	0.80
Ton total	1.01	1.05	3.18	1.72	0.00	0.00	0.00	2.34	4.65	4.45	6.27	2.53

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Chapter 20

ENVIRONMENTAL IMPACTS ON MARINE BENTHIC COMMUNITIES IN AN INDUSTRIALIZED CARIBBEAN ISLAND-TRINIDAD AND TOBAGO

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ABSTRACT

The twin island state of Trinidad and Tobago is the Caribbean's southern-most island state located between 10^0 to 11^0 North latitude and 60^0 to 61^0 West longitude. Like most of the Caribbean small-island states, Trinidad and Tobago's coastal waters are an economically important natural resource and at the same time, a vulnerable one. Trinidad and Tobago is a producer of oil and natural gas with the energy sector being by far, the most important contributor to the country's GDP, Government revenues and foreign exchange. Crude oil production averaged 122,902 barrels per day (bbl/d) in 2004, while natural gas production averaged 2,938 million standard cubic feet per day (Ministry of Planning and Development Central Statistical Office, 2007). The petrochemical sector (at Point Lisas Industrial Estate, west coast of Trinidad) has continued to expand in line with natural gas production producing methanol, ammonia, urea, and natural gas liquids. In this respect, Trinidad and Tobago is the 5th largest exporter of liquid nitrogen gas (LNG) in the world, and the single largest supplier of LNG to the U.S.A. (between 70-75% of all LNG imported into the U.S.A, Ministry of Planning and Development Central Statistical Office, 2007). Coupled with this, La Brea on the south-west coast of Trinidad is home to the world famous Asphalt or Pitch Lake. This coastal area in Trinidad has one of the highest known natural seepage rates on earth - an estimated 100 barrels per dy per 2560 km^2.

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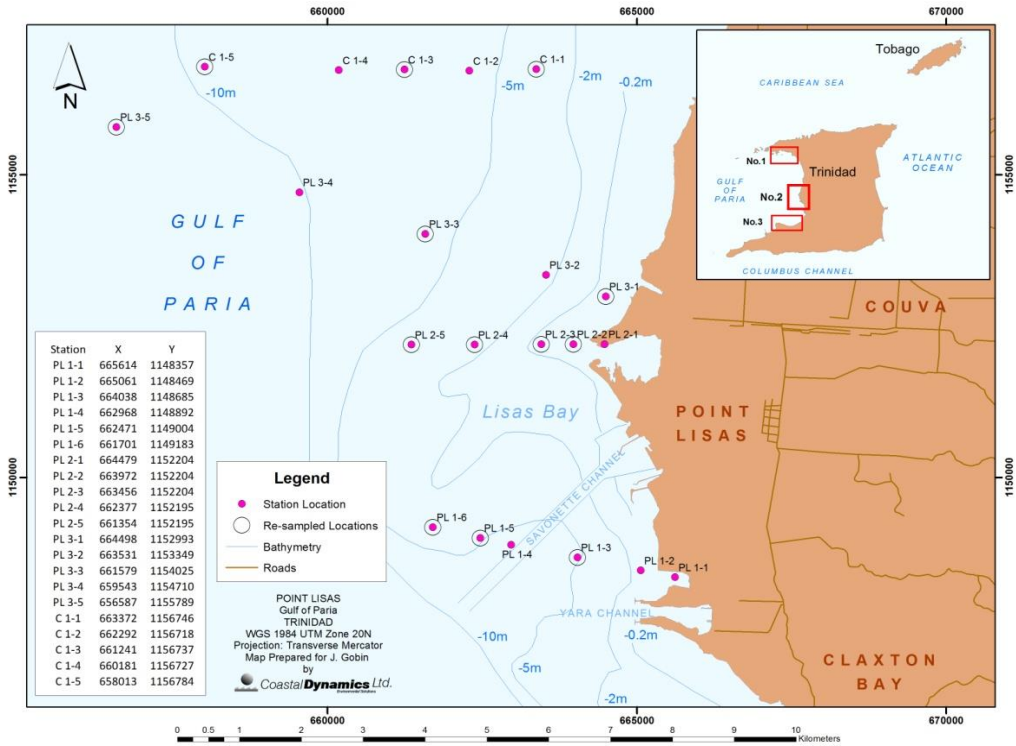
INTRODUCTION

Macrobenthic research and marine species diversity give a good indication of the nature of the environment at that location. Benthic organisms are effectively sessile living in the bottom sediment and they tend to act as integrators of the effects of various kinds and levels of disturbances including pollutants etc. Other marine organisms (e.g., fish and larger macrofauna) feed on the benthos, hence the status of the benthic community needs to be maintained if the quality of the environment (including our rich marine resources) is to be preserved. Against existing baseline information, knowledge of changes in abundances, species compositions and diversities of benthic organisms may be attributable to environmental impacts. In this respect, benthic research is very important towards assessments of environmental impacts on overall marine communities. This research is especially valuable to nations such as Trinidad and Tobago which has a history of maritime oil exploration and production.

BRIEF REVIEW OF LOCAL BENTHIC ECOLOGICAL ASSESSMENTS

For Trinidad and Tobago and the Gulf of Paria (west coast area, Figure 1), one of the earliest published descriptions of macrobenthic data was as a result of an extensive survey carried out in 1952 to 1953 (Van Andel and Postma 1954). This information provides significant baseline data since it was acquired prior to the drilling of the very first offshore wells (by Trinidad Northern Areas Ltd.) at Soldado. Later, the Institute of Marine Affairs (IMA) carried out several macrofaunal surveys in the coastal areas of the Gulf of Paria. The first of these was in the area of the Point Lisas Industrial Estate (PLIPDECO/IMA 1982, Vol. 5) (Figure 1, Inset Map, No.2). That macrobenthic survey (including chemical and geological data) was not strictly pre-construction since there were already a few existing (and operational) sites at that time e.g., the Brechin Castle sugar factory and Federation Chemicals –FEDCHEM (now Trinidad Notrogen (TRINGEN). Such information is still important baseline data however, since the present day operations now include approximately 94 industries - petro-chemical related sites and factories as well as a number of light industries. Results (at that time) indicated that while the inshore coastal areas at Point Lisas were typical of coastal estuarine conditions, there was already evidence of a certain amount of environmental stress. A “hot spot” area close to FEDCHEM, was identified where a number of stations were already abiotic for macrobenthic organisms.

In a baseline survey of the Scotland Bay area (Figure 1, Inset Map, No. 1), Chaguaramas (Alkins and Kenny 1980), the marine biological communities studied included benthic fauna and flora. Scotland bay at that time supported a wide range of fairly small communities of corals, a sea grass bed, a mixed coral/sponge/algal community, an algal community, an intertidal and sublittoral mud community and a sand and rock community. The authors described interesting community features and suggested that they should be protected from overuse or pollution. The coral communities in particular, had already been described earlier as “under physiological stress as they are growing on the edge of their salinity tolerance range” (Kenny 1975).



Station	X	Y
PL 1-1	665614	1148357
PL 1-2	665061	1148469
PL 1-3	664038	1148685
PL 1-4	662968	1148892
PL 1-5	662471	1149004
PL 1-6	661701	1149183
PL 2-1	664479	1152204
PL 2-2	663972	1152204
PL 2-3	663456	1152204
PL 2-4	662377	1152195
PL 2-5	661354	1152195
PL 3-1	664498	1152993
PL 3-2	663531	1153349
PL 3-3	661579	1154025
PL 3-4	659543	1154710
PL 3-5	656587	1155789
C 1-1	663372	1156746
C 1-2	662292	1156718
C 1-3	661241	1156737
C 1-4	660181	1156727
C 1-5	658013	1156784

Figure 1. Map showing macrobenthic stations sampled (circled = re-sampled) in the Point Lisas coastal area.

Another detailed macrobenthic survey (including chemical and sediment geological data) was carried out in the coastal area extending from Diego Martin to Port of Spain (Figure 1, No. 1) during 1982 (Agard 1984). Over both a wet and dry season, forty seven (47) stations were sampled for macrofaunal content. The most abundant species was *Streblospio benedicti* (a polychaete) a good indicator of organic pollution (Pearson and Rosenberg 1978). Its' distribution was restricted to the Sea Lots/Laventille area where there were overloaded sewage treatment ponds with overflow pipes (Agard 1984). The amphipod *Ampelisca parapanamensis* which is very sensitive to low level pollution was the second most abundant species. Its' distribution was ubiquitous except for the areas dominated by *S. benedicti* and where there were elevated levels of petroleum hydrocarbon.

In general, Agard (1982) found that the spatial effects of pollution (high sediment organic content and low dissolved oxygen) on the benthic macrofauna at the Sea Lots/Laventille area, were far more pronounced than the temporal variation due to seasonal climatic change. The author described the presence of 5 ecological zones (ranging from very polluted, polluted, semi-healthy 1, semi-healthy 11 and healthy) each of which had its own characteristic faunal assemblage. This study provides good baseline information with which further monitoring results can be compared especially with respect to the presence or absence of the tentative indicator species identified: *S. benedicti* and *A. parapanamensis*.

Another comprehensive macrobenthic survey was carried out by the IMA in the area extending from Pointe-a-Pierre to La Brea (Figure 1, Inset Map, No. 3). This area is subject to chronic natural oil seepage and oil spillage from oil production activities (Agard and Gobin 1992; Agard, Gobin and Warwick 1992). Three (3) major station groups (based on

hierarchical classification and multidimensional scaling ordination) showed concordance with differences in water depth and sediment type. Abundance/Biomass comparison plots indicated that macrobenthic communities near the oil refinery were grossly to moderately stressed while those close to the Trinidad Pitch Lake (one of the largest natural oil seeps in the world were not). Contamination occurs here at La Brea on what is probably an evolutionary time scale from one of the world's largest hydrocarbon seeps (Wilson et al., 1974; Geyer and Giammona, 1980). This is in contrast with contamination over ecological time from an oil refinery at Pointe-a-Pierre, a few kilometers away (Agard et al., 1988). Results near the La Brea oil seep suggest a diverse benthic macrofauna (Government of Trinidad and Tobago, 1984 and Agard and Gobin 1992, Gobin et al., 2012). It is thought that these organisms may rely on hydrocarbon degrading bacteria as a food source (Zobell and Feltham, 1938, Gobin et al., 2012).

There have been 2 other studies carried out by Nansingh (1993) and Price (*in prep*). Nansingh (1993) sampled quantitatively for macrofauna in the intertidal zones of 4 different areas of the Gulf of Paria – Caroni and Carli Bay (mudflat) areas, Granville and Williams Bay (sandy beach areas).Stations were sampled using cores at the 4 stations with additional mangrove root samples at Caroni Swamp. The mud flat stations reflected the greater diversity of species compared to the inner swamp area. Based on his results Nansingh and Jurawan (1999) described vulnerability of coastal areas and suggested that a potential oil spill could impact on these habitats with severe consequences. The authors applied a coastal environmental rating (an index of 1 implied "least" sensitive while 10 was "most" sensitive) and rated the studied areas as follows: Caroni swamp -9, Williams Bay-7, Granville Bay-7 and Carli Bay-7. Price (MPhil Thesis *in prep*) sampled the benthic macrofauna associated with seagrass beds at Williams Bay (Chaguaramas, Trinidad) William's Bay and St. Peter's Bay in Trinidad and compared them with La Guira Bay and Bon Accord Lagoon in Tobago.

Environmental Impacts on Macrobenthos in the Point Lisas Industrial Estate (PLIE) Coastal Area

Trinidad and Tobago is a highly industrialised country with 2 very large industrial estates: Point Lisas Industrial Estate (PLIE) and La Brea Industrial Estate. The Point Lisas Industrial Estate (PLIE) is today, the heart of Trinidad and Tobago's petrochemical sector. The Estate is a world-class facility, covering 860 hectares and represents an investment of over US $2 billion (http://www.plipdeco.com/main/index.php?page=estate-management-overview). The PLIE is home to 94 companies involved in a range of activities dominated by the petrochemical sector.

As the largest oil and natural gas producer in the Caribbean, Trinidad and Tobago's hydrocarbon sector moved from an oil dominant to a mostly natural gas based sector in the early 1990s.Trinidad and Tobago now houses one of the largest natural gas processing facilities in the Western Hemisphere- the Phoenix Park Gas Processors Limited (PPGPL). With 11 ammonia plants and 7 methanol plants, Trinidad and Tobago is the world's largest exporter of ammonia and the second largest exporter of methanol (IHS Global Insight 2013).

Following the original Point Lisas Industrial Estate survey (PLIPDECO/IMA 1982, Vol. 5), a detailed benthic survey was carried out during 1984 to 1986 in a similar coastal area of Point Lisas (Gobin 1988). This quantitative study of polychaetes (Annelida- which contribute

approx. 70% of total macrofauna here) was carried out to determine the distribution and abundance of these communities in the coastal area of the Estate. These distributions were evaluated with respect to proximity and effects of potential "polluting effluents" or disturbances. The polychaetes were well distributed over the study area except for the well known indicators of "organic pollution" *Capitella capitata* and *Streblospio benedicti* which were confined to some inshore stations. Additionally at some inshore stations there were elevated levels of zinc, petroleum hydrocarbons, ammonia and temperature (max. 41^0C). There was also a marked seasonal shifting of sediment at some stations (e.g., at Couva River mouth) accompanied by some patchy species distribution. A general conclusion of that study (Gobin 1988) was that there appeared to be no marked deterioration in the Point Lisas coastal area since the original study (PLIPDECO/IMA 1982). This was based on the fact that the "stressed stations" appeared to be at the same locations for both studies (although 8 years apart) with elevated levels of "pollutants" being confined still mainly to inshore areas.

With the continued expansion of developments and increased activities at the Point Lisas Industrial Estate, increasing volumes and variety of effluents continue to enter the Gulf of Paria. These continue to potentially pose serious threats to the quality of this environment which is a major fishing ground. These effluents have already been suggested as responsible for "contaminating" and "polluting" environmental status (Agard, Gobin and Warwick 1993). A more recent project approximately 25 years later (by the author J. Gobin 2010) attempted to evaluate changes in the benthic communities in the area. This is the first study of its kind in the history of benthic community analyses for Trinidad and Tobago.

MATERIALS AND METHODS

A subset (comprising of 13) of the original 25 stations which had been previously sampled in May 1985 (Gobin 1988) were re-sampled during May 2010 (Figure 1). The same methodology was used for both macrofaunal surveys (1985 and 2010). Benthic sediment grab samples were taken using a 0.025 m^2 van Veen grab at each of the thirteen (13) stations (2008). At each station, 3 replicate grab samples were collected and each sediment sample was sieved and washed (using sea water) through a .05 mm^2 sieve mesh. The fauna retained was stained using Rose-bengal (a proteinaceous dye) to facilitate sorting and preserved in a 10% formalin solution. All 39 samples were transported back to the laboratory where they were re-sieved and gross sorted, separating the polychaetes from all other taxa. All polychaetes were identified as far as possible to species level using the relevant taxonomic keys. All other taxonomic groups were identified to Family or Genus or species, as far as possible also using relevant keys. All identifications were carried out by the author (J. Gobin) for both surveys (1985 and 2010).

Species abundances, richness and SWI (Shannon-Wiener Index) diversities were calculated (Shannon and Weaver 1963) towards an overall understanding of the distribution of the benthic organisms at the stations (for 1995 and 2010 data). The macrobenthic data were analysed using the PRIMER (Plymouth, UK) multivariate analyses programmes (after Field, Clarke and Warwick 1982; and Agard, Warwick and Gobin 1993). Similarity matrices were used to produce plots (MDS- multidimensional space) in which similarity between stations is represented as physical distances (Field, Clarke and Warwick 1982) - for both 1995 and 2010 data.

GENERAL RESULTS

In keeping with the macrobenthic review focus of this paper- only some results of this study are presented here. In the 1985 samples, a total of 3140 polychaete individuals belonging to 70 species were identified. Overall in 1985, the dominant polychaete was *Capitella capitata* (an indicator of high organic content- at station D) with *Armandia maculata* (Ophelidae) being the next. In the absence of any major changes one would expect about half the number of individuals and species for approx. half the number of samples (13). In the 2010 samples the number of individual polychaetes was severely reduced to 412 belonging to 43 species. The dominant polychaete was *Paraprionospio pinnata* (of the Spionidae). Species diversity (for polychaetes) values recorded at each station for the 1985 and 2010 survey are presented in the following figure (Figure 2). Stations 1.3 to C 1.5 (13 in total) have been re-labelled incrementally as: A to M on these plots.

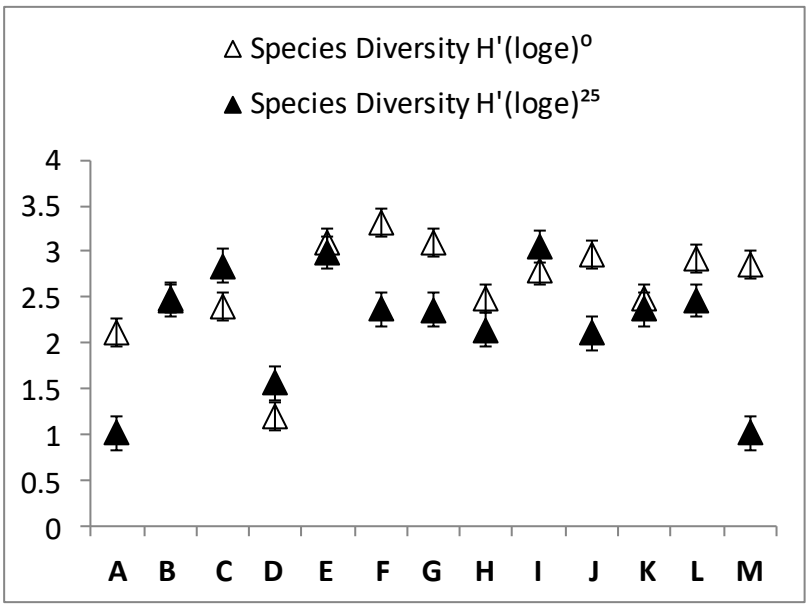


Figure 2. Species Diversity (H') at stations (A to M) sampled (with replicates combined) for 1985 (0 yrs = ^{0}) and for 2010 (25 yrs later = ^{25}).

Overall the benthic polychaete species have clearly decreased markedly in terms of species diversity at 7 of the 13 re-sampled stations, twenty-five years later. At least 3 stations (B, E and K) however reflected similar diversity for both sampling times. At the same time, three (3) stations C, D and I have increased species diversity after 25yrs. Station F had the greatest species diversity in 1985 with stations E and I having the highest in 2010. After 25yrs of continued industrial activity the macrobenthos in the Point Lisas area is still reasonably diverse.

DISCUSSION

Trinidad and Tobago's west coast has been reasonably very well surveyed in terms of its macrobenthic faunal species and their diversities (PLIPDECO/IMA 1982, Gobin 1988, IMA 1994 etc.). In addition to the number of studies reviewed (above), there have been a large number of surveys which contribute to the "grey literature." For example, there have been a number of macrobenthic assessments as components of Environmental Impact Assessments (EIAs)- a requirement by the local Environmental Management Authority (EMA) for proposed development activities. A large number of these have been done as pre-drilling surveys for oil and gas exploration activities and were concentrated on the East and North-east offshore areas of Trinidad. The author (J. Gobin) has also contributed (as the only specialist Benthic Ecologist) in Trinidad and Tobago) to the majority (> 40) of these assessments between 1995 and 2007 (e.g., (i) 2005 Gobin, J. *Benthic Ecology Section of Document prepared for British Gas, TT: the North Coast Marine Area (NCMA) Report*; (ii) 1998 Gobin, J. *An Environmental Impact Statement on the effects of Siltation in the area of Atlantic LNG, Point Fortin. Benthic Ecology Section of a report prepared for Atlantic LNG, Point Fortin, by CANE and Associates Ltd*. etc.) There have been very few post-development (e.g., post exploration drilling) surveys carried out in these areas although these have been proposed within the various EIAs' terms of reference (TORS). This is of course quite unfortunate, since continued monitoring surveys should follow baseline studies in order to provide key information on "real" changes (as a result of the activities) in macrobenthic species compositions and distributions compared to "predicted" changes (as is typical of EIAs).

This re-survey (25 years later) provides some significant benthic macrofaunal data (and the first of its kind) for Trinidad and Tobago and the Point Lisas Industrial coastal area.

Although only a small component of a more complex environment, these results confirm that macrobenthic species (polychaetes in this instance) are reasonably hardy organisms and are therefore good indicators of the state of the environment. The use of the type of data (polychaete species level) is supported by Warwick et al., (1990) and Somerfield and Clarke (1995) who confirm that aggregation patterns at different taxonomic levels are very useful for determining patterns of impact. Results suggest that in general, species diversities have markedly decreased at some of the stations over the years in those areas of the Gulf of Paria which were re-surveyed (25 years later). This was not surprising given the major expansions that have occurred at the PLIE and the increased volumes and contaminants being released in this coastal area. According to UNDP/EMA (1998) some pollutants in liquid effluent that are of greatest concern in Trinidad and Tobago are: "Total Dissolved Solids (TDS), mainly from the ammonia production sector in the Point Lisas Industrial Estate; Nitrogen (N) from the urea plant at PLIE (72%); Oil and Grease mainly from the ammonia production sector in the PLIE and Metals in effluent from the steel mill (at PLIE)."

The recent Global Environmental Outlook Report (GEO 5 2012) describes those damaged ecosystems globally as "great damage to the very ecosystems that support human livelihoods". The report further predicts that ecosystems will take centuries to recover from damages if they recover at all (http://www.un-ngls.org/spip.php?article3907). Global and large scale disturbances over time (e.g., pollution, logging/farming, climate shifts etc.) have already altered "original baselines" of marine benthic ecosystems. In studies such as this,

recovery of the variables are therefore only being measured on a contemporary timescale. Jones and Schmitz (2009) tested the prediction of "irreparable harm" using a synthesis of recovery times compiled from 240 independent studies reported in the scientific literature. They provided startling evidence that most ecosystems globally can (given human will) recover from very major perturbations on timescales of decades to half-centuries.

Results of a similar (to this study) comparative tropical study (Webber and Webber 1998) also described increases in nitrate concentrations and increases and changes in the plankton community associated with excessive eutrophication of Kingston Harbour waters (Jamaica) over a 20 year period (1970s to 1990s). Much earlier predictions for Kingston Harbour, Jamaica (Wade 1976) was a- much deteriorated and possibly abiotic coastal area. This prediction was also made for this Point Lisas coastal area (IMA archival data, PLIPDECO/ IMA 1980). Results of this study confirm a reasonable diverse benthic community.

In consideration of disturbance and effluent contaminants in the PLIE area the benthic communities may be considered in terms of benthic macrofaunal resistance and/or resilience. Resistance implies that the macrofauna are able to to some extent to tolerate these disturbances, while resilience defines the speed at which an ecosystem can return to its former state following a disturbance. Coastal ecosystems tend to be naturally resilient and Jones and Schmitz (2009) suggest that "recovery may be independent of the magnitude of perturbation and instead, idiosyncratic to the ecosystem type". Community composition and ecosystem function may change very little where environmental impacts are such that organisms can acclimate to the change or at least tolerate it, for some time. Results of this study confirm macrofaunal resistance and/or resilience.

This high recovery potential for benthic communities was described for the Baltic sea as being due to very strong seasonality of the ocean, strong physical disturbance and short generation times of most coastal animal species (Hällfors et al., 1981). The author (J. Gobin) has confirmed that approximately 7 to 9 months is the average time needed for coastal species in Trinidad and Tobago to regenerate following dredging activities (Gobin, archival data). However, all organisms have limits to what they can temporarily or permanently tolerate, and when change exceeds some of these limits, the ecosystem functioning and in turn community compositions are likely to change. Monitoring surveys to determine or chart further changes to benthic communities are therefore a key recommendation for the PLIE and the oil and gas East coast area of Trinidad and Tobago.

It is well documented that small island developing states (SIDs) face special environmental hazards and problems not which of the least are impacts due to climate change. At the same time, most SIDs lack human resources in critical fields such as science and technology, governance and economics which are all required for a holistic and systems approach to sustainable development (http://www.unep.org/pdf/Emerging_issues_for_small_ island_developing_states.pdf 2014). For example, virtually all Pacific island countries lack adequate skilled and trained workforce (http://www.ilo.org/wcmsp5/groups/public/@asia/ @ro-bangkok/documents/projectdocumentation/wcms_120577.pdf).

This is interestingly not the case in Trinidad and Tobago which has a number of very well trained scientists, technologists, governance and economics professionals. Additionally there are 2 Universities (University of the West Indies and University of Trinidad and Tobago) as well as an Institute of Marine Affairs – institutions which can provide guidance and advice as well as a number of key Environmental and Maritime divisions and research departments (within Ministries). Trinidad and Tobago is however typical of a SIDS economy which relies

on a relatively narrow base of commodity; in this case oil and gas resources. This economic dependency has created a scenario where *scientific findings and data do not mix with oil and gas exploration*! In other words, Trinidad and Tobago's economic driver takes precedence over scientific findings and the "paper" requirements for surveys and studies detailing "impacts on the benthic communities" within the EIA requirement (as submitted by the various multinational and local companies) is exactly that! The author is not aware of any company being refused permission to carry out exploration activities in the waters of Trinidad and Tobago based on marine environmental concerns! Other scholars (Burdge and Vanclay 2010) were similarly not optimistic about the ability of EIAs and SIAs (Socio economic Impact Assessments) to make substantial changes in the politics of development decisions

Given the recent fluctuations in oil prices and the growing unsustainability of supplies, renewable energy is an alternate area with great potential for consideration by Trinidad and Tobago. At the same time, this much needed economic diversification will result in less degradation to the overall marine environment and the associated macrobenthic biodiversity. This even if the some components of the marine environment appear to be resilient or tolerant.

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Chapter 21

PREDICTION OF *ZOSTERA MARINA* SHOOT GROWTH, LEAF PRODUCTION, LEAF AREA AND SHOOT WEIGHT USING THE SHEATH LENGTH

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ABSTRACT

We examined the possibility of using the length of the outermost sheath (OSL) to predict values of different variables of *Zostera marina* (eelgrass), as our previous observations indicated a strong correspondence between eelgrass length structures (leaves and sheaths) and its biological and morphometric variables, i.e., growth, production, and leaf area. We first determined leaf growth and production by means of the Ibarra-Obando and Boudouresque (1994) technique, and then used correlation matrixes to explore the relationship between length of shoot leaves and sheaths, and the following variables: shoot growth (SG), leaf production (LP), shoot weight (SW = sum of weights of all leaves and sheaths in a shoot), and shoot leaf area (SLA) in order to predict them, based on a single shoot structure easy to measure in the field. Our results indicate that the OSL

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is a reliable and easy technique to measure a variable that can be used to predict the variables mentioned above on two-week and monthly periods. Then we must emphasize the importance to developing predictive equations for each study region as the only way to prevent over or underestimation of the variables of interest.

INTRODUCTION

Assessment of plant growth and production has been the object of much research, both in terrestrial and aquatic environments (reference). The trend through time has been toward a simplification of the methods used (reference). Examples of a simple and useful technique comes from agronomy, where the growth patterns or plant density indicates the seed production in some cultivars; or from forestry, where engineers use tree length and trunk circumference (diameter at breast height) to calculate tree growth, basal area, biomass or wood production (Husch et al., 1982; Brokaw, and Thompson, 2000). Snedaker and Snedaker (1984) extended these techniques to mangroves and other plants. Relatively simple techniques have been developed for aquatic plant biomass assessment (Maceina et al., 1984; Downing and Anderson, 1985), some of which are based on morphometric relationships (Daoust and Childers, 1998). For seagrasses, rather complicated mathematical models have been used to understand seasonal variations in *Z. marina*'s distribution, biomass, and production (Verhagen and Nienhuis, 1983; Bach, 1993; Bocci et al., 1997, Echavarria-Heras et al., 2012, 2013). Recently has been development some techniques to measure seagrass growth and others biological variables using statistical and allometric tools (Short and Duarte 2001; Gaeckle et al., 2006, Echavarría-Heras et al., 2013).

Seagrasses' vegetative morphology is rather simple (den Hartog, 1970). Vegetative shoots are produced through lateral shoot production. Each shoot has a series of leaves bound to their sheath, which correspond to rhizome node in a 1:1:1 (Leaf: sheath: rhizome node) relationship. This means that there is more than one sheath in each shoot. As a consequence, a strong relationship exists between these structures and growth and production dynamics (Bak, 1980; Short, 1983; Duarte, 1991; Ibarra-Obando and Boudouresque, 1994; Meling-López and Ibarra-Obando, 1997; Short and Short, 2000; Gaeckle et al., 2006).

Allometric models have been developed to assess eelgrass leaf production under natural (Solana-Arellano et al., 1991; Echavarria-Heras et al., 2013) or laboratory conditions (Hamburg and Hoffmann, 1986), and shoot length has also been estimated using biometric relationships (Bak, 1980). Ibarra-Obando and Boudouresque (1994) proposed using the length of the outermost sheath as a morphological index of leaf growth and production. The main differences between this and traditional techniques (i.e., Kentula and McIntire, 1986), is the use of the first node instead of the ligule, as the reference point to assess leaf growth. The possibility of using the length of the outermost sheath as a morphological index of shoot growth and production was tested by Meling-López and Ibarra-Obando (1997), who during an annual cycle marked eelgrass shoots simultaneously with a traditional technique (Kentula and McIntire, 1986) and with the Ibarra-Obando and Boudouresque (1994) technique. During summer, a high and significant correlation between techniques was found, for shoot growth and production, which was not observed during winter because Kentula and McIntire's technique is more sensitive to high values and/or variance among measurements (Meling-López and Ibarra-Obando, 1997).

The observation that strong relationships exist between eelgrass morphological structures, that these relationships can be expressed in statistical terms, and that they reflect not only leaf growth and production but shoot growth and production as well (Ibarra-Obando and Boudouresque, 1994), allowed us to use regression equations to predict these and other variables, like leaf area, and shoot weight (we refer shoot weight as the sum of weights of all leaves and sheaths in a shoot) by measuring a single structure. Here we consider whether the sheath might be this structure. The sheath has two advantages: 1) It is less fragile than the leaves, and remains intact for longer periods of time, and 2) its measurement in the field is easy.

With this background, we formulated the hypothesis that shoot growth, leaf production, shoot weight, and leaf area of *Z. marina* can be assessed using regression equations that only incorporate the length of the outermost sheath. These equations would have the advantage that just the measurement of a single shoot structure would be required. Therefore, the objective of this study was to develop and validate these equations. We proceed in three different stages. The first one corresponded to the calculation of the regression equations. The second one was the validation of the equations for two-week periods, and the third one, the development of monthly predictive equations.

MATERIALS AND METHODS

Z. marina vegetative shoots were collected at San Quintín Bay, Baja California (30 24' N; 115 56' W) during August-December 1992, and February-August 1993. At every low spring tide (every two weeks), at a depth of -0.1m MLLW (Mean Low Water), 21 shoots at random were marked following Ibarra-Obando and Boudouresque (1994) technique. Marking new shoots and harvesting marked shoots were simultaneous processes in every field visit. A total of 459 vegetative shoots were marked during the two-week study period.

Shoots were marked with a hypodermic needle at the level of the ligule, and the length of the outermost sheath (OSL) recorded. We refer to the outermost sheath as the oldest one attached to the shoot (Figure 1). Once in the laboratory, shoots were dissected, and only the above ground parts were considered. Notice that in the laboratory leaves and sheaths were numbered from next to oldest (1) to youngest (5), acknowledging that fully developed sheaths are only present in the oldest leaves. As a consequence, the 1:1 proportion (Leaf: sheath) is not always present (Figure 1). The following variables assessed:

Sheath length. It is the distance (cm) from the first node to ligule. The OSL was recorded again in the lab to determine if there had been any increase in length during the incubation time. Results indicated there were no changes in length during this period (results not shown). The length of the other sheaths present in the shoot was also recorded.

Leaf growth. For marked leaves the distance (cm $time^{-1}$) from the ligule to the needle mark was measured, and to it, the OSL subtracted. This means that the remaining lower portion of the marked leaves represented new growth. In the case of new leaves, their whole length was considered new growth. Whole leaf lengths were also measured to estimate the proportion represented by growth in each instance.

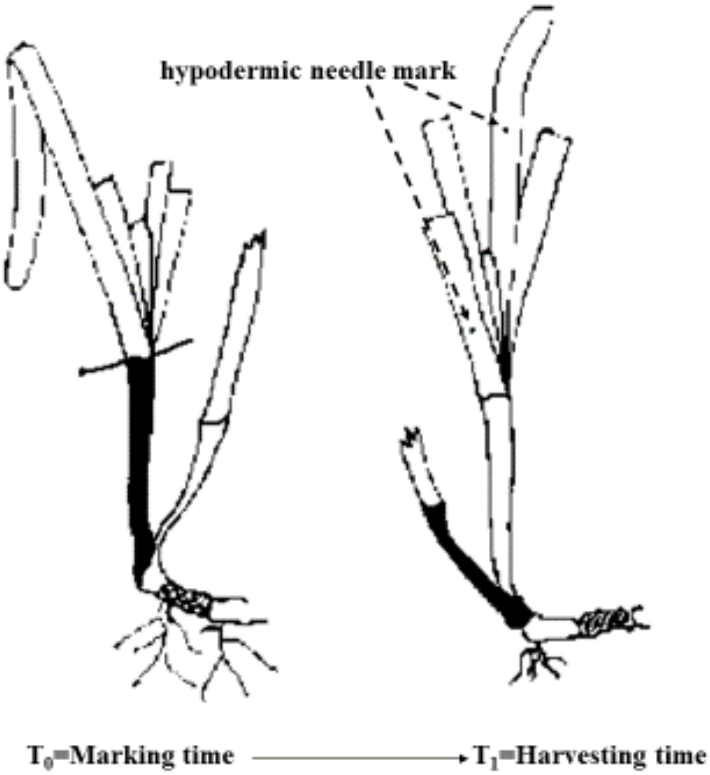

Figure 1. Description of field and laboratory work for the Ibarra-Obando and Boudouresque (1994) technique with *Zostera marina* vegetative shoots. At the marking time (T_0) shoots are marked at the level of the ligula with a hypodermic needle, and the length of the outermost sheath (OSL), indicated in black, is measured Two weeks later (T_1) marked shoots are harvested. Dots in the leaves correspond to the needle mark. Once in the laboratory, shoots are dissected, and individual leaves and sheaths are separated. The oldest leaf has already been lost. Leaves and sheaths are numbered from next to oldest (1) to youngest (5), acknowledging that fully developed sheaths are only present in the oldest leaves.

Leaf production (LP). Leaf growth expressed in weight (g DW time^{-1}).

Sheath growth. This variable was considered proportional to LP. The percentage of leaf length represented by its growth was assigned to its sheath (cm time^{-1}). The portion of the sheath representing new growth during the incubation period was also identified in its lower portion.

Shoot Growth (SG). The sum of growth for all leaves and sheaths in a single shoot during the incubation period expressed in length units (cm time^{-1}).

Shoot Weight (SW). The sum of both leaf and sheath growth in a single shoot during the incubation period expressed in weight units (g DW time^{-1}).

Shoot Leaf Area (SLA). The sum of individual leaf lengths multiplied by their widths (cm^2 time^{-1}). The sheaths were not included.

For all the variables mentioned above two-week averages were obtained and used to explore different combinations of correlation matrices to select the most appropriate shot structure to be used. Results not only showed significantly and high correlations between the different variables, but also in combination with some of the selected length structures (results not shown). It was decided to use only those length structures that showed high and significant correlations with SG, LP, SW, and SLA, and which were easy and fast to measure

both in the field and in the laboratory. These structures were leaves and sheaths of the outermost sheath (OSL), and those in positions 1 and 2. Resultant regressions were later used as predictive equations.

To validate the above-obtained regression equations, a series of two-week samplings took place from October 1993 to March 1995, following the same procedure described above for field and laboratory. Results from the field and laboratory (observed values) were then compared with those estimated from the previously developed equations (calculated values).

Using the two-week data from August-December 1992, and February-August 1993, we calculated monthly average values for all the variables described in the first stage, and the two kinds of data sets (observed and calculated) were compared and analyzed statistically with the same procedure as above. High and significant correlations were found, allowing the calculation of monthly predictive equations, which were validated through monthly sampling from June 1996 to November 1997. SW values were calculated using the biomass (g DW m^{-2}) and density (shoots m^{-2}) relationship. It was found that the observed data were not statistically different from the calculated values, allowing the development of monthly regression equations for SG, LP, SW, and SLA.

As samples were independent and random, and data sets followed the normal distribution and presented variance homogeneity, parametric statistics were applied (Hays and Winkler, 1970; Zar, 1984) using the STATISTICA 6.0 (StatSoft) program. To test whether the whole set of homologue series (observed and calculated) were significantly different or not, an ANOVA test was used. Total averages of the two sets of data (observed and calculated) were compared with a t-student test. We considered the general assumptions for regression and correlation analysis. We used the r^2 value instead the r value to find out whether the observed values were significantly different from the calculated ones, considering them different if the r^2 value was not significant and/or smaller than 0.5, and no different if the r^2 value was significant and bigger than 0.5. In all cases, the confidence level was set at 95%.

RESULTS

Leaves length showed high correlation with their corresponding sheaths ($r^2 = 0.92$; $p < 0.01$), that was also valid for contiguous leaves and sheaths (Table 1). The dataset for two-week periods indicated that the sheath represented, in average, 17% of leaf length (0.69 ± 1 SE), varying between 15% during winter and 25% during summer (Figure 2). Individually, the sheath can represent up to 33% of its corresponding leaf length, with this high percentage corresponding to summer. For intermediate leaves the relationship is lower, as the sheath is not yet fully developed while internal leaves do not present a sheath; this indicates that leaves develop and mature earlier than sheaths. In this last analysis described above, we did not consider the outermost leaf and sheath, as the oldest leaf falls apart during the incubation period.

Leaves and sheaths presented their maximum length during summer-autumn and their minimum values during winter-spring (Figure 3). These seasonal trends can also be observed in shoot growth, leaf production, shoot weight, and shoot leaf area (data not shown). SG, LF, SW, and SLA were highly correlated with leaves and sheaths. Shoot growth and leaf area showed their highest correlation with sheath number 1 ($r^2 = 0.9390$, and 0.9326 respectively;

p< 0.051), while leaf production and shoot weight were highly correlated with sheath number 2 (r^2 = 0.9047, and 0.8691 respectively; p< 0.05). Correlation values with the outermost sheath were lower (Table 2), despite this fact, we selected the length of the outermost sheath (OSL) as the structure that could reflect SG, LP, SW, and SLA, as its length remains constant through time (growth has ceased). As already mentioned the sheath also has the advantage that measurement of its length, both in the field and in the lab, is easy.

Table 1. Correlation matrix for *Zostera marina* leaves and sheaths length. Only values for the most external or oldest structures (leaves and sheaths) are presented, as r^2 values were the highest ($p < 0.05$)

	Outermost Sheath	Leaf 1	Sheath 1	Leaf 2	Sheath 2
Outermost Sheath	X				
Leaf 1	0.9376	X			
Sheath 1	0.9677	0.9162	X		
Leaf 2	0.8872	0.9156	0.9361	X	
Sheath 2	0.8131	0.7270	0.8835	0.7812	X

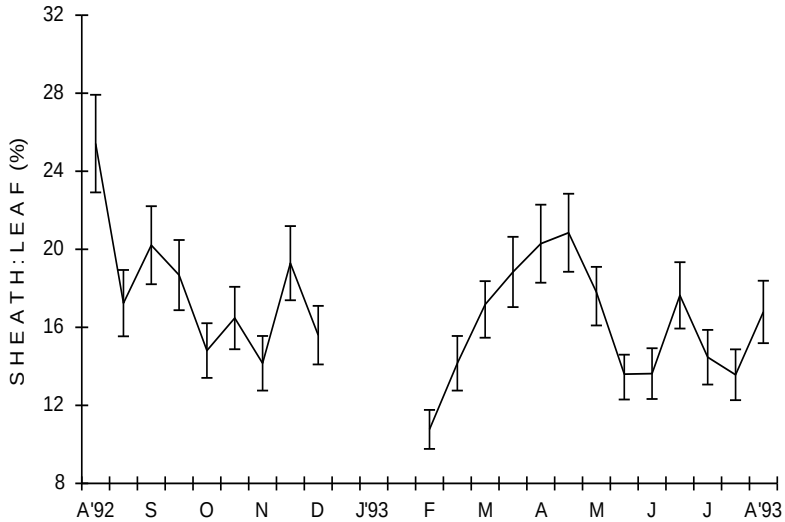

Figure 2. *Zostera marina* leaf length: sheath length throughout the two-week study period. Bars represent ± 1SE.

Table 2. Correlation matrix for the assessed variables on *Zostera marina* vegetative shoots. Only correlations with the oldest structures (leaves and sheaths) are shown, as r^2 values were the highest ($p < 0.05$)

	Shoot Growth	Leaf Production	Shoot Weight	Shoot Leaf Area
Outermost Sheath	0.8955	0.6892	0.7839	0.9252
Leaf 1	0.8619	0.6282	0.7106	0.8788
Sheath 1	0.9390	0.7650	0.7968	0.9326
Leaf 2	0.8881	0.6980	0.7099	0.8680
Sheath 2	0.9220	0.9047	0.8691	0.9128

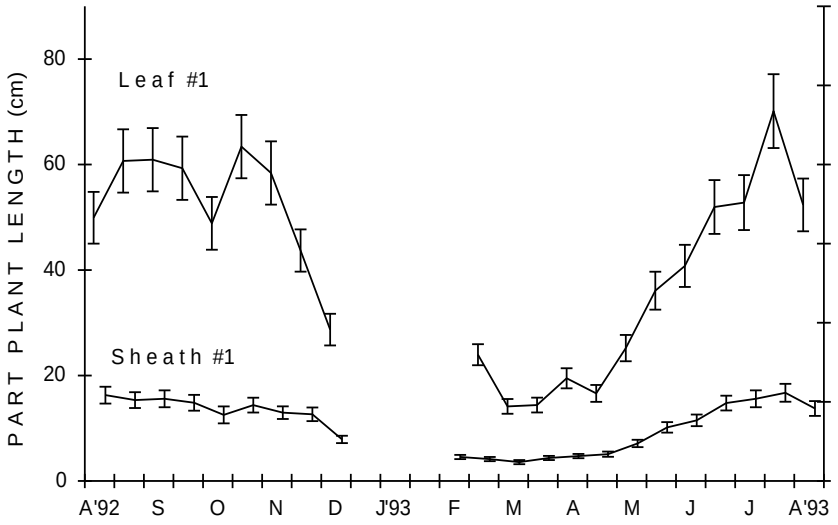

Figure 3. Averages for the two-week periods for leaf #1 and sheath #1 length (cm).

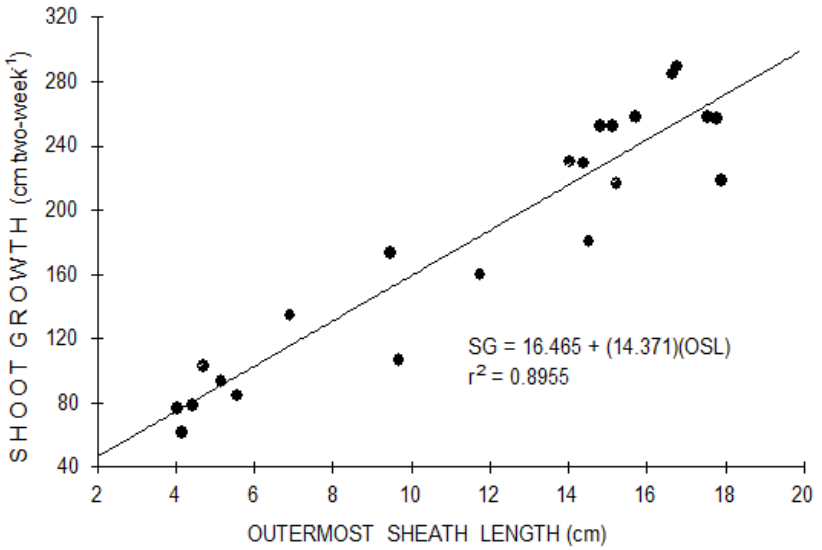

Figure 4. The regression line for Shoot Growth SG (cm two-week^{-1}), and Outermost Sheath Length OSL (cm). Standard error of estimate (S. E. E.) = 1.7; $p < 0.05$.

Table 3. Correlation matrix (r^2 values) among the measures of *Zostera marina* biological variables ($p < 0.01$)

Variables	Shoot Growth	Leaf Production	Shoot Weight	Shoot Leaf Area
Shoot Growth	X			
Leaf Production	0.8796	X		
Shoot Weight	0.8818	0.9387	X	
Shoot Leaf Area	0.9554	0.8392	0.8814	X

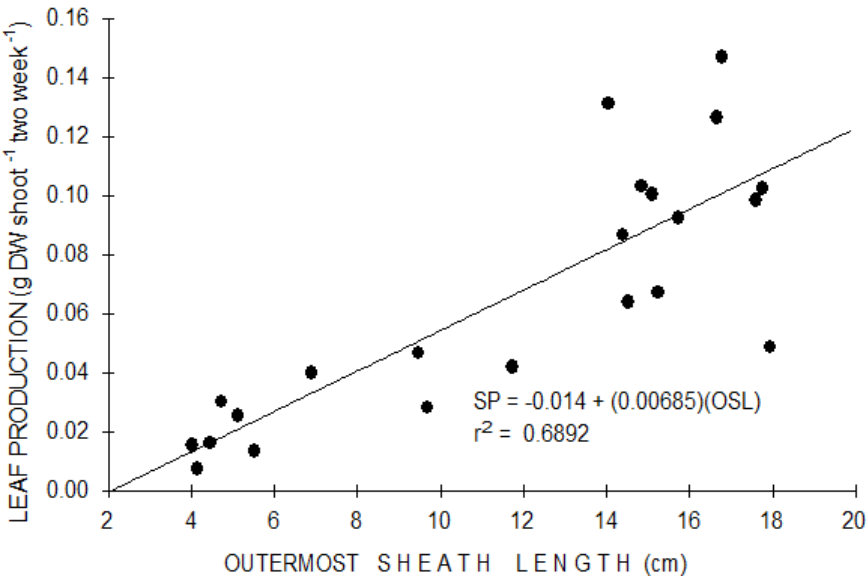

Figure 5. The regression line for Leaf Production SP (g DW shoot^{-1} two week^{-1}) and Outermost Sheath Length (OSL) (cm). Standard error of estimate (S.E.E.) = 1.1; $p < 0.05$.

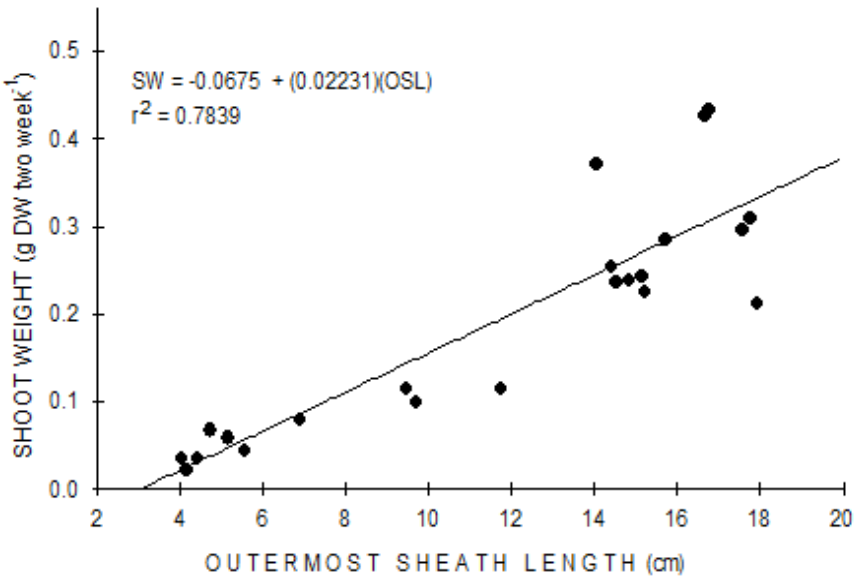

Figure 6. The regression line for Shoot Weight SW (g DW two week^{-1}) and Outermost Sheath Length (OSL) (cm). The standard error of estimate (S. E. E.) = 1.1; $p < 0.01$.

The predictive equations obtained were all significant ($p < 0.01$) and showed high correlation values (Figures 4, 5, 6, and 7). We also noticed that a high correlation existed between these variables (Table 3). These last correlations represent Thus, an alternative way to predict these variables.

Table 4. Equations developed to predict monthly values (mo^{-1}) of the assessed variables: Shoot Growth (SG), Leaf Production (LP), Shoot Weight (SW), and Shoot Leaf Area (SLA), with respect to the Outermost Sheath Length (OSL). Correlations (r^2) and Standard Error of the Estimate (SEE) are given ($p < 0.01$)

Variable to be predicted	Equations (Y = a + bX)	r^2	SEE
SG (cm $shoot^{-1}mo^{-1}$)	SG = 16.223 + 28.23 OSL	0.9003	1.72
LP (g DW $shoot^{-1}mo^{-1}$)	LP = -0.018 + 0.00729 OSL	0.7957	2.02
SW (g DW $shoot^{-1}mo^{-1}$)	SW = -0.1289 + 0.04525 OSL	0.8966	1.37
SLA (cm^2 $shoot^{-1}$ mo^{-1})	SLA = -35.29 + 18.108 OSL	0.9591	1.08

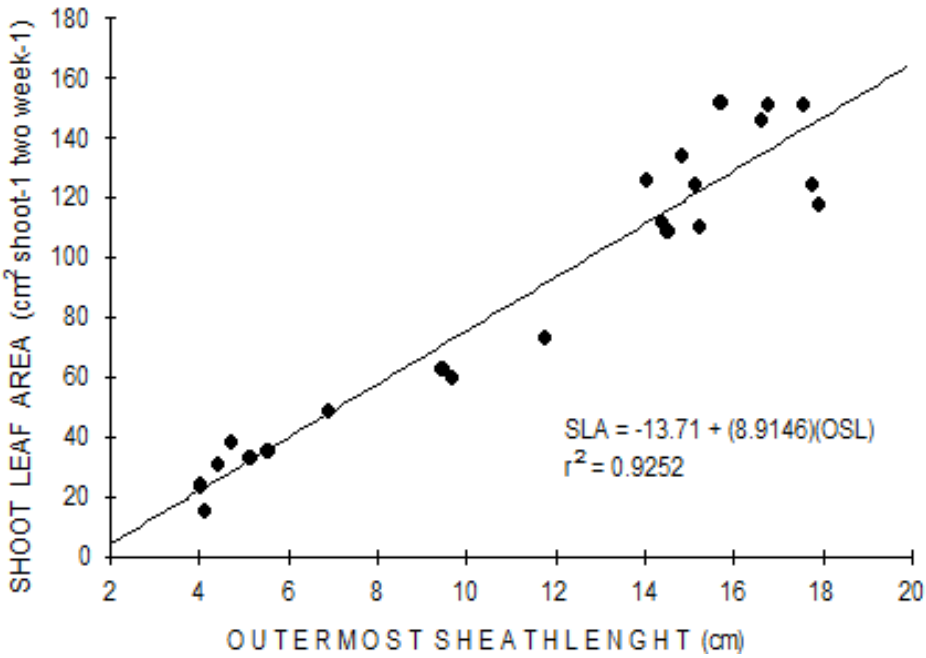

Figure 7. The regression line for Shoot Leaf Area (SLA) (cm^2 $shoot^{-1}$ two $week^{-1}$) and Outermost Sheath Length (OSL) (cm). The standard error of estimate (S. E. E.) = 1.4, $p < 0.01$.

When the observed values for the two-week period were compared with the calculated ones, no significant differences were observed ($p > 0.05$). In a similar way, the t-student test indicated no significant difference ($p > 0.05$) between the total averages of the two series of data. This result was confirmed by the correlation values among the series of data ($r^2 = 0.8$; $p < 0.01$), indicating that there existed indeed a strong correspondence between observed and calculated data (Figures 8, 9, 10, and 11).

Equations to predict monthly values for each variable are presented in Table 4, all being significant ($p < 0.01$). It can be noticed that the fit was improved due to the reduction of variance. SW values obtained during the monthly samplings from August 1996 to November 1997, were compared with the calculated values for SW from the predictive equation (Figure 12). Neither the ANOVA nor the t-student test indicated significant differences between the two sets of data or their averages ($p < 0.05$).

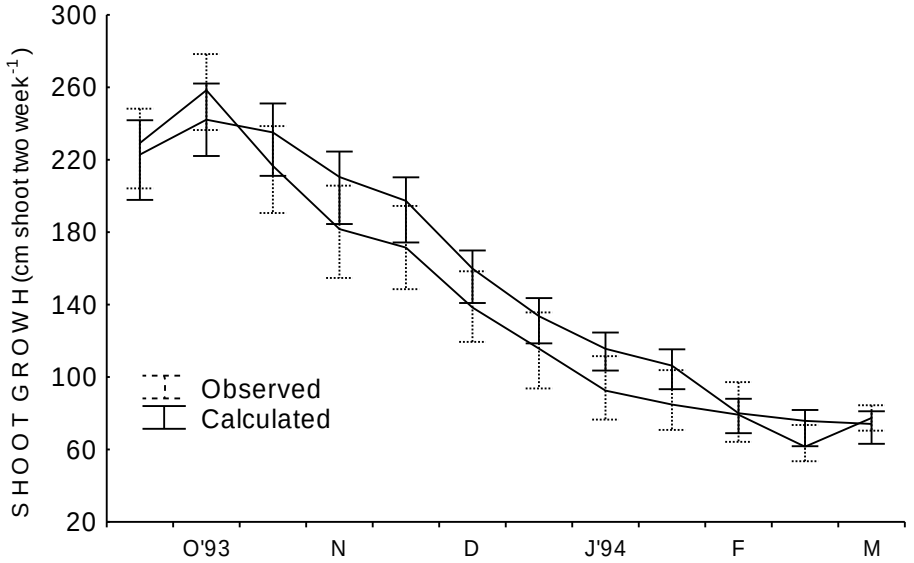

Figure 8. Comparison of observed and calculated Shoot Growth (SG) values (cm shoot^{-1} two week^{-1}) from October 1993 to March 1994. Average (142 and 154), highest (258 and 242), lowest (62 and 74), and SE (19.5 and 18.7), respectively from each data series. Bars represent ± 1SE.

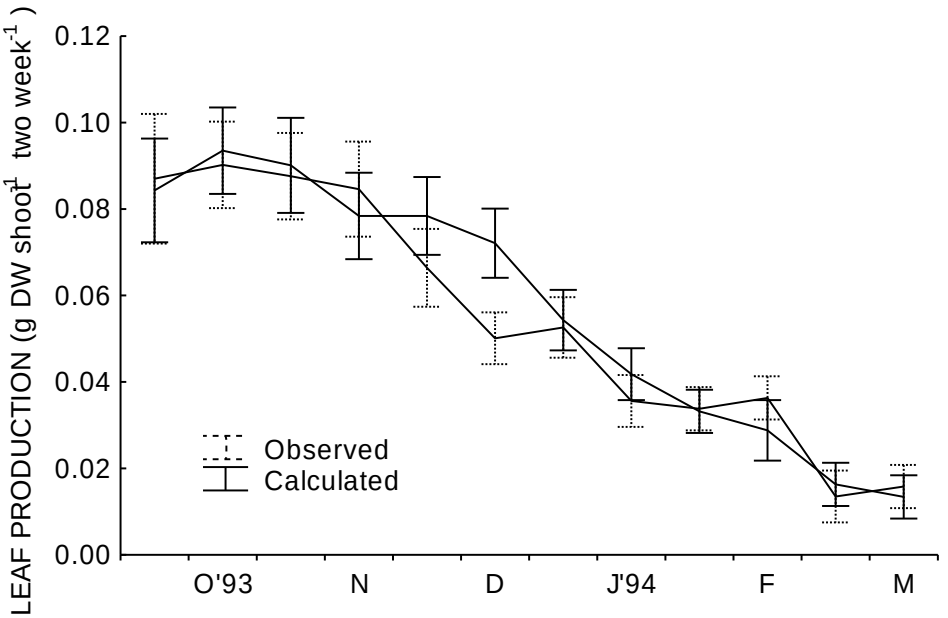

Figure 9. Comparison of observed and calculated values for Leaf Production (LP) (g DW shoot^{-1} two week^{-1}) from October 1993 to March 1994. Average (0.054 and 0.57), highest (0.090 and 0.094), lowest (0.013 and 0.013), and SE (0.0085 and 0.0085), respectively from each data series. Bars represent ± 1SE.

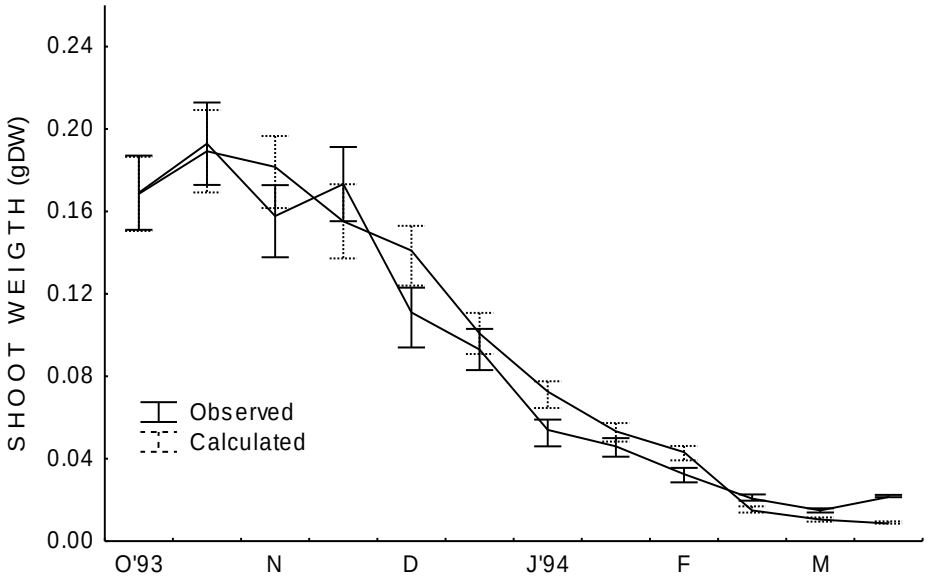

Figure 10. Comparison of observed and calculated values for Shoot Weight (SW) (g DW shoot^{-1}) from October 1993 to March 1994. Average (0.13 and 0.189), highest (0.19 and 0.18), lowest (0.019 and 0.013), and SE (0.019 and 0.020), respectively from each data series. Bars represent ± 1 SE.

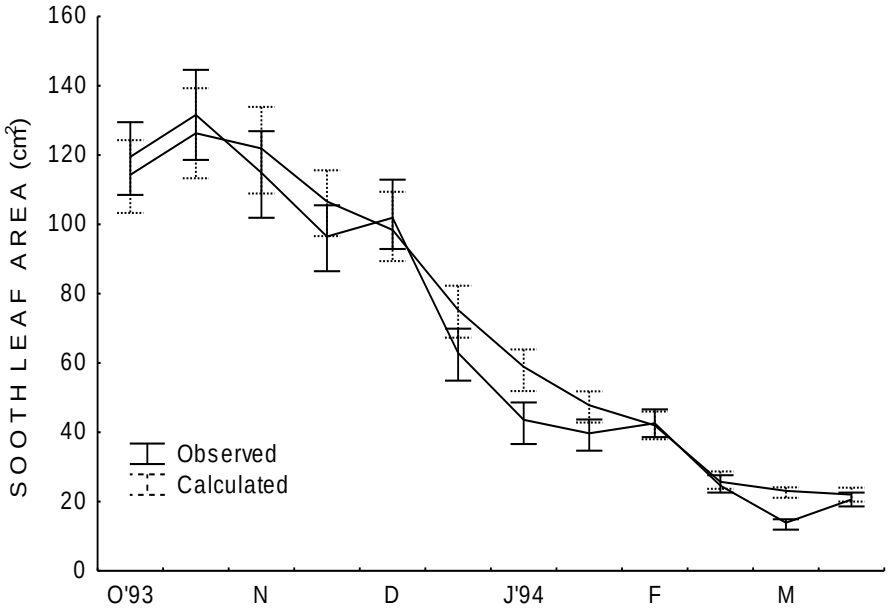

Figure 11. Comparison of observed and calculated values for shoot leaf area (cm^2) from October 1993 to March 1994. Average (67.7 and 71.9), highest (131.6 and 126.3), lowest (13.9 and 22.0), and SE (12.3 and 11.6), respectively from each data series. Bars represent ± 1 SE.

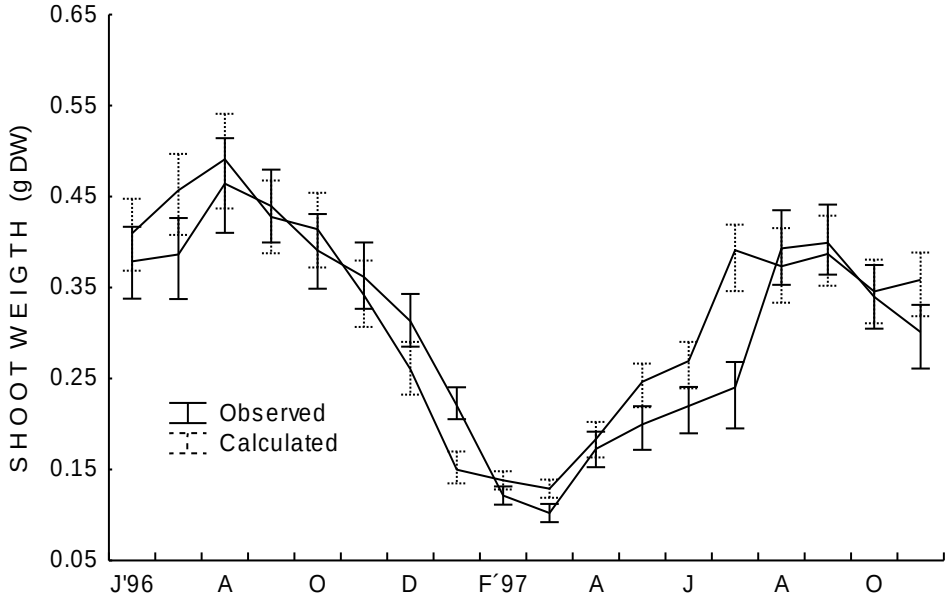

Figure 12. Comparison of observed and calculated values for Shoot Weight (SW) (g DW) from June 1996 to November 1997. Average (0.302 and 0.321), highest (0.464 and 0.491), lowest (0.102 and 0.129), and SE (0.25 and 0.26), respectively from each data series. Bars represent ± 1 SE.

DISCUSSION

In this study, we have explored whether the length of the outermost sheath could be used to predict *Z. marina* shoot growth and leaf production. Our results indicate that, although higher correlation values corresponded to leaves or sheaths in position 1 and 2 (Figure 1 and Table 1), and these structures could have been used as well, the use of the OSL is more convenient as it prevents shoot destruction. We validated our statistical models for two-week and monthly time intervals, finding no significant difference between observed and estimated values.

Using statistical techniques, not only we have confirmed Ibarra-Obando and Boudouresque (1994), and Meling-López and Ibarra-Obando (1997) results for SG and LP estimation but have also been able to predict two more variables: shoot weight and shoot leaf area. To transform SW estimates to biomass values we just need to incorporate shoot density values (Meling-López and Ibarra-Obando, 1999). Rapid assessment of biomass, growth and production are necessary, as these variables are considered basic in understanding seagrass beds structure (den Hartog, 1970), and to monitoring *Z. marina* beds (Gaeckle et al., 2006).

The fact that a strong correlation exists between all the selected variables (Table 3) represents another alternative to predict one variable from the other. Indeed, Poumian-Tapia and Ibarra-Obando (1999) have mentioned that eelgrass biomass in San Quintín Bay could be estimated from LAI (Leaf Area Index) values but this procedure is more time consuming, as all the leaves in a shoot need to be measured. Instead, the sheath method requires that only the OSL be measured.

The use of regression equations as a technique to estimate values for the previously mentioned *Z. marina* variables have the advantage of presenting a fast, reliable and non-destructive technique; however, these equations could be site specific and may need to be developed for every location. If it is true, this will require fieldwork to obtain values for those variables to be predicted. Although initial field work is required to establish the relationship between sheath length and leaf growth, Gaeckle et al., (2006) showed that a set of four periodic measurements is adequate. Once the regression equations have been developed, they would need to be validated with extra fieldwork, but once the relationship is established between sheath length and leaf growth, measurement of sheath length alone yields an accurate assessment of current leaf growth any time during the year (Gaeckle et al., 2006). Although the proposed sheath technique might seem tedious, complicated and time-consuming, once the regression equations have been developed**,** field work will be considerably reduced, and the lab work could be not necessary, and sampling can be extended to new locations, areas and depths, allowing comparisons,

The result indicates that two-week and monthly equations can be used to predict SG and LP, SW and SLA in an easy and reliable way, and that the OSL is a good indicator of eelgrass dynamics, instead most of the techniques used to asses both leaf growth and shoot production in Z. Marina, Lake leaf marking or plastochron are expensive, destructive, more lab time consuming (Kentula and McIntire, 1986, Ibarra-Obando and Boudouresque 1994, Short and Duarte, 2001), and the data are difficult to explore or needs an expensive statistical program as Echavarría-Heras et al., (2012, 2013) and Solana-Arellano et al., (1991, 2008).

Long-term monitoring of eelgrass growth and biomass is important to capture seasonal and yearly trends (Gaeckle et al., 2006), but both of them are hard to measure accurately (Short and Duarte, 2001), the technique we are proposed is a reliable form to save time and explore data in an easy way.

As seagrasses in general present a similar simple morphology (den Hartog, 1970; Duarte, 1991), equations like the ones we have developed for Z. marina on the Pacific coast of Baja California should be feasible to develop for other seagrass species, and locations. If we consider the possibility of a different growth dynamics in each geographic location, then the probability exists that the sheath length represents a different proportion of the total leaf length and as a consequence of SG, LP, SW, and SLA as well. Then we must emphasize the importance to developing predictive equations for each study region as the only way to prevent over or underestimation of the variables of interest.

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Chapter 22

DISTRIBUTION AND ECOLOGY OF THE PACIFIC LOBSTERETTE *NEPHROPSIS OCCIDENTALIS* FAXON, 1893 (CRUSTACEA, DECAPODA, ASTACIDEA), ON THE CONTINENTAL SLOPE OFF WESTERN MEXICO

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ABSTRACT

The geographic and bathymetric patterns of distribution of *Nephropsis occidentalis*, the only marine decapod crustacean of the infraorden Astacidea known from the eastern Pacific Ocean, were studied on the continental slope off the Mexican Pacific, and environmental drivers of those patterns were investigated. Samplings were performed using an Agassiz dredge and a benthic sledge in three main areas: the Gulf of California, the west coast of the Baja California Peninsula and the southern Mexican Pacific. Twelve sampling cruises were performed from 1991 to 2014, by which 171 hauls were obtained between 522 and 2309 m. Temperature, oxygen, salinity and total organic matter from sediments were sampled simultaneously, and data of surface production were recorded. The relationship between the patterns in abundance of *N. occidentalis* and potential environmental drivers was explored through generalised linear models. In addition, all previously published and unpublished records of the species were compiled and bathymetric patterns of distribution along latitude were explored. Within Mexico, 85 specimens of *N. occidentalis* were captured over a narrow bathymetric range, mainly between 1000 and 1300 m, at oxygen concentrations above hypoxia. Because of its high level of activity, this species is likely excluded from hypoxic waters. Males and females were of similar size and larger individuals were restricted to depths greater than 1100 m. The bathymetric patterns of distribution of *N. occidentalis* were associated with oxygen and temperature, probably because of the interdependence between aerobic capacity and temperature tolerance in ectothermic animals. In addition, surface production enhanced

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aggregations of *N. occidentalis* four months later. In the Mexican Pacific, *N. occidentalis* occurs in environmental conditions with 0.22-0.87 ml O_2 l^{-1} and 3.4-4.4 ºC. Including unpublished records, a total of 61 localities where the species occurs were compiled and a continuous distribution of *N. occidentalis* along the continental slope of the Eastern Pacific from Mexico (ca. 27ºN) to Chile (ca. 33ºS) was confirmed. The first depth of appearance of *N. occidentalis* slightly decreased southwards and somehow followed the bathymetric distribution of the OMZ, thus confirming the effects of such structure on the distribution of the species. Finally, fishery potential of the species is briefly addressed and compared to what is known about similar deep-water species worldwide.

Keywords: Astacidea, *Nephropsis occidentalis*, oxygen minimum zone, Eastern Pacific, deep-sea ecology

INTRODUCTION

The family Nephropidae is a relatively diverse taxon in the world's oceans, with 21 species in the Atlantic, 34 species in the Indian Ocean, and 31 species in the Pacific (Wahle et al., 2012). Within the Pacific Ocean, this family presents a very heterogeneous distribution, with as many as 30 species occurring in the western part vs. none in the central Pacific, and only one, *Nephropsis occidentalis*, in the eastern Pacific (Wahle et al., 2012). This species also constitutes the only marine Astacidea species occurring in this region. Moreover, the own genus *Nephropsis* is rather diverse in the Indo-West Pacific (9 species) and the Atlantic (5 species) (Chan, 2010), but not in the eastern Pacific.

Species from the genus *Nephropsis* are burrowers and possess a robust first pair of chelae and small unpigmented eyes (Schiff & Hendrickx, 1997; Chan, 1998) (Figure 1). *Nephropsis* spp. are mostly distributed from ca. 135 to 2560 m depth, with the majority of species occurring between ca. 500 and 1250 m. From a morphological point of view, within the Nephropidae, the genus *Nephropsis* is closer to *Nephropides* than to the rest of the family's genera (Ahyong, 2006). However, phylogenetic relationships based on molecular analysis indicate that *Nephropsis* is closer to *Metanephrops* (Wahle et al., 2012).

Nephropsis occidentalis, commonly known as the Pacific lobsterette, was described by Faxon (1893) (Figure 1A) based on material collected off Acapulco and the Tres Marias Islands. It is a relatively large (up to 135 mm of total length: Hendrickx, 2003) benthic astacid lobster distributed from the southern Gulf of California and the west coast of the Baja California Peninsula to Valparaíso, Chile (Bahamonde, 1959; Manning, 1970; Holthuis, 1991; Hendrickx, 1995; Hendrickx, 2003). It has been recorded at depths from 270 to 1310 m, but in Mexico the bathymetric distribution of this species is restricted to depths between 1050 and 1310 m, below the core of the oxygen minimum zone (Hendrickx, 2003).

Oxygen minimum zones (OMZs) occur worldwide and are large oceanic areas where dissolved oxygen concentration in the water is below 0.5 ml l^{-1} (Stramma et al., 2010). Those severely affect the distribution of marine benthic communities and, within OMZ cores, where conditions of severe hypoxia take place, macro- and megafaunal organisms are virtually absent (Levin, 2003; Gooday et al., 2009). At the lower boundaries of OMZs, where oxygen concentration increases gradually, megabenthic and benthopelagic organisms feature massive aggregations (Diaz & Rosenberg, 1995; Levin, 2003; Hendrickx, 2012a) and a rapid taxonomic replacement with depth is observed related with the size and swimming capacity

of taxa (Wishner et al., 1990; 1998; Murty et al., 2009). Below those boundaries, abundance of megafauna decreases with depth despite the continuous increase in dissolved oxygen in the water. Both the patterns in oxygen concentration in the water an in food availability have been repeatedly discussed as the main drivers of fauna bathymetric distribution. However, the works available to date have mainly been performed at a community level, and intraspecific patterns of distribution in OMZ areas and autecology of key species have barely been addressed (Jeffreys et al., 2012; Hendrickx & Papiol, in press).

The largest OMZ of the world's oceans is located in the eastern Pacific (Díaz & Rosenberg, 1995; Levin, 2003) and it distributes from ca. 60ºN to 30ºS (Helly & Levin, 2004). The amplitude of this OMZ varies with latitude, its lower boundary being found at shallower depths southwards, and its intensity being lowest at northern latitudes. Maximum thickness and intensity are observed at latitudes from ca. 25ºN to 8ºN, encompassing the continental margins of Mexico, Guatemala, El Salvador, Costa Rica, and Panama, where the OMZ ranges from ca. 100 to 1000 m depth (Helly & Levin, 2004; Fuenzalida et al., 2009). Below the OMZ off the Mexican Pacific, considerably abundant and diverse communities of benthic invertebrates have been reported across several studies (Zamorano et al., 2007; Méndez, 2007; Massin & Hendrickx, 2011; Hendrickx et al., 2011; Zamorano & Hendrickx, 2012). In this area, *N. occidentalis* is a typical member of the decapod crustaceans' community, which is dominated by some large benthic (e.g., *Heterocarpus affinis*, *Lebbeus scrippsi*) and benthopelagic (e.g., *Acantephyra brevicarinata*, *Nematocarcinus* spp.) shrimps, and large to medium-sized squat lobsters (e.g., *Galacantha diomedae*, *Munidopsis depressa*) (Hendrickx, 2001; 2012a; Papiol & Hendrickx, in press).

Despite its potential as a fishery target (Retamal, 1977; Holthuis, 1991), little additional knowledge on the biology and ecology of *N. occidentalis* is available and, to our knowledge, only some information on the morphometrics of the species has been published (Hendrickx, 2003). A similar situation takes place for the rest of *Nephropsis* spp., for which knowledge is limited to sparse documents dealing with their distribution (Pequegnat & Pequegnat, 1983; Macpherson, 1991; Cartes et al., 2007), morphometrics (Dineshbabu, 2008), and physiology (McAllen et al., 2005).

During exploratory cruises off the west coast of Mexico performed from 1991 to 2014 a relatively large series of specimens of *N. occidentalis* was captured. The present contribution reports the material not cited in Hendrickx (2003) and provides information on the distribution and ecology of this species along the Pacific coast of Mexico. In addition, it integrates the information of the published and unpublished records of *N. occidentalis* across its entire range of distribution. Finally, the fishery potential of the species is briefly addressed comparing it with that of other astacid lobsters.

MATERIAL AND METHODS

The Mexican Pacific

Within the TALUD project, 12 cruises were performed on the Mexican Pacific slope from 1991 to 2014 (Table 1). A total of 170 stations were explored in three main areas: the southern Gulf of California (GC), the western coast of the Baja California Peninsula (WBaja),

and the southern Mexican Pacific (SMPac). Samples were obtained within and below the OMZ core (defined herein as the fringe where oxygen concentration is <0.5 ml l^{-1}), from ca. 700 m to ca. 2200 m in both the GC and the WBaja, and from ca. 850 m to ca. 2200 m in the SMPac.

Sampling of Biological Data

Two different fishing gear were used for sampling: on the 1991 cruise (TALUD III) a total of 12 hauls were performed using an Agassiz dredge (horizontal length 2.50 m; vertical height 1 m), and in the rest of the surveys samples were obtained using a benthic sledge (horizontal length 2.35 m; vertical height 0.9 m) (see Hendrickx, 2012b). Both sampling gear were equipped with an outer collecting net of ca. 5.5 cm (2 1/4") stretch mesh and an inner net of ca. 2.0 cm (3/4") stretch mesh. Considering the similarity of the two gear and the low mobility of the target species, results obtained by both sampling gear were analysed jointly. All samplings were carried out on board of the R.V. "*El Puma"* at an average speed of 1.75 knots and the time on the bottom of each haul was on average 30 minutes.

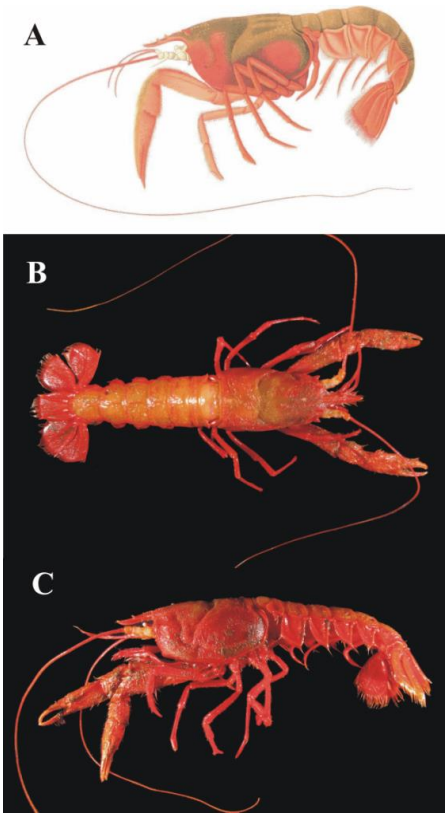

Figure 1. (A) Original illustration of *Nephropsis occidentalis* (from Faxon, 1895);
(B) *Nephropsis occidentalis*, dorsal view (TALUD) (EMU-9830). C. Same, lateral view.

Once identified, all specimens of *N. occidentalis* were sexed, counted, measured (carapace length, *CL* in mm; ± 0.1 mm) and weighed (± 0.1 g) at the laboratory. Most specimens were preserved in ethanol and deposited in the Regional Collection of Marine Invertebrates (EMU) at UNAM in Mazatlán, Mexico.

Sampling of Environmental Data

Depth was measured with an EdoWestern analogic (TALUD III-VIII) or a Simrad digital (TALUD IX-XVIB) recorder. Temperature, salinity, and oxygen (the latter two only from TALUD VII and on) were measured around 20 m above the sea bottom (20mab) with a Seabird 19 CTD equipped with an oxygen sensor at each station. Rosette-mounted 10 litres Niskin bottles were also deployed and dissolved oxygen content was estimated with the Winkler method (Strickland & Parsons, 1972) during all samplings. Oxygen values obtained with the two methods (CTD and Winkler) were usually similar. Winkler data were used in the statistical analysis and those obtained with the CTD were plotted vs. depth.

Table 1. Date, depth range, minimum and maximum latitude and longitude, number of hauls performed and number of *Nephropsis occidentalis* specimens collected during the oceanographic cruises conducted within the TALUD research project

Cruise	Dates	Latitude range (N)	Longitude range (W)	Depth **range** (m)	# of hauls	# individuals
TALUD III	17-23 August 1991	22°35'48" 26°10'18"	106°37'42" 109°58'24"	712-1360	12	3
TALUD IV	23-28 August 2000	21°58'24" 25°53'59"	106°28'53" 110°11'17"	778-2250	15	4
TALUD V	13-17 December 2000	21°59'14" 25°46'01"	106°28'30" 109°42'39"	730-2140	13	11
TALUD VI	13-17 March 2001	22°00'01" 25°45'49"	106°28'06" 109°53'59"	770-1600	14	25
TALUD VII	5-10 June 2001	22°00'24" 26°06'30"	106°31'36" 110°10'30"	740-1600	15	7
TALUD VIII	16-19 April 2005	24°24'24" 26°03'42"	109°25'06" 110°50"6"	700-2200	10	0
TALUD IX	11-15 November 2005	24°23'48" 26°04'42"	109°17'36" 110°47'42"	626-2309	13	0
TALUD X	9-15 February 2007	26°35'16" 28°14'50"	110°21'10" 112°26'50"	820-1865	16	0
TALUD XII	28 March-2 April 2008	16°49'18" 19°30'27"	100°30'52" 105°26'20"	865-2125	18	12
TALUD XIV	7-11 April 2011	28°16'03" 29°08'53"	112°17'40" 113°25'28"	522-1165	7	0
TALUD XV	29 July-5 August 2012	22°58'15" 27°05'42"	110°27'54" 114°35'30"	750-2136	15	23
TALUD XVIB	20 May-3 June 2014	28"28'18" 31°48'00"	115°24'06" 117°13'00"	710-2093	22	0

Sediment Sampling and Analysis

Sediments were sampled by means of a Smith-McIntyre dredge or a modified USNEL box core at each sampling station and samples of the top 3 cm were obtained and stored at 4-8°C. At the laboratory, total organic matter contents of sediments (TOM_{sed}), calculated as the difference between dry weight (DW: 60°C to constant weight) and ash weight (500°C in a furnace for 2 h), were obtained and used as indicators of food availability for benthos (see Beaulieu, 2002).

Phytoplankton pigment concentration (PPC, mg Chla m^{-3}) values were obtained from http://reason.gsfc.nasa.gov/Giovanni and were used as an estimate of surface primary production in the area. This was used to assess the response of bathyal decapod crustaceans to surface production processes (Cartes et al. 2004). Monthly average PPC values recorded for each locality on the date of sampling (PPC_{sim}) and 1-6 months before (PPC-1 to PPC-6) were used in order to evaluate the time lag between surface processes and the faunal response.

Data Analyses

Population Structure

Geographical patterns of distribution of *N. occidentalis* collected during the TALUD survey in western Mexico were evaluated by plotting the presence/absence of the species on a map. Abundance and biomass of *N. occidentalis* were standardized to a common swept area of 1 hectare (ind ha^{-1} and g ha^{-1}, respectively). In order to determine the depth distribution of the species, abundance and biomass patterns with depth were analysed for the Mexican Pacific as a whole, grouping samples every 100 m. Statistical differences in bathymetric patterns of abundance and biomass of *N. occidentalis* were tested. All data were tested for normality using the Kolmogorov-Smirnov test. For data that were normally distributed, ANOVA were used. For data that did not satisfy the assumptions of normality, even after transformation, a non-parametric Kruskal-Wallis or Mann-Whitney U test was used.

Sexual and bathymetric differences in *CL* were tested for the whole Mexican Pacific using a Mann-Whitney U and a Kruskal-Wallis test, respectively.

Environmental Variables

The bathymetric patterns of near-bottom temperature (T_{20mab}), salinity (S_{20mab}), and oxygen (O_{20mab}), and of TOM_{sed} in each sampling area where the species was present were examined. In addition, environmental maximum and minimum thresholds of distribution of the species were recorded.

Drivers of Population Structure

In order to identify which variables explained the patterns of distribution of *N. occidentalis*, values of abundance at all depths were compared with independent explanatory variables by means of generalized linear models (GLM). Abundance values were log-transformed and the distribution family used was Gaussian with identity link function. The models were computed by adding single terms based on minimizing Akaike's Information Criterion and only including variables that were significant ($p < 0.05$). Environmental variables included were: T_{20mab}, O_{20mab}, PPC_{sim}, PPC-1 to PPC-6, and TOM_{sed}.

All statistical analyses were carried out with STATISTICA 10 (StatSoft Inc.) and R 3.1.2 (http://www.r-project.org/) softwares.

Worldwide Distribution

Previously published and unpublished records of catches of *N. occidentalis* worldwide were compiled and the geographic distribution of the species was explored. The bathymetric distribution of the species in relationship with latitude was investigated and compared with latitudinal patterns of dissolved oxygen concentration in the water described by Helly & Levin (2004).

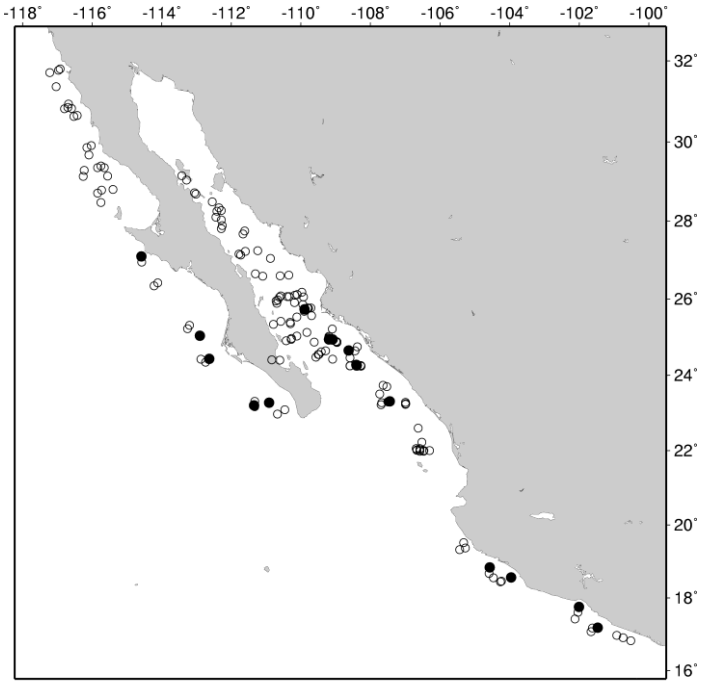

Figure 2. Position of the samplings performed during TALUD cruises with presence (●) and absence (○) of *Nephropsis occidentalis* indicated.

RESULTS

The Mexican Pacific

Population Structure

A total of 85 specimens of *N. occidentalis* were collected in the Mexican Pacific in 18 hauls out of the 170 initially performed (Table 2). Northernmost latitude of collection of *N. occidentalis* was 27.1°N, off Baja California (Table 2; Figure 2).

Within the Gulf of California, *N. occidentalis* was neither collected on the peninsular slope nor at latitudes above 26°N. Therefore, samples obtained above 27.1°N off Baja California (TALUD XVI-B cruise; see Table 1) and samples obtained at the peninsular slope and at the northern sampling sites within the Gulf of California (TALUD VIII, IX, X, XIV) were not considered for abundance and biomass estimations and comparisons. Seventeen of the hauls (= 94%) containing *N. occidentalis* were obtained between 980 and 1310 m, and only two specimens were caught in one haul located at 772-786 m (Table 2). Bathymetric analyses of abundance and biomass for the Mexican Pacific revealed greatest values of both parameters between 1100 and 1300 m (Figure 3). Moreover, on the continental slope of the Gulf of California, all specimens were caught between 1100 and 1300 m, and no specimens were present between 1000 and 1100 m. Main Kruskal-Wallis test for bathymetric changes in abundance ($H_{13,101}$ = 42.55; P < 0.001) and biomass ($H_{13,101}$ = 43.02; P < 0.001) were significant. However, the large number of hauls without *N. occidentalis* provided high intra-

samples variance which probably led to the lack of statistical significance when testing for bathymetric differences in abundance and biomass across 100 m depth intervals.

All specimens collected were adults. Sex ratio (M : F) was 1:0.65. Overall, sizes of males and females were similar (Mann-Whitney U test, $n_F = 34$, $n_M = 51$, $P = 0.80$) and ranged from 18.3 to 42.3 mm, 84% of the specimens ranging from 24 to 40 mm (Figure 4a). The size of individuals changed with depth (K-W test $H_{3,85} = 10.83$; $P < 0.05$), larger individuals ($CL >$ 35 mm) being restricted to depth greater than 1100 m (Figure 4b).

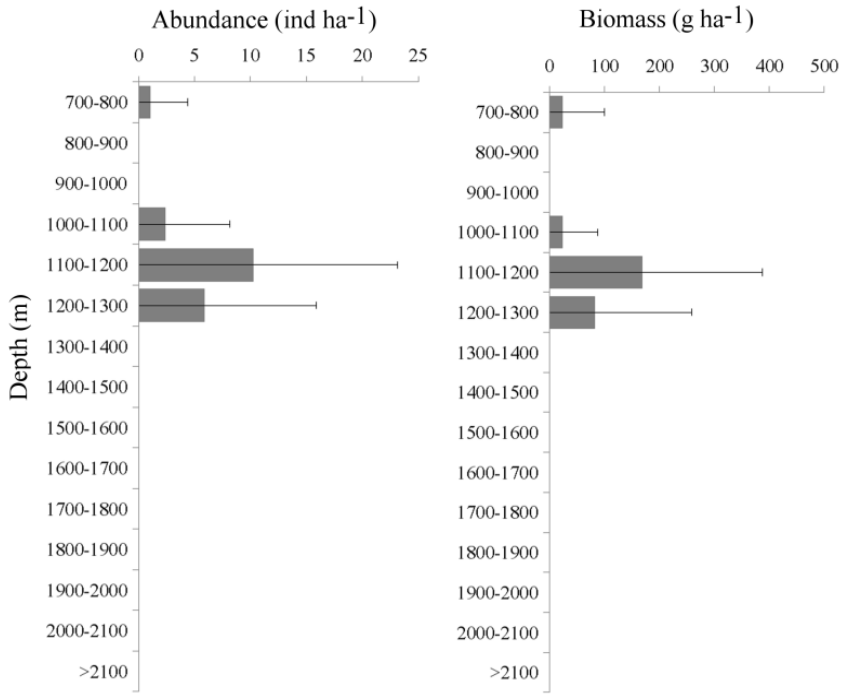

Figure 3. Bathymetric patterns in abundance and biomass of *Nephropsis occidentalis* collected in the Mexican Pacific considering the entire bathymetric interval sampled during the survey.

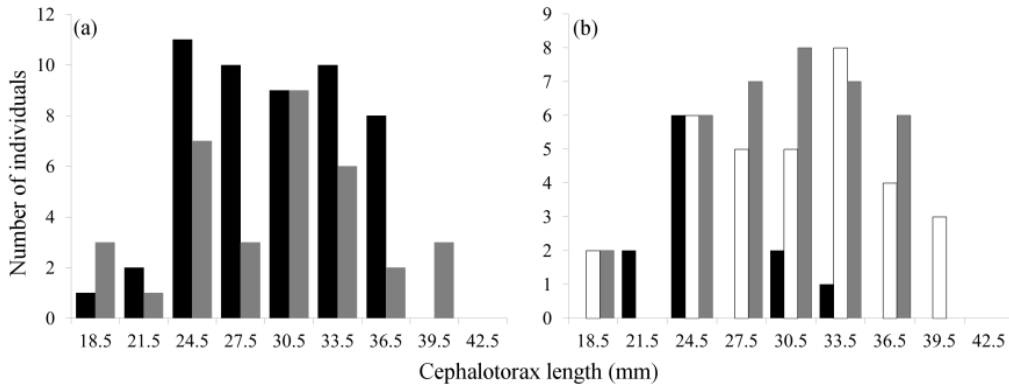

Figure 4. Cephalotorax length distribution (a) of males and females of *Nephropsis occidentalis* (black = males; grey = females) and (b) by depth range (black = 1001-1100 m; white = 1101-1200 m; grey = 1201-1300 m).

Table 2. List of specimens (number and size range by sex) of *Nephropsis occidentalis* collected during the TALUD cruises. Catalogue number of each lot, sampling location, date and depth are also indicated

EMU	Locality	Position	Date	Depth (m)	Males		Females (ovigerous)	
		Latitude (N) Longitude (W)			N	*CL* range (mm)	N	*CL* range (mm)
Gulf of California								
4048	Off Bahía Santa María	24º38'48" 108º37'48"	Aug 19, 1991	1198	1	25.4	2	31.1-40.0
5517	Off Ensenada del Pabellón	24º16'24" 108º24'18"	Dec 15, 2000	1180-1200	5	25.3-37.3	4(1)	28.7-42.3(37.1)
5518	Southern Gulf of California	24º56'24" 109º05'36"	Aug 26, 2000	1200-1274	0		1	33.5
5534	Off Ensenada del Pabellón	24º15'18" 108º24'06"	Aug 25, 2000	1245	3	33.2-35.0	0	
5535	Southern Gulf of California	24º56'18" 109º11'48"	Dec 16, 2000	1280-1310	1	25.1	1	33.3
5536	Gulf of California	23º18'36" 107º27'56"	Mar 14, 2001	1050-1160	0		1	39.6
5966	Off Ensenada del Pabellón	24º16'12" 108º23'42"	Jun 7, 2001	1160-1180	4	24.3-35.0	3	25.1-32.6
5967	Off Isla Lechuguilla	25º43'50" 109º53'59"	Mar 17, 2001	1240-1270	6	31.7-35.5	2(1)	30.5-34.1(34.1)
5968	Southern Gulf of California	24º56'18" 109º06'42"	Mar 16, 2001	1190-1270	1	26.5	2	25.6
Southern Mexican Pacific								
8733	Off Lázaro Cárdenas	17º45'16" 102º00'29"	Mar 30, 2008	1198	2	30.6-34.7	1	20.0
8734	Off Guerrero	17º11'03" 101º28'05"	Mar 28, 2008	1180-1299	1	29.1	1	19.5
8735	Off Michoacán	18º33'43" 103º57'45"	Abr 1, 2008	1058-1088	2	21.4-24.2	2	21.7-24.2
8736	Off Colima	18º50'19" 104º34'14"	Abr 2, 2008	1101-1106	2	36.8-37.3	1	35.8
Western Baja California								
9826	Off western Baja California	24º 25'48" 112º38'06"	Jul 30, 2012	1212-1235	3	29.3-29.7	0	
9827	Off western Baja California	23°16'42" 110º54'55"	Aug 5, 2012	980-1036	3	24.4-26.3	4(1)	24.8-34.9(34.9)
9830	Off western Baja California	25º02'12" 112º54'06"	Jul 30, 2012	1210-1245	3	18.3-32.1	1	26.7
9829	Off western Baja California	23°12'02" 111º20'50"	Aug 4, 2012	1118-1150	3	27.5-33	4	20.3-30.7
9828	Off western Baja California	27°05'42" 114º35'30"	Aug 1, 2012	772-786	2	23.2-28.4	0	
Not in the collection								
	T VI-34 Off Isla Lechuguilla	25º43'50" 109º53'59"	Mar 17, 2001	1240-1270	9		4(1)	

Environmental Variables

In the three areas where *N. occidentalis* was present, at depths greater than 700 m T_{20mab} decreased with depth while both O_{20mab} and S_{20mab} increased with depth (Figure 5), the latter increasing very slightly. O_{20mab} started increasing shallower on the slope off WBaja than in the GC and the SMPac. Patterns of T_{20mab} were similar in the three areas, but S_{20mab} was in general lower off WBaja. TOM_{sed} was not available for 15 samples (i.e., 9%). For the rest of data, both in the GC and the SMPac greatest values of TOM_{sed} were found at the shallowest depths sampled (ca. 700-900 m) (Figure 6). Below those depths, TOM_{sed} values were stable. Off the WBaja, TOM_{sed} values did not follow any bathymetric pattern and were generally lower than in the other two areas.

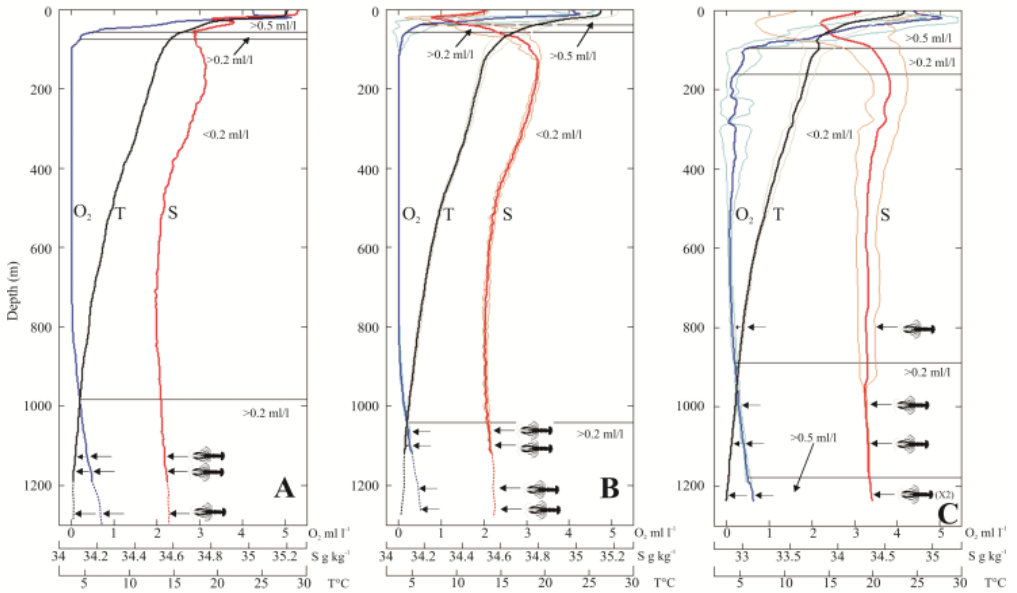

Figure 5. Bathymetric profiles of average (and standard deviation) temperature, salinity and oxygen measured for the three areas in which *N. occidentalis* was collected: (a) Gulf of California (Talud VII St. 19), (b) southern Mexican Pacific (TALUD XII St. 10, 13, 23, 28), and (c) western Baja California Peninsula (TALUD XV St. 8, 5C, 13, 2, 24). Upper and lower boundaries of the 0.2 and 0.5 ml l^{-1} dissolved oxygen concentrations (horizontal lines) OMZ and presence of *Nephropsis occidentalis* (arrows) indicated.

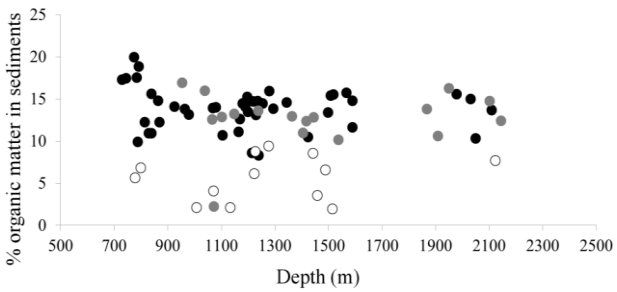

Figure 6. Percentage total organic matter in sediments by area (black = continental slope of the Gulf of California; grey=southern Mexican Pacific; white = western Baja California).

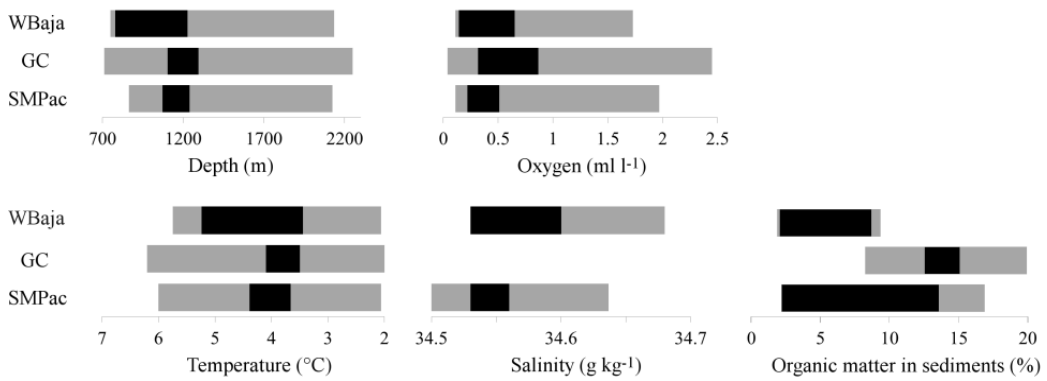

Figure 7. Ranges of depth, oxygen, temperature, salinity and organic matter in sediments sampled (light grey) and ranges corresponding to captures of *N. occidentalis* (98% of individuals captured: black; remaining 2% captured: dark grey). WBaja = western Baja California; GC = Gulf of California; SMPac = southern Mexican Pacific.

Ranges obtained for environmental parameters (Figure 7) indicate that *Nephropsis occidentalis* occurred over a relatively narrow oxygen range (0.14-0.87 ml l^{-1}), although most specimens (98%; the two specimens collected at 779 m not being considered) were present at $O_{20mab} > 0.22$ ml l^{-1}. Lowest oxygen concentration tolerated by *N. occidentalis* was similar in the three areas analysed. T_{20mab} where *N. occidentalis* were collected ranged from 3.4 to 5.2°C (98% of specimens between 3.4 and 4.39°C). S_{20mab} were not available for the continental slope of the GC. In the other areas, *N. occidentalis* was collected at S_{20mab} between 34.53 and 34.60 g kg^{-1}. Regarding TOM_{sed}, both in the WBaja and at SMPac *N. occidentalis* was found from the lowest values measured (ca. 2%) to almost maximum values obtained (8.7% and 13.6%, respectively). In the GC, *N. occidentalis* appeared at greater values of TOM_{sed}, from 12.6% to 15.1%.

Drivers of Population Structure

The GLMs performed on the abundance data considering all samples explained 15% of the total variation (Table 3), with O_{20mab}, T_{20mab} and PPC-4 as the main drivers of abundance patterns of distribution.

Worldwide Distribution

A total of 61 records of *N. occidentalis* (Tables 2, 4) were compiled, 37 of which were unpublished records. Those confirmed that *N. occidentalis* is an endemic species of the eastern Pacific. The species is continuously distributed from the west coast of Baja California (27.1ºN), Mexico, to Valparaiso (ca. 33ºS), Chile, with confirmed captures from off the coasts of El Salvador, Nicaragua, Costa Rica, Panama, Ecuador, and Peru (Table 4; Figure 8). Considering all captures, *N. occidentalis* is restricted to depths greater than 700 m along its distribution range, except for two specimens collected at 533-569 m off Peru and a single specimen collected at 270 m off Valparaiso, Chile (33ºS), coinciding with its southernmost distribution limit.

The analysis of the bathymetric distribution of *N. occidentalis* with latitude revealed that shallowest depths of occurrence slightly decreased from north to south (Figure 9). Between latitudes of ca. 27°N to ca. 11.5ºN organisms were found at ca. 1000-1300 m; between 11.5ºN and 11.5ºS most samples were obtained at ca. 800-1000 m. At latitudes around 17ºS organisms were distributed along a wider depth range, being observed from 800 to ca. 1250 m. The bathymetric distribution of the species somehow matched the profile of the OMZ described by Helly and Levin (2004) (Figure 9), this being especially noticeable in the U-shaped pattern of distribution from 27.1ºN to ca. 6.5ºN and in the relatively shallow samples collected at ca. 4ºS at 551 m (depth range of haul 533-569 m) and at ca. 33ºS at 270 m.

Table 3. Generalized linear models performed on abundance values of *Nephropsis occidentalis*

	Estimate	*p*	*F*	% Explained deviance
Intercept	1.24	<0.01		
O_{20mab}	-0.49	<0.01	0.023	5.39
T_{20mab}	-0.20	<0.05	0.016	6.08
PPC-4	0.13	0.07	0.08	3.23
Total explained deviance		14.70%		
AIC		99.58		

O = oxygen; T = temperature; 20mab = 20 m above the seabed; PPC-4 = phytoplankton pigment concentration four months before the sampling; AIC = Akaike's information criterion.

Table 4. List of worldwide records of *Nephropsis occidentalis*, other than those of the TALUD survey

	Depth range sampled (m)	Depth range captures (m)	Number of individuals captured	Number of hauls captures	Source
Mexico	22-3482	945-1236	32	5	(1) (2)
El Salvador	57-1368	1054	Unk	1	(3)
Nicaragua	122-1471	784-1116	Unk	5	(3)
Costa Rica	119-1530	836-1212*	Unk	6	(3)
Panama	108-1467	954	Unk	1	(3) (4)
Ecuador	500-1500	835-977	2	2	(5)
Peru	200-1500	700-1300	62	23	(6) (7)
Chile	Unk	ca. 270- 1000	5	3	(8) (9)

Unk = unknown. * = Depth range sampled for two hauls not known.

Source: (1) Faxon, 1893; (2) Manning, 1970; (3) J. López pers. comm.; (4) R. Vargas, pers. comm.; (5) Cornejo-Antepara, 2010; (6) Macpherson, 1990; (7) Kameya et al. 1997; (8) M. Retamal pers. comm.; (9) G. Guzman pers. comm.

DISCUSSION

The distribution of a species depends on the influence of a variety of interacting abiotic and biotic factors both at the macro- and the mesoscale (Boschi, 2000; Sexton et al., 2009). Below OMZs, the interaction between dissolved oxygen concentration and food availability

has often been discussed as the main driver of species' bathymetric distributions (e.g., Levin et al., 1991; Levin et al., 2009; Mosch et al., 2012; Levin & Sibuet, 2012). However, few studies have established direct relationships between fauna patterns and environmental drivers, especially regarding benthopelagic megafauna. The few existing works have usually been performed at the community level (Murty et al., 2009; Papiol & Hendrickx, in press), and studies on population distribution and ecology are even scarcer (Jeffreys et al., 2012; Hendrickx and Papiol, in press).

In this chapter the patterns of distribution of *Nephropsis occidentalis*, a benthic lobsterette living below the OMZ of the Eastern Pacific Ocean (Hendrickx, 2003), were analysed at the mesoscale level off Mexico, and the environmental factors controlling those patterns were explored. In addition, information on the distribution of *N. occidentalis* at the macroscale was compiled, and bathymetric patterns of distribution along latitude were investigated.

Mexican Pacific

Population Structure

Nephropsis occidentalis is a relatively large benthic decapod crustacean with a unimodal size distribution of the population off the Mexican Pacific ranging from 18.3 to 42.3 mm *CL*. Maximum size coincides with the specimen already reported by Hendrickx (2003), which constitutes the largest specimen known to date.

This lobsterette mainly inhabited depths between 980 and 1310 m, with oxygen concentrations above hypoxic values (i.e., > 0.2 ml l^{-1}; Kamykowski & Zentara, 1990), along the whole Mexican Pacific. Megafauna, such as crabs and shrimps, are rare where oxygen drops below 0.1 ml l^{-1}. Low swimming capacity benthic decapods (e.g., *Munidopsis* spp.; McAllen et al., 2005) with low metabolic rates (Company & Sardà, 1998) proliferate below the OMZ cores at water with oxygen concentration below 0.2 ml l^{-1} (e.g., Wishner et al., 1990; Murty et al., 2009; Papiol & Hendrickx, in press), and more mobile taxa with higher metabolic demands for oxygen are usually found in water with higher oxygen concentration. Although *N. occidentalis* is a benthic species with little natatory ability, *Nephropsis* is a very active genus (McAllen et al., 2005). Therefore, high metabolic requirements of *N. occidentalis* probably exclude it from hypoxic water and its distribution is restricted to depths greater than ca. 1000 m, where water is more oxygenated. Surprisingly, two individuals were recorded in a haul performed at depths of 772-786 m and oxygen concentration of 0.14 ml l^{-1}. Temporal excursions into less oxygenated waters for escaping predators or finding food have been observed in other species (Gooday et al., 2009) and seem a plausible explanation for this finding, as some Nephropidae are known to be adapted to short periods of hypoxia (Eriksson et al., 2013).

The peak in abundance of *N. occidentalis* in the lower limit of the OMZ core and its distribution within a narrow depth range of ca. 300 m is somehow parallel to the pattern of distribution observed for decapod crustaceans below OMZs in other regions (e.g., Wishner et al., 1990; Murty et al., 2009). Aggregations of organisms below OMZ cores have been attributed to the large accumulation of food in these areas. The restriction of *N. occidentalis* within such a narrow depth range could be driven by the parallel restriction of its food source to this stratum. It is likely that *N. occidentalis* is sustained by benthic and benthopelagic

macrofauna that peak in narrow bands below OMZ cores (Wishner et al., 1995; 2008; Mosch et al., 2012), as the species' powerful chelipeds and the presence of polychaetes, decapod crustaceans, and fish remains in their stomachs (authors' unp. data) suggest it is an active predator on macrofauna. The paucity of macrofaunal prey at greater depths (Wishner et al., 1995; Levin et al., 2000; Mosch et al., 2012) could cause the parallel disappearance of *N. occidentalis*.

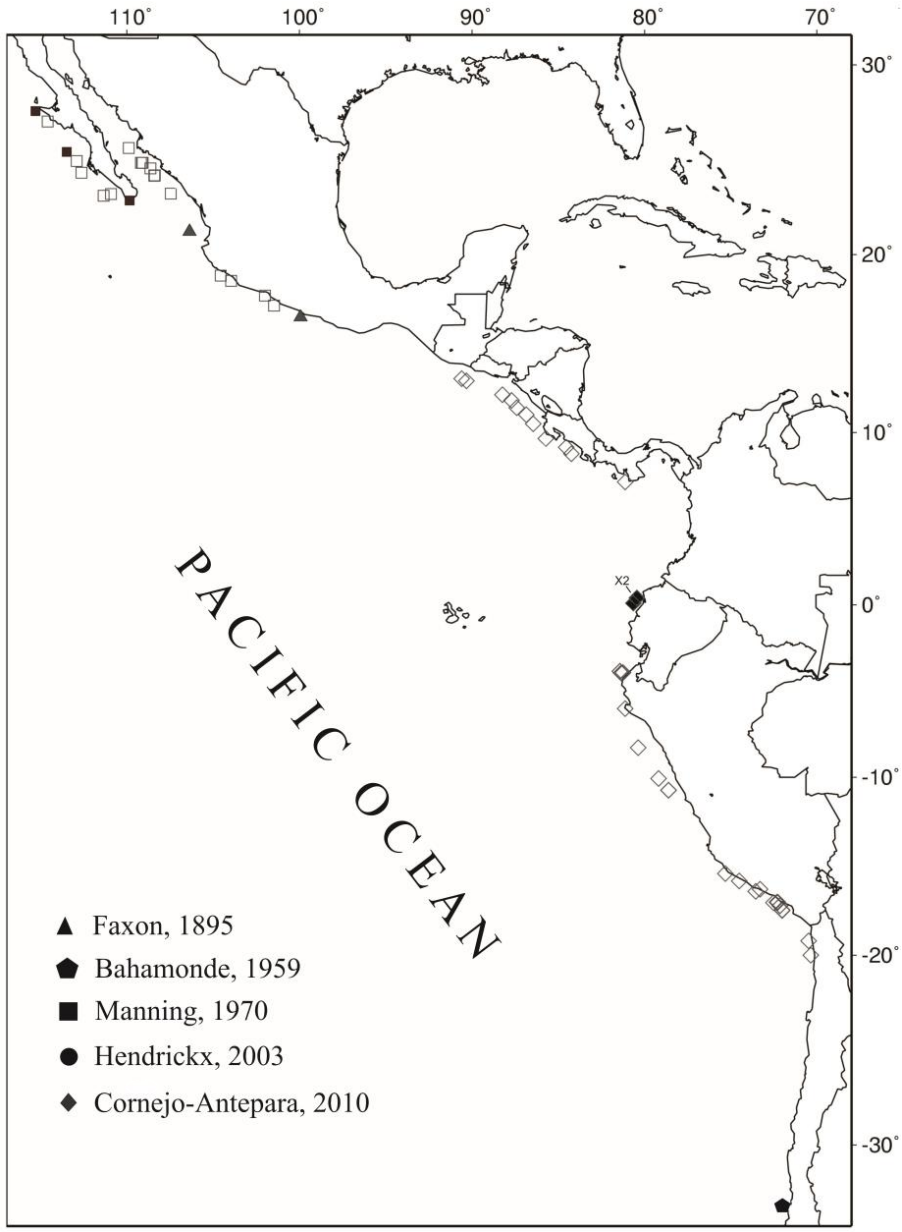

Figure 8. Distribution of *N. occidentalis* in the eastern Pacific including previously published records (solid symbols) and additional records (open symbols). See Tables 2 and 3.

Although the low number of organisms available forces cautious interpretation of the data, results suggest that within the narrow bathymetric range of distribution of *N. occidentalis* there was size structure in which larger individuals were restricted to depths greater than 1100 m. Bathymetric size segregation within a given habitat is usually attributed to food availability together with how species exploit existing food resources (Rowe, 1971; Carey, 1981). The depth pattern observed in *N. occidentalis* could be associated with the presence of different potential food sources (i.e., prey items) through the depth gradient (Wishner et al., 1995; Levin et al., 2009) coupled with ontogenic variations that could take place in the diet of *N. occidentalis*, as reported for other Nephropidae (e.g., *Metanephrops thomsoni*; Choi et al., 2008).

Notwithstanding the relatively large range of sizes collected, all specimens obtained during the TALUD surveys were adults. Although the selectivity of the gear mesh size is biased towards capturing medium to large-size individuals, small specimens (i.e., <15 mm total length) of other species (e.g., *Galacantha diomedeae*, *Munidopsis* spp.,) have been regularly collected using the same net (authors' pers. observ.). The bathymetric distributions of smaller specimens of *N. occidentalis* as well as their recruitment patterns remain therefore unsolved, as occurs with the majority of decapod crustacean species from below OMZs. Little is known about the occurrence of larvae or juveniles of deep-water decapod crustaceans in general and specially within or below the OMZ off the Mexican Pacific (see Hendrickx & García-Guerrero, 2007; Thatje et al., 2005). Besides, only three ovigerous females were obtained, ranging from 34.1 to 37.1 mm CL, which is very close to the maximum known size of the species (42.3 mm CL). These eggs-carrying females were collected in March, August and December, an indication that reproduction probably occurs all year-round.

Environmental Drivers of the Distribution of the Pacific Lobsterette

A large amount of variation was not explained by the models obtained in our study, probably due to the complex and very patchy distribution of megafauna in the Mexican Pacific (Méndez, 2007; Papiol & Hendrickx, in press), which is likely enhanced by the clustering of burrows observed in some species of Nephropidae (Dybern & Höisaeter, 1965). Besides, the availability of information on some additional variables (e.g., salinity, prey availability, or $\delta^{13}C$ of sediments) that have been identified as important drivers of decapod crustaceans' distribution in other studies (Murty et al., 2009; Papiol et al., 2012; Fanelli et al., 2013) could contribute to increase the amount of variation explained in our models. However, combined effects of both oxygen and temperature partially controlled the distribution of *N. occidentalis* over the depth gradient. As mentioned above, metabolic rates associated with activity level of a species somehow define its minimum oxygen thresholds. Coherently, the active *N. occidentalis* is excluded from hypoxic environments. Also, the positive influence of temperature on metabolic rates (Childress et al., 1990) likely reinforces the exclusion of *N. occidentalis* from shallower, less oxygenated, warmer waters. In addition, aerobic capacity and temperature tolerance are interdependent processes within most ectothermic animals. There are both high and low species-specific critical temperatures that lead to tissue hypoxia through limited ventilation and circulation performance (Frederich & Pörtner, 2000; Pörtner, 2001; Ekau et al., 2010). In the Mexican Pacific, the optimum temperature range for aerobic performance of *N. occidentalis* seems to be between ca. 3 and 5°C at dissolved oxygen concentrations of between 0.87 and 0.22 ml l^{-1}.

Within OMZ areas, the interaction between oxygen and food availability has often been discussed as main driver of species' distribution. In the present chapter the foremost importance of food was not patent, most probably due to the lack of adequate food indicators (e.g., $\delta^{13}C$ composition of sediments or biomass of potential prey; Murty et al., 2009; Fanelli et al., 2013). However, food inputs generated at surface favoured aggregations of *N. occidentalis* four months later. The relationship between fauna patterns and surface production is coherent with the occurrence of short food chains (Fanelli et al., 2013) that have been proposed below OMZ cores (Wishner et al. 1995). Zooplankton taxa potentially inhabiting the lower boundary of the OMZ (Wishner et al., 1995; 2008; Roullier et al., 2013) likely channel the fresh organic matter sinking from surface to megafauna (Fanelli et al., 2011).

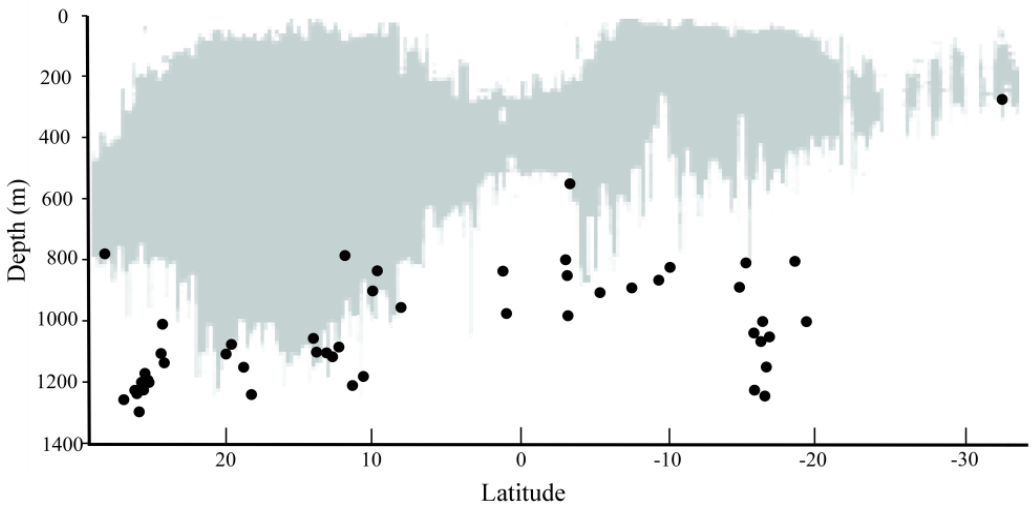

Figure 9. Bathymetric distribution (●) of *Nephropsis occidentalis* along a latitudinal gradient from Mexico to southern Chile. The depth range of the OMZ corresponding to upper and lower values of ≤ 0.5 ml l^{-1} is indicated (grey) (adapted from Helly & Levin, 2004).

Worldwide Patterns of Distribution

The latitudinal range of distribution of *N. occidentalis* was not expanded with respect to previous knowledge, but the 37 additional records compiled in the present chapter confirmed that the species is continuously distributed all along a very large section of the continental slope off western America, from ca. 27º40'N off Baja California, Mexico, to ca. 33ºS, off Valparaiso, Chile. Most works dealing with invertebrate zoogeographic regionalization at the macroscale have been performed on littoral and shelf areas (e.g., Brusca & Wallerstein, 1979; Boschi, 2000; Castillo & Guiñez, 2000), and some studies have been performed at hydrothermal vents (Won et al., 2003; Wolff, 2005) and seamounts (Samadi et al., 2006). Despite the relative scarcity of deep-water samplings, Wicksten (1989) investigated zoogeographic provinces based on decapod crustaceans occurring from 50 m to depths greater than 1500 m. Both shallow and deep-water studies coincided in the segregation of the ocean margin from California to central Chile into several provinces characterised by different species (Wicksten, 1989). *Nephropsis occidentalis* is distributed along this entire coast, and thus its distribution is considered particularly wide.

To our knowledge, *N. occidentalis* has not been reported further north or south of the range reported herein, thus some biotic and/or abiotic factors must be preventing the species' expansion beyond these geographical limits (Sexton et al., 2009). Among those factors, the most important usually listed are the geological history of each area, topographic barriers, physical-chemical gradients, and biological factors (Briggs, 1974; Vermeij, 1978; Wicksten, 1989). The northern and southern limits of distribution of *N. occidentalis* are related with the inflow of low salinity intermediate water masses (i.e., the North Pacific Intermediate Water, NPIW: Fiedler & Talley, 2006; and the Eastern South Pacific Intermediate Water, ESPIW: Schneider et al., 2003). The inflow of the NPIW in the north is coherent with the lower salinity and its greater oscillations observed in the southern Baja California in this study (see Figure 5). We hypothesize that the changes in the salinity due to the presence of haline fronts could be above the tolerance limit of *N. occidentalis*, constituting a barrier for the dispersal of the species. Small changes in the physical properties of the environment especially affect species living in the deep sea, where the environment is usually stable (Gage & Tyler, 1991). Although the salinity minima of both water masses (NPIW and ESPIW) are located shallower than the range of distribution of *N. occidentalis*, they could still be affecting the distribution of their planktonic larvae.

Inside the Gulf, the distribution of *N. occidentalis* was limited to the continental side of the slope. The sedimentary and less precipitous character of the continental shelf and slope contrasts with the steep and calcareous rocky nature of the peninsular coast (Shepard, 1950; Thunell, 1998), and may provide more suitable habitat for burrowing decapods such as *N. occidentalis*.

Our results (both by Winkler and CTD) suggested a deeper location of the lower boundary of the OMZ ($O_2 = 0.5$ m l^{-1}) for the Mexican Pacific than that reported by Helly & Levin (2004). Nevertheless, the latitudinal pattern of the shallowest depth of collection of *N. occidentalis* was somehow parallel to that of the lower OMZ boundary, with a southward trend of shallower collection of *N. occidentalis*. This distribution pattern is consistent with the above discussed importance of oxygen concentration as a limiting factor for *N. occidentalis* proliferation below the OMZ. In addition, *N. occidentalis* was usually distributed over narrow bathymetric ranges, likely due to the edge effect at the OMZ lower boundary, as described above. In contrast, at latitudes of 15-19ºS *N. occidentalis* occupied a relatively wide bathymetric range. There is no clear explanation for this. The OMZ edge effects caused by the food accumulation and rapid transformation may be significantly reduced in this area where the OMZ is less intense, and there could be no need for *N. occidentalis* to adapt to this particular landscape. However, many other environmental (biotic or abiotic) factors could be causing this different distribution, and further studies would be required to clarify this issue.

Fishery Potential

With a worldwide increasing interest for new fishery resources in deep waters (Roberts, 2002; Norse et al., 2012), many species of decapod crustaceans that had been traditionally ignored are now regarded as potentially important. The "langoustines", "lobsterettes" or "scampi" are no exception to this and recent surveys have been undertaken in order to evaluate the stocks and gather information on their biology (Lynch & Garvey, 2005; Choi et al., 2008; Paramo & Saint-Paul, 2012; Robey et al. 2013). In the specific case of the genus

Nephropsis, Pequegnat & Pequegnat (1983) reported three species in the Gulf of Mexico. Densities (orgs ha^{-1}) varied considerably according to depth and from one species to another. *Nephropsis agassizii* was collected in depths of 900-1600 m with maximum density (ca. 200 orgs ha^{-1}) observed at 950 m. *Nephropsis aculeata* occurred in depths of 350-1350 and ranked second in population density (maximum of ca. 100 orgs ha^{-1} at 500 m). The third and less abundant species, *N. rosea* (ca. 25 orgs ha^{-1}), was also the shallowest (500-750 m) with population peak at 700 m. Guéguen (2000) reported *N. aculeata* (321-491 m depth) and *N. rosea* (547-854 m depth) as two of the major species of decapod crustaceans from off the coast of Guyana, with catch per hour of trawl comprised between ca. 23 and 637 g. *Nephropsis aculeata* has also been reported as an abundant species off the Mississippi river and East Florida, with captures of up to 40 kg h^{-1} (Holthuis, 1991). This species is distributed at depths of 137-824 m in the Gulf of Mexico, Caribbean Province and extended to the north off Massachussets. Another species that has recently caught the attention is *Nephropsis carpenteri*, occurring around India and Japan. This forms part of the by-catch of the deep-sea shrimps' fishery (e.g., *Aristeus*, *Solenocera*, *Metapenaeopsis*, *Heterocarpus*) off Chenia, India, and represents up to 40% of total catch with maximum size of 154 mm total length (Thirumilu, 2011). Morphometric relationships and fishery aspects of the Indian Ocean lobsterette *Nephropsis stewarti* have been documented by Dineshbabu (2008) for the SW coast of India. The species is fished between 250 and 500 m and yields an average annual catch of 23.3 t, with a sharp decrease after a peak of captures in 2001-2002, and is processed together with scylarids lobsters and mostly exported. A survey in the same area in 2006, however, indicated that *N. stewarti* might have a very limited importance because catches are restricted to a rather reduced area (Jayaprakash et al., 2006). Small catches of *N. stewarti* of up to 0.5 kg h^{-1} of trawl have also been reported off Madagascar (Crosnier & Jouannic, 1973). Although the maximum size of this species is greater than that of *N. occidentalis* (total length 113 mm in males, 158 mm in females), almost 90% of the catch corresponded to specimens with a length range of 80-105 mm, similar to what has been observed with *N. occidentalis*.

Because information related to the abundance and distribution of *N. occidentalis* in the eastern Pacific is still very limited, it is difficult to evaluate if a profitable fishery could be initiated. Holthuis (1991) referred to a potential interest for this species off the coast of Chile and Retamal (1977) reported this lobsterette as a common by-catch of the *Heterocarpus reedi* fishery. Almost 40 years after this report by Retamal (1977), no fishery has apparently been developed for *N. occidentalis* off Chile (G. Guzman, pers. comm., 2014; M. Retamal, pers. comm., 2014) and captures of this species have been incidental, with only a few specimens available in museums collections (Table 2). Although it is likely that *N. occidentalis* still forms part of the *H. reedi* fishery (that produced a total capture of ca. 4,350 t $year^{-1}$ from 2006-2010) (Wehrtmann et al., 2012), no specific information seems to be available on the potential use of this lobsterette in the area or on its ecology (P. Arana, pers. comm. 2014).

Bottom trawling is the major fishery in deep-sea habitats (Kaiser et al., 2002), and technical advances in the last 6 decades have allowed the exploitation of these habitats (Thiel, 2003; Clark et al., 2007; Benn et al., 2010). For example, in the Mediterranean Sea, deep-water bottom trawling targeting *Aristeus antennatus* (400-800 m) is common (Cartes & Sardà, 1989; Cartes et al. 2008). However, it is well known that trawl fisheries have an enormous environmental impact, which is especially notable on deep-water communities (Gray et al., 2006; Althaus et al., 2009, Maynou & Cartes, 2011), and any intention to switch part of the current fishery activity in that direction should be carefully evaluated. Besides, in

the particular case of western Mexico, targeting deep-water crustaceans or other deep-water species (e.g., Holothuroidea, Macrouridae) occurring below the OMZ would imply deployment of fishing gear at depths greater than 700-800 m in most areas, a costly operation with few guarantees of sustainability.

OUTLOOK

The investigation on the distribution and ecology of decapod crustaceans living below OMZ areas is still in its early stages, and further studies are not only necessary but urgently required in order to understand the role of each species within the communities. Additional multidisciplinary studies encompassing samplings of biotic and abiotic data are essential in order to better understand the distribution of *N. occidentalis*. However, considering that both oxygen and temperature are drivers of the bathymetric distribution of deep-water decapods, there is a potential susceptibility to the global pattern of expansion of OMZs resulting of climate change. Consequently, the study of deep-water communities in these areas should be considered a priority.

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Chapter 23

SEA TURTLES AS ECOSYSTEM INDICATORS: SITUATION IN MEXICO

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ABSTRACT

Sea turtles are migratory vertebrates that are linked to many coastal and marine habitats in different parts of the world depending on the stage of their life cycle. These agencies have given them a higher value as a sentinel species by characteristics such as longevity, size, resilience to environmental changes of the seas, and even changes induced by humans as environmental pollution. One of the main risks to sea turtles is drowning in gill nets or trawl fisheries used in flake or shrimp; it has been extensively analyzed in different regions of the country and the world. However, the effects of environmental pollution on the health of sea turtles is the least studied. The environment has been altered dramatically in recent years by rising temperatures, increased severity, and frequency of storms and rising sea level, where most human settlements have been deforested and changed land use for urban, port and tourist developments. In the case of turtles, these changes affect species differentially throughout his life. For example, climate change and temperature variations can affect the sex ratio of the hatchlings and nesting patterns since they depend on healthy beaches to lay eggs, so the migration patterns of females could be affected significantly by climate change. The dependence of sea turtles of seagrass meadows, mangrove forest, rhodolith beds, Sargassum forest, coral reefs and deep ocean to live, give them the importance of indicators of the environmental health of these ecosystems. Understanding how pollution and climate change may affect these ecosystems not only benefit conservation programs of populations of sea turtles, but also the diversity associated with them. On which they depend on millions of people living along the shores of the world using marine resources and ecosystem services for their economic activities and food safety.

INTRODUCTION

Several studies claim that over-exploitation of fishery resources, climate change, environmental pollution, and the resulting acidification of the oceans, threatening marine ecosystems. Some of the most common contaminants derived from human activity are pesticides, herbicides, chemical fertilizers, detergents, hydrocarbons, sewage, plastics and other solids. Many of these pollutants accumulate in the deep ocean, except plastic that accumulates mostly on the surface, where they are ingested by small marine organisms introduced in the global food chain (Beltran et al. 2005). The threat of extinction faced by sea turtles worldwide has meant that today all species are included in Appendix I of the resolutions of the Convention on International Trade in Endangered Species of Flora and Fauna Threatened (CITES 2001). The International Union for Conservation of Nature and Natural Resources (IUCN 2006) lists all species and subspecies of sea turtles in danger of extinction, with three of them in critical condition: the olive ridley (*Lepidochelys kempii*), hawksbill turtle (*Eretmochelys imbricata*) and the leatherback turtle (*Dermochelys coriacea*), while the Endangered Species Act of the United States and the Inter-American Convention for the Protection and Conservation of Sea Turtles (IAC) regards them as threatened or endangered. In Mexico, the degree of threat and protection of sea turtles is established in the Mexican Official Standard NOM-059-ECOL (Official Journal, September 2010) while regulations for handling and care laboratory are reflected in NOM -162-SEMARNAT (February 2012).

A species that can illustrate the serious threat that hangs over these agencies is the hawksbill turtle. Exploitation that has made this kind of the value of its shell has caused a reduction in global population by over 80% in the last 105 years, and it is expected that this reduction will continue in the near future due to the activities of gathering of eggs in nesting areas, capture of juveniles and adult individuals for consumption, incidental killing in fishing gear (Gardner and Nichols 2001) or ship strikes both fishing tourism, intensely still happen in different areas where these organisms are distributed. The concern generated by this decline is reflected in different publications like Groombridge and Luxmoore (1989), the volume dedicated in 1999 by the journal Chelonian Conservation and Biology at depth examination of this kind, the "Status Listing Justification for the Hawksbill Turtle (*Eretmochelys imbricata*) as Critically Endangered on the 1996 IUCN Red List of Threatened Animals "by Meylan and Donnelly (1999) and" Status of the Hawksbill Turtle (*Eretmochelys imbricata*) in the Caribbean region "by Meylan (1989).

Currently, it is considered that only five populations exist hawksbill more than 1,000 nesting females annually in the Republic of Seychelles (Indian Ocean), Mexico, Indonesia and two in Australia. Four of these populations (Indonesia, Seychelles and in Australia) show a declining trend. The government of Australia has described the hawksbill populations in its territory as vulnerable; they are considered as the largest populations in the world (CITES 2001). In the last document prepared by the group of experts of sea turtles IUCN / SSC, it is mentioned that worldwide, only Mexico remains positive trends in population recovery (Guzman-Hernandez, 2005) as a result of more than ten years of protection. Cases like that of Mexico show that populations of this species may react positively to programs of long-term preservation (Frazier 2000).

BIODIVERSITY OF SEA TURTLES IN MEXICO

Mexico is considered one of the countries with the highest diversity of reptiles in the world and is considered as an important example sea turtles (Carabias et al. 2005). In the coast are six of the seven species in the world: the olive ridley (*Lepidochelys kempii*) that nests on the coasts of Tamaulipas and in small quantities in the coast of Campeche; hawksbill turtle (*Eretmochelys imbricata*) nesting on the coast of Campeche, Yucatan, Quintana Roo and another Pacific and Atlantic is the largest (Mortimer or Marquez or both) population, the green turtle (*Chelonia mydas*) whose records are in the Gulf of Mexico, Caribbean Sea and the Pacific; the loggerhead turtle (*Caretta caretta*) and the leatherback turtle (*Dermochelys coriacea*) nesting in the Caribbean and Pacific coasts. Since 1993, year in which the dramatic decline of the latter species was observed, actions were initiated along the Mexican Pacific coast looking for possible causes of this decline. For it was signed in 2003 the Tri Convention for the Recovery and Conservation of the Leatherback Turtle in the Eastern Pacific with the main objective to design and establish measures for the conservation and recovery of the stock of Eastern Pacific Leatherback and habitat which it depends, based on the available scientific evidence into account the environmental, socioeconomic and cultural characteristics of the parties. In that agreement it provides that, for the recovery of lutes, should be given full attention to terrestrial factors (alteration and destruction of nesting beaches, illegal collection of eggs, etc.) and marine (mainly the result of fishing activities and pollution), as well as playback or presence of the olive ridley (*Lepidochelys olivacea*) and the black turtle (Chelonia agassizii) whose nests are recorded in the Pacific (Marquez 2002; Guzman-Hernandez 2005; Sarti et al. 2007).

THREATS TO SEA TURTLES

Turtles have certain characteristics that make them particularly vulnerable to environmental disturbances such as:

CLIMATE CHANGE

In our country, it lives about 10% of the diversity of the planet, so it is considered a megadiverse country. The causes of this loss of biodiversity may be falling into two broad categories: 1) immediate causes: habitat destruction and fragmentation, overexploitation of wildlife resources, invasive species, pollution, and 2) structural causes: human population growth and consequent increase in resources mainly for food (Hawkes et al. 2006).

Urban and tourism development that has been taking place in coastal areas where turtles lay their eggs has caused problems at that stage of the life cycle. Buildings such as hotels, breakwater, fillers or sand extraction sites; as well as the elimination of the natural vegetation of the dunes, causing high erosion or soil depletion and affects suitable for sea turtles (Figure 1) environmental conditions.

Figure 1. Playa El Suspiro Turtle camp at Cabo San Lucas, BCS, under the effects of tropical storm Paul 2013.

Figure 2. Contamination by vegetation carried with the water in Playa el Suspiro, Los Cabos B.C.S. México after the storm "Norman" September 2012.

Van Houtan and Halley (2011) showed evidence supporting the damage of climate change on sea turtles. One of the consequences of climate change will increase the frequency and intensity of storms and hurricanes; events seriously harm sea turtles (Van Houtan and Bass 2007). There will also be a loss of their nesting sites as a result of sea level rise caused by climate change (Reece et al. 2013). The damages suffered by turtles are not limited to the influence of the marine environment, but also to the land portion due to the need of land areas

to spawn. Global changes in land use and poor logging practices, biodiversity impact and generate a large amount of organic waste that is carried by rivers to the sea (Fitzherbert et al. 2008). This shift logs and other organic waste can cause side impacts on multiple ecosystems (Foley et al. 2007). One of those ecosystems is the sandy beach where waste spewing from ocean currents and sea barriers are parallel accumulate. An excess of this type of plant residues can alter the nesting sea turtles and the survival of newly hatched individuals on their way from the nest to the sea (Figure 2). However, detailed studies of the effect of organic matter accumulated on beaches on nesting behavior and survival of nests and hatchlings of sea turtles are still as rare as needed on nesting beaches (Quinones et al. 2007).

Pollution

Jakimska et al. 2011b mentioned that turtles are exposed to many pollutants. Pollution is a negative outcome associated with the socioeconomic and technological development of humanity as one of the biggest problems that this development present is the generation of chemicals in various industrial activities have accelerated in recent decades. Some of these substances are released into the environment as byproducts of other processes and are used in different farming and crop protection (Kampalath et al. 2006). The seas and coasts are most affected since most of these chemicals eventually reach the sea. The seas not only receive the discharge of sewage and agricultural activities, but they are also dumped radioactive waste and much rubbish (and Vanegas Espina 2005). Currently there are some studies that have explored the effects of various pollutants on the health of marine turtles and their populations; among them we can mention the research conducted by Fitzgerald (2004) and Act-Quinonez et al. (2009) who determined the presence of heavy metals in marine turtles in northwestern Mexico; Morales-Rodriguez and Cobos-Gasca (2005) used as a biomarker to *Eretmochelys imbricata*, omnivorous species up to the beaches of Campeche each year to lay their eggs, to evaluate the temporal variation of the concentration of DDT and DDE between 1999 and 2000. Rosales et al. (2011) worked on understanding the role of chorioallantois in retaining organochlorine pollutants (pesticides) and their relation to other environmental variables: plasma sediment and olive ridley sea turtle (*Lepidochelys olivacea*) Baja California South. While Labrada and colleagues (2011) are evaluated the health status of the eastern Pacific green turtle (*Chelonia mydas*) inhabiting the coast of Baja California Sur, through physiological biomarkers. Thus, we can see that all these research highlights the extent of contamination affecting sea turtle populations.

Pesticides

Persistent organic pollutants (POPs) are organic compounds mostly man-made and characterized by its presence worldwide, its stability, the slower biodegradation, their accumulation in fatty tissues and its long half-life, van de Merwe and colleagues in 2010 showed damage *Chelonia mydas* embryos because of Persistent Organic Pollutants (POPs). Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCs) and dichloro-diphenyl-trichloroethane (DDT) and its derivatives have contaminated many environments, including oceans (Moore et al. 2002;. Labrada et al. 2011), spread over all geographic regions

because of its intensive use in agricultural and industrial activities. PCBs, as persistent organic compounds with a relative mobility, are among the chemicals that can reach any region that this is remote (Moore et al. 2002; Keller et al., 2004). Currently, atmospheric transport is recognized as the leading cause of dispersion and the primary source of pollution in the polar regions and the open sea. However, CBs continue to be detected in different environmental samples and animal tissues besides their use continues in some countries development due to lack of effective regulation (Godley et al. 1999, McKenzie et al. 1999; Lazar et al. 2011; Rosales et al. 2011).

Hydrocarbons

The study of oil pollution in the oceans and coastal areas, faces two opposite aspects of human activities: 1) the alteration of marine and coastal ecosystems caused by mining operations, refining, transportation, storage and use of oil as the main source of energy, and 2) the undeniable need to preserve and protect marine resources for our current uses and future generations (NAS 1985; Botello 2005). The exploration and extraction of hydrocarbons at sea pose a serious threat to sea turtles to stop their activities foraging and nesting habitat destruction seriously. Dredging not only destroys habitat but incidentally may cause serious injury or even kill sea turtles. The oil in the skin and shell of a sea turtle can affect breathing functions and salt gland and blood chemistry. When the oil slick reaches the beach, this is pasted into each grain of sand and rock affecting the turtles on the beach, at sea or in the sand where they lay their eggs. The latter is polluted and affected the development of embryos, on the other hand, at hatching, the chicks are also contaminated by the oil is on the beach (Figure 4). Ingestion of pellets tar is also of concern that the tar balls are the second most common type of debris ingested (NMFS / FWS 1992, 1998).

Spills in the Gulf of Mexico del Pozo Ixtoc-I off the coast of Campeche deserve special measurement. This spill was considered the largest in the world, because for nine months over 3'100,000 barrels of crude oil in marine waters (Botello 2005) were spilled. The oil spill from the platform of British Petroleum (BP) in April 2010 in this area affected the five species of turtles Lora (Kemp's ridley sea turtle), white (*Chelonia mydas*) loggerhead (*Caretta caretta*), leatherback (*Dermochelys coriacea*) and hawksbill (*Eretmochelys imbricata*). Of the seven known species of sea turtles living today, five live in the Gulf of Mexico (Fomix 2010). Sea turtles are not only damaged by direct losses. The daily consumption of petroleum products from shipping, burning coal and wood, as well as combustion motor, producing similar polycyclic aromatic hydrocarbon content of crude oil (Botello 2005).

Heavy Metals

The accumulation of heavy metals in marine life is of great interest because of the potentially toxic effects that these elements may cause, especially predators located in the highest parts of the food chain (Law-Quinonez et al. 2011) as the turtles.

Sewage and Garbage

It found the presence of debris such as plastic, Styrofoam, string, rubber, glass, metal, paper and woven in the stomachs of sea turtles are being transported by sea currents fibers. When ingested, these wastes can obstruct the digestive tract, causing health problems such as infections or death (Lazar and Gračan 2011).

Damage Caused by the Lights on Nesting Beaches

Alteration of nesting habitat has increased significantly (Witherington 2000). The beaches have been the pressure of growing demand (Chacon & Arauz 2001) for the development of tourism activities, construction of hotels, restaurants and residences (Lutcavage et al. 1997, Parra 2002), involving changes in the physical structure and an increase artificial light (Chacon & Arauz 2001). It has been documented that artificial lighting on nesting beaches affect sea turtles and produces disorientation of hatchlings emerging who diverted their way to the sea to follow the light signals (Kamrowski et al. 2012).

Other threats caused by the presence of man in the turtle habitat are the presence of domestic animals such as pigs, cats and dogs can become predators of eggs and chicks, and the use of motorized vehicles on beaches which crush eggs and hatchlings in addition to compact the sand difficult and output spawning nests (Day et al. 2007). Anthropogenic impact and beach erosion significantly impact the complex and delicate coastal and marine ecosystems, contributing significantly to the reduction of nesting habitat and therefore on populations of these organisms, since the females return to their natal beaches to nest (Márquez et al. 2007).

With these processes of biomagnification (being predators stops) and bioaccumulation (being lived animals), we can estimate the type and degree of exposure that are subject populations and from there information of interest for conservation and/or survival. It is known that sublethal exposures for extended periods of time can cause long-term toxic effects, such as reproductive disorders, decreased the ability of the immune defense system against infections and infestations, nervous and behavioral alterations and an increased risk of developing certain cancers (López-Castro et al. 2010; Law-2013 Quinonez et al.). These effects have been described in a considerable number of species, both terrestrial and marine, which leads us to believe that similar effects are also observed in marine turtles (García-Fernández et al. 2009; Páez-Osuna et al. 2010).

We start by defining marine pollution, biomarkers and sentinel species. Many of these terms can refer to several concepts, however, use the more accepted. Marine pollution is the introduction of substances and energy by the entry of water used for cooling industrial plants in the marine environment, which is harmful to live resources, it constitutes a risk to human health, harm marine activities as fishing damages the quality of sea water and reduces recreational activities (definition proposed by the experts of the United Nations (GESAMP 1980). The biomarkers are living organisms that are exposed to contaminants, or that may be useful for predict future damage and even may themselves present harmful effects. The context in which a biomarker falls to be interpreted in the biochemical, physiological, morphological or behavioral level (Badii-Zabeh et al. 2005, Cajaraville et al. 2000). The

species bioindicators used to detect environmental changes through a stress response that can be extended to other groups. They are used to reflect the biotic or abiotic state of the environment, reveal evidence of impacts caused by environmental changes or to indicate the diversity of other species, groups or communities in an area (Tabor and Aguirre 2004; Badii-Zabeh et al. 2005, Rendon 2005). Tabor and Aguirre said in 2004 that the use of biomarkers in the diagnosis of alteration of ecosystems has several advantages: a) the response of biomarkers can indicate the presence of pollutants biologically available, b) with a suitable battery set or biomarkers You can ascertain the presence of pollutants that had not been considered, c) can detect intermittent pollution events and integrate in time and space exposure, d) they can detect induced by complex mixtures of pollutants effects. In many cases, the use of biomarkers is much easier and cheaper to implement than other options, such as chemical analysis (Handy et al. 2003). Mayer et al. 1992 states that it is necessary to know the biochemistry and physiology of the organism, set the size and reproductive status, which is simple sampling, which is available in sufficient number and ages, know their trophic level and mainly delivering social and ecological importance. This is necessary to give a complete diagnosis of the condition of the body, and that serves to monitor environmental problems.

They can give examples of sea turtles as sentinels, so we have the leatherback turtle that may be a biomarker of changes in the short and long term food networks and the availability of resources in the overexploited marine ecosystems (Saba et al. 2008; Fossette et al. 2010). They are used to monitor the effects of dredging (McRae 2014).

Sea Turtles: Sentinels of Climate Change: Temperature determines the sex of the turtles. An increase in the average temperature could cause a bias in the sex ratio that could be used as evidence of climate change. The increase of air temperature will advance the date of nesting (Weishampel et al. 2004). The use of stable isotopes to identify the foraging area for turtles. Changes of places to eat in conjunction with the measurement of other variables can be indicators of climate change (Rodriguez Baron 2010; Strang et al., 2012.).

Sea Turtles: Sentinels of Pollution Contaminants Biocumulan (Jakimska et al. 2011a, 2011b.). Since a correlation was found between fibropapilloma tumors and highly polluted areas, it has been proposed to the disease as contamination indicator (Aguirre et al. 2004). The fibro papilloma tumors can cause weakening and eventually death of turtles green (C. mydas). From the 1980s, the incidence increased and has become the biggest threat to the green turtle (Gamache and Horrocks 1991) and has already been detected in other species.

There is evidence for maternal transfer of persistent organic pollutants into the eggs so that they can be used as indicators of pollution (Alava et al. 2011; Sakai et al., 1995).

Perfluorinated compounds (PFCs) are water-repellent properties oil are hardly degraded in the environment and can bio Cuma arse. They pose a threat to many organisms because they are toxic and endocrine disruptors. Evidence of their presence in turtles (Guerranti et al. 2013). Vitellogenin as a biomarker of endocrine disruption in turtles (Zaccaroni et al. 2010). Gonadal development partnership between the turtle and the concentration of the herbicide atrazine (De Solla et al. 2006).

Sea turtles are considered as potential indicators of the health of the marine, coastal habitats and local environments in which they live in temporary, annual or even decadal scales (Aguirre and Lutz 2004; Wallace et al., 2004). The longevity of individuals of sea turtles, the residence time on land or water, and the variety of marine habitats by traversing along his life story, return to these organisms susceptible to exposure to multiple stressors and Pollutants (Milton and Lutz 2003). Several studies have explored the vulnerability of the

turtles to these two factors, being that this will depend directly on their longevity (Milton and Lutz 2003; Aguirre et al. 2006)., Ecological niche, including diet and feeding strategy (Gardner et al. 2006 ; Kampalath et al. 2006), concentration and duration of exposure (Talavera-Saenz et al. 2007) and metabolic processes and homeostatic (Gardner et al. 2003;... Sakai et al. 2000).

Sea turtles are becoming one of the most popular icons of the environment. Taking advantage of his charismatic image, a remarkable variety of stakeholders including scientists, conservationists, community-based organizations, corporations and governments have sought to use sea turtles to motivate people to consider issues surrounding the health of the marine ecosystem, warning while a potential danger situation as demonstrating the presence of contaminants in the environment in which they live and the degree of exposure, the sustainability of fisheries, the potential benefits of protected areas, indigenous cultural value (often involving nationalism and cultural) pride, biodiversity and the desirability of cooperation and the multilateral management of shared marine and terrestrial species (Eckert and Hemphill 2005). In the context of the use of sea turtles as sentinel species it has been proposed to generate databases on parameters for: how to make environmental monitoring using sea turtles using data and analysis eggs in search of contaminants, and which it is an effective technique that causes little damage to the populations studied (Alava et al. 2011, Schneider et al. 2011). Use of tissue carapace has proven useful in the study of pollutants (Komoroske et al. 2011). Analysis of blood samples to detect contaminants such as lead, selenium and mercury (Burger et al. 2009) and has the advantage of being a minimally invasive technique (Labrada et al. 2011, Lara-Uc et al. 2011). Tissue analysis of recently dead turtles can be useful (da Silva et al. 2014.) To make the following analysis: in vitro culture of stem cells, for example, liver cells green turtle has potential in monitoring and evaluation of mercury contamination (Wang et al. 2013). Determining the affinity trophic by stable isotopes (Rodriguez Baron 2010; Strang et al., 2012). Antioxidant enzyme activity. This may be a good biomarker of contamination in turtles (Labrada-Martagón et al. 2011). The search for biomarkers that reveal the exposure of organisms to endocrine disruptors, for example, the presence of vitellogenin in males. Satellite tracking to monitor populations (Schofield et l. 2013), and the unborn tracking (Mansfield et al. 2014).

Advantages and disadvantages of using turtles as bioindicators. Is it easy sampling? A collecting permit is required; this is handled with the undersecretary for environmental protection management (Directorate General of Wildlife) specifying the objectives of the study and whether it is a research project or teaching purposes. They should have experience in sampling either blood, tissue, etc. Because if you do not have to experience results that are issued are be as reliable, on the material used, the shape of the blood collection, in which means of transport the fabrics are, as time has shown to the analysis from which the sample was drawn until delivery to the laboratory. A feature is a sentinel species that react very quickly to environmental disturbances. However, to be ancient turtles, the impact of these disturbances on the size of the populations occurs slowly so it does not reflect the effects of the unrest on short-lived species (disadvantage). At the same time a long-lived species can bioaccumulate more polluting than short-lived species, the latter exacerbated by being the top predators turtles.

Challenges

Develop a reliable method for sexing turtles and to estimate the proportions of sexes. As very long-lived, the impact of environmental stressors on the population size may be delayed. Such delay would cause the indirect environmental stressor effects could not be detected on time in species with a short life.

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Chapter 24

SIMILARITY IN MOLLUSK ASSEMBLAGES ASSOCIATED WITH CONSPICUOUS HABITAT FORMERS IN NORTHWEST MÉXICO

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ABSTRACT

Mollusks mesograzers are an important ecological factor in subtidal environments. We analyzed conspicuous habitats from Gulf of California and Mexican Pacific littoral in northwest to answer: 1) How are formed the associated mollusks assemblages? 2) Is taxocenosis structure maintained over time and space inside habitats? 3) How does biodiversity change between habitats? Mollusks were found in critical habitats *Zostera marina*, rhodoliths and *Sargassum* forest, and invasive red seaweed *Acanthophora spicifera*. We made clusters about their presence in different geographical sectors and surveys using qualitative and quantitative data. 75 small species were recorded. Rhodoliths was the habitat with higher richness (25) and diversity, as well eelgrass beds (24). The grouping shows one assemblage that contains localities with eelgrass, the second rhodoliths, and the third seaweeds. Small gastropods *Acteocina*, *Alabama*, and *Barleeia* were essential components in all hosts. The structure changed over time and between habitats. Results suggest similar assemblages in eelgrasses and distant regards brown/red seaweeds as well rhodolith beds. This study highlights the importance of *Zostera marina* beds by evidence of similarity even localities inside and outside gulf; coralline beds by preserving atypical diversity and brown/red seaweeds as transitional microhabitats and temporary corridors subject to further change. These interactions are more complex than estimated, but this contribution helps to understand these critical habitats as biological corridors and implications on mollusk distribution.

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INTRODUCTION

Eelgrass and seaweed constitute a sort of biodiversity as micro-habitats, are significant for recruitment, settlement, and development of fishes and invertebrates (Morse 1992; Russo 1999; Christie 2009), in which mollusks are part of mesograzers inhabiting and one of the major groups interest of them. In addition to the structure complexity for shelter, another reason is food availability (Albano & Sabelli 2012). Sometimes mesograzers enhance host condition by removing epiphytes or redirecting nutrients to the sediments (Fong et al. 2000; Rakocinski et al. 2008), another reason to highlight its importance is because they are contributors to secondary production. Despite this, studies remain poor in assessed spatial composition and variation of this group on subtidal habitats (Wernberg et al. 2008).

In the Gulf of California and Pacific Coast, researchers have documented marine and brackish-water molluscan biodiversity in geographical scaling using the whole of the substrates (Hendrickx et al. 2007), another, in particular, localities but overall fauna (Quiroz-Vazquez et al. 2005;Hinojosa-Arango y Riosmena-Rodríguez et al. 2004; Foster et al. 2007). This region contain several relevant ecosystems: *Sargassum* forest, rodolith and eelgrass beds actually are considered as critical habitats (Steller et al. 2003; Riosmena-Rodríguez 2009; Ávila et al. 2010; López-Calderón et al. 2013; Hinojosa-Arango et al. 2014) by establishment and development for abundant associated fauna and their ecosystemic services (Hinojosa-Arango & Riosmena-Rodríguez 2004; Foster et al. 2007; Riosmena-Rodríguez & Medina-López 2010; Suarez-Castillo 2014), therefore, arises the need to analyze predominantly these ecosystems.

Recently, in addition to the historical or natural process of colonization (Muñíz-Salazar et al. 2005), the anthropogenic factor (shipping and contamination) is influencing the species presence and dispersal around the world, as the actual situation of the invasive seaweed *Acanthophora spicifera* (Russell 1992; Schaffelke & Hewitt 2007), which was observed inside the gulf in 2006 by first time (Ávila et al. 2012) and has since then spread and established throughout Bahía de La Paz, making it one of the most conspicuous ecosystems and potentially invasive along the gulf.

We observed that one of the most significant and fluctuant taxa associated whit this conspicuous habitat in Mexican Pacific Northwest is Mollusca, however, recently beginning to be studied. The aim of this study analyzes if there a similarity in mollusk assemblages between the most distinct habitats recorded in northwest Mexican Pacific by the response: 1) How are formed the mollusk assemblages each habitat? 2) Is taxocenosis structure maintained over time and space inside habitats? 3) How does biodiversity change between habitats? This research is to understand the mollusk taxocenosis dynamic inside the distinct habitats recorded, which involve critical habitats and the most invasive seaweed by the moment.

STUDY SITE

Peninsula coast and the Gulf of California maintain the border of geographical distribution of *Zostera marina* along northeast Pacific (Muñíz-Salazar et al. 2005; Riosmena-Rodriguez et al. 2012), as well *Sargassum* (Suarez-Castillo et al. 2013) and the major rhodolith beds (Steller et al. 2003; Ávila & Riosmena et al. 2011). The invasive seaweed

Acanthophora spicifera by the moment is distributed in Bahía de La Paz (Ávila et al. 2012). Pacific Coast communities are not only separated geographically from communities in the Gulf of California but are also exposed to different environmental conditions (Cabello-Pasini et al. 2003). The Gulf of California is a semi-enclosed sea where the exchange with Pacific waters occurs only in the south of the gulf via cyclonic circulation (Castro et al. 2000). We analyzed localities at these critical habitats, as well the invasive species *A. spicifera*. Sampling was conducted at different sites each locality, from 1-5 m depth by SCUBA, to collect most of the mobile organisms for concurrent studies.

Samples were taken from *Zostera marina* in San Ignacio (SI): 26º55' N; 113º10' W North (N), 26º49' N; 113º15' W, Media (M), 26º42' N; 113º60' W, Pitahaya (P). Bahía Magdalena in *Zostera* (BMZ): CFE, Las Tijeras (T1), Cancún (C1), El Muerto (EM1), Punta de Gato (PG1). Sonora in *Zostera* (SZ): Punta Arenas (PA1), Coniic (C1), Bajo Callero (M1), Punta Xpanams (PX), Punta Chueca (SR), Punta Tormenta (PT), Palo Fierro (PP), Estero San Miguel (SM), Punta Ohna (PO), El Álamo (BA), Bajo Media Luna (ML), Bajo Canal (MC). Rhodolith beds from Bahía Magdalena (BMR) 24º15' N; 111º30' W (CFE) and 25º20' N; 112º15' W (PED). *Sargassum* forest throughout Bahía de La Paz (BLP): L1 (24º45'37"N; 110º40'24" W), L2 (24º35'52" N; 110º44'48" W), L3 (24º28'29" N; 110º41'37" W), L4 (24º18'59" N; 110º38'24" W) and L5 (24º12'15" N; 110º32'6" W) in June 2011 and May 2012. Invasive seaweed *Acanthophora spicifera* in Ensenada de La Paz (ELP) 24º12'07" N; 110º17'59"' W.

Field Sampling

Samples were taken every two months from April to August. 2010 in *A. spicifera* from holdfast using a sampling square of 0.00625 m^2 over three beds, nine replicates each one. June 2011 and May 2012 from *Sargassum* in 5 beds, sampling 15 replicates each site from the holdfast. June 2011 in two rhodolith beds, 20 replicates each one using a square of 0.0625 m^2 (same for the next three localities). October 2009, March, April and August 2010 from *Z. marina*, Sonora in 12 sites, sampling three replicates each one from the rhizome and the sediment surface (same for the next two localities). June, April, and September 2009 and December 2010 from *Z. marina,* San Ignacio in 4 sites, three replicates each. June 2011 from *Z. marina*, Bahía Magdalena in 5 locations, three replicates each.

Laboratory Analyses

The samples were separated by sieved through a 0.5 mm mesh and preserved in ethyl alcohol (70%). Each sample were examined for the record and counted all organisms possible. Mollusks were sorted quantitatively and identified as a group possible using particular references. Some species required further identification.

Feeding Habits and Feeding Guilds

The next feeding habits and guilds were considered: carnivores (C) herbivorous (H) Omnivorous (O). Deposit feeders (D) feeding on organic particles contained in the sediment, filter feeders (F) intercepting nutrient particles with their gills and/or mucous strings, Suctorial parasite (Sp), Grazer (Gr) feeds by scraping, either on algae or sessile animals, and

Browsing (feeds by tearing or gathering particular items (Br), we selected sorts of Rueda et al. (2009). Trophic information was mined from the literature for each species wherever possible. If the feeding behavior of a particular species was unknown, it was assumed to feed in a similar manner to species within the same major group.

Data Analysis

Mollusk taxocenosis structure and dynamics were analyzed by the number of specimens (N), the number of species (S), the Shannon index (H', using the log formula) and Pielou's evenness index (J'). Before all data were converted to m^2 for comparison. After were tested for KS normality and homogeneity of variances by Cochran's test, and log-transformed as necessary. Component Principal Analyses (CPA) were made each habitat to detect and compare the most variable data corresponding with the species. We performed a cluster analyzes and non-metric multidimensional scaling (MDS) in different geographical sectors and surveys using qualitative and quantitative data (presence/absence and Ind m^{-2}) and the Jaccard similarity index. Before analysis, data were the fourth root transformed abundance data, to minimize the effect of extremely abundant species. The SIMPER routine was then used to locate which species contribute most to the differences between habitats or, conversely, which species contribute most to the similarities within the same group. Multivariate analyzes were performed using R software and PRIMER.

RESULTS

Fauna Composition

An almost total of assemblages were dominated by mollusks in most of the surveys (Figure 1), except in *Sargassum*, the transitional habitat in BLP, which usually is occupying by crustaceans, and the next abundant group is polychaetes. We recorded 42 families and 73 species of mollusks across all sites and time surveys (Appendix 1). The material was identified as level possible. Species abundance was different between sites inside habitat along time, due to the location, but we chose the total abundance all surveys for the show the principal components and abundant species each locality (Figure 2). In most of the habitats dominated the carnivorous guild, except in Sonora due to the high abundance of the detritus feeder *Caecum* in soil (Figure 1).

Similarity and Taxocenosis Structure

Main species contribution consistently accounted for greater than 70% of the total dissimilarity among the different habitats and localities (Table 1). The taxocenosis in eelgrass beds and brown/red seaweed is characterized by high abundance of less dominant species than rhodolith beds (Figure 2).

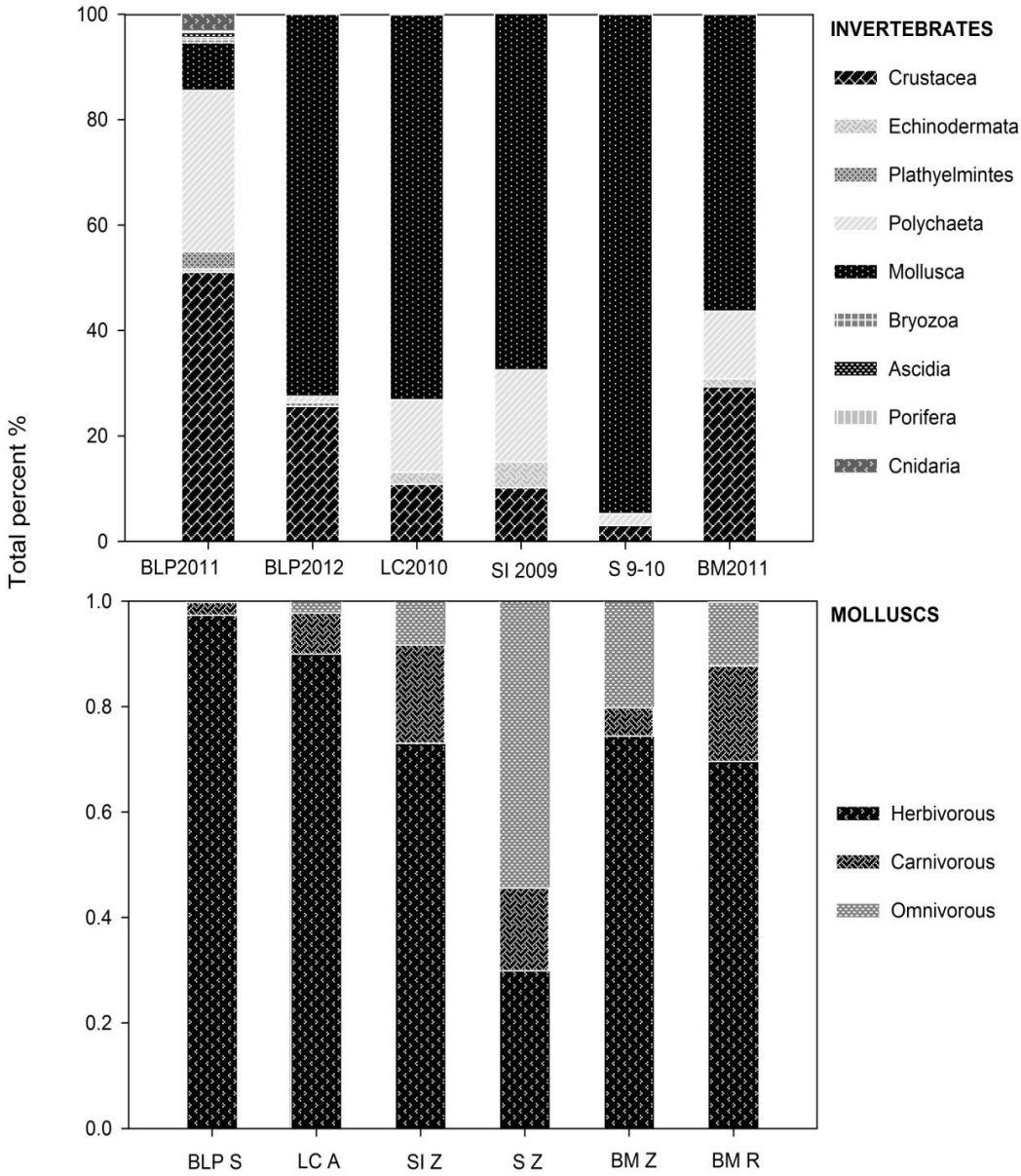

Figure 1. Contribution of Invertebrates in the different habitats: Bahía de La Paz *Sargassum* (BLP S), Roca Caimancito *Acanthophora* (LC A), San Ignacio *Z. marina* (SI Z), Sonora (S Z) and Bahía Magdalena in *Z. marina* (BM Z) and Bahía Magdalena in rhodolith beds (BMR).

Taxocenosis structure in *Acanthophora* was relatively consistent over time but differ between sites, the greatest abundances were found to in this invasive seaweed (Figure 2), *Assiminea* were the most abundant species in this habitat. Rhodolith bed shows less abundance but greatest S, J' and H'; the species recorded in this habitat were entirely different to eelgrass taken the same date and lagoon, only three species share both. BLP contains fewer species and only the dominant *Alaba supra lira ta* (Figure 2). *Alabimia, Barleeia, Acteocina, Caecum* and *Alaba* were the most related taxa in almost all habitats. Several particular taxa

less abundant were found only inhabiting eelgrass beds, even one locality inside gulf and two outside as well: *Alabina*, *Nassarius*, *Anachis*, *Neopilina*, *Cerithiopsis*, *Mitrella*, *Turbonilla*, *Elephantelum*, *Hipponix*, *Calyptraea*, and *Biv 1* (Table A1).

The mollusk assemblages grouped among similar habitats represent by eelgrass and distant respect to seaweed beds (Figure 3). Taxocenosis composition in *Sargassum* was near to *Acanthophora* group; these localities are cited in Bahía de La Paz both. Clustering in rhodolith beds was far to the other groups since most of the species were only found in these habitats. A significant nMDS ordination is detected; stress values for the analyzes were 0.146, the plots show some clustering of habitats (Figure 4). SZ is near to SIZ and BMZ, even if these last is situated outside gulf.

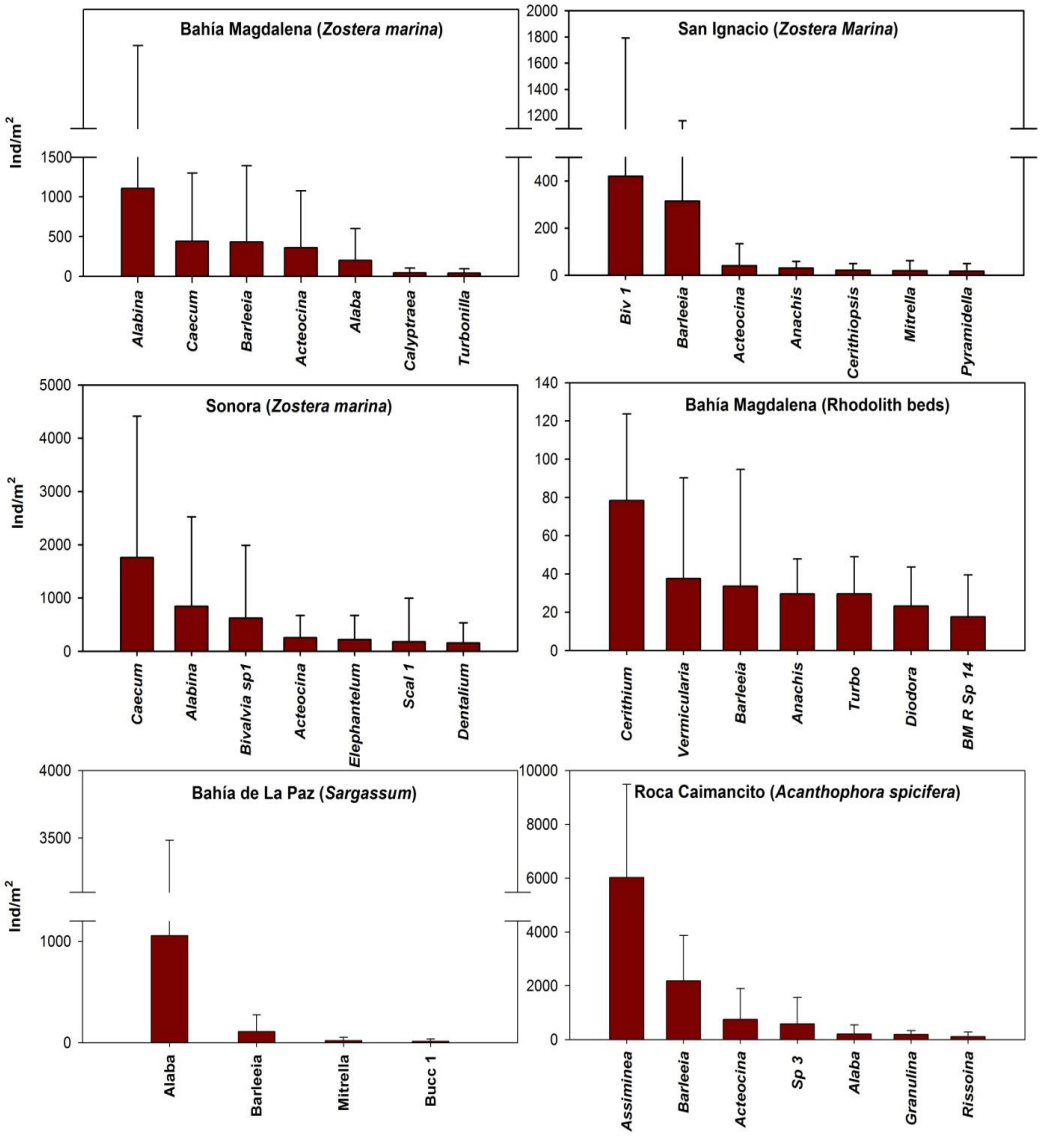

Figure 2. Representative species each habitat, selected by high abundance and principal components after Component Principal Analyses each habitat.

Table 1. Results of SIMPER analysis on the percent contribution of benthic categories, testing dissimilarity between zones

Category	Contribution %	Cumulative %	Category	Contribution %	Cumulative %
Groups: BM Z & SI Z			Groups: BM Z & BM R		
Average dissimilarity = 88.14			Average dissimilarity = 93.31		
Alabina	20.95	20.95	*Alabina*	19.28	19.28
Barleeia	16.05	46.92	*Alaba*	16.78	36.05
Calyptraea	9.92	56.42	*Caecum*	8.42	44.47
Acteocina	9.5	64.27	*Barleeia*	7.68	52.16
Groups: BM Z & S Z			Groups: BLP S & LC A		
Average dissimilarity = 87.49			Average dissimilarity = 95.11		
Caecum	26.85	26.85	*Assiminea*	48.52	48.52
Alabina	22.63	49.48	*Barleeia*	17.28	65.8
Biv 1	10.14	59.63	*Alaba*	9.86	75.66
Acteocina	7.2	66.83	*Acteocina*	4.79	80.46
Groups: S Z & SI Z			Groups: BLP S & S Z		
Average dissimilarity = 92.73			Average dissimilarity = 95.11		
Caecum	30.4	30.4	*Caecum*	29.39	29.39
Alabina	16.57	46.97	*Alabina*	14.8	57.88
Biv 1	15.44	62.4	*Alaba*	13.68	68.57
Barleeia	5.83	68.24	*Biv 1*	10.69	73.8

DISCUSSION

Olds et al. (2012) have shown that connectivity is essential in conservation planning and this is extremely difficult to find in many planning process in marine protected areas. Also is a consideration lacking in environmental impact reports who needs to evaluate the synergic impacts of assemblages in any given project. Strong differences were found among the major habitats studied in relation to feeding habits and in relation to diversity (Figure 1). Steller et al. (2003) have found strong differences between Rhodolith beds and sandy areas, our analysis extend this observations to other habitats. Seagrasses meadows and Rhodolith beds seems to be the most diverse areas but less abundant (Figure 5) comparing among them.

Foster et al. (2007) have found rich and diverse biota associated to *Sargassum* forest in Bahia Concepcion and our results are contrasting with less species and less abundant. This situation might be related to geographical position because our sampling is from Bahia de La Paz. However, when comparisons with the *Zostera marina* meadows are made strong similarities among sites are clearly present. In the case of Rhodolith beds it is clear that no significant differences have been found in the associated species from what we know. Our study is unique because no one has compare this ecosystems before, we have found indicators for further analysis with the whole invertebrate community.

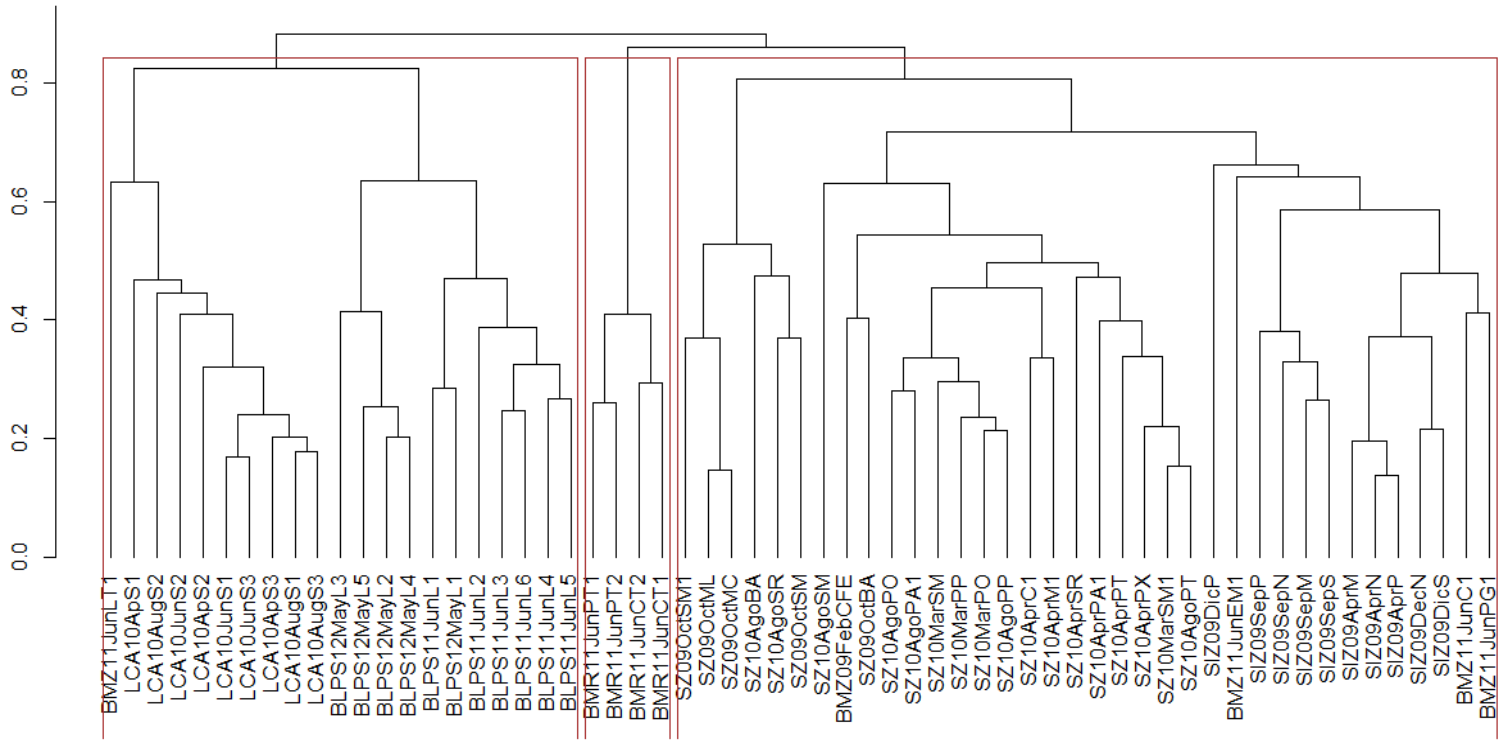

Figure 3. Cluster analyzes. The key is arrangement in the next order: localities: BLP (Bahía de La Paz), Roca Caimancito (LC), Bahía Magdalena (BM), Sonora (S) and San Ignacio (SI); hosts: *Zostera marina* (Z), *Acanthophora spicifera* (A), *Sargassum* (S) and rhodolith beds (R); year, month and sites inside each locality.

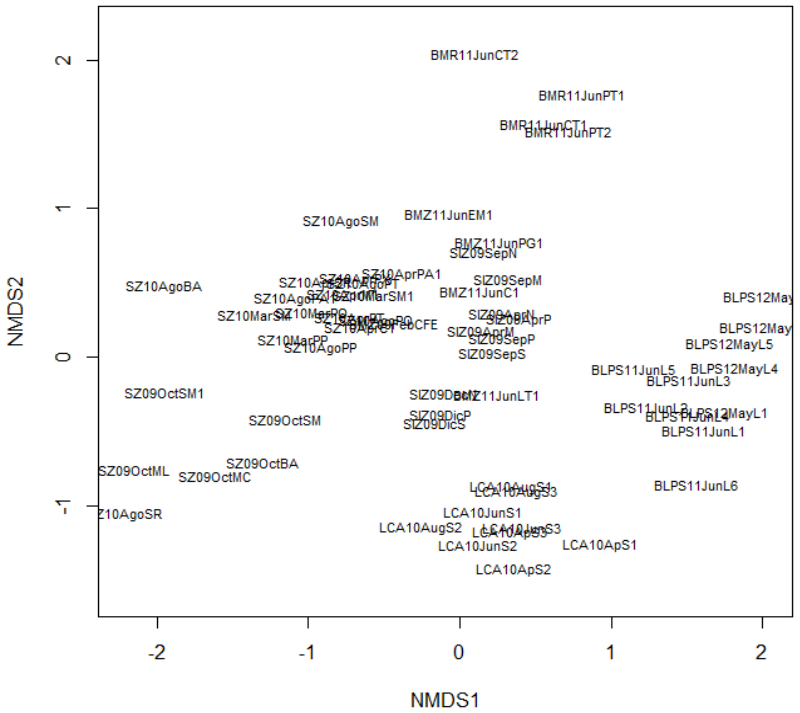

Figure 4. Nonmetric Multidimensional Scaling (nMDS) biplot on the Jaccard similarity matrix of transformed benthic taxocenosis data (the fourth root transformed abundance data) showing physical means for all habitats and sites. Bahía Magdalena *Zostera marina* (BM Z), San Ignacio *Zostera marina*, Sonora *Zoster Marina* (S-Z), Bahía de La Paz *Sargassum* (BLP A), Bahía Magdalena rhodolith beds (BM-R) and Roca Caimancito *Acanthophora spicifera* (LC A).

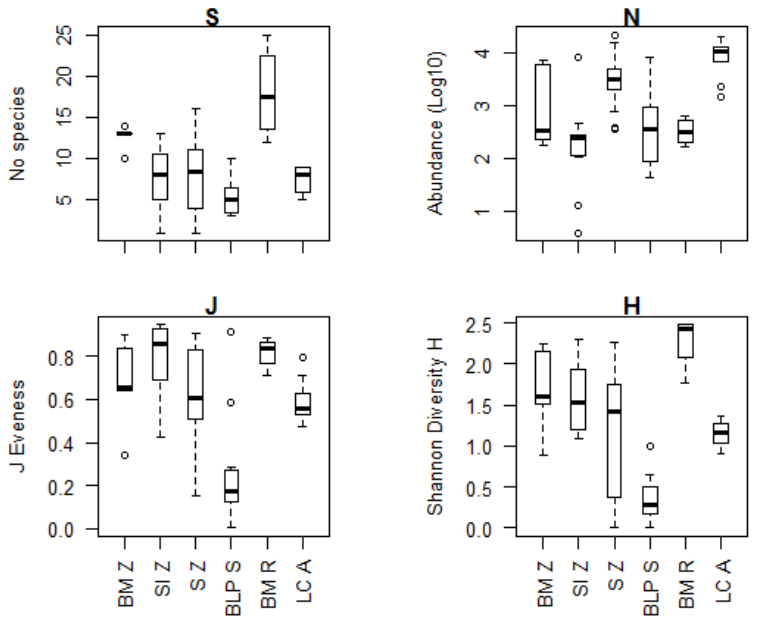

Figure 5. Ecological attributes. Bahía Magdalena *Zostera marina* (BM Z), San Ignacio *Z. marina*, Sonora *Z. marina* (S-Z), Bahía de La Paz *Sargassum* (BLP A), Bahía Magdalena rhodolith beds (BM-R) and Roca Caimancito *Acanthophora spicifera* (LC A).

APPENDIX

Table A1. Mean abundance (Desvest) by m^2 of species associated with different habitats. Bahía Magdalena (BM), San Ignacio (SI), Bahía de La Paz (BLP) and Roca Caimancito (LC)

Genus	BM Zostera		SI Zostera		Sonora		BM Rod		BLP		LC	
Dentalium	13	(20)	0	(0)	157	(377)	0	(0)	0	(0)	0	(0)
Neopilina	4	(10)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Alabina	1,105	(2,165)	6	(14)	843	(1,682)	0	(0)	0	(0)	0	(0)
Cerithium stercusmuscarum	0	(0)	0	(0)	0	(0)	78	(45)	0	(0)	0	(0)
Cerithiopsis	7	(14)	22	(28)	81	(146)	0	(0)	0	(0)	21	(46)
Epitonium	4	(5)	0	(0)	0	0)	0	(0)	0	(0)	0	(0)
Alaba	199	(402)	1	(4)	103	(318)	3	(5)	1,055	(2,429)	212	(340)
Buccinidae 1	0	(0)	0	(0)	182	(813)	0	(0)	0	(0)	0	(0)
Vermicularia	0	(0)	0	(0)	0	(0)	38	(53)	0	(0)	0	(0)
Turritella	0	(0)	0	(0)	0	(0)	10	(7)	0	(0)	0	(0)
Assiminea	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	6,022	(3,470)
Caecum	442	(857)	0	(0)	1,763	(2,647)	5	(10)	0	(0)	54	(110)
Elephantelum	14	(25)	0	(0)	220	(452)	0	(0)	0	(0)	0	(0)
Crucibulum	0	(0)	0	(0)	0	(0)	8	(7)	0	(0)	0	(0)
Calyptraea	43	(58)	3	(5)	0	(0)	0	(0)	0	(0)	0	(0)
Hipponix	0	(0)	3	(8)	50	(218)	0	(0)	0	(0)	0	(0)
Rissoina	0	(0)	0	(0)	0	(0)	17	(18)	0	(0)	110	(168)
	0	(0)	0	(0)	0	(0)	0	(0)	13	(23)	0	(0)
Anachis	16	(14)	31	(29)	127	(258)	30	(18)	0	(0)	0	(0)
Mitrella	32	(56)	20	(42)	0	(0)	4	(6)	20	(34)	0	(0)
Granulina	20	(45)	17	(46)	0	(0)	0	(0)	0	(0)	190	(143)
Nassarius	22	(26)	17	(15)	36	(100)	8	(8)	0	(0)	0	(0)
Oliva	0	(0)	0	(0)	3	(14)	0	(0)	0	(0)	9	(27)
Pilsbryspira nymphia	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Acteocina	359	(718)	41	(93)	255	(421)	0	(0)	0	(0)	748	(1,151)
Haminoea	0	(0)	0	(0)	0	(0)	0	(0)	3	(11)	0	(0)
Haminoea sp2	2	(3)	0	(0)	28	(51)	0	(0)	0	(0)	0	(0)
Turbonilla	39	(56)	6	(8)	8	(22)	0	(0)	0	(0)	6	(17)
Pyramidella	2	(5)	17	(32)	0	(0)	0	(0)	0	(0)	0	(0)
Theodoxus	1	(2)	0	(0)	0	(0)	6	(6)	0	(0)	0	(0)
Diodora inaequalis	0	(0)	0	(0)	0	(0)	23	(20)	0	(0)	0	(0)
Tricolia	0	(0)	0	(0)	0	(0)	0	(0)	6	(10)	0	(0)
Fissurellidae sp1	6	(7)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Tegula	14	3(2)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Turbo funiculosus	0	(0)	0	(0)	0	(0)	30	(19)	0	(0)	0	(0)
Turbo	0	(0)	0	(0)	0	(0)	10	(12)	0	(0)	0	(0)
Barleeia	432	(963)	314	(846)	0	(0)	34	(61)	110	(166)	2,183	(1,687)
BLP Sp 4	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
BLP Sp 5	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
BLP Sp 6	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
BLP Sp 7	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
BLP Sp 8	0	(0)	0	(0)	0	(0)	0	(0)	1	(3)	0	(0)
BLP Sp 9	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
BM R Sp 8	0	(0)	0	(0)	0	(0)	2	(3)	0	(0)	0	(0)
BM R Sp 15	0	(0)	0	(0)	0	(0)	7	(12)	0	(0)	0	(0)
BM R Sp 10	0	(0)	0	(0)	0	(0)	2	(3)	0	(0)	0	(0)
BM R Chione	0	(0)	0	(0)	0	(0)	2	(3)	0	(0)	0	(0)
BM R Sp 14	0	(0)	0	(0)	0	(0)	18	(22)	0	(0)	0	(0)
BM R Sp 9	0	(0)	0	(0)	0	(0)	9	(11)	0	(0)	0	(0)
BM R Sp 21	0	(0)	0	(0)	0	(0)	10	(21)	0	(0)	0	(0)

Genus	BM Zostera		SI Zostera		Sonora		BM Rod		BLP		LC	
BM R Sp 22	0	(0)	0	(0)	0	(0)	1	(2)	0	(0)	0	(0)
BM R Sp 23	0	(0)	0	(0)	0	(0)	1	(2)	0	(0)	0	(0)
BM R Sp 24	0	(0)	0	(0)	0	(0)	2	(3)	0	(0)	0	(0)
BM R Sp 25	0	(0)	0	(0)	0	(0)	1	(2)	0	(0)	0	(0)
LC Sp 5	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	79	(104)
LC Sp 6	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	47	(114)
LC Sp 7	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
LC Biv 1	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	22	(34)
Sp 3	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	581	(990)
Gast 4	0	(0)	0	(0)	47	(218)	0	(0)	0	(0)	0	(0)
Gast 8	0	(0)	0	(0)	3	(14)	0	(0)	0	(0)	0	(0)
Gast 11	0	(0)	0	(0)	1	(5)	0	(0)	0	(0)	0	(0)
Gast 12	0	(0)	0	(0)	19	3(2)	0	(0)	0	(0)	0	(0)
Cardita	0	(0)	0	(0)	0	(0)	6	(5)	0	(0)	0	(0)
Tagelus	0	(0)	0	(0)	1	(5)	0	(0)	0	(0)	0	(0)
Tellina	1	(2)	2	(5)	0	(0)	3	(5)	0	(0)	0	(0)
Chione	6	(14)	0	(0)	59	(96)	6	(6)	0	(0)	0	(0)
Arca	0	(0)	0	(0)	3	(14)	0	(0)	0	(0)	0	(0)
Pecten	4	(8)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Bivalvia sp1	0	(0)	420	(1,372)	624	(13,64)	0	(0)	0	(0)	0	(0)
Bivalvia sp2	0	(0)	9	(16)	6	(27)	0	(0)	0	(0)	0	(0)
Biv 3	0	(0)	0	(0)	4	(18)	0	(0)	0	(0)	0	(0)
Biv 13	0	(0)	0	(0)	99	(328)	0	(0)	0	(0)	0	(0)

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Chapter 25

A CRITIQUE OF THE ENVIRONMENTAL IMPACT STUDIES IN MÉXICO BASED ON TWO STUDY CASES

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ABSTRACT

By means of behavioral studies it has been determined for the first time in Mexico the critical habitat of a semi-resident group of coastal dolphin *Tursiops truncatus* (bottlenose dolphin) in Ensenada de La Paz and the south-southeast of Bahia de La Paz. In this zone it is more frequent to observe small groups of mothers with calves and juvenile dolphins. Through the years it has been recorded a habitat shift of these cetaceans induced by environmental and anthropogenic factors: loss of southwest basin in Ensenada de La Paz due to silting, dredging and tropical cyclones; similarly a reduction of the feeding activity has been observed in the northwest basin due to fisheries and the increase of nautical traffic which overlaps with the main transit route of bottlenose dolphins near El Mogote, thus affecting their conduct. The disturbance could increase because of dredging realized in 2012 by the touristic development Paraiso del Mar to build a dock for a marina in El Mogote, affecting particularly zones where females teach juvenile dolphins to feed next to mangroves. Unfortunately, the ecological, social and economic importance of these coastal wetlands it is not considered, subject to a stress and risk increase in the last decades due to non-regulated deforestation and dredging, as a consequence of the mentioned touristic development for the construction of a golf course, and affecting the mangrove zone. It is imperative a decree by SEMARNAT stating Ensenada de La Paz and south of Bahía de La Paz as a critical habitat for dolphins, based on the scientific information available; given the current threats on this Ramsar site and its communities, the lack of acknowledgement as a critical zone, and the need to be included in the environmental impact evaluation for coastal developments. By including

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them as part of the environmental policy other species will be protected too, establishing general management plans for its conservation, building agreements with environmental authorities, researchers, government agencies, society organizations, fishermen, and general public.

Keywords: bottlenose dolphin, critical habitat, mangrove, Ensenada de La Paz, conservation

INTRODUCTION

The Article 2 of the Mexican Constitution ensures that natural resources are owned by the nation (and its citizens) have developed concessions to the implementation of productive projects (whether public or private) where it could impact the ambient. Based on this constitutional mandate the General Law of Ecological Balance seeks to preserve ecosystems and their processes to human actions. These processes are extracting water from wells, rivers and lakes through land use for different purposes to the way we use the oceans with its highly productive ecosystems. When a country assumes a model of sustainable development you are able to meet current needs without putting at risk the resources continue to be used long term for the same population. To this end, they require environmental instruments to support us in order to determine whether the activity to be developed could have an impact on ecosystems or significantly affect its processes. This is key, you can use the environment but what you intend to do has to be friendly to the future development and not necessarily destroy the natural capital that exploits.

Although its origins are in the 70's, the instrument used in Mexico to assess the potential impacts of human activities began to be used effectively in 2000 arising from both changes in environmental policy and the commitments the country signing international treaties. It is when it is proposed that all development projects will have to submit an Environmental Impact Statement taking two forms: private or regional which is related to the size of the project. There is also what is called Preventive Report being used in very specific cases. Although it seems that these manifestations seem to name only assess potential impacts on the environment which would also have to consider are social, economic, cultural and even political aspects (since the project in question could cause some instability). It is also necessary to consider that even developed in two modes have to keep in mind the potential synergistic impacts both the quantity and the size of other nearby projects that can be generated.

To give an award to a particular is very necessary that the Environmental Impact Statement develop a detailed description of the environment or ecosystems in the area of interest. This is a key point for any project since being a concession to a certain time what could hope for is that development does not cause a drastic change in the environment where the grant is completed changes are as small as possible. However, there is no classification of terrestrial ecosystems consistent between different secretariats in the SEMARNAT (i.e., that each agency secretariat terrestrial ecosystems are recognized differently). However, the seriousness of the matter is that on aquatic ecosystems seems to be marine or freshwater is the biggest difference possible. There is an inclusive classification of all marine ecosystems leaving defenseless internationally recognized for any administrative or procedural protection of ecosystems in Mexico but we have not yet dared to protect (including seagrass beds, maerl

beds, kelp/Sargassum forests, marsh meadows, hydrothermal vents, gorgonian forests, etc ... among many others). This generates a lot of controversy of how to develop an environmental statement if you do not have a complete catalog of the "environment" of the country. Perhaps this is related to the number of professionals in the marine sciences have been trained and employed in different spheres of the productive life of the country.

But as it is assumed that the MIA will seek to assess whether the processes to be carried out to make an impact on these ecosystems should know some of its dynamic aspects. These should be at least biodiversity (species in the area), ecological processes (reproduction, recruitment, breeding or feeding, etc. ...) and participation in geological processes (stability of beaches, soil, sediment origin, etc. ...). Now if this is not well understood in developing the EIS then it is not possible to propose measures to compensate or mitigate the impacts making it impractical to say that a project is feasible or not, right? It is something that needs to be worked intensively gathering critical information that you have in the country on this issue both in research centers and universities, it is not possible that information of this nature is generated and is not used. Although there are very serious academic exercises like those made by the National Commission for the Use and Conservation of Biodiversity (CONABIO) to understand the Natural capital to understand their gaps or omissions not covered there are still some corners that need to be evaluated priority.

But consider that these deficiencies are already covered in these fundamental inputs that the process requires. The next thing to ask for this instrument to fulfill its roles is: who makes the demonstrations and who evaluates them. It is disturbing to know that is supposed to be to draw up a register of providers of this service are properly credited to the demonstrations but this one is at the state level (where differences to be accredited are evident), in some cases to levels City (counted) but we could not find a single federal standard or features should have this provider. Subsequently, although SEMARNAT you have a direction to make assessments of these studies it seems that they must have a wide range of professionals to cover the topic of each and every one of the evaluations. However, it does not seem to be the case and has not touched me that these offices require advice or support from academics who might have information on the project or subject knowledge to support decision making. This would be necessary to conduct some sort of survey data verification so as to delineate dispute that projects might have. The figure of the expert is not well defined at both the federal and state levels. It requires a profile of each position to ensure that the process will be well qualified professionals who achieve the goal of having fewer disputes arising in these processes.

Within this instrument it has been considered that the public consultation and the reports are objective elements where you can clarify any doubts regarding the project. But experience with public consultations in Baja California Sur (and we are sure in other parts of the Republic) tell us that this has been a media circus than an exercise of reflection on the true intentions of the petitioner. We think there should be other elements from previous assessment that the petitioner could use to assess the chances of their proposal well before starting to invest in studies that did not represent support decision making. Then you end up with the wrong idea about the interest in the protection and conservation of the environment losing sight of the nuances that this may have on the approaches of each organization. In this area at country level absent the professional associations in Marine Sciences (perhaps with the honorable exception of Oceanographers organizations in the North state) that could be guarantors in the process of evaluation. Perhaps the key point is that it has not properly stimulated private organizations environmental assessment (like has happened with Civil

Society Organizations) profiles which are desirable for the development of the EIS. This would stimulate the development of professionals in the area of knowledge and would provide with the necessary feedback to professional associations, removing the temptation to involve public institutions that, through your profile, may have conflicts of interest to develop these demonstrations.

A well implemented MIA monitoring may be evaluated to assess the observed impacts against the predicted and to adjust or modify the planned mitigation in each process. Clearly this represents an additional cost that often do not want to pay but if you want to know the state of the ecosystems as a project (or several projects) is necessary to have this ongoing assessment. That is why in other countries environmental monitoring program that measures are developed, registers and makes available to the public a series of crucial data in the development environment in the region. These are also used by insurance companies working with large companies assuring its activities related compensation and any potential impact.

Thus, the paradigm of sustainable development are not the windows of opportunity that should have different organizations or companies. But the way they achieve, or not accredit an environmental impact study due to lack of renovation of processes and the limited view of its scope. It would be important that the permanent dialogue that should have all sectors in the country could discuss adjustments to existing procedures. They may implement alternative assessment systems that represent relative cost differentials and the size of the project but at the same time give certainty to investors, CSOs and government processes that will achieve the objectives of environmental policy. The experience gained in these collective processes might achieve that decisions are at least controversial and politicized that current net profit for the ecosystem. Because of that we are presenting here 2 study cases in La Paz Bay who serve as examples to show how the actual model is not working and the urgent need of a review.

DOLPHINS, MANGROVES AND EL MOGOTE

Mangrove structure, functioning and permanence are singular, given that it is an ecosystem located at the threshold between marine and terrestrial environments. On one hand it is dominated by oceanic factors like tidal regime, marine currents, salinity, temperature, and nutrient availability; on the other hand atmospheric factors like humidity, wind, temperature and irradiance determine its physiological status and survival. From an anthropocentric point of view, mangroves experience significant changes due to land use modifications, deforestation, and pollution (1). Unfortunately, anthropogenic agents have had a determinant effect on this coastal ecosystem, reducing its spatial coverage. It is estimated that in the last two decades, approximately 50% of the mangroves of the world have been lost (Santamaría-Gallegos et al., 2011). FAO (2007) determined that the worldwide coverage of mangrove was 150,000 km^2 between 1980 and 2005. A recent assessment by Giri et al. (2011) states that worldwide mangrove coverage in year 2000 was 137,360 km^2, 12% less that estimated by FAO.

From an ecologic and economic point of view, mangrove of the Baja California Peninsula are acknowledge as valuable ecosystems, and protected by the Mexican legal framework. Despite of this, currently they are highly threatened by the construction of real estate development with urban and tourist purposes: golf facilities, marinas and roads. In order to

carry out mangrove conservation in Mexico it is required that they are considered in management plans, that authorities enforced the law strictly during the inspection and authorization of building activities along the coastal zone, and that public policies at the federal, state, and municipal level acknowledge the economic value of the environmental services provided by mangrove forests.

The municipality of La Paz, Ensenada de La Paz, and Bahia de La Paz in its Eastern portion have 14 wetlands with mangrove forests (Mendoza-Salgado et al., 2011). Inside Ensenada de La Paz the sandy bar known as El Mogote has two wetlands with mangrove forests: Zacatecas and El Mogote. Zacatecas mangrove has been subject to a management proposal by federal authorities to be preserved for being the habitat of least tern (*Sternula antillarum*) an endangered migratory bird species that uses this habitat as reproductive area (Amador & Mendoza, unpublished data; Mendoza 1994; Mendoza-Salgado et al., 2011). While mangrove El Mogote has a series of mangrove forests separated by dune zones, the total length along the shore of these mangroves is 6 km. Mangrove El Mogote constitutes the central subject of this chapter, given that mangroves along El Mogote form a fundamental ecological connection with a population of coastal dolphins known as tursiones (*Tursiops truncatus*) in Ensenada de La Paz.

It is known that between 1973 and 1981 Ensenada de La Paz lost more than 44 ha of mangrove, 21.5% of its total coverage (Mendoza-Salgado et al., 1984), the purpose of this deforestation was to obtain firewood, build roads to access the coast by mangrove-oyster fishermen, and to develop recreational areas (Mendoza-Salgado et al., 2011). Mendoza-Arrambídez et al. (2007) developed and applied a Coastal Environmental Quality Index (ICAC) at Bahia de La Paz, which shows that a wide spectrum exists in terms of conservation status of coastal wetlands with mangrove forests. These values go from good environmental quality (ICAC = 58%) to an environmental alert status (ICAC = 31%) (Mendoza-Salgado et al., 2011). Unfortunately, more that 30% of the surveyed sites show environmental alert indexes, thus mangrove forest at those sites have ecophysiological problems in its remineralization capacity of organic matter (Mendoza-Salgado et al., 2011).

Lack of community awareness is another serious problem for the preservation of mangrove forests, because a large sector of the population thinks of this coastal ecosystem as illegal dumps, breeding ground for mosquitoes, free source of firewood, and bad odor. It is necessary to strengthen a dissemination campaign in all levels of society so that people know that these forests are fragile ecosystems on which hundreds of resident and migratory species rely on, and where key biogeochemical processes take place that have an important role in the recycling of carbon, nitrogen, and phosphorous through the terrestrial-marine system. This promotion of environmental culture will bring a new approach to social conscience: healthy mangrove forests mean a reduction of CO_2 in the atmosphere, healthy fisheries, biodiversity preservation and a higher environmental quality for Bahia de La Paz.

Dolphins (*Tursiops truncatus*) are known as bottlenose dolphins or toninas, they distribute in almost every ocean, except at higher latitudes (Leatherwood & Reeves, 1983), they are gregarious animals that form herds (Walker, 1981; Leatherwood & Reeves, 1982; Mead & Potter, 1990). Females reach maturity between 5 and 12 years old, while male does it between 10 and 15 years old (Sergeant et al., 1973; Mead & Potter, 1990; Wells, 1991). Gestation in these animals last approximately 12 months (McBride & Kritzler, 1951; Hansen, 1990; Wells, 1991; Mann & Smuts, 1999), females give birth in any season of the year, with one calf every 2 or 3 years (Shane et al., 1982). A strong binding forms between mother and

calf that lasts 3 to 10 years; calves do not interact with males (Shane et al., 1986; Wells et al., 1987; Buckstaff, 2004). Social organization in dolphins is based on age and sex and the association mother - calf and between adult males is very stable. Size and composition of groups it is related with different parameters, characteristics, requirements and attributes of each animal *per se*, intra- and inter-specific relationships, availability and use of resources. It also varies seasonally, according to the structure of habitat, activity patterns, food availability, reproductive stage, and protection against predators (Caldwell, 1955; Wells et al., 1980, 1987; Shane et al., 1986; Ballance, 1990; 1992; Wells, 1991; Mann & Smuts, 1999; Connor et al., 2000; Bearzi, 2005).

Coastal zone is the home range for bottlenose dolphins, reaching permanent social groups (Wells et al., 1980; Ballance, 1985). The preference for this zone could be a result of prey abundance supported by coastal systems and the protection offered by this zone against predators. In this zone bottlenose dolphins reproduce, breed, and feed their offspring, therefore their presence in the coastal zone can be used as an indirect indicator of productivity. Bottlenose dolphin distribution is also related to other factor such as bathymetry, tidal regime, type of bottom, and prey diversity, thus they can be found in a wide range of environments (Wells et al., 1980; Irving et al., 1981; Shane et al., 1986; Ballance, 1992; Wilson et al., 1997; Hastie et al., 2004). They feed on fish of 5 to 30cm in length; their diet includes individuals of the families Mugilidae, Scianidae, Clupeidae, Scombridae, Batrachoidae and Haemulidae, among others. Also octopus, squid, shrimp, crab, and in some cases sharks can be present in their diet (Gunter, 1942; Barros and Odell, 1990; Corkeron et al., 1990; Corkeron and Ross, 1990; Mead and Potter, 1990).

Human impact has increased on coastal ecosystems in the lasts decades, mainly because of anthropogenic activities and settlement developments such as fisheries, tourism, marine traffic (Wells et al., 1980; Shane et al., 1986; Wilson et al., 1997; Barco et al., 1999; Bristow and Rees 2001; Nowacek et al., 2001; Prideaux, 2003; Lemon et al., 2006). As a consequence of these worldwide impacts a measure adopted for the protection and conservation of bottlenose dolphins is the declaration of critical habitats, formed by regions of high presence of bottlenose dolphins where feeding, socializing, and resting activities take place (Higham and Lusseau, 2004; Hoyt, 2005). This declaration promotes protection of core areas to preserve critical habitat for the species as well as their prey and marine ecosystems; establishing that only non-invasive research and periodic environmental surveys are allowed, conducted by specialist in the species studied or under their supervision (Hoyt, 2005). Habitat use pattern is different between localities, so it is important to know species behavior and its relation with environmental and anthropogenic factors in order to establish a critical habitat as a function of feeding, socializing, resting, and breeding areas (Wells and Scott, 1997; Nowacek et al., 2001; Ingram and Rogan, 2002; Higham and Lusseau, 2004; Pierpoint and Allan, 2004).

For northwest Mexico, research focused on bottlenose dolphins in the Gulf of California and Pacific Ocean has focused on subjects like community structure, distribution, abundance, morphology, genetics, feeding, home range, movements, behavior, group organization, distribution, size and population structure, photoidentification, residence patterns, reproduction and birth, human impacts and strandings. In Ensenada de La Paz and south of Bahia de La Paz there is a semiresident population of bottlenose dolphins that can be observed almost the whole year and that has been one of the population most studied in Mexico since 1987 through 2006 (Acevedo-Gutiérrez, 1989; Marcín-Medina, 1997; 2010;

Díaz-Gamboa, 2001; Rojo-Arreola, 2002; Valadéz-Suárez, 2002; Salinas-Zacarías, 2005). In 2005 and 2006 a survey was conducted regarding its behavior (Figure 1) and it was determined that this region represents a portion of its critical habitat (Figure 2). Several of their activities take place near the coastline of El Mogote (feeding, breeding, reproduction, socialization, resting), including the outer margin to the Bahia as well as the inner margin to the Ensenada. It is worth mentioning that this is the first time in Mexico that a critical habitat is determined for coastal dolphins. These cetaceans can be observed in groups from 2 to 40 individuals, however small groups (2 to 10 animals) predominate, formed by females with calves and juvenile dolphins which are the ones observed inside the Ensenada; constituting a breeding lagoon (Figures 3 and 4). Its distribution range goes from 1 to 5 m from the coastline and from 1 to 7 m depth, shallow waters where they protect their offspring. Mothers teach their calves to hunt by playing, the mother and the calf throw a fish several in the air until the calf learns to eat it. It is important to mention that juvenile dolphins do not have and innate hunting instinct, it is an acquired trait taught only by its mother. Such a conduct has been frequently observed in the Ensenada channel and in shallow waters near the El Mogote mangrove; small fishes inhabit this area and represent an easy prey. It should also be highlighted that reproductive activity has been observed mainly in the Ensenada channel and in Ensenada mouth (Marcín-Medina, 2010).

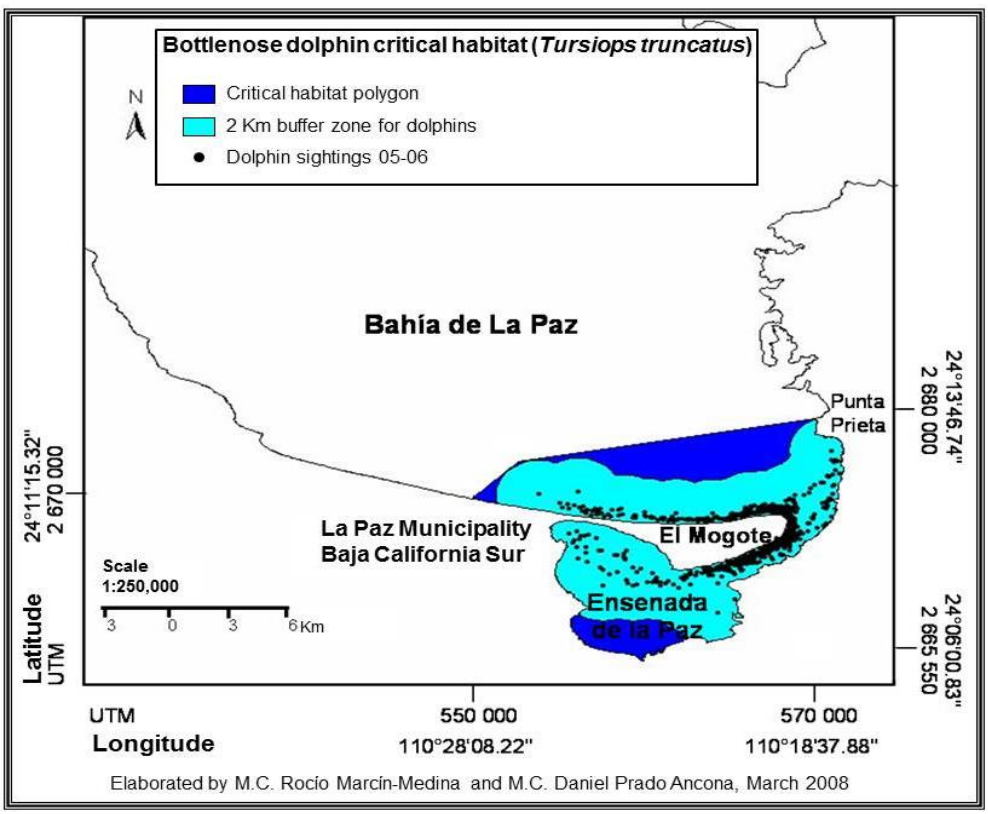

Figure 1. Critical habitat polygon and buffer zone base on bottlenose dolphins (*Tursiops truncatus*) sightings and behavioral studies during 2005 and 2006 in Ensenada La Paz and southeast of Bahia de La Paz (Marcín-Medina, 2010).

Figure 2. Mother and calf traveling in the Ensenada de La Paz channel.
Picture by Alberto León-Gómez-Villacorta.

Figure 3. Juvenile dolphins near to El Mogote in Ensenada channel. Picture by Rocío Marcín-Medina.

This behavioral study was compared with a previous one made on 1995-1996 using the same methodology in Ensenada La Paz (Marcín-Medina, 1997; 2010), finding a habitat shift in 2005-2006 induced by environmental factors and anthropogenic disturbances (Figure 4). Southeast basin was lost (zone 1) due to sedimentation associated with tropical storms, hurricanes and dredging; reduced feeding activity in the northwest basin (zone 2) as a consequence of fisheries and increase in marine traffic superimposed on bottlenose dolphins main route near El Mogote; this affects dolphins' behavior particularly the mothers. This species is very sensitive to noise, in July 2005 it was recorded that a group of females with their calves were feeding when dredging activities of marina Singlair in Fidepaz began (zone 1). Dolphins were scared by sound and never returned to the zone, at least during the whole

year that the survey continued; it is an example of negative effects caused by noise pollution which implies loss of habitat (Marcín-Medina, 2010).

In La Paz city there has been an increase in mega-projects to create more touristic facilities (hotels, marinas) in the last 12 years. Among them there are the "Costa Baja" development in Bahia de La Paz, and the "Paraiso del Mar" development in Ensenada de La Paz; the latter was planned to occupy an area of 504.31 ha with 3,922 residential units, 2,050 hotel rooms in a 6 stories building, two 18-holes golf courses, a desalination plant, 5 wastewater plants, an outer marina with capacity for 535 docking stations, a pier with a building at the end, and a boardwalk (39 ha of marine development in a zone adjacent to Ensenada de La Paz). Clearly, this marina will obstruct the secondary navigation channel of the bottlenose dolphins, and the circulation of vessels will increase the noise pollution in their main transit route, specially where females teach their calves how to feed. Therefore, the construction of this marina poses a threat inside the critical habitat for this species protected by NOM-059-SEMARNAT-2001.

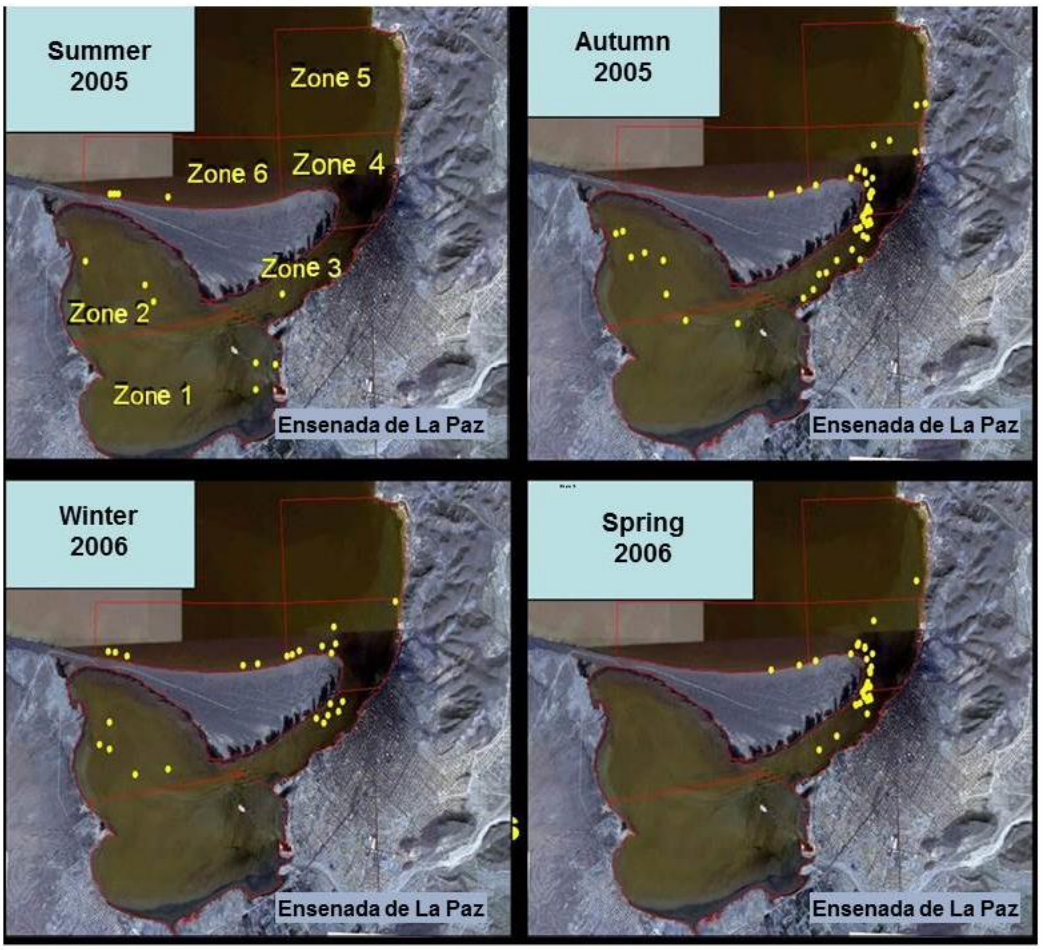

Figure 4. Bottlenose dolphin seasonal feeding zones in Ensenada and south of Bahia de La Paz in summer 2005 and spring 2006. Points represent dolphin feeding location (Marcín-Medina, 2010).

Grounds where this mega-project is planned (over 500 ha) were owned by the state, but in February 2004 a land use change was authorized from forests grounds and several vegetal species were removed from the area. In the written project it was stated that mangrove forest will be removed only in an area of 1.19 ha in order to build both the dry marina and the touristic marina, and that a buffer fringe 100 m wide will be established between the remaining mangrove forest and every construction made by this mega-project. This was not so, even when a group of scientific researchers (including some authors of this chapter) sent letters to the SEMARNAT delegacy (Secretary of Environment and Natural Resources) at La Paz and to the President of Mexico to denounce the damage on mangroves and probably dolphins as well, one month later DGIRA (General Directorate for Environmental Risk Impact) at SEMARNAT authorized the MIA (Environmental Impact Declaration) of this mega-project without the public consultation process required by law. In the resolution of the MIA it was consider that deforestation of 1.19 ha of mangrove forest did not put at risk the ecosystem and that petitioner's proposal for restoration of Enfermeria and El Grande estuaries (only the latter located at El Mogote, where the mega-project is planned) will be enough to mitigate the impact. As for the impact of vessel traffic on the feeding zone of dolphin community DGIRA only manifests that petitioner will be conditioned to develop a protection program for the environmental imbalance and a "survey program of the project's environmental performance" including: indicators for the incidence of nautical activity on main components of marine biota in the area with emphasis on the marine mammal population (*Tursiops truncatus*) present in Ensenada de La Paz, and to include as an indicator the results of the survey program for water quality. Include concrete preoperative actions to the activities of the touristic marina anticipating possible affectations that can occur due to nautical activities inherent to the mega-project to marine biota preservation. Emphasizing on transit routes, feeding and refuge zones for marine mammals, fishes like whale shark (*Rhincodon typus*) and manta (*Manta birrostris*). All this created discontent among some researchers and civil society organizations (OSC), thus a decision to appeal on a long environmental litigation to impugn the authorization of the MIA, defending organisms, values, and ecosystem services of El Mogote was made.

The OSC "Ciudadanos Preocupados A.C." on July 2005 showed the review resource for the violation to the NOM-022-SEMARNAT-2003 for the mangrove affectation, violation to the NOM-059-SEMARNAT-2001 for the detriment to species like whale shark, breeding zones without mitigation measurements, and for considering that proposed measures by the petitioner of the MIA are insufficient to mitigate the damage to Enfermeria and El Grande estuaries. SEMARNAT resolution was unfavorable, stating that the arguments were unfounded without sustenance and insufficient to declare the nullity of the contested resolution. In October 2006 "Ciudadanos Preocupados A.C." replied recurring to an annulment of judgement before the TFJFA (Federal Court of Fiscal and Administrative Justice) where it was requested the impugnment of this development for the violations of NOM-059-SEMARNAT-2001 and NOM-022-SEMARNAT-2003 and because DGIRA did not considered the existence of a presidential mandate declaring protected forest zone the grounds surrounding the city and port of La Paz, publish on August 24, 1938 in the DOF (Federation Official Diary). The mandate includes El Mogote and forbids the use and extraction of live wood, only dead wood extraction is allowed. In February 2008 "Paraiso del Mar" realized without clearance dredging activities and installation of sewage pipelines on the shore of La Paz city where Reforma Street is located. In June of the same year they

repeated the operation, this time where Nayarit Street is located. Although official complaints were stated no authority took action nor penalized those actions in any way. In response, judges from S11M (Metropolitan Hall #11) of TFJFA emitted the record 32183/06-17-11-3 on April 3rd, 2010 which cancels the clearance granted by DGIRA to the real-estate mega-project "Paraiso del Mar" regarding environmental impact. They were no longer authorized to perform any operation or development including the marina and its environmental effects given that the permission previously granted violated protection regulations for mangrove forests (NOM-022-SEMARNAT-2003) ignoring that the 500 ha grounds for such project are located in a zone declared as a wetland of international importance by the Ramsar convention (site #1816). Petitioners presented two legal appeals which were both denied by PJF (Federal Judicial Power), meanwhile it was expected that SEMARNAT through DGIRA emitted a resolution against "Paraiso del Mar" thus denying any development to this mega-project. Surprisingly SEMARNAT disrespectfully ignored judges' decision violating legal order, while real-estate enterprise continued working. Despite all above mentioned, during March 2011 Paraiso del Mar started illegally to dredge, construct and operate of the marina in the Ensenada de La Paz channel adjacent to the mangrove zone (Figures 5 and 6). Although organizations like CEMDA (Mexican Center of Environmental Right), AICMMARH, A.C. (Research and Conservation Association of Marine Mammals and their Habitat), and ConCiencia denounced this activities to SEMARNAT at local and federal level, as well as to PROFEPA (Federal Attorney for Environmental Protection), neither authority took action to stop such activities. One of PROFEPA's guidelines established on June 1st, 2009 states that when an enterprise performs an activity that requires environmental clearance without authorization it will be closed. Nevertheless this development continued with the construction of a pier for 25 vessels. After several months the activities ceased because of the complaints; nowadays the hotels zone, and the golf course still operate; and the residential zone is inhabited. In March 2014 Guillermo Haro Belchez, head of PROFEPA, stated that this project will be reviewed and that if it does not comply with the required specifications it could be closed or even be demolished (2).

In October 2008 another mega-project named "Entremares" presented its MIA in the modality regional. This development was planned at El Mogote adjacent to "Paraiso del Mar", with a property size of 390.76 ha to construct touristic, residential and hotels infrastructure. Development included construction of a system of tidal channels, a docking for 440 vessels, an 18-holes gulf course, shooting practice field, commercial and residential lots, hotels, duplex houses, single-family houses, villas, commercial areas, clubhouse, with a total of 3,420 housing units or its equivalent to 6,840 hotel rooms. Tidal channels will serve for vessels to circulate and dock inland. In order to develop this project, the petitioner required a deforested area of 285.99 ha, 73.2% of the total surface of its property. Tidal channel system would be formed by smaller channels in an area of 4.3 ha in the salitrales zone and/or in the restriction zone specified in the numeral 4.16 of the NOM-022-SEMARNAT-2003 (minimum of 100 m to the mangrove fringe). The larger navigation channels forming the touristic-residential complex would cover an area of 37.68 ha, including two main flow channels inside Ensenada de La Paz 50 m wide on the sea, and 30-40 m wide on the land with a depth of 2-3m. In the adjacent lots to the tidal channels, floating docks is planned with 9 to 18 m long for a total of 440 docking facilities. For this mega-project DGIRA did make a public consultation and a public meeting in November. Different opinions were exposed against this development like damage to mangroves, Marcin-Medina delivered data regarding the critical habitat of

dolphins and the impact of vessels on its main transit route where frequent feeding and socializing activities take place. In November 2009 in the resolution made by DGIRA, 7 state and federal agencies opposed to this project, like CONANP (Natural Protected Areas Commision) in consideration of articles 3 and 4 of Ramsar Convention did not consider feasible any deforestation, digging, and dredging activities for the opening and construction of channels for the "Entremares" mega-project inside the Ramsar polygon wetlands Mogote-Ensenada de La Paz. In an unprecedented action, the DGVS (General Directorate for Wildlife) a branch of SEMARNAT, declares for the first time the damage to dolphins stating that the petitioner does not indicate the dates when the field surveys were realized, which could limit the identification of environmental impacts. For example, information about the presence of bottlenose dolphins and how they utilize the region was presented, but it was not considered which would be the impacts from vessel use over bottlenose dolphin's populations, as well as for whale sharks, given the fact that the mega-project plans the construction of piers inside the navigation channels; thus it is to be expected that these vessels do not only use the projected channels but the whole Ensenada de La Paz region as well. Despite all these arguments, DGIRA approved the mega-project with some conditions; one of them was land use change, which was denied by the municipality of La Paz. Meanwhile organizations like AICMMARH, A.C., CEMDA, Tiburon Ballena Mexico, and ConCiencia pointed out the risks that this project represented to local species; these opinions where written in the annulment of judgment before the Federal Court of Fiscal and Administrative Justice. In September 2012 the TFJFA permanently nullify the Environmental Impact Authorization (AIA) of "Entremares" and determined that the petitioner did not consider in the project the possible damage caused to endangered species, like California sea lions, coyotes, gray foxes, and raccoons, all of them inhabitants of the forested protected zone El Mogote, located in front of La Paz city in Baja California Sur. Petitioner appealed legally but in November 2013, the Eleventh Collegiate Tribunal confirmed the nullity of the AIA, resolving to deny the appeal for considering that the 1938 decree that declares the port of La Paz area as a forested protected zone, is valid as a natural protected area (ANP) in the category "protection area for flora and fauna", according to LGEEPA (Ecologic Equilibrium General Law).

Figures 5 and 6. Dredging for marina Paraiso del Mar (A) in March 2011. Already dredged zone and pier with docking for 25 vessels (B). Both pictures by R. Siddharta Velázquez Hernández.

MARINA AZUL AND THE ACCUMULATIVE AND SYNERGIC IMPACTS

Another case is the fact that during the development of the Environmental Impact Study of the Project Marina Azul the consultant did not made a good evaluation because some relevant issues were not taking in consideration. Direct, synergistic, cumulative and residual ecological corridor in the southern Bay of La Paz impacts because we believe there are omissions, methodological faults, flaws and gaps in the Environmental Impact Statement filed. In the case of omissions, you can make a direct reference to the literature review of the relevant area as a list of species cannot be restricted to the exclusive area of the project and should be included in the zone of influence of the project as They focused on the development zone but not in the region. This project was categorized as REGIONAL so the information should be presented as part of the MIA. The document was presented with a list of some pretty grim and updated references.

It represents an omission the fact that there was a collection of diverse taxa marine, coastal and land that should be considered in the report. Within my count there are about 3000 species inhabit ecosystems corridor Bay La Paz-Bay Window (considering the Archipelago of Isla Espiritu Santo and Isla Cerralvo) where they have at least 50 endemic species and at least 500 species NOM ECOL are 059. There are at least 8 ecosystems (mangrove forests, rocky reefs, coral reefs, forests of seaweed, maerl beds, seagrass, sacrocaule scrub and marsh grasses) that did not show appropriate information distribution in the region. The project is located in the fishing 7 Bay of La Paz and represents an important area for fishermen in the region. The document does not present a single analysis of the situation of coastal fisheries. information relevant to the project are ignored as the winds relationship with respect to the movement of marine sediments pair raised. The Pleistocene terrace which covers almost 50% of the project was not considered. It is not considered the presence of invasive species in the project area (at least 2 species of invertebrates in the area and at least 4 of macroalgae in the vicinity) that could increase your coverage by leaks resulting nitrogen-rich water Golf Equestrian Club and pastures. So this project could well be causing direct impacts to ecologically and economically important populations.

The study data have methodological errors as the data collection were not raised using widely accepted standards in academia so that the data presented have presented interpretative vices. This is particularly evident in the methodology for determining the coverage of corals, macroalgae, density of vertebrates and fish for sea area but could set the same standard for the land area. The major mistakes I find in these data are in the design of sampling as no samples were statistically significant aftershocks in which clearly show that the project area is homogeneous in this biological corridor. Having no appropriate sampling design and not having a robust database we could not calculate the residual impact that could estarse generated as part of the development.

This Environmental Impact Statement has gaps in information and in the vicinity of this project there are 6 projects already approved and some of them already in operation shown in Figure 7: Maravia (adjacent), Punta Blanca (adjoining), El Judio (next), Azul del Cortes (next), The Saltito (next) and Vista Cerralvo (near and longer active). There is at least one mining concession adjacent and in addition to the desalination plant proposed in this project, there are two more: one in Maravia (adjacent) and another planned for the south of the CNA

project. This set of projects having at least 4 sea which would add to the already operational in 5 Peace and plan tourist Megacruceros input. In addition there are two thermal power projects in close and marine aquaculture area. All these projects will promote the increase in population along with the frequency of visitors to the ANP: Sloop Gulf Islands and ANP in a first stage but most certainly to other areas such as Cabo Pulmo ANP by increased traffic in the calculation boats least 2 thousand boats continuously circulating. This was not evaluated as part of the MIA and displayed synergistic and cumulative impacts that have not been evaluated or resolved herein. All these projects represents the increase in population which also put pressure on the aquifer of La Paz, La Ventana and Dam Buena Mujer.

Figure 7. Overall view of Marina Azul project and related approved projects and desalinization plants.

CONCLUSION

The way in which Mexican environmental authorities like SEMARNAT have authorized touristic-nautical developments like Paraiso del Mar and Entremares in La Paz, B.C.S. shows a clear systematic violation of our environmental law (LGEEPA) resulting in the deforestation of mangrove forests, modification of the coastline and damage to protected species.

There are 5 wetlands inside Ensenada de La Paz with mangrove forests; El Mogote is one of them affected by deforestation, garbage, construction for touristic activities, and dredging of water channels. As it could be observed, main threat is the establishment of touristic facilities like Paraiso del Mar or Entremares, the potential impact of the latter was higher; because the petitioner pretended to remove the mangrove to build the inner channels so residents could board their vessels outside their houses. El Mogote is bordered by red and black mangrove, is considered a highly fragile environment, acknowledge as Ramsar site #1816 on February 2nd, 2008 (Wetlands Mogote-Ensenada de La Paz).

Ensenada de La Paz and south of Bahia de La Paz are part of the critical habitat for groups of female's bottlenose dolphins, which they use during most of the year for feeding as well as nursing, protect them against predators, socialize and rest. One potential threat that can affect this protected species is coastal touristic nautical development, with an increase in the number of constructions and marinas along the coast. The latter will influence habitat quality due to the increase in the number of vessels traveling in the zone, with a concomitant increase in marine noise; hearing system is the most important sense for the development of different biological activities in cetaceans.

In Mexico critical habitats are mentioned in articles 63 and 64 of the LGVS (General Law for Wildlife), those articles were reformed by the Commission of Environment, Natural Resources and Fish from the Republic Senate (DOF, 2010), stating that SEMARNAT has the authority to declare critical habitats by means of a secretariat agreement, based on scientific information of species. Critical habitats are specific areas in which organisms develop their essential biological processes (feeding, reproduction, socialization, and resting). Physic, chemical or acoustic pollution, as well as risk of collision with vessels can affect populations of coastal marine mammals. Therefore SEMARNAT with the aid of researchers specialized in such species and their behavior must decrete zones as critical habitats and consider management measures for the conservation of species. Without a doubt, one of these zones is Ensenada de La Paz and south of Bahia de La Paz, that should be legally acknowledged as a critical habitat for dolphins; this will help for the protection and conservations of the whole ecosystem. By means of management programs, human activities that could impact the critical habitat of bottlenose dolphins must be regulated; this impact is higher for mothers with calves and juvenile dolphins, which according to the environmental laws of our country should be protected.

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Chapter 26

THE KEY ROLE OF THE SPECIES *HEDISTE DIVERSICOLOR* (POLYCHAETA, NEREIDIDAE) IN ESTUARINE ECOSYSTEMS

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ABSTRACT

The ragworm *Hediste diversicolor* (O. F. Müller, 1776) is a widespread species of coastal lagoons and estuaries from Morocco to Scandinavia. Population dynamics of *H. diversicolor* from the Atlantic coast of Morocco were studied in the Bou Regreg Estuary (Gillet, 1993), in the Souss Estuary (Aït Alla et al., 2006); in Spain, in the Bay of Cadiz (Arias & Drake, 1995); and in Portugal in the estuarine systems (Sprung, 1994; Fidalgo e Costa et al., 1998; Abrantes et al., 1999). In the North Atlantic Ocean, *H. diversicolor* was studied in the Severn Estuary (Mettam, 1979; Mettam et al., 1982); in the North Sea, in the Ythan Estuary, Scotland (Chambers & Milne, 1975); in North East England (Olive & Garwood, 1981); and in the North Norfolk (Nithart, 1998). *H. diversicolor* is a major link in food webs and of economic importance as bait for fishing in several European countries. This species was chosen because it has a key role in the functioning of estuaries, a major link in benthic food webs and through sediment reworking by bioturbation activity. In France, the population dynamics of *H. diversicolor* was first studied in the Loire Estuary (Gillet, 1990; Gillet & Torresani, 2002) and the bioturbation activity (Gillet et al., 2012). This is a preliminary work to a large scale research program on *H. diversicolor* in different estuaries with the PNETOX '*National Program of Ecotoxicology*'. In the framework of the French National Program of Ecotoxicology, environmental quality was assessed in the multi-polluted Seine Estuary and the comparatively clean Authie Estuary, France (2002-2004), by determining contaminant levels in water, sediments and the infaunal worm *H. diversicolor* (Durou et al., 2003; Amiard-Triquet et al., 2007; Gillet et al., 2008). A scientific project comes under the

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framework of the International network Nereis Park Experiment gathering 27 laboratories from all over the world. The experiments were carried out concurrently in spring 2007 using a common protocol to study the bioturbation activity of *H. diversicolor* (Gillet et al., 2012; Gilbert et al, in press).

Keywords: *Hediste diversicolor*, Polychaeta, estuaries, population dynamics, bioturbation

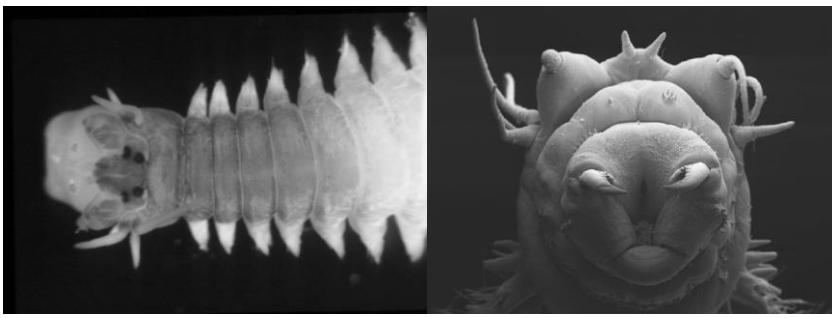
1. INTRODUCTION

The endobenthic ragworm *Hediste diversicolor* (O. F. Müller, 1776) is a widespread species of coastal lagoons and estuaries from Morocco to Scandinavia. Population dynamics of *H. diversicolor* from the Atlantic coast of Morocco was studied in the Bou Regreg Estuary (Gillet, 1993), in the Souss Estuary (Aït Alla et al., 2006); in Spain, in the Bay of Cadiz (Arias and Drake, 1995); and in estuarine systems of Portugal (Sprung, 1994; Fidalgo e Costa et al., 1998; Abrantes et al., 1999). In the North Atlantic Ocean, *H. diversicolor* was studied in the Severn Estuary (Mettam, 1979; Mettam et al., 1982); in the North Sea, in the Ythan Estuary, Scotland (Chambers and Milne, 1975); in North East England (Olive and Garwood, 1981); and in the North Norfolk (Nithart, 1998). *H. diversicolor* is a major link in food webs and of economic importance as bait for fishing in North Atlantic Ocean. This species was chosen because it has a key role in the structure and functioning of estuaries. It plays a major link in benthic food webs and through sediment reworking by bioturbation activity. In France, the population dynamics of *H. diversicolor* was first studied in the Loire Estuary (Gillet, 1990; Gillet and Torresani, 2002) and the bioturbation activity (Gillet et al., 2012). This is a work to a large scale research program on *H. diversicolor* in different estuaries through the PNETOX 'National Program of Ecotoxicology'. In the framework of the French National Program of Ecotoxicology, environmental quality was assessed in the multi-polluted Seine Estuary and the comparatively clean Authie Estuary, France (2002-2004), by determining contaminant levels in water, sediments and in the infaunal worm *H. diversicolor* (Durou et al., 2003; Amiard-Triquet et al., 2007; Durou et al., 2007; Gillet et al., 2008). A scientific project comes under the framework of the International network Nereis Park Experiment gathering 27 laboratories from all over the world. The experiments were carried out concurrently in spring 2007 using a common protocol to study the bioturbation activity of *H. diversicolor* (Gillet et al., 2012; Gilbert et al, in press).

2. MATERIALS AND METHODS

A survey within the French National Program of Ecotoxicology was carried out in 2002, 2003 and 2004 to study the responses of *H. diversicolor* populations to the impact of pollution in the multi-polluted Seine Estuary comparatively to the reference Authie Estuary (non-contaminated site). Samples of *H. diversicolor* (Figure 1) were collected in both sites: Authie Estuary (Station: 50°22,217'N; 1°36,484'E) and in the Seine Estuary, (Station: 49°26,823'N; 0°14,628'E) in 2002 – 2004 (Figure 2). Four quadrats at 25 cm^2 to a depth of 20 cm were taken in order to calculate density and biomass of the target organisms. Quadrat sediment was placed on mesh wire pan and sieved. *H. diversicolor* samples were measured

for fresh weight and then placed in ethanol tubes. Each individual was then tested to measure the length of L3 (Gillet and Torresani, 2003; Ait Alla et al., 2006; Durou et al., 2007). L3 corresponds to the length measurement of the first three segments: prostomium, peristomium and first chaetiger. The length of these first three segments is positively correlated with the fresh weight and used to determine trends in size within the population. The size frequency histograms were treated by the SAEM (Simulated Annealing Estimating Mixture). The algorithm EM (Dempster et al., 1977; Redner et al., 1984) was used for the identification of parameters of a mixture and replaces the methods based on the analysis of the modes of the histograms. An amelioration of the algorithm EM consisted to base on methods of recuit simulated where result the name SAEM (Simulated Annealing E, M), to introduce one random perturbation of the probabilities -a posteriori- of affectation (Soubiran et al., 1991; Celeux et al., 1995). This method presents numerous developments to study the size frequency histograms to determine cohorts within a population (Robert, 1996).



(a) (b)

Figure 1. Anterior region of *Hediste diversicolor* in binocular microscope (A) and frontal view in scanning microscope (B).

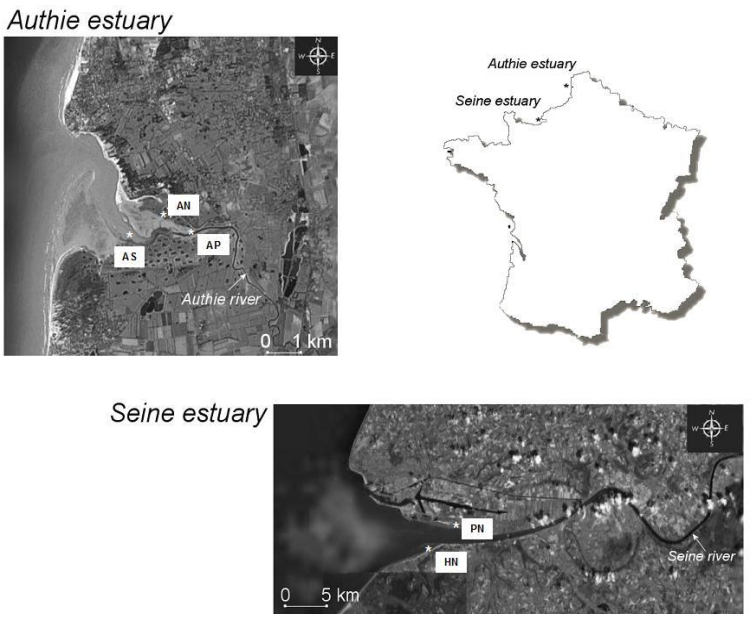


Figure 2. Locations of sampling site in the Authie and Seine estuaries, France.
National Program of Ecotoxicology.

3. POPULATION DYNAMICS OF *HEDISTE DIVERSICOLOR*

In 2002, the density of *H. diversicolor* ranged from 1,084 to 3,584 individuals m^{-2} in the Authie Estuary and from 136 to 920 ind m^{-2} in the Seine Estuary. In 2003, the densities varied from 912 to 3,328 ind.m^{-2} in the Authie Estuary and from 80 to 288 ind.m^{-2} in the Seine Estuary. A decrease of the density was observed in the Seine Estuary with 920 ind.m^{-2} in February 2002 and only 80 ind.m^{-2} in November 2003. In 2002, the biomass of *H. diversicolor* showed temporal variations with a maximum of 32.66 g.m^{-2} in July and lower values in February with $\overline{B}$ = 21.55 g.m^{-2} in the Authie Estuary. The biomass varied from 3.42 g.m^{-2} in February to 0.68 g.m^{-2} in September in the Seine Estuary. In 2004, the density varied from 672 to 3,512 ind.m^{-2} in the Authie Estuary and from 120 to 192 ind.m^{-2} in the Seine Estuary. Biomass varied from 3.94 g.m^{-2} (dry weight) in February to 26.77 g.m^{-2} in August in the Authie Estuary and from 0.56 g.m^{-2} in February to 1.25 g.m^{-2} in August in the Seine Estuary. Density and biomass are greater in the uncontaminated site of the Authie Estuary than in the contaminated site of the Seine Estuary (Figure 3). When the data was compared to mean historical values (from 2002 to 2015) collected overlapping the site of collection (radial 3 and 5) (Figure 4) values showed that the highest recorded density was 1,293 ± 850 ind.m^{-2} in 2001 and the lowest recorded value was 25 ± 12 ind.m^{-2} in 2010. Results showed that the average density for *H. diversicolor* was higher than previous collections between the years 2009-2013. The density of *H. diversicolor* was also compared to historical data collected at Authie and Seine estuaries in February (2002, 2003 and 2004) (Gillet et al., 2008). Results showed that the density of individuals collected was lower than values observed in the previous study when compared to Authie, as demonstrated in Figure 4.

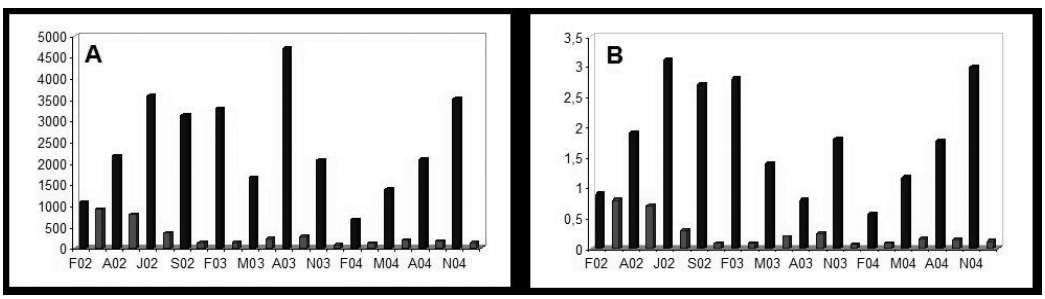


Figure 3. Density (A) and biomass (B) of *Hediste diversicolor* in the Authie and Seine estuaries, France. National Program of Ecotoxicology carried out in 2002-2004 (Gillet et al., 2003).

With the same size, the individuals from the Seine Estuary have a low biomass. For the individuals from the Authie Estuary, the relationship between Dry Weight (DW) and length (L3) was $DW = 4.2205\ L3^{2.9832}$ with r = 0.66 (N = 57 individuals). For those from the Seine Estuary, the relation between Dry Weight and L3 was $DW = 0.4697\ e^{1.7209L3}$ with r = 0.38 (N = 32 individuals) (Figure 5).

The size frequency histograms of the length L3 were separated into size classes from 0.3 to 4.3 mm (Figure 6). Most of the size frequency histograms were plurimodal with 2 or 3 cohorts. In 2002, we observed three cohorts C1, C2 and C3 in February in the Authie Estuary.

From April to September, there were two cohorts C2 and C3 then two cohorts C3 and C4 in the Authie Estuary. In the Seine Estuary, we observed two cohorts C2 and C3 in February but only one cohort C3 in April and July and two cohorts C3 and C4 in September 2002. The individuals of *H. diversicolor* from the Authie Estuary belonged to eight different cohorts instead of six cohorts for those from the Seine Estuary. The life time for a cohort was 13 months in the Authie Estuary and varied from 6 to 11 months in the Seine Estuary (Figure 7). These differences of the number of cohorts could be attributed to the effect of pollution on the population of *H. diversicolor* (Durou et al., 2007).

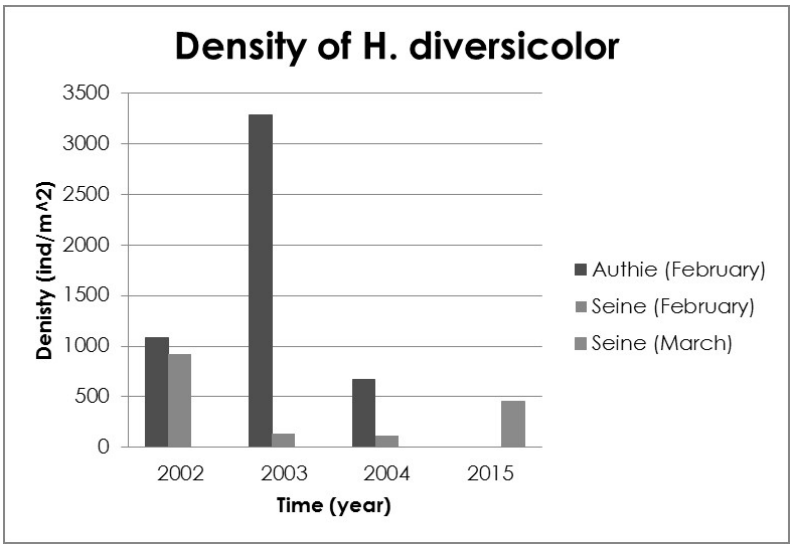


Figure 4. Comparison of *H. diversicolor* density to historical data acquired from Seine (polluted) and Authie (non-polluted) Estuary.

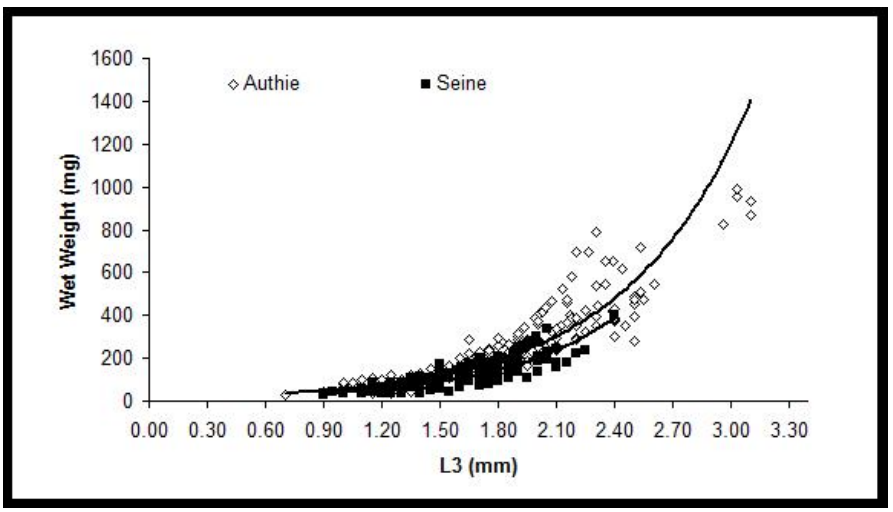


Figure 5. Comparison of individual size and weight of *H. diversicolor* from Seine (polluted) and Authie (non-polluted) Estuary.

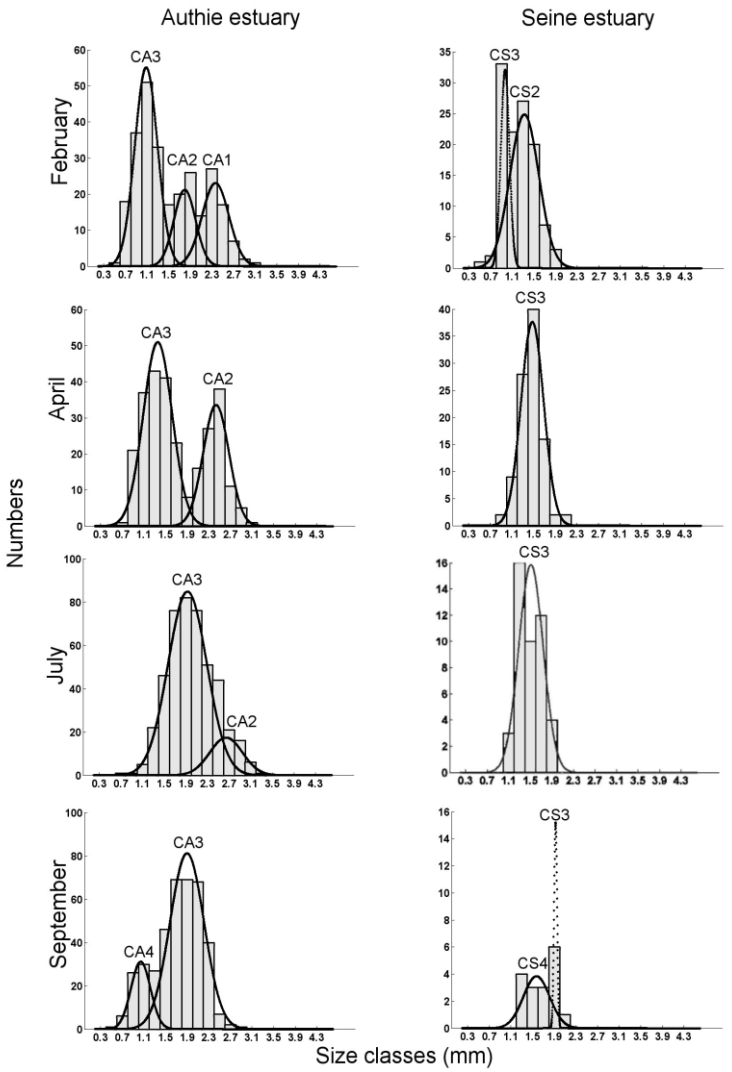


Figure 6. Comparison of size frequency histograms of *H. diversicolor* from Seine (polluted) and Authie (non-polluted) Estuary (2002).

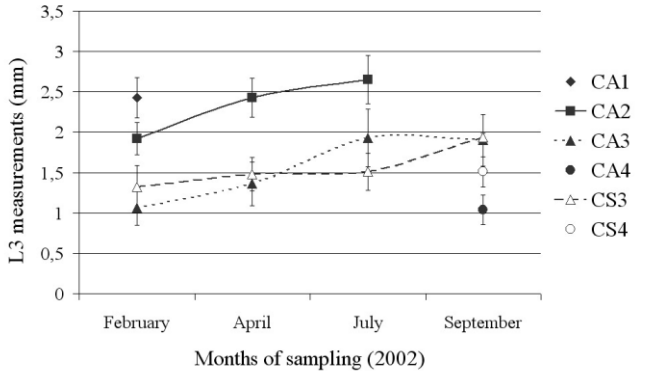


Figure 7. Number of cohorts of *H. diversicolor* from Seine (polluted) and Authie (non-polluted) Estuary (2002).

In 2002, the mean size L3 of the cohorts varied from 1.04 mm to 2.65 mm in the Authie Estuary and from 1.32 mm to 1.94 mm in the Seine Estuary. In 2003, the mean size L3 of the cohorts varied from 1.08 mm to 2.27 mm in the Authie Estuary and from 1.27 mm to 1.82 mm in the Seine Estuary. In 2004, the mean size L3 of the cohorts varied from 1.01 mm to 2.41 mm in the Authie Estuary and from 1.07 mm to 1.77 mm in the Seine Estuary. The size of individuals was lower in the Seine Estuary than in the Authie Estuary. These differences of the mean size could be attributed to the effect of pollution on the population of *H. diversicolor* (Durou et al., 2007).

Comparison with Other Estuaries

The density of the population of *H. diversicolor* observed in the Seine Estuary was similar to those observed in the Loire Estuary in 1987 with 800 to 3,200 ind.m^{-2} (Gillet, 1990) and in 1999 with 300 to 2,560 ind.m^{-2} (Gillet and Torresani, 2003). The biomass observed in the Authie Estuary was higher than biomass observed in the Loire Estuary in 1987 with 16 g.m^{-2} (Gillet, 1990). In the Seine Estuary, density and biomass decreased from 2002 to 2003 and seemed to show a regression of the population of *H. diversicolor*. The lower densities of *H. diversicolor* were comparable with densities from polluted parts of the Tees Estuary (Gray, 1976). In the Ems Estuary, North Sea, Essink et al., (1985) found decreasing densities of *H. diversicolor* toward a point of discharge of organic waste. They suggested two causes: mortality and migration. Such a migration was observed in the oued Souss Estuary in Morocco by Aït Alla et al., (2006). Two recruitment periods were detected each year. These results were similar to the data of Gillet (1990) and Gillet and Torresani (2003) in the Loire Estuary. In Southern regions, the populations of *H. diversicolor* presented a longer spawning period and two recruitment periods in spring and autumn Abrantes et al. (1999), Aït Alla et al., (2006). In Northern regions, the populations of *H. diversicolor* had a short spawn and only one recruitment period, usually in spring or summer. Thus migration and low recruitment were likely to be involved in the disappearance of the worms in the Seine Estuary. Pollution might affect the recruitment of juveniles, the number of cohorts and the growth of individuals (Durou et al., 2007).

4. BIOTURBATION ACTIVITY OF *HEDISTE DIVERSICOLOR*

This scientific project comes under the framework of the International network Nereis Park Experiment gathering 27 laboratories from all over the world (Figure 8). The experiments were carried out concurrently in spring 2007 using a common protocol to study the bioturbation of *H. diversicolor* (Figure 9). Sediments and polychaetes were sampled in 18 locations around the world from May to July, 2007. Based on a common experimental protocol, surface sediments (0-5 cm depth) were sampled and handled similarly in the 18 participating laboratories, i.e., sieved through a 1-mm mesh to remove large animals and debris, and then homogenized. Subsamples of sediments were collected for organic matter (OM) content and for sediment grain size determination. *H. diversicolor* was used by 16 laboratories. All laboratories obtained worms at natural sampling sites. The worms were

weighed individually and sorted to obtain samples with a biomass representative for local natural populations. They were kept in homogenized sediments with aerated water, under a 12: 12 dark: light cycle and at *in situ* temperature until experimental use. Four PVC or Plexiglas core tubes (height: 20 cm; internal diameter: 10 cm) were filled with homogenized sediments (15 cm deep sediment column) and incubated for a week in experimental containers under the previously described conditions. Four worms were introduced in each of three cores, leaving the last core as a control. Worms were allowed to establish burrows for 7 days before the addition of particle tracers. Two g of fluorescent inert particles (63-125 μm; Partrac Ltd., Glasgow, UK) were suspended in water into a tube and homogeneously deposited through the overlying column on the surface of each sediment column (resulting in a visible layer of ≤ 1 mm thick). After 10 days of incubation, the overlying seawater was removed and the experimental cores were carefully sectioned into 0.5-cm thick layers from the surface down to 2 cm depth, and 1-cm thick layers down to 15 cm. The sediment from each layer was freeze-dried, homogenized and shipped to EcoLab (Toulouse, France) for further analysis. At EcoLab sediment subsamples were processed for counting of luminophores under UV-light (light peak at 365 nm; digital camera Olympus C-2500L; image analysis software Image-Pro Plus).

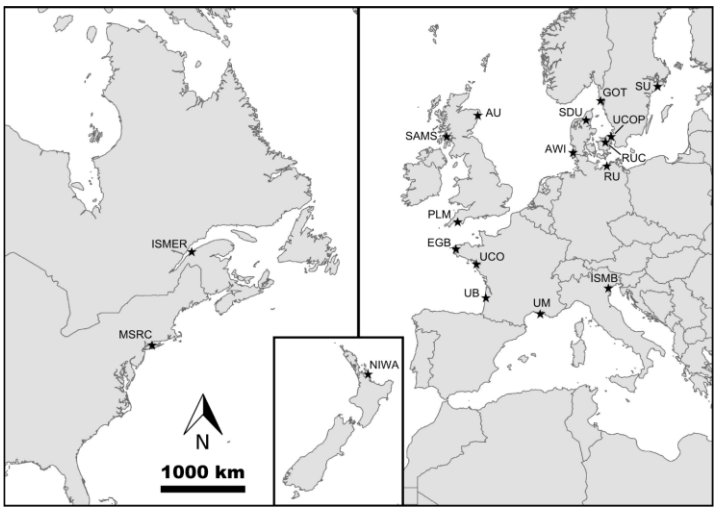


Figure 8. Locations of sampling sites for the 18 laboratories involved in the Nereis Park Experiment (Gilbert et al., in press).

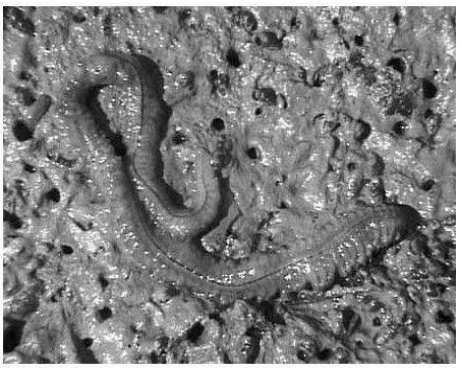


Figure 9. Reworking activity of *Hediste diversicolor* in the sediment of estuarine ecosystem.

4.1. Bioturbation Activity of *Hediste diversicolor* in the Loire Estuary

In the Loire Estuary, samples were collected in the intertidal zone of the Saint-Nazaire Bridge (Figure 10). During the field experiment, we estimated the surface area of the burrow walls (Sb) value varied from Sb = 0.26 m2.m-2 to 2.18 $m^2.m^{-2}$ (mean Sb = 0.75 $m^2.m^{-2}$), and the pumping rate (Pr) value from Pr = 0.9 $l.d^{-1}m^{-2}$ to 7.7 $l.d^{-1}$ m^{-2} (mean value Pr = 2.7 $l.d^{-1}m^{-2}$). The density and the biomass of *H. diversicolor* populations largely controlled the amount of reworked sediment (Figure 11).

In the laboratory, we estimated the pseudo-diffusive mixing, D_b value varied from D_b = 1.2 to 1.6 (mean D_b = 1.5). The non-local transport (r) from the upper layers to the bottom of the tubes varied from r = 1 to 7.2 (mean r = 4.3) (Figure 11). A positive relationship could be established between D_b and the temperature while on the contrary a negative relationship could be established between the D_b and biomass. It seems that small individuals were more efficient reworkers than bigger ones.

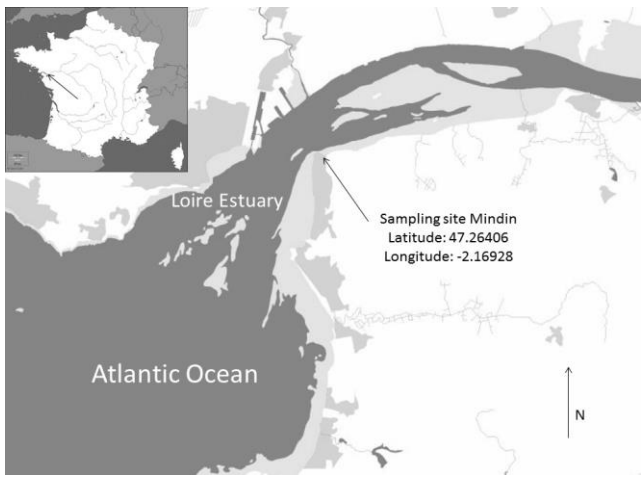


Figure 10. Location of the sampling site in the Loire Estuary, France for the Nereis Park Experiment.

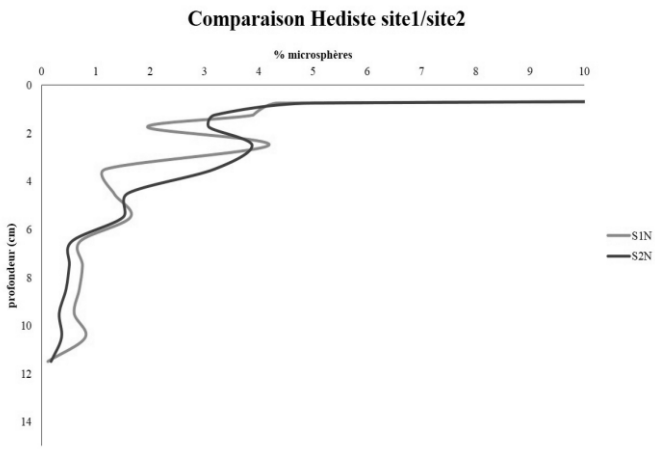


Figure 11. Luminophore profiles obtained with the gallery-diffusor model *H. diversicolor* in the Loire Estuary, France (Gillet et al., 2003).

4.2. Bioturbation Activity of *Hediste diversicolor* in the Nereis Park Experiment

The results provided by this study allowed ranking of the global sediment reworking intensity by *H. diversicolor* populations between different geographical locations. In an attempt to investigate the possible causes of this ranking, correlations between sediment mixing proxies (MPD, D_b and r) and the registered experimental, biological and environmental parameters were tested. The apparent biodiffusion-like coefficient D_b and the non-local coefficient r allowing the best fit with the modelled profile according to the least square method were obtained for each experimental luminophore profile (Figure 12). In addition, the maximum penetration depth of luminophores (MPD) was used as a proxy to estimate sediment reworking depth.

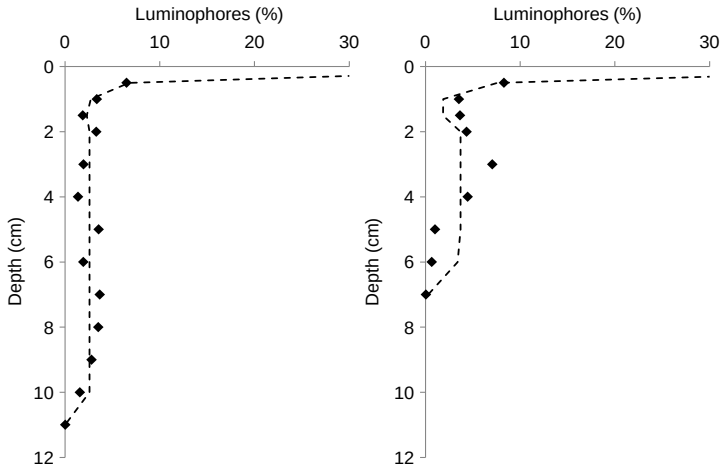


Figure 12. Luminophore profiles (diamonds) and corresponding modelled profiles (dotted lines) obtained with the gallery-diffusor model *H. diversicolor* in the Nereis Park Experiment (Gilbert et al., in press).

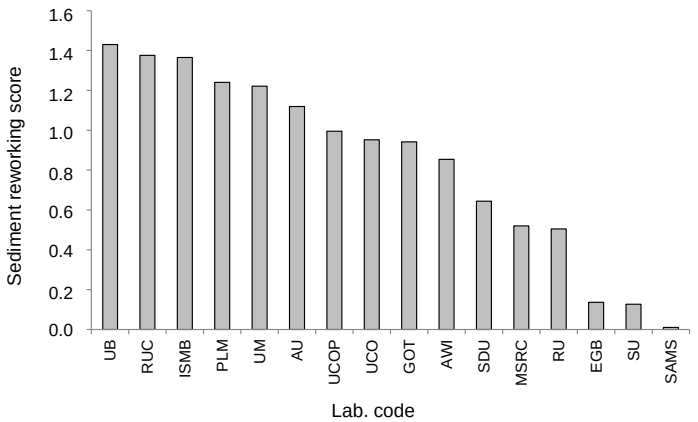


Figure 13. Sediment reworking score integrating the values of both the biodiffusion-like coefficient *Db* and non-local coefficient *r* obtained for the sixteen *Hediste diversicolor* laboratory experiments involved in the Nereis Park Experiment (Gilbert et al., in press).

The global sediment reworking intensity among experiments (i.e., geographical locations) involving *H. diversicolor* was ranked according to the sediment reworking scores integrating the values of both the biodiffusion (D_b) and non-local (r) coefficient. The highest score (i.e., the highest global sediment reworking intensity = 1.42) was found for UB (France) and the lowest for SAMS (Scotland) (score = 0, i.e., the lowest values of D_b and r for the whole study) (Figure 13).

However, beyond the specific results obtained on *H. diversicolor* sediment reworking, this inter-laboratory work highlighted the usefulness of joint experiments in functional ecology. Indeed, in order to investigate some process at a global scale while integrating a set of biological and environmental parameters which differ locally over more or less large gradients, this comparative approach had good merits in comparison to what could be achieved with a single experimental study at one location to assess the same range of factors, due in part to the numerous experimental conditions to be considered which would require the set up an extremely complicated experimental design. Covering large, natural ranges in such a comparative study compensates for the correlative nature of the results obtained. Due to its high physiological plasticity, the ragworm *H. diversicolor* appears to be able managing burrow construction and sediment reworking within a wide range of soft bottoms. The sediment reworking intensity by this species has been shown to be quite variable at a global scale and to be governed by both biological and environmental parameters.

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Chapter 27

HOX-GENES IN THE ONTOGENESIS OF POLYCHAETES

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ABSTRACT

The basic plane and evolution of bilateral animals (Bilateria) are closely associated with Hox-genes. These genes usually exist in the genome in the form of clusters, gene complexes with a conservative position of individual genes inside them. These genes encode transcription factors containing a homeodomain, and their expression is ordered along the main body axis in accordance with the rule of colinearity. This means that the position of a gene in the cluster determines its spatial and, sometimes, temporal expression in the embryo. In vertebrates and arthropods, which are studied in most detail, Hox-genes define the morphological distinction between different regions of the body (tagmas). Boundaries of Hox-genes expression often coincide with the borders of tagmas. In addition, these genes also function in adult organisms, where they are involved in regeneration processes and the maintenance of tissue homeostasis. In recent years, there has been an surge of interest in the study of Hox-genes in animals that are not traditional models of molecular biology. Polychaetes are the most intriguing objects of such studies. They belong to a poorly studied evolutionary branch of Bilateria, the Lophotrochozoa, which is enough to make them attractive for both molecular and developmental biologists. Among polychaetes, there are both families with morphologically specialized segments grouped into tagmas (Chaetopteridae) and families with numerous identical segments (Nereididae). There are polychaete species with impressive abilities to regeneration of the head and the tail and those incapable of regeneration at all. In addition, the life cycle of polychaetes often includes the larva, which may be essentially different from an adult worm. How do the Hox-genes work in so different systems? Can we compare some of their functions with those of homologous genes in insects and vertebrates? What is their place in the hierarchy of molecular regulators controlling developmental processes in polychaetes? If we answer these questions, many pieces of the jigsaw puzzle representing the earliest evolution of bilaterian animals would fall into

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place. In this review, I present an up-to-date information about Hox-genes in polychaetes and make some cautious assumptions about the ancestral functions of the Hox-cluster.

Keywords: Annelida, Hox-genes, UrBilateria, larval development, regeneration

1. INTRODUCTION

In this chapter, I describe Hox-genes of polychaetes, their structural organization and functional features in the ontogenesis. Among hundreds of evolutionary conservative gene families, Hox-genes are especially important for understanding the relationship between individual development and macroevolution. Edward B. Lewis, an American geneticist scientist (Nobel Prize in Medicine 1995), was the first to discover this relationship. In 1987 he published the results of his long-term studies of the segmentation control pattern in *Drosophila* in *Nature* (Lewis, 1987). He used mutation analysis to consider the structure and the function of specific *bithorax* locus (a gene complex bithorax or BX-C). Calvin Bridges, who worked in the laboratory of Thomas Hunt Morgan, isolated and described a strange *bithorax* mutation at this locus in *Drosophila* as early as in 1915. This was a classic homeotic mutation, with the third thoracic segment being partially transformed into the second one. In this case, the anterior part of the halter became the anterior part of the wing (Bridges and Morgan, 1923).

Lewis knew nothing about the nature of factors encoding the BX-C sequence but made several important conclusions, most of which proved true. Some of them are as follows:

1) BX-C genes form a cluster, a complex of genes, which resulted from tandem duplications of an ancestor gene with subsequent mutational divergence of its offspring;
2) The position of the gene on the chromosome is directly connected with the spatial order of the gene's activation (this link is now referred to as colinearity);
3) *Diptera* originated from primitive four-winged ancestors, while insects originated from primitive arthropods, which had legs on all abdominal segments. In the course of evolution, "leg-suppressing" genes emerged, which suppressed the development of legs on abdominal segments. "Halter-promoting" genes suppressing wing development on the third thoracic segment also evolved. The loss-of-function mutation in BX-C may lead to the recapitulation of the primitive state, i.e., the emergence of four-winged and many-legged flies.

Now we know that insects evolved no new Hox-genes. Instead, "old" genes assumed new control functions of the development program. In general, however, the logical reasoning suggested by Lewis proved correct.

After the publication of this seminal article, the researchers obtained a tool directly linking the molecular control of development and phylogenetic evolution. A new science, evolutionary developmental biology or EvoDevo, was born.

BX-C sequences and other *Drosophila* genes causing homeotic transformation were found, cloned and sequenced independently by three research teams in California and Indiana (the USA) and Basel (Switzerland) (cit. ex Papageorgiou, 2007). As Lewis had predicted, these genes appeared to be serial homologs. They encode transcription factors with a

distinctive DNA-binding protein domain, the *homeodomain*. The nucleotide fragment encoding it, the *homeobox*, consists of 180 base pairs. The name *Hox*-genes is derived from "homeotic" and "homeobox." In *Drosophila* they are organized in two complexes, ANT-C and BX-C. A set of homologous Hox-genes was later found in vertebrates (Ranginib et al., 1989; Godsave et al., 1994; Burke et al., 1995). Their mutations also result in spatial shifts of structures located along the body axis. It was suggested that Hox-genes originated not in insects but much earlier, in the Precambrian (ca. 600 million years ago), when the last common ancestor of chordates and arthropods supposedly lived.

The analysis of Hox amino-acid sequences of homeodomains and flanking areas in animals from different evolutionary branches dramatically altered the phylogeny of bilateral animals (de Rosa et al., 1999). The evolutionary tree of Bilateria is divided near the basement into two branches, *Deuterostomia* (Chordates, Hemichordates, Echinoderms) and *Protostomia*. The latter, in turn, are divided into *Ecdysozoa* (Arthropoda, Onychophora, Nematoda, Priapulida and others) and *Spiralia* (Brachiopoda, Nemertea, Annelida, Mollusca, Platyhelminthes, Rotifera and others) (de Rosa et al., 1999). The name Ecdysozoa derives from the Greek word *ecdysis* meaning molting. All ecdysozoans are covered by hard cuticle, and their growth is accompanied by periodical molting, when old covers are discarded and the animal grows quickly or, rather, expands, until the new covers harden. *Spiralia* branch is named after the type of early embryonic cleavage. This branch is very heterogeneous. It include Lophotrochozoa, Platyhelminthes, Gastrotricha, Syndermata and Gnathostomulida (Struck et al., 2014). Lophotrochozoa are an especially intriguing group for evolutionary biologists because of the great variability of body organization plans. An octopus, a murex and an oyster belong to the same taxon Mollusca but this is hard to believe judging from their appearance. The term Lophotrochozoa derives from two other zoological terms: Lophophorata and Trochophora. Lophophorata (Brachiopoda, Bryozoa and Phoronida) are animals with the lophophore, an organ of suspension feeding. Earlier the group Lophophorata was included in Deuterostomia. The trochophore is a spherical ciliated larva typical of the Lophotrochozoa (Annelida, Mollusca).

This new "molecular" phylogeny fitted well the previous phylogenies based on 18s rDNA (Field et al., 1988, Aguinaldo et al., 1997) and mitochondrial DNA (Cohen et al., 1998). A comparative study of Hox-genes sequences suggested that the common ancestor of bilateral animals (located at the basement of all the three evolutionary branches) already had at least seven Hox-genes (de Rosa et al., 1999). Modern studies of metazoan evolution focus on the morphology of this common ancestor, its origin and the reasons behind a great evolutionary radiation of its descendants.

In the last decade, mechanisms underlying morphological evolution have mostly become clear. Remarkable new methods, made it possible to fine-tune the functioning of a certain gene, to turn it off at a certain moment of development, to stimulate its ectopic expression and even to edit the genome *in vivo* (CRISPR/Cas9 technology). However, our knowledge about the functions of Hox-genes and even the patterns of their expression in different branches of Bilateria is very uneven. While the functions of Hox-genes in Deuterostomia and Ecdysozoa are studied in detail (at least as concerns vertebrates and arthropods), similar studies of Lophotrochozoa arejust starting. The set of molecular tools for controlling morphogenesis in these animals is same as used in studies of fruit flies or humans. The difference is in the strategy of using these tools. Studies of Hox-genes in Lophotrochozoa can provide fascinating insights into regulatory evolution and epigenetics.

2. POLYCHAETES: WHAT ARE THEY?

Before starting to discuss the Hox-genes of polychaetes, it is necessary to make clear what these animals are. The phylum Annelida was previously divided into two monophyletic branches, Polychaeta and Clitellata (Clitellata = Oligochaeta + Hirudinea) (Rouse and Fauchald, 1997). This division was based on a convincing body of morphological evidence. However, the classification of annelids changed dramatically as soon as nucleotide and amino-acid sequences were applied. At first, there were just a few genes and gene families, then mitochondrial genomes became available, then scientists had at their disposal expressed sequence tags (ESTs) libraries and even whole transcriptomes read with the aid of NGS platforms (Illumina) (Zrzavy et al. 2009; Shen et al., 2009; Struck et al., 2011; Weigert et al., 2014, Andrade et al., 2015). This approach revealed several counter-intuitive connections within Annelida and showed that their classification was much more complex than previously thought (Figure 1).

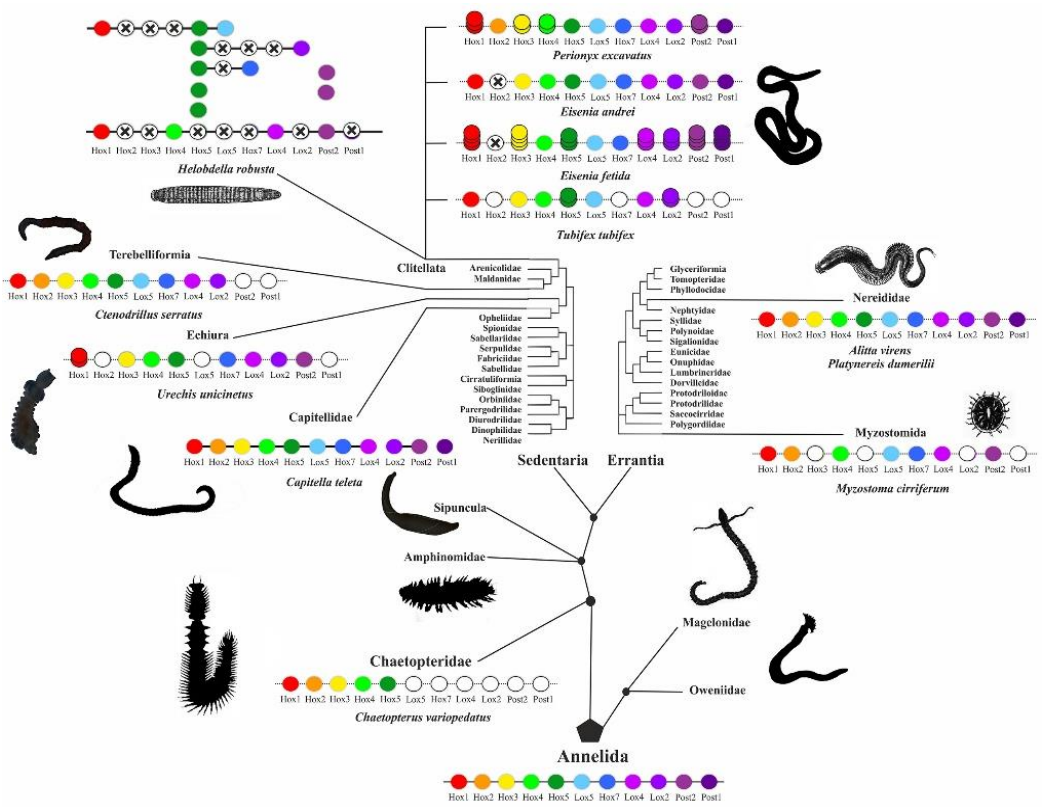

Figure 1. Annelid relationships based on combined results from larger phylogenomic studies. The stem of phylogenetic tree is divided to two big branches: Sedentaria and Errantia. The data about the compound and the structure of Hox-clusters are distributed very irregularly. White circles mean genes, for which there isn't data. Circles with X mean lost genes. Heavy lines mean proved physical connection between genes. The line's absence means proved absence of connection. Dotted lines mean hypothetic connection between genes (according to two reviews (Weigert and Bleidorn, 2016; Barucca et al., 2016) and several original articles (Irvine and Martindale, 2000; Andreeva et al., 2001; Frobius et al., 2008; Simakov et al., 2013; Zwarycz et al., 2015).

It appears now that polychaetes are paraphyletic. The term "polychaetes" fell out of taxonomic register, becoming the synonym of annelids (Struck et al., 2011; Weigert and Bleidorn, 2016). We continue, however, to use the name "polychaetes" for sea annelids from two different monophyletic branches, Errantia and Sedentaria, because this use is firmly rooted in tradition. Importantly, these two branches had a common ancestor in the remote past. Petrified mandibles (scolecodonts)resembling the jaws of modern eunicid polychaetes (Errantia) have been found in strata at the border of Late Cambrian and Early Ordovician, i.e., they are at least 485 million years old (Weigert and Bleidorn, 2016). The last common ancestor of Errantia and Sedentaria might have lived even earlier. Some of its traits were recapitulated based on phylogenetic reconstructions and paleontological data from Middle-Cambrian sediments of Burgess Shale (Middle Cambrian; ~508 mya) (Struck et al., 2011; Parry et al., 2015). This was a macroscopic epibenthic worm with paired palps, biramous parapodia and simple (capillary) bristles. It might also have had aciculae (supporting chitinous rods within parapodia) (Struck et al., 2011) but this is uncertain. Annelids from the Burgess Shale have no aciculae (Parry et al., 2015), while a fossil worm from earlier sediments has them (Liu et al., 2015).

Errantia and Sedentaria are the crown group of Annelids, with the largest number of species and the greatest diversity of shape. There are among them both segmented and unsegmented worms, forms with a simple life cycle and with complex life cycles involving several larval stages. Besides these two groups, Annelida also includes six basal branches: Oweniidae, Magelonidae, Chaetopteridae, Sipuncula, Amphinomida, and Lobatocerebrum (Weigert and Bleidorn, 2016). There are also both segmented and unsegmented worms among them. This circumstance complicates considerably the search for the ancestor of the crown group of annelids.

3. SET AND STRUCTURE OF HOX-CLUSTERS IN POLYCHAETES

Hox-genes of annelids are the best studied among Lophotrochozoa. However, superimposing the data about the Hox-cluster set and the transcription pattern of Hox-genes on the phylogenetic map, we can see that they are fragmentary (Figure 1). Clitellate are studied better than other lines (Wysocka-Diller et al., 1989; Aisemberg et al., 1993; Snow and Buss, 1994; Cho et al., 2004, Cho et al., 2012; Simakov et al., 2013; Zwarycz et al., 2015; Endo et al., 2016), which is probably due to an easier maintenance of cultures of terrestrial and freshwater annelids. At the same time, molecular phylogeny and the fossil record indicate that the Clitellata evolved in late Paleozoic, not earlier than in the Permian period. They are very different from the ancestor of the crown group and basal annelids (Parry et al., 2015).

Almost full sets of Hox-genes were cloned from *Urechis unicinetus* (Sedentaria, Echiura), *Ctenodrilus serratus* (Sedentaria, Cirratuliformia) and *Myzostoma cirriferum* (Errantia, Mizostomida) (Barucca et al., 2016) but there are no data about their expression yet. The first five Hox-cluster genes were cloned from the only studied representative of the basal group, *Chaetopterus variopedatus* (Irvine and Martindale, 2000). Their transcription patterns are well-known, and we will discuss them later.

The most complete data are available for two polychaetes from the Errantia branch (*Alitta virens* and *Platynereis dumerilii;* Family: Nereididae) and one polychaete from the Sedentaria

branch (*Capitella capitata;* Family: Capitellidae). Though two families from nearly a hundred cannot, of course, represent the entire crown group of annelids, the studied species were at least not from the same branch.

The full set of Hox-genes characteristic of Lophotrochozoa was first described in *Alitta virens* (formerly *Nereis virens*) (de Rosa et al., 1999). In *Alitta* genome there are eleven Hox-genes: *Hox1*(PG1), *Hox2*(PG2), *Hox3*(PG3), *Hox4*(PG4), *Hox5*(PG5), *Lox5*(PG6−8), *Hox7*(PG6−8), *Lox4*(PG6−8), *Lox2*(PG6−8), *Post2*(PG9+) and *Post1*(PG9+). This set is considered as an ancestral complement of Mollusca, Brachiopoda and Annelida (Barucca et al., 2016). In the second nereid polychaete (*Platynereis dumerilii*) only nine genes were initially cloned (Kulakova et al.2007; Pfeifer et al., 2012) but missing orthologs were later found (*Hox7*, *Lox4*) (Bakalenko N., unpublished data). The size and the structure of Hox-cluster in nereid polychaetes has not been described in detail yet. According to preliminary data obtained by pulsed field electrophoresis (PFE) (Andreeva et al., 2001), the Hox-cluster of *Alitta* is situated in a single locus not exceeding 2.4 – 2.5 Mb. This indicates that Hox-genes are not scattered in the genome, i.e., the cluster is not atomized as it is in the urochordate *Oikopleura* or the octopus (Seo et al., 2004; Albertin et al., 2015). However, the resolution of PFE does not allow one to determine the exact size of the cluster and the pattern of its organization (whether it is organized, disorganized or split *sensu* Denis Duboule, 2007). In *Platynereis* Hox-genes are also localized on one chromosome and, moreover, in one linkage group with some "extended" Hox-genes (*eve* and *mox*) and genes from EHGbox (*en, hb9/mnx1, gbx*). These genes, which often flank Hox-clusters in Deuterostomia, are considered as a part of an ancient synteny between the ancestral gene clusters from the Mega-cluster of homeobox-containing genes of the Antennapedia class (ANTP-class) (Pollard and Holland, 2000; Hui et al., 2012).

A similar set of Hox-genes was found in the polychaete *Capitella capitata* (Frobius et al., 2008). The structure of the cluster is studied in detail. It is localized in three different scaffolds. In the first scaffold in the region of 243 kbp genes *CapI-lab* (PG1), *CapI-pb* (PG2), *CapI-Hox3* (PG3), *CapI-Dfd* (PG4), *CapI-Scr* (PG5), *CapI-lox5* (PG6−8), *CapI-Antp* (PG6−8), and *CapI-lox4* (PG6−8) are placed. The remaining genes, *CapI-lox2* (PG6−8) and *CapI-Post2* (PG9+), occupy 21.6 kbp in the second scaffold. *Post1* lies within the third scaffold surrounded by non-Hox sequences. This suggests that Post1-ortholog is not part of the Hox-cluster in *Capitella*. All Hox-genes in this polychaete have the same transcriptional orientation and have no ORFs between them. If we summarize Hox-coding areas, the total size of the cluster will be 345 Kb. The genome of *Capitella* has been sequenced (Simakov et al., 2013) but the gap between scaffolds is unfortunately still present. This probably indicates that there is a region difficult to read and to assemble or that the distance between Hox-cluster fragments is very large.

It is interesting that the studied species of Clitellata (oligochaetes and leeches) often have Hox-cluster reconstructions leading to the loss or duplication of certain genes (Simakov et al., 2013; Barucca et al., 2016). For example, leeches have no *Post1* orthologs, and *Hox2* is present only in the earthworm *Perionyx excavatus*. Another earthworm, *Eisenia fetida*, has multiple duplications possibly involving the entire genome. Owing to them, the number of Hox-genes in the genome is increased up to 28 (Zwarycz et al., 2015).

Orthologs of the first five Hox-genes of polychaetes were found in Deuterostomia and Ecdysozoa. These genes seem to have been inherited from the common ancestor of Bilateria, the UrBilateria (de Rosa et al. 1999). They are the most conservative Hox-genes. The central

Hox-genes of annelids (*Lox5*, *Hox7*, *Lox4* and *Lox2*) have no definite orthologs among those of vertebrates (*Hox6*, *Hox7*, *Hox8*) and arthropods (*Antp*, *Ubx*, *abd-A)* (de Rosa et al. 1999; Hueber et al., 2013; Frobius et al., 2008). To solve complicated phylogenetic relationships within this group of sequences, a detailed analysis of signature amino acid motifs was necessary (Hueber et al., 2013). It revealed that the last common ancestor of Protosomia had at least two central Hox-genes, Antp/Hox7-like and abd-A-like. The first sequence is the most conservative; it was inherited from the ancestor of Protostomia/Deuterostomia (Hueber et al., 2013). Antp/Hox7-like gene gave rise, by independent duplications, to *ftz* and *Antp* in Arthropoda and to *Lox5* and *Hox7* in Annelida. The second central gene, abd-A-like gene, evolved shortly before the division of UrProtostomia into Ecdysozoa and Spiralia. This ancestral gene gave rise to *abd-A* in Arthropoda and to *Lox4* and *Lox2* in Annelida. It is interesting that the key gene responsible for the development and evolution of insects, *Ubx*, evolved relatively late in the arthropod line, probably by duplication of the ancestral *abd-A* sequence (Hueber et al., 2013). The affinity between the central Hox-proteins of Deuterostomia and Protostomia can be traced at the level of the single sequence type, Antp/Hox7. It suggests that UrBilateria had one central Hox-gene, Antp/Hox7, which was duplicated and diverged in a specific manner in each evolutionary branch.

The posterior genes of annelids are *Post1* and *Post2*. They are representatives of PG9+ and are characteristic of Spiralia. Until recently, *Post1* was believed to have evolved after the duplication of the *Post1/Post2* ancestral sequence at the level of the last common ancestor of Mollusca, Brachiopoda and Annelida. This belief was based on the fact that only one posterior gene similar to *Post2* was found in the genome of nemerteans and flatworms (Barucca et al., 2016). However, a single *Post-1* gene and four *Post-2* paralogs have recently been reported from *Schmidtea mediterranea* (Tricladida) (Currie et al., 2016). It is possible that *Post1* evolved earlier than previously thought but has been lost repeatedly.

Thus, orthologs of central and posterior genes duplicated and diverged independently in each evolutionary branch of Bilateria (Deuterostomia, Ecdysozoa and Spiralia). This makes them useful phylogenetic markers for studies of animals with an uncertain taxonomic position (Chaetognatha, Rotifera etc.). Besides, structural evolution of the Hox-cluster is inseparable from its functional evolution. Below I will show how the expression of Hox-genes is associated with morphogenesis in polychaetes.

4. Expression Patterns of Hox-Genes

4.1. *Chaetopterus variopedatus*

Hox-genes transcription patterns in polychaetes were first studied in *Chaetopterus variopedatus* (Irvine and Martindale, 2000; Peterson et al., 2000). This polychaete has a world-wide distribution. It builds U-shaped burrows and gathers food particles by specialized organs while pumping water through the burrow. Though it belongs to basal annelids, *Chaetopterus* has a very complex heteronomous organization. The body of the adult worm has three functionally and morphologically different parts, tagmas (A, B and C). Tagma A consists of a presegmental prostomium and a peristomium, which are fused together. The peristomium has ten chaetae-bearing segments, setigers (A1-A9). They have certain

morphological differences (A4 bears a bundle of dark spines, while A9 bears neuropodial uncini) (Irvine et al., 1999). Tagma B consists of five specialized segments (B1-B5) used for the collection of food particles by filtration and the pumping of water through the burrow. Each of these segments has a unique morphology (Figure 2a). Tagma C consists of a variable number of uniform segments, where gametes are formed.

The function of five genes cloned in *Chaetopterus* (*CH-Hox1*, *CH-Hox2*, *CH-Hox3*, *CH-Hox4* and *CH-Hox5*) intrigued the researchers a lot. First of all, it was important to understand how the transcription of the Hox-genes is related to the time when a segment is set up along the body axis and with the morphological boundaries of the tagmas. If the Hox-genes of *Chaetopterus* followed the rule of spatial colinearity characteristic of vertebrates and arthropods, this would mean that this rule probably operated in their common ancestor. The pelagic larva of *Chaetopterus* was another intriguing aspect. Is the Hox-gene cluster used for its formation? If so, what happens during the formation of definitive structures?

The place, the time and the intensity of transcription were determined by the WMISH (Whole Mount In Situ Hybridization) method (Irvine and Martindale, 2000) and by the quantitative assessment of the amount of CH-Hox-genes transcripts in the organism (Peterson et al., 2000). All Hox-genes in *Chaetopterus* appear to start functioning in the posterior zone of the larva long before any visible segmentation. Topologically, this zone corresponds to the growth zone. All larval and definitive segments are formed later. *CH-Hox1* and *CH-Hox3* begin to transcribe after 24 hpf (L1), the initial transcription level of *CH-Hox3* being lower than that of *CH-Hox1*. *CH-Hox4* and *CH-Hox5* are activated after 48 hpf (L2), and *CH-Hox5* transcription level at this stage is lesser than that of *CH-Hox4*. At later stages, the signal intensity for all these genes become equal. This might indicate the presence of a temporal gradient (Irvine and Martindale, 2000), and quantitative analysis confirmed this assumption (Peterson et al., 2000). Thus, 3'-associated genes of *Chaetopterus* are activated according the rule of temporal colinearity with the only exception: *CH-Hox2*, which has the maternal matrix, is present in embryo from the start (Peterson et al., 2000). The authors note that other animals with teloblastic growth (leeches, crustaceans, insects with a short germ band) have no such early Hox-expression in the growth zone. This may be a characteristic feature of polychaetes or an ancestral condition, that was lost in the studied representatives of other groups (Irvine and Martindale, 2001).

By the time the larva reaches stage L5, the areas of Hox-genes expression are not limited by the growth zone anymore and spread to the setigers. As a result, *CH-Hox1*, *CH-Hox2*, *CH-Hox3*, *CH-Hox4*, and *CH-Hox5* have anterior boundaries in segments 2, 1, 3, 4, and 5, respectively. The rule of spatial colinearity obviously applies to CH-Hox-genes. The only gene that violates both rules (temporal and spatial colinearity) is *CH-Hox2*.

During larval growth and development, CH-Hox-genes begin to be involved in the formation of the nervous system, in parapodia and in the superficial ectoderm of segments. Besides, *CH-Hox1* and *CH-Hox2* are expressed at the foregut/midgut boundary. This domain persists for *CH-Hox1* until the metamorphosis and is localized at the caudal end of the pharynx (Irvine and Martindale, 2000).

The transcription boundaries of CH-Hox-genes are related to the morphological boundaries of the tagmas. It is especially true of the posterior boundaries of transcription. So, the posterior boundary of *CH-Hox1* and *CH-Hox2* is located at the ninth segment, where tagma A ends. The posterior boundary of *CH-Hox5* is located at the level of B2 segment, coinciding with the anterior morphological boundary of subsequent palette-bearing segments

B3-B5, which generate the water flow. The anterior boundaries of all the five genes pass through relatively homogeneous tagma A. However, a certain correlation is observed here as well. A4 is the only setiger where *CH-Hox3* domain is not covered by *CH-Hox4* as well as the only segment bearing the bundle of dark spines.

Unfortunately, there are still no data about central and posterior genes of Hox-cluster in *Chaetopterus*, which was Hopefully, a recent discovery that *Chaetopterus* belongs to the basal annelids (Weigert and Bleidorn, 2016) will rekindle the interest of researchers in this object.

4.2. *Capitella teleta*

The polychaete *Capitella teleta* (formerly known as *Capitella sp. I*) belongs to Sedentaria. Unlike *Chaetopterus*, it has a feeble tagmization. The body of this polychaete is divided into the thoracic and the abdominal part, with all the segments being similar. However, chaetae on the thoracic and the abdominal segments are morphologically different, the thoracic ganglia are placed more closely that abdominal ones, FMRF-amid immunoreactive neurons lie only in the fifth segment (de Jong and Seaver, 2016). Embryogenesis and larval development in *Capitella* occurs inside the brood tube. A female produces 100-250 synchronously developing offspring. Non-feeding free-swimming larvae come out of the tube on the 8th-9th day after fertilization (Stage 9). After a short pelagic stage, larvae begin their metamorphosis: they lengthen, loose ciliary bands (the prototroch, the telotroch, and the neurotroch), settle and begin to feed. Subsequent growth is ensured by the prepygidial (subterminal) growth zone. A juvenile polychaete has 13-14 segments. It matures within 8-10 weeks, reaching the size of 55-65 segments. Embryonic and larval development of *Capitella* was described in detail, the fate of single blastomeres was traced, and the cell lines were mapped (Seaver et al., 2005; Meyer et al., 2010). Many of its developmental molecular markers were cloned. All this information makes *Capitella* much easier to work with (Seaver and Kaneshige, 2006; Frobius and Seaver, 2006; Shimeld et al., 2010; Meyer et al., 2015). In addition, it can regenerate its posterior body part.

Larval morphogenesis in *Capitella* is noteworthy. Its sophisticated larva consists of 13 segments (Seaver et al., 2005). According to the BrdU-labeling data, initial larval segments are formed in the bilaterally-symmetric growth zone, which is placed ventro-laterally (Seaver et al., 2005). This growth zone, covering almost the entire length of the embryonic anterior-posterior axis, produces the first ten larval segments within a short time period (~24 h). The remaining larval segments (A2-A4) originate from the posterior growth zone. They are formed slowly, one segment a day. Late pelagic larva emerges from the brood tube and does not undergo further segmentation until the end of metamorphosis (Frobius et al., 2008). Several questions arose in connection with *Capitella*. How is the expression pattern of Hox-genes related with the positional segment identity in a polychaete with a poorly defined tagmosis? Is larval expression different from the postlarval one? Do annelids from different evolutionary branches share common traits of the Hox-genes transcription pattern?

All these questions were answered in 2008, when the expression of all Hox-cluster genes in *Capitella*, from the early larval stage to the third day after metamorphosis, was analyzed by WMISH (Frobius et al., 2008). All larval CapI-Hox-genes transcripts seem to form wide gradients with a different intensity in the nervous system and in the setiger ectoderm. Their

anterior expression boundaries are located along the anterior-posterior body axis according to the rule of spatial colinearity (Figure 2b). The only gene that violates this rule is *CapI-pb* (PG2). It begins to work in the subesophageal ganglion at T1 level, while the boundary of *CapI-lab* (PG1) lies at T2 level. Posterior boundaries of most genes are localized at the level of the last larval segments and the ectodermal growth zone. This rule also has exceptions. So, *CapI-Hox3* is probably the only gene that works in the mesodermal part of the growth zone, while CapI-*Hox5* is the only gene with a distinct larval posterior boundary at the level of thoracic segments T7-T8. In the late larva, all Hox-genes are down-regulated, with a weak transcription persisting in the nervous system and the growth zone.

CapI-lab, *CapI-pb*, *CapI-Hox3* begin to transcribe almost simultaneously, before segmentation. After this, *CapI-Dfd* and *CapI-Scr* become consecutively activated, then *CapI-lox5*, *CapI-Antp*, and *CapI-lox4* and finally, in the end of larval stage, *CapI-lox2* and *CapI-Post2* (Frobius et al., 2008). Generally, the rule of temporal colinearity holds. However, in some cases the transcription signal of 5'-genes is brighter and more complex than that in the 3'-neighbours at the same stage. For example, the patterns of *CapI-lox5* and *CapI-Post2* are brighter and more detailed than those of *CapI-Scr* and *CapI-lox4*/*CapI-lox2*, respectively.

Besides an axial transcription in the nervous system and the ectoderm, additional domains have been found for some *Capitella* Hox-genes. So, *CapI-lab*, *CapI-pb*, *CapI-Hox3*, and *CapI-Antp* are expressed in the presumptive foregut (Frobius et al., 2008). The expression of *CapI-lab* begins in the dorsal part of the stomodaeum and later is localized in the esophagus; *CapI-pb* is transiently expressed in the dorsal part of the pharynx; *CapI-Hox3* is weakly detected in the pharynx and in the esophagus; *CapI-Antp* is weakly and transiently expressed in the pharynx, more ventrally than *CapI-pb*.

CapI-lab is transiently expressed in two cell clusters in the cranial epidermis of the larva in the middle of development, while *CapI-Hox3* and CapI-Antp are weakly expressed in the brain. *CapI-Post1*, falling out of the Hox-cluster, contributes to the specification of chaetae-bearing cells, chaetoblasts (Frobius et al., 2008).

This sophisticated and dynamic pattern of expression reduces dramatically by the start of the worm's postlarval life. Spatial expression domains of all Hox-genes are weakened and become restricted by the CNS (Central Nervous System) ganglia. Moreover, transcription of all genes except *CapI-Hox3*, *CapI-Dfd* and *CapI-lox5* disappears in the growth zone (Frobius et al., 2008; de Jong and Seaver, 2016). The anterior boundaries of expression generally remain stable (the expression of *CapI-Antp* shifts anteriorly by half a segment in the juvenile worm). It is interesting that the posterior boundaries of Hox-genes expression between the thorax and the abdomen are established precisely at this stage. Those of *CapI-lb*, *CapI-Hox3*, *CapI-Dfd*, *CapI-Scr*, and *CapI-lox5* are located, in juveniles, at the anterior edge of T9, and that of *CapI-Antp*, at the posterior edge of T9. *CapI-lox2* and *CapI-Post2* have anterior boundaries at A1 (Frobius et al., 2008).

4.3. *Alitta virens* and *Platynereis dumerilii*

Nereid polychaetes *Platynereis dumerilii (Pdu)* and *Alitta virens (Avi)* (formerly known as *Nereis virens*) belong to Errantia. Unlike *Chaetopterus* and *Capitella*, they are homonomously segmented worms, that is, all their segments are similar. The number of segments is nearly 70 in an adult *Pdu*and slightly more than 200 in an adult *Avi*. These worms

grow by formation of new segments in the prepygidial growth zone. They breed once in their lifetime, producing numerous offspring (~2000 in *Pdu* and about a million in *Avi*). Both species are capable of caudal regeneration.

The ontogenesis of *Pdu* and *Avi* includes two consecutive larval stages: a spherical lecithotrophic trochophore and a segmented nectochaete (Figure 2c). The nectochaete sinks to the bottom and after a long pause (5 days of development for *Pdu*; 16–17 days of development for *Avi*) begins to form postlarval segments from the subterminal (prepygidial) growth zone. The temperature optimum for the development of a normal larva is different in these two species (18^0C *Pdu*; 10.5^0C *Avi*). On the average, *Pdu* develops three times faster than *Avi*. The presence of two larvae with an essentially different organization (segmented and non-segmented) in the ontogenesis of these polychaetes has attracted much attention of embryologists, zoologists and development biologists (Wilson, 1892; Fischer et al., 2010; Starunov et al., 2017).

After the discovery that the genome of nereids contained 11 Hox-genes (de Rosa et al., 1999), the scientists began to suspect that these genes might work in a different way than in other segmented animals. It was unclear whether Hox-genes were involved in larval morphogenesis. If they were, a large set of regionalizing genes looked obviously redundant for a four-segment nectochaete and even more so for a spherical trochophore. It was also unclear what Hox-genes were doing at the postlarval stage. Indeed, what is such a large Hox-cluster needed for, if all body segments are equal?

Most Hox-genes in *A. virens* and *P. dumerilii* seem to be expressed during metamorphosis of the spherical trochophore into the nectochaete (Kulakova et al., 2007). The expression is colinear to anterior-posterior axis of the larval body and involves segmental ectoderm, neuromeres of the neural cord and the pygidium, i.e., all derivatives of the primary somatoblast (2d-micromere). Based on the general larval expression pattern, these genes may be classified into the four groups.

The first group contains *Hox1, Hox4, Hox5, Lox5* and *Post2*. Early expression of these genes does not fade during the development and appears colinear. There are reasons to think that they are used in the regionalization of the larval body. The anterior expression boundaries of *Hox1, Hox4, Hox5,* and *Lox5* are consecutively bound to the boundaries of three chaetae-bearing segments and their neuromers (Figure 2c). *Post2* expression marks the pygidium and pygidial cirri (Kulakova et al., 2007).

Hox2 and *Hox3* belong to the second group. They are activated in 2d derivatives much earlier than other Hox-genes. Their dynamic transcription accompanies the complex process of the establishment of the definitive axis in the larval body. Later their expression pattern becomes similar to the well-ordered axial expression pattern of the genes from the first group. At this time, the anterior boundary of *Hox2* expression lies at the level of the cephalic segment, i.e., more anteriorly than that of *Hox1* expression. The anterior expression boundary of *Hox3* passes at the level of the first chaetae-bearing segment, coinciding with the *Hox1* boundary. In the end of the metamorphosis into the nectochaete, the expression of *Hox2* and *Hox3* fades gradually and becomes limited by the subterminal zone (the future growth zone of the juvenile worm).

The third group is formed by central Hox-genes (*Hox7, Lox4* and *Lox2*), which are not activated until the nectochaete stage. They work in a thin subterminal zone. In the beginning of postlarval growth, they become involved into the patterning of new segments.

The last group is represented by a single gene, *Post1*, which lost its Hox-function and is responsible for the formation of chaetal sacks (Kulakova et al., 2002).

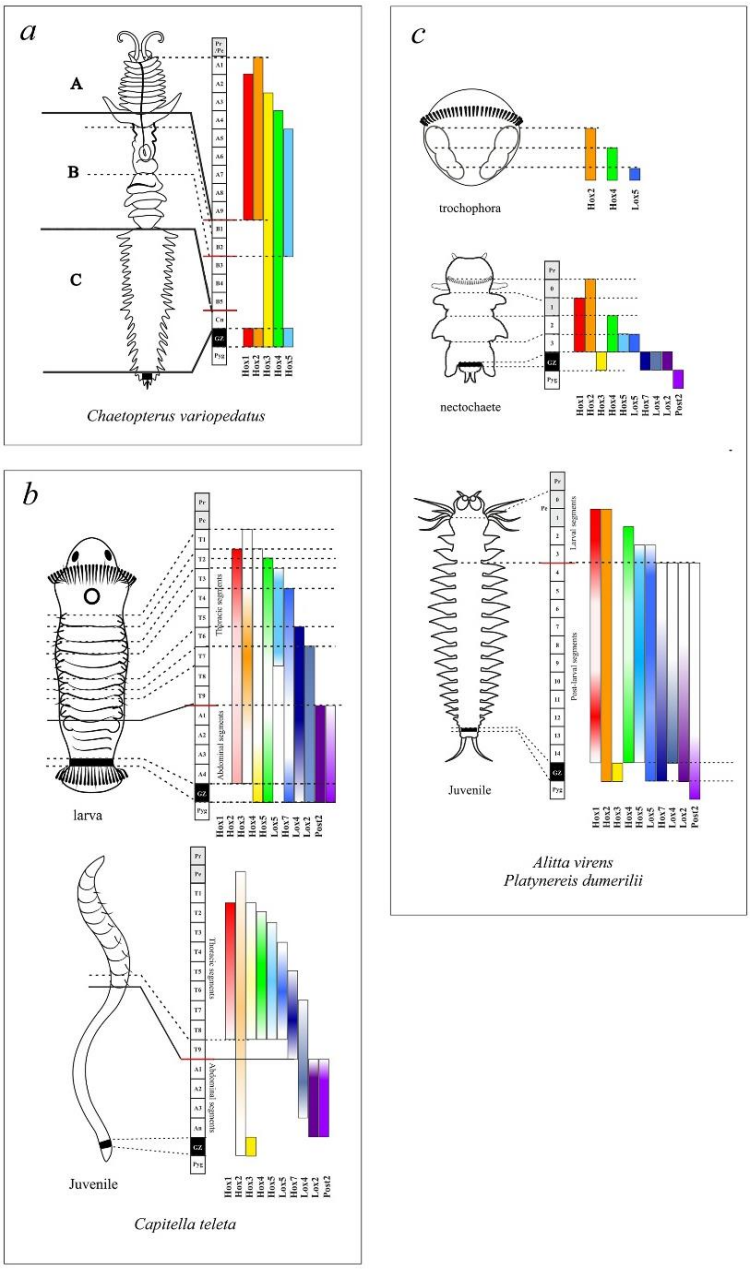

Figure 2. The scheme of Hox-gene transcription domains in studied polychaetes. The explanation is within the text. Solid lines mean boundaries of tagmas in *Chaetopterus* and *Capitella.*

Hox-genes of some nereids have additional expression domains, as do Hox-genes of *Capitella* and *Chaetopterus*. For example, *Hox1* and *Hox2* genes are expressed in the pharynx and at the esophagus-midgut boundary. Besides, *Pdu-Hox1* (but not *Avi-Hox1*) is expressed in the apical tuft (Kulakova et al., 2007; Bakalenko et al., 2013).

The expression of postlarval Hox-genes in *Avi* is studied in detail (Bakalenko et al., 2013). Data obtained by our research team indicate that in *Pdu* Hox-genes are involved in the same processes, and their patterns do not differ much from those in *Avi* (Novikova, Bakalenko, unpublished data). All Hox-genes of nereids operate during postlarval ontogenesis. Their expression (except that of *Hox3*) is represented by wide differently directed gene-specific gradients in the superficial tissues of segments, neural ganglia and parapodia.

Hox3 orthologs are expressed in juvenile nereids only in the ectodermal growth zone. An intermediate pattern is described for *Avi-Hox2*. This gene has a strong transcription in the mesodermal growth zone and a weak one in some ectodermal clusters positioned on the median line of each chaetae-bearing segment (Bakalenko et al., 2013).

The postlarval expression of Hox-genes in nereids is very different from the larval, hinting at the presence of two different morphogenetic programs of the body construction: one for the larva and another for the worm. The first program is responsible for the formation of the larval oligomerous body according to the rule of spatial colinearity. Each of the four segments has its own stable Hox-code. Anterior boundaries of transcription become established in larval segments and remain unchanged throughout the lifetime of the worm.

The second program forms a polymer, constantly growing body, which has no tagmas. The only difference between postlarval segments is their position in relation to the body ends. Transcription gradients of Hox-genes change proportionally with the worm's growth, in other words, they are zoomed. This suggests that the same segment is placed in a different Hox-context in relation to the growth zone (Figure 3). Each postlarval segment expresses a full set of Hox-genes but these genes are expressed at different times, and the expression boundaries are unstable.

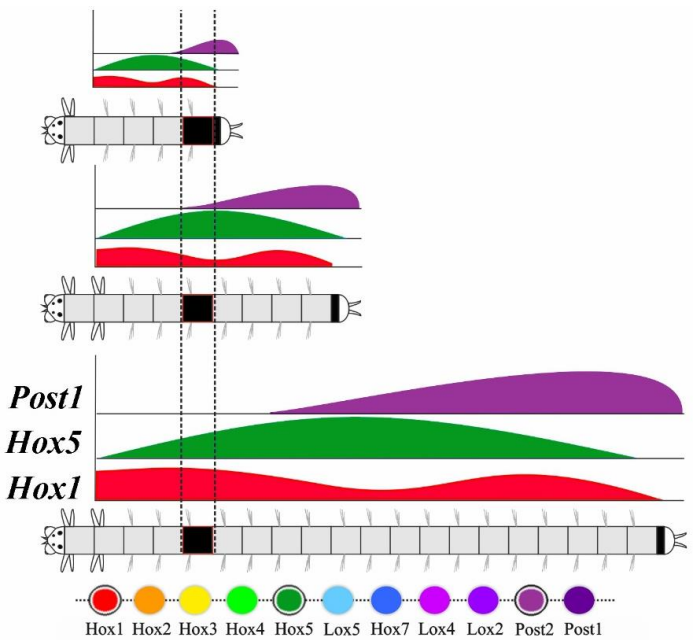

Figure 3. Hox-genes expression gradients scheme in postlarval body of nereid polychaetes. These gradients change in proportion to worm's growth. The same segment gets into different "Hox"-contexts depending on its position toward the head and toward the pygidium.

Such a complex system must be there for some purpose. It is possible that a juvenile polychaete uses Hox-genes to assign and maintain the position value of each segment of the many. The polychaete "numbers" segments with the use of the Hox-protein set to control their growth and quantity. If this is true, then a position failure would affect the functioning of Hox-genes. Indeed, in *Avi* most of the Hox-genes quickly reorganize their pattern during regeneration (Novikova et al., 2013). Expression boundaries of these genes rapidly shift into new spatial coordinates of the regenerating worm to restore normal proportional relations between the domains of their activity. It was found that a position failure smoothens within less than 8 hours. A segment which is nearest to the cut line acquires a "Hox-code" of the posteriormost segment. After that, a new structure begins to form from the cells of the regeneration blastema: a pygidium and a new growth zone (Novikova et al., 2013).

It is still unknown how often polychaetes use Hox-cluster for the maintenance and the restoration of position values in a regenerating body but there are reasons to think that this is not a unique phenomenon among nereids. For example, a shift of anterior expression boundaries for three genes, *CapI-lox4*, *CapI-lox2* and *CapI-Post2*, occurs during regeneration in *Capitella* (de Jong and Seaver, 2016). Crucially, in this case *CapI-lox4* and *CapI-Post2* shift to the thoracic area, which never occurs in the normal condition. The shift happens at a different time for different genes. The stronger the failure in the normal position of the Hox-gene expression, the earlier transcription begins. All this evidence points to the existence of a common mechanism of position value restoration in polychaetes, even those divided by 500 million years of evolution.

5. WHAT DO POLYCHAETES TELL US ABOUT URBILATERIA?

Our attempts to reconstruct traits of the common ancestor of bilateria with the help of extant model objects are in some respects similar to an attempt to reconstruct the acorn's appearance on the basis of an oak's saw cut. Nevertheless, the search of similarities and differences in development programs of polychaetes, arthropods and vertebrates is certainly not useless. Within the gene regulatory network we can identify circuits so closely related to the bilateral body architecture that they are unlikely to have ever changed.

The Hox-cluster is one of such hyper-conservative functional control levers of bilaterian development. Its most prominent feature is colinear transcription. Spatial colinearity or traces thereof are almost always detected, even if the cluster is scattered (atomized) within the genome (Seo et al., 2004). Temporal colinearity is strictly associated with the cluster's continuity (Monteiro and Ferrier 2006) and is obviously present only in vertebrates, with their compact and ordered clusters (Denis Duboule called them "organized" — Duboule, 2007). Not only colinearity but even local exceptions can tell us about the ancestral condition of the cluster and control ways at the dawn of bilaterian evolution.

In the basal polychaete *Chaetopterus*, spatial and temporal transcription colinearity of the first five Hox-genes in larval segments was shown. In *Capitella*, spatial colinearity of Hox-expression is preserved at all ontogenesis stages but strict temporal colinearity is doubtful because the stages of development in this polychaete have too broad time boundaries, and a detailed analysis of the dynamical Hox-pattern is impossible. Besides, *Capitella* has a gap in the Hox-cluster (Frobius et al., 2008). In nereid polychaetes, Hox-expression was shown to be

divided into two stages. Some of the genes (*Hox1, Hox2, Hox3, Hox4, Hox5, Lox5* and *Post2*) pattern the four-segmented body of the larva, demontsrating spatial but not temporal colinearity. These genes, together with the remaining *Lox7*, *Lox4* and *Lox2*, begin to work in juvenile nereids according to absolutely different rules (Kulakova et al., 2007; Bakalenko et al., 2013). If we assume that *Chaetopterus* is indeed a basal polychaete, this state of things appears to be secondary. On the other hand, we do not know how the entire complex of Hox-genes in *Chaetopterus* works at the stage of segmented larva. Neither do we know anything about the work of the CH-cluster in postlarval segments (tagma C).

An ontogenetic difference in the expression patterns of Hox-genes in nereids is obvious. It provokes a question about the presence of functional traces connecting the structural evolution of the Hox-cluster with that of developmental programs.

According to molecular phylogeny data, the last common ancestor of Bilateria had at least seven Hox-genes: five anterior genes (*PG1-5*), one central gene (Hox6-8/Antp/Hox7) and one posterior gene (Hox9-14/Abd-B/Post2-1) (de Rosa et al., 1999; Hueber et al., 2013). If this is true, the formation of the segmented larva in *Avi* and *Pdu* is accompanied by the expression of Hox-genes from the most ancient paralog groups (Kulakova et al., 2007).

Single exceptions from the colinearity rule and additional domains of some Hox-orthologs transcription were found in polychaete larvae. Such a secondary involvement of genes into an unusual morphological program is referred to as co-option (True and Carroll, 2002). For example, *Post1* is expressed in chaetae-bearing sacs in nereids and *Capitella* (Kulakova et al., 2002; Frobius et al., 2008). The evolutionary age of this co-option is comparable with that of the common ancestor of brachiopods and annelids, because in the brachiopod *Terebratalia transversa* the Hox-gene *Post1* was also transiently detected at the late gastrula stages in four ectodermal chaetal sacs of the larva (Schiemann et al., 2017).

The second important exception from the the rule of spatial colinearity is a floating boundary of *PG2* expression. *CH-Hox2* has an anterior expression boundary rostral to that of *CH-Hox1*. The same relationships between PG2-orthologs is present in *Capitella*, *Alitta*, and *Platynereis* (Frobius et al., 2008; Kulakova et al., 2007; Bakalenko et al., 2013). In the amphioxus *Branchiostoma floridae* (Cephalochordata), Hox-genes are activated consecutively but *Amphi-Hox2* violates the rule of spatial colinearity, its anterior expression border being shifted anteriorly of *Amphi-Hox1* (Wada et al., 1999). This feature was noted for Hox2-orthologs in all studied vertebrates (Krumlauf, 1993; Prince et al., 1998). In *Drosophila pb* domain also gets out of the common register but its anterior boundary lies more posteriorly than that of *lab* and *Dfd* (Rogers and Kaufman, 1997). Interestingly, Hox-genes studies in Cnidaria (Hydrozoa, *Clytia*) suggest that *PG2* and *PG3* may have evolved earlier than *PG1* (Quiquand et al., 2009). If so, then an early and a more anterior expression of *Hox2* and *Hox3* may be a trace of their true ancient function. Some anterior Hox-genes of polychaetes are involved in the foregut development program. Surprisingly, in vertebrates *HoxB1-HoxB5* genes are expressed in overlapping domains in the developing foregut (Bogue et al., 1996), while in the lancelet *Amphi-Hox2* is expressed in the preoral pit (Wada et al., 1999).

Thus, some Hox-genes have several conservative pan-bilaterian functions, which are not related directly with their axial regionalizing activity within a united vector tool, the Hox-cluster. Calling these functions “co-options,” we might be missing the point. They could have emerged at the earliest stage of bilaterian evolution. If so, the co-option of these genes is a coordinated expression within the Hox-cluster rather than the specification of separate cell lines. In all probability, UrBilateria already possessed time- and space-ordered Hox-genes

transcription, which depended on the continuity of the cluster. Were this not the case, there would not have been undivided clusters in different animal lines. On the other hand, such a coordinated common regulation was accompanied by individual regulation of separate, probably the most ancient genes. All exceptions to the colinearity rule (including a complete change of Hox-function or an additional pan-bilaterian transcription domain) most often concern *PG1-PG5*.

The germinal layer in which the Hox-cluster of UrBilateria first became active is another important question. In echinoderms and vertebrates, Hox-genes start their work in the mesoderm (Arenas-Mena et al., 2000; Barak et al., 2012), whereas in arthropods they do so in the ectoderm (Hughes and Kaufman, 2002). In some cases the expression of Hox-genes begins almost simultaneously in the ectoderm and the mesoderm (Brena et al., 2006). Spiralia might shed some light upon this question. During the development of *Capitella*, Hox-genes are sequentially activated in the larval ectoderm (Frobius et al., 2008). In *Chaetopterus*, Hox-patterning is more complex. Hox-genes are activated in the posterior part of the L1 larva, according to the temporal colinearity rule. Later, as the larva elongates, Hox-expression translates into a spatial colinear pattern in the nervous system (Irvine and Martindale, 2000). We have recently discovered an early cryptic expression of *Hox2*, *Hox4* and *Lox5* in mesodermal bands of *Avi* (Kulakova et al., 2017). This short-time expression is initiated in the embryo and the early trochophore (Figure 2c). It is colinear in space and time and occurs before the expression of the same orthologs in the ectoderm. The difference between the time of initiation for each of the three genes is approximately two hours, and a synchronous culture of slowly developing larvae is necessary to reveal the start of transcription. A similar early transcription stage can probably be identified in other polychaetes, too. Besides, an early staggered expression of the Hox-genes *pb*, *Hox3*, and *Dfd* was found along the anterior-posterior axis of the developing larval mesoderm in two brachiopod species, *Terebratalia transversa* and *Novocrania anomala* (Schiemann et al., 2017). Indications of the colinearity of Hox-expression in brachiopods can be seen only at the level of the mesoderm.

6. HOX-GENES AND LARVAL DEVELOPMENT

The boundaries between larval and postlarval ontogenesis in polychaetes are not always obvious. The stage of the trochophore, an unsegmented ciliated larva, is relatively clear. Larvae of this type are called primary. They consist of a few cells, which specialize early into relatively few cell types (Peterson et al., 1997). Trochophore larvae of the studied polychaetes (*Chaetopterus*, *Platynereis*, *Alitta*) do not use the Hox-cluster for body patterning. A similar situation has been described for the sea urchin and nemerteans, which activate the axial transcription of the Hox-cluster only during metamorphosis and only in the presumptive tissues of the developing definitive body (Arenas-Mena et al., 2000; Hiebert and Maslakova, 2015). It has been suggested that the regulation programs responsible for the development of primary bilaterian larvae may have emerged at the time when the Hox-cluster still included few genes, which did not take part in the regionalization but were involved into tissue-specific differentiation (Davidson, 2001).

Polychaetes may also have a secondary segmented larva, whose organization is similar to that of the definitive organism. Secondary larvae of all studied polychaetes use the Hox-

cluster for the regionalization of the body. Both *Capitella* and nereid polychaetes turn on Hox-genes colinearly at the stage of the segmented larva. However, larval and postlarval ontogenesis of these phylogenetically distant polychaetes is not similar. *Capitella* has no trochophore, and its secondary larva consists of many segments formed in two growth zones (Seaver et al., 2005). Some of these segments form almost simultaneously in the lateral growth zone, while others form consecutively in the subterminal growth zone. The secondary larva of nereids (the nectochaete) consists of a few segments, all of which derive from the ventral somatic plate.

Unexpectedly, the expression patterns of Hox-genes were found to be similar in juveniles of *Alitta* and late larvae of *Capitella*. In both cases, these genes are transcribed as wide gradients and many of them mark a growth zone. The parallel with the expression patterns of ortholog genes is visible (Figure 2bc). A heterochronic shift seems to have transferred a nereid-like definitive development program to the larval period of *Capitella* development. In this case, the postlarval program of *Capitella* appears to be an independent add-on, which is absent in nereids. Another possibility is that the definitive stage was closer to the postlarval stage of *Capitella*. In this case, Hox-genes were involved in specification of single neural ganglia, and their wider functions are shifted to the larval stage. If so, the nereid ancestors lost the definitive stage, and the breeding time shifted to an earlier stage. This idea is supported by the fact that in Rotifera and Chaetognatha (basal Spiralia) Hox-genes are involved almost exclusively in the linear neuron specification (Papillon et al., 2005; Frobius and Funch, 2017). In this light, a complex secondary larva of *Capitella* and a juvenile nereid are homologous stages of the life cycle, which intercalated between the primary larva and the definitive stage. In nereids, the definitive stage was lost during evolution.

Evidence from the studied polychaetes shows that the same animal may use different developmental programs during its ontogenesis: an embryonic program for the formation of the primary larva, a larval program for the formation of the secondary larva, and a postlarval program for the formation of the definitive body. All the above-mentioned features of polychaetes' ontogenesis indicate a high modularity of their developmental programs. Ontogenetic modules in polychaetes consist of Gene Regulatory Networks (GRNs), which interact weakly and can evolve independently. They are, in fact, separate epigenomes packed into a common genome. Unlike epigenomes of different cell lines (for example, those operating in mammalian fibroblasts), they have a complete hierarchic structure, which is not constrained by epigenetic limitations. This hypothesis perfectly explains the remarkable somatic embryogenesis of polychaetes. In these animals, heterochronies and heterotopies of the developmental program involve all levels of the GRNs hierarchy and therefore can shift entire ontogenetic modules. If these shifts occur in space, this is a good prerequisite for somatic embryogenesis. If they occur in time, a new ontogenetic stage may emerge or an old one may be lost.

A vast morphological variety of Spiralia is a direct consequence of their multi-level ontogenesis, which is evolutionarily flexible at each level. The marks of structural and functional evolution of the Hox- genes are tracked quite well. The more objects from various groups are studied in this respect, the better understanding of evolutionary ways we may hope to achieve.

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Chapter 28

DYNAMICS OF THE STRESS PROTEIN CONTENT IN THE WHITE SEA MUSSEL *MYTILUS EDULIS* L. IN COURSE OF SALINITY ADAPTATION

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ABSTRACT

Mussels inhabiting the littoral/sublittoral zones of seas and oceans are regularly exposed to osmotic stresses. Although the mussels are rather prevalent and of a high economic value, little is known about their capacity to adapt to changes in environmental salinity, especially in terms of cell and molecular mechanisms of salinity adaptation. It is still not clear, whether changes in the environmental osmolarity (water salinity, concentration of various ions and/or organic osmolytes) do cause stress (heat shock) proteins to emerge in the cells, particularly, the heat shock protein of 70 kDa (Hsp70).

This article comprises a study on the adaptation to alterations of the environmental salinity in the White Sea mussel *Mytilus edulis*, the research having been conducted for several years and involving both long-term acclimation and exposure to stress salinity.

In the first series of experiments, the mollusks were acclimated to salinities within the range of their salinity tolerance (14-35‰). The composition and level of HSPs in their gill epithelium were examined by the method of immunoblotting; constitutive stress proteins of about 70 and 40 kDa were revealed. A mass-spectrometry analysis confirmed that the revealed mussel protein with a molecular weight of about 70 kDa belongs to heat shock proteins of the 70 kDa family. After a long-term (11-14 days) acclimation to salinities at the limits of the salinity tolerance range (14‰ and 35‰), the level of Hsp70 in gill epithelium cells was higher than that in the control group mussels contained in normal sea water of 24-26‰. Hsp70 induction was also observed in the cells of isolated gills that had been exposed to salinity shock. These results suggest that the chaperone system of mussel gills can react to salinity changes more or less independently, and is able to function when not controlled by the whole organism.

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The second series of experiments was performed on mussels acclimating to a salinity of 10‰, which is outside of their salinity tolerance range. Osmolarity of the extravisceral liquid of the mussels was measured in course of acclimation. This study showed that the induction of stress protein synthesis in *M. edulis* is a response to salinity changes in the internal medium of the mollusks, but not to the changes in their surrounding medium.

The adaptive changes of the Hsp70 content in mussel gills took different time depending on the salinity gradient. The increased level of Hsp70 in the acclimated/ adapted mussels as compared to the intact ones allows to conclude that this stress protein participates in the adaptation of the White Sea mussel to the salinity changes.

Keywords: stress proteins, Hsp70, *Mytilus edulis*, mussel, salinity, adaptation

INTRODUCTION

This work deals with the influence of the salinity changes on the content of the heat shock protein (Hsp70) in the White Sea mussels *Mytilus edulis* after their acclimation to low and high salinities.

Several issues prompted us to unite some of our theoretical and experimental data in this research. These issues are considered to be of large importance when studying the behaviour of the stress (heat shock) proteins that accompanies the adaptation of an organism to a factor.

No need to mention that in the recent decades much attention has been paid to the function of heat shock proteins (HSPs) in the adaptation process. Numerous, namely tens of thousands, studies have been carried out on various organisms, from microorganisms and protozoa to human beings, demonstrating that the adaptation to temperature is accompanied by an induction of heat shock proteins, first of all, of the Hsp70, a protein of 70 kDa molecular weight (see reviews: Feder and Hofmann, 1999; Margulis and Guzhova, 2000, 2009; Hochachka and Somero, 2002; Ermylova, 2007).

However, until now studies on the acclimation to the changes of the environmental salinity and on the adaptation to such changes have been incomparably fewer than those on the temperature adaptations. It is still poorly understood whether the Hsp70-family proteins play any role in the adaptation of an organism to altered salinity. Even when studying marine intertidal organisms, including mussels (*Mytilus galloprovincialis*, *M. trossulus*, *M. californianus*), authors give preference to temperature as an abiotic factor affecting the mollusks (Hoffman and Somero, 1996; Helmuth and Hoffman, 2001; Buckley et al., 2001; Halpin et al., 2002; Sagarin and Somero, 2006; Fitzgerald-Dehoog et al., 2012).

It seems that not only does temperature dominate salinity in the current researches due to a tradition to connect “heat shock proteins” particularly with the “heat”, but also because of the popular “global heating” and “climate changes” issues.

When examining the constitutive level, expression, or induction of a stress protein in the setting of acclimation and/or adaptation, it is impossible to ignore the fact that each species has its own tolerance range (a set of environmental conditions within which it can best survive and reproduce) of every abiotic factor. It is important to whether know or determine this range before choosing the values of a stress factor, as the organism experiences stress in the conditions approaching the upper or lower limits of the tolerance range. We even assume that the evidence of induction of the stress proteins may be used as a marker of the limit points of the tolerance range (Goodkov et al., 2010). These data were deemed crucial in the

1970s discussion of the step-wise acclimation and of possibilities to widen the tolerance range (Alderdice, 1971; Khlebovich and Kondratenkov, 1973); in the last decades, though, this aspect has been considered in occasional works only (Smurov and Fokin, 2001; Filippov and Filippova, 2006; Komendantov et al., 2006; Komendantov and Smurov, 2009), some of such studies also turned out not to be able to avoid the popular topic of the climate changes (Tomanek, 2008; Zerebecki and Sorte, 2011).

Previously, the Hsp70 induction had been registered in isolated gills of the *Mytilus edulis* mussels from the White Sea placed into water with a reduced salinity (Kharazova, 1999). This fact is important for investigations of the *M. edulis* reaction to any harmful impact, as this species tends to pressurise its mantel cavity in stressful conditions. Our intention was to prove if an isolated gill can react to the salinity conditions independently from the whole organism, and thus to confirm or reject a possibility to use the isolated gills in the experiments on the salinity acclimation and salinity stress.

It is worth mentioning that the results of investigations on the salinity effect in mollusks have been rather contradictory. For example, *Mytilus edulis* mussels from the North Sea (salinity of 29‰) had a higher constitutive content of Hsp70 than those inhabiting the Baltic Sea with its 6‰ salinity (Brown et al., 1995). The stress proteins level in the euryhaline clam *Potamocorbula amurensis* from the San Francisco Bay was lower at a salinity of 0.3‰ than at 27‰ (Werner and Hinton, 2000); the reduced environmental salinity inhibited the temperature-caused induction of Hsp70 (Werner, 2004). However, Hsp70 content remained unchanged by the salinity alterations in *Mytilus galloprovincialis* from the Mediterranean (Li et al., 2009). In a study of the relative sensitivity to pollution in two populations of mussels, *M. edulis* from the Baltic Sea and that from the North Sea, the stress response to the salinity changes in those two populations was compared to that in water with the diesel oil infused in it along with the salinity changes. It appeared that respiration and NH4-N excretion change synergistically when the salinity decreases in the Baltic mussels, but not in the North Sea mussels. A more pronounced decrease of O/N-ratios in the Baltic mussels at ambient salinities indicates that these are more sensitive to the additional stress of oil pollution combined with the already existing salinity stress (Tedergen and Kautsky, 2012). In a study performed by O.A. Muraeva and colleagues (2015) on proteomics of the gastropod mollusk *Littorina saxatilis* exposed to the experimental hypoosmotic stress, it was observed that the expression level of the 70 kDa protein increased and reached its maximum on the 3rd to 5th day of exposure. However, this protein had not been mass-spectrometrically characterised, and for this reason the authors did not discuss neither its chaperon function, nor its role in the adaptation to salinity in the *Littorina* in the study mentioned.

It is clear from the information above that there is little data on the role of the heat shock protein when an organism adapts to the environmental salinity. As the salinity changes have a gross influence on the marine inhabitants, different strategies of their chaperone system and expansion/speciation capacities may be revealed. The mechanism that triggers the salinity adaptations having already been discussed (Berger, 2005), further data on the participation of the heat shock protein in this mechanism are required.

MATERIAL AND METHODS

Experiments were performed during several years, each time from July to September, at the White Sea Biological Station, Zoological Institute, Russian Academy of Sciences, located in the Chupa inlet of the Kandalaksha Bay in the White Sea.

The *Mytilus edulis* L. mussels were collected at a depth of 1–2 m. The water temperature varied from 7 to 15°C, and the salinity ranged from 24 to 26‰, which was within the common seasonal fluctuations in this area (Babkov, 1998). The mussels were placed into aquariums with seawater of a 24–26‰ salinity in isothermal cameras at 10 ± 1°C. Some animals (control group) or their isolated gills were constantly contained in these conditions. Others (or their isolated gills) were acclimated for various periods (from several hours up to 14 days) to water with a different salinity (14-35‰ in the first series and 10‰ in the second series) at 10 ± 1°C. In the case of the low-salinity acclimation, samples were taken on the 1st, 5th, 9th, and 14th day of keeping the mussels in the 10‰ water. The aquarium water was changed every 2 days; the mussels were not fed. Several hundred mussels were used in the experiment.

The mussels tolerance to the altered seawater salinity was estimated by the number of specimens that had sealed the mantle cavity under the unfavourable salinity conditions. For this purpose, the animals were placed in crystallisers with seawater of a salinity from 0 to 50‰ and, in 1 h, the number of such mussels (the percentage of the total number of experimental mollusks with open shells and siphons) was calculated. Thus, the range of salinity tolerance in the mussels (Figure 1) was determined.

SPs were identified in the homogenates of gills from 3-5 mussels per experimentation point. The term “dissected gills” (DG) is employed, when a gill of an acclimated or a control mollusk was used in an experiment, while the term “isolated gills” (IG) means that the gill itself was treated by a control or stress salinity, separately from the whole organism.

The tissue was homogenised in a glass homogeniser with a Teflon pestle in the extraction buffer (20 mM Tris_HCl, pH 7.5, 20 mM NaCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride to inhibit protease activity). The homogenates were either immediately centrifuged at 12000 rpm for 20 min or frozen at –20°C to obtain samples, and then thawed and simultaneously centrifuged. In the further assays, supernatants were used.

The protein composition was assayed by an SDS-electrophoresis in 10% PAAG in the Tris-glycine Laemmli system. The supernatant was mixed (3 : 1) with 4 × Laemmli buffer (1% SDS, 5% β-mercaptoethanol, 10% glycerine). The probes were boiled at 100 °C in a water bath for 3–4 min. The electrophoresis was performed in the Mini-PROTEAN BIORAD System at 10–12 mA for 1–1.5 h and then at 20–25 mA for 2–2.5 h. Before the electroblotting, calibrating electrophoreses were carried out, and a control gel staining was performed after the protein transfer to the cellulose; after the electrophoresis/electroblotting, the gels were fixed in a mixture of formaldehyde, ethanol and acetic acid, stained with 0.25% Coomassie Brilliant blue R 250 solution for 1 h, and differentiated by 7% acetic acid. Also, an equal amount of the gill material was used to level the protein content loaded for the electrophoresis. In addition, the gel loading control was done by cutting and staining the lower part of the gel (the zone containing proteins with the molecular weight varying from 30

to 10 kDa), from which the analyzed proteins were transferred to the nitrocellulose membrane.

To reveal the HSPs, the separated proteins were transferred onto nitrocellulose by electroblotting (Towbin et al., 1979). The nitrocellulose was incubated with monoclonal antibodies SPA 822 obtained against chicken Hsp70 (Stressgen Technologies, Enzo Life Sciences), cross reactive in a wide variety of uni- and multicellular organisms. The antibody binding on the nitrocellulose was monitored by the enzyme reactions of secondary antibodies conjugated with the alkaline phosphatase (Sigma, Aldrich, United States), as well as of the secondary biotin-conjugated antibodies (Sigma-Aldrich, United States) and the alkaline phosphatase-conjugated ExtrAvidin (Sigma-Aldrich, United States). The molecular weight of the separated proteins was identified with the ColourBurst (Sigma-Aldrich, United States) markers of the protein molecular weight and the Prestained Protein Ladder (10–170 kDa) (Fermentas, Lithuania).

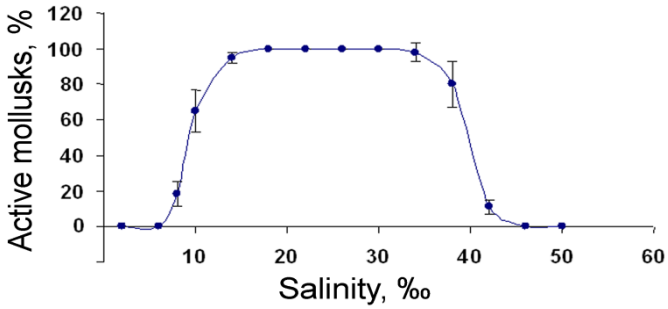

Figure 1. Mussels activity in water of various salinity. Vertical bars – 0.95 confidence intervals.

The protein bands visualised by Coomassie staining corresponding to the 70 kDa protein were excised and prepared for mass spectrometry (Shevchenko et al., 1996). The mass–spectrometric assay was performed on a MALDI TOF Bruker reflex IV mass spectrometer with positive ions and in the reflectron mode. The peptide spectra were treated with the Swiss PROT database (http://prospector.ucsf.edu).

The salts content in the extravisceral liquid of the mollusks was determined using an electric conductivity outfit (Khlebowich and Berger, 1965). The extravisceral (mantle cavity) liquid samples were first diluted 200 times. The conductivity outfit was graduated using sodium chloride solutions with concentrations varying from 5 to 30 mg/L. The obtained data were statistically processed. The table contains mean arithmetic values for the results of 4 – 7 measurements and the corresponding 95% confidence intervals.

RESULTS

First, the range of the mussel resistance to the salinity was estimated. It was found that the siphons and shells of the animals remained open at a wide range of salinities, from 14 to 35‰ (Figure 1). At a salinity below 8 and above 42‰, all animals shut their shells, thus sealing their mantle cavity. Further experiments on the mussels acclimation to the environmental salinity were based on these observations. In this series of experiments the

water salinity varied between 35 and 14‰, i.e., was within the range where 100% of the animals remained active, or, in other words, within the salinity tolerance range of the species *M. edulis*.

The immunoblotting with antibodies to Hsp70 revealed constitutive stress proteins with molecular weights of 70 and 40 kDa in the gill epithelium of the control mussels (Figure 2, *lane* 1). The electrophoretic mobility of one of these proteins corresponded completely to that of the marker protein of the Hsp family with a molecular weight of 72 kDa. The mass-spectrometry assay confirmed that the protein revealed by the immunoblotting was Hsp70. MALDI-TOF mass spectra demonstrated the presence of the proteins identified with the Swiss PROT database as mouse, rat, and pig HS71L; hamster, bovine, mouse, rat, goat, and human HSP72, and mouse HS71A and HS71B. Since all these proteins belong to the Hsp70 family, which is highly conservative in various species (Feder and Hofmann, 1999; Margulis and Guzhova, 2000; Hochachka and Somero, 2002), we may suggest that the mussel 70 kDa protein also belongs to this family.

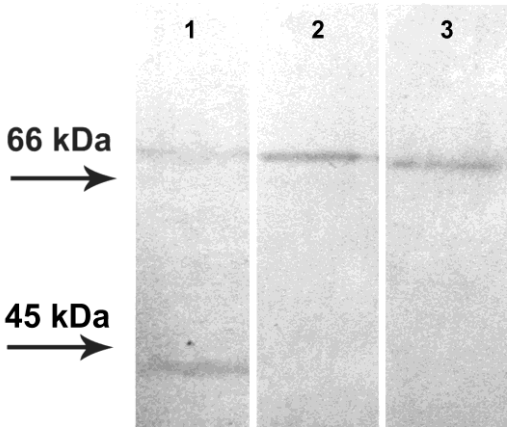

Figure 2. Heat shock proteins in the cells of gill epithelium of *Mytilus edulis* in control mussels and in mussels after their long-term acclimation. *Lanes: 1* – control mussels, maintained in control salinity 24 – 26‰; *2* and *3* – 14 days acclimation in 14 и 35‰ respectively.

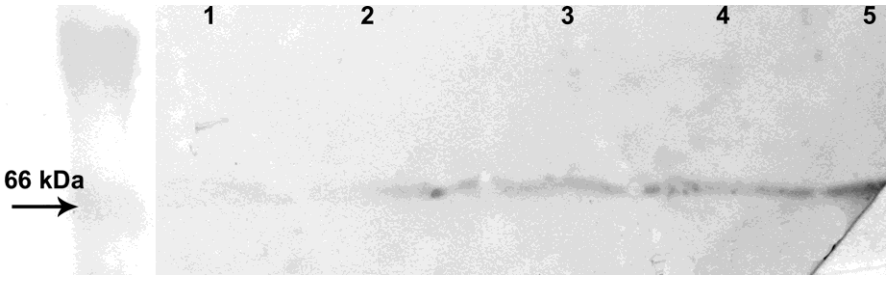

Figure 3. Hsp70 in gill epithelium of control mussels, mussels after long–term (11 days) acclimation and isolated gills of mussels after reciprocal salinity shocks. *Lanes: 1* - control mussels maintained at salinity 26‰, *2* - after acclimation to salinity 14‰, *3* - after acclimation to salinity 35‰, *4* - isolated gills from mussels acclimated to 14‰ after salinity shock (35‰, 1 h), and *5* - isolated gills from mussels acclimated to 35‰ after salinity shock (14‰, 1 h).

After a long-term (14 days) acclimation to the salinities of 14 and 35‰, the Hsp70 level in the mussel gill epithelium increased. This increase, as compared to the control animals, was evidenced in the specimens acclimated both to 14 (Figure 2, *lanes* 1, 2) and 35‰ (Figure 2, *lanes* 1, 3). Similar data were obtained in another year – the mussels had enhanced the expression of Hsp70 (Figure 3, *lanes* 2, 3) after having been acclimated to the salinity of 14 and 35‰ for 14 days.

To examine the reaction of the isolated gills to the salinity stress, the isolated gills of the mussels which had acclimated to 14 and 35‰ salinity for 11 days were placed for 1 h into water with a cross-changed salinity (35 and 14‰, respectively). In both cases, the Hsp70 content increased (Figure 3, *lanes* 1, 4, 5).

In the other experiments performed in two different years, the isolated mussel gills were placed into water of 14, 24 (control), and 35‰ salinity for 1, 3, and 24 h (Figure 4, a, b). An increased level of Hsp70 was observed in the isolated gills contained in the water of 14‰ salinity for 3 and 24 h (Figure 4a, *lanes* 1, 3, 4 and 4b, *lanes* 1, 2 and 4, 5). The content of Hsp70 did not change neither in the gills placed into water of the same salinity but for a shorter period (1 h) (Figure 4a, *lanes* 1, 2), nor in water of 35‰ salinity. Salinity of 35‰ even reduced the expression of Hsp70 as compared to control: it was most evident after an exposure for 3 h (Figure 4a, *lanes* 1, 6 and 4b, *lanes* 1, 3).

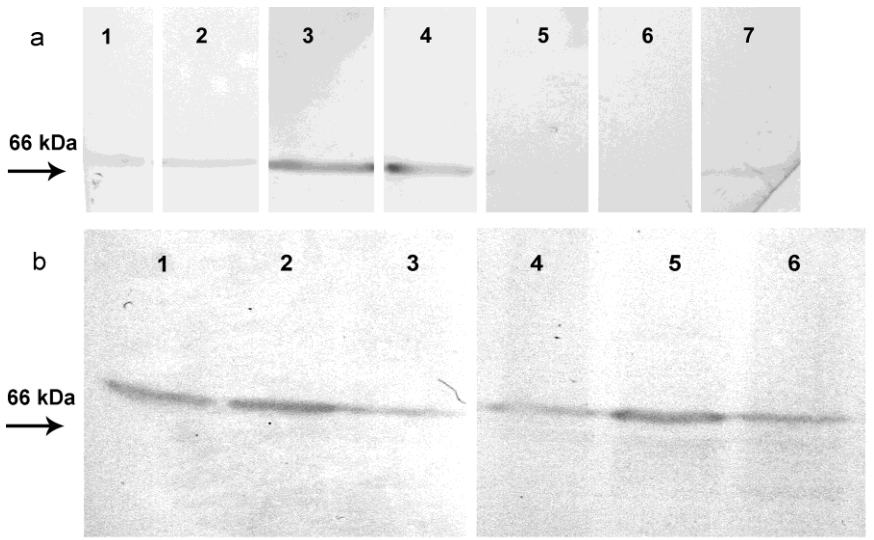

Figure 4. Hsp70 in the gill epithelium of the *Mytilus edulis* after maintaining their isolated gills in control conditions and in stress salinities in two (a, b) different seasons. a – *lanes*: 1 – isolated mussel gills after 24 hrs maintenance in control salinity; 2–4 – isolated mussel gills after their maintenance in 14‰ during 1, 3 and 24 hrs, respectively; 5–7 – isolated mussel gills after their maintenance in 35‰ during 1, 3 and 24 hrs, respectively; b – *lanes*: 1 – 3 hrs in control salinity; 2 – 3 hrs in 14‰; 3 – 3 hrs in 35‰; 4 – 24 hrs in control salinity; 5 – 24 hrs in 14‰; 6 – 24 hrs in 35‰.

The second series of experiments was performed in order to reveal whether the Hsp70 amount in the gill epithelium cells of the *M. edulis* mussel changes in course of the acclimation to the low salinity conditions (10‰), such conditions having provoked an isolation reflex in the mollusks examined. We also aimed to find out, in case if such changes take place, when they occur and how they correlate with the osmolarity of the internal

medium of the mussels (the mantle cavity liquid). Upon exposing the mussels to a salinity outside of their salinity tolerance range, we again counted the active mollusks in the low salinity water; the number of mollusks that remained active upon being moved from the 25‰ salinity water (control) to diluted seawater with a salt content varying from 14 (lower limit of salinity tolerance range, Figure 1) to 0‰ is shown in the Table 1.

The data obtained confirm that the White Sea mussels are able to actively exchange water and salts with the environment at water salinity levels of 14‰ and higher. At the salinity levels ranging from 8‰ to 12‰, only a certain portion of the experimental mollusks was active, while others isolated themselves from the environment. At lower salinity levels, all the mollusks obturated their tubes and closed their shells. These results are in agreement with those published earlier (Berger and Lukanin, 1985; Podlipaeva and Berger, 2012).

Table 1. Dependence of the number of active mussels on water salinity

Salinity, ‰	14	12	10	8	6	2	0
Active mollusks percentage, %	100	95±2	63±5	16±3	0	0	0

Note: Arithmetic means are provided for the results of 4–7 measurements and the corresponding 0.95 confidence intervals.

Changes of the salt content in the extravisceral (mantle cavity) liquid of the mussels placed into the 10‰ salinity water (Figure 5) were analyzed. The analysis showed that the mollusks had for a long time retained a high salt content in their mantle cavity liquid, by blocking to a certain degree the exchange of water and salts with the surrounding seawater. In the control group, the mollusks were kept in seawater of a 25‰ salinity, and the salt content in their mantle cavity liquid was 25 ± 0.4 g/L. On the fifth day of the acclimation to 10‰ salinity, the salt content in the mantle cavity liquid was still significantly higher than in the surrounding medium, namely, 15 g/L (Figure 5), which corresponds to the 15‰ seawater salinity. The difference between the salt concentrations in the external and internal media diminished gradually until the ninth day when the concentrations finally became equal. By this time, all the mollusks that were kept in the water with the salinity levels of 10‰ had partly opened their shells and were filtering seawater. The salt content in their mantle cavity liquid was 10 g/L.

The immunoblotting and subsequent enzymatic reaction revealed a weak double band corresponding to the polypeptides with a molecular weight of about 70 kDa (Figure 6a, *lane* C) in the samples of the dissected gills obtained from the mollusks that had been kept in water of the control salinity. In this series of experiments, we did not detect the protein with the molecular weight of about 40 kDa that had earlier been found in the dissected gills of the control mollusks and had not subsequently been observed neither after the exposure to osmotic shock or long-time acclimations to an increased/ decreased salinity, nor in the isolated gills (Podlipaeva and Berger, 2012). This may have been caused by changes in abiotic and/or biotic factors that occurred since the earlier seasonal observations. Hsp70 is also represented in all the analyzed samples by a double band (Figures 6a, 6b), a phenomenon that has not been previously mentioned in the mussels, but, for example, was observed in the *Tetrahymena* (Podlipaeva et al., 2008). It also may be a result of using the biotin-conjugated antibodies.

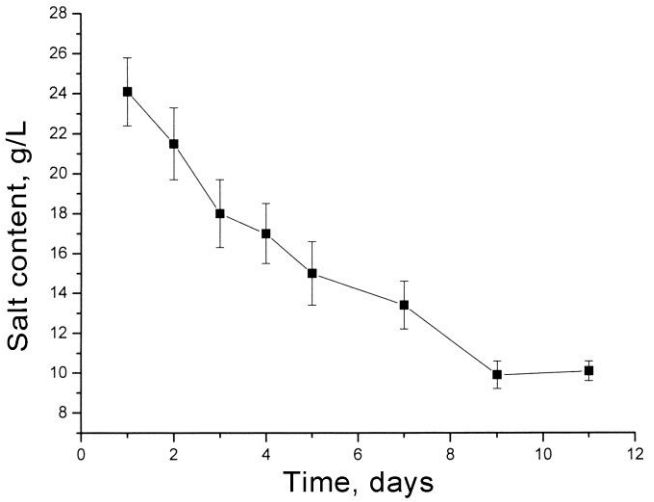

Figure 5. Salt content in the mantle cavity liquid of mussels in the course of acclimation to 10‰ during 11 days. Vertical bars – 0.95 confidence intervals.

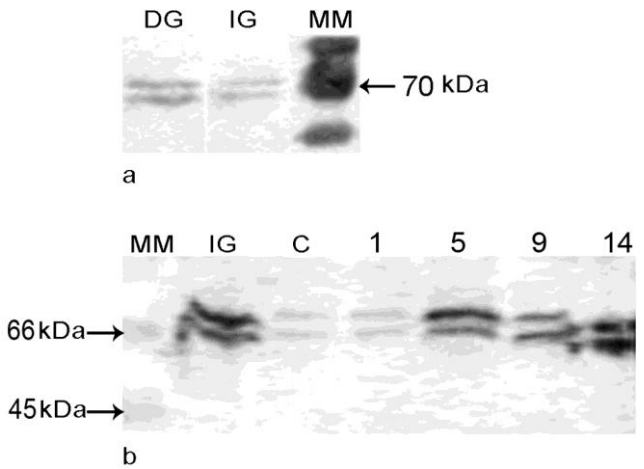

Figure 6. Hsp70 in the gill cells of mussels kept in 25‰ (control) and 10‰. *a* – *lanes*: DG—Hsp70 in the dissected gills of the mussels which were kept for 1 day in control salinity (25‰); IG—Hsp70 in the isolated gills which were placed into the water of control salinity (25‰) for 1 day; MM—molecular weight marker; *b* – *lanes*: MM—molecular weight marker; IG—isolated gills placed into salinity 10‰ for one day; C—control (24–25‰, dissected gills); 1, 5, 9, and 14— mussels (dissected gills) after being kept in 10‰ salinity for 1, 5, 9, and 14 days, respectively.

After 1 day of keeping the mussels in water with a salinity of 10‰, no perceivable changes were observed in the Hsp70 content in the cells of the dissected gill epithelium, as compared to the control (Figure 6b, *lanes* C and 1). At the same time, in the isolated gill cells, a noticeable induction of the Hsp70 expression was observed 1 day after the gills being placed in the water of the same (10‰) salinity (Figure 6b, *lanes* IG and C). In an independent series of experiments, we have demonstrated that the Hsp70 content in the epithelium of the isolated gills placed into the water of control salinity (25‰) did not exceed the Hsp70 content in the dissected gills of the mollusks kept under the same salinity conditions (Figure 6a, *lanes*

DG and IG). Then, on the fifth day of acclimation to 10‰ salinity, we clearly observed the Hsp70 induction in the cells of the gill epithelium (Figure 6b, *lanes* C and 5), and on the 14th day of acclimation the Hsp70 content reached its maximum level (Figure 6b, *lane* 14). The data obtained do not provide grounds to claim that the Hsp70 content increases in the gills of the mussels acclimated to 10‰ salinity in a linear mode; it may only safely be said that the Hsp70 content indeed increases within the period from the 1st to the 14th day of acclimation (Figure 6b, *lanes* 1, 5, 9, and 14).

DISCUSSION

We found that the cells of the mussel gill epithelium had constitutively expressed 70- and 40 kDa proteins. Electrophoresis, immunoblotting with antibodies to Hsp70, and mass spectrometry identified the mussel 70 kDa protein as a member of the Hsp70 protein family.

Some preliminary evidence is available for the other protein with a molecular weight of about 40 kDa, which was identified in one series of experiments only by the same antibodies to Hsp70 in intact mussels, but not in isolated gills. Previously, a heat shock protein of 38 kDa had been reported to be present constitutively and induced as a result of the temperature acclimation in the mollusks of the genus *Tegula* (Tomanek and Somero, 2000), and induction of the stress protein of about 40 kDa as a result of thermal stress had also been revealed in the red seaweed *Porphyra* (Podlipaeva et al., 2014).

In the mussels, after a long-term acclimation to salinities very close to the limits of their natural tolerance range, the Hsp70 content in the cells of the dissected gill epithelium increased (Figures 2, 3). This shows that the heat shock proteins of the 70 kDa family are involved in the salinity adaptation of the osmoconformers, such as mussels.

As for the experiments using the isolated gills exposed to water of different salinities, they are in a good accordance with the experiments on the Hsp70 induction in the isolated mussel gills acclimated to variable environmental salinity (Kharazova, 1999), and at least three conclusions can now be drawn. First: we can ascertain that in some conditions (acute test, p.e.) isolated mussel gills can be used in the experiments with salinity changes together with dissected gills of the mollusks examined. Second: the chaperone system of the mussel gill is rather autonomous and functions more or less independently of the control from the whole organism.

This second conclusion confirms the autonomous adaptation in the mollusks and other osmoconformers in terms of: inorganic ion content regulation, volume regulation, variations in the adaptive rearrangements of RNA, protein synthesis, isozyme spectra, and others (Berger, 1986; Berger and Kharazova, 1997).

Our third conclusion from the data obtained is that different time is needed for the adaptive changes in the content of the stress proteins in the mussel cells, depending on the salinity gradient. These changes occurred in 3 h at 14‰ salinity (Figure 4, a, b). At a higher salinity (35‰) and with a more extended exposure (24 h), no changes in the amount of Hsp70 were observed, although it was increased in the dissected gill after a longer (11–14 days) acclimation of the mussels to the same salinity.

It looks like the factor dose impact on the length of the period required for the Hsp70 induction might provide an explanation why, in some cases, no stress proteins were registered

to participate in the salinity adaptation in various hydrobionts (Black and Bloom, 1984; Li et al., 2009). Further data are needed to confirm this assumption. However, it is rather clear that, to study the response of the cell chaperone system to environmental variations in salinity, both acute experiments and an analysis of the long-term adaptive changes are required. The advantage of such an approach was shown, for example, in a research of the stress protein content in the mollusks of the *Tegula* genus exposed to a long-term thermal acclimation (Tomanek and Somero, 2000).

In course of the acclimation of the White Sea *M. edulis* to low salinity, a nonlinear dynamics of the Hsp70 content was detected (Figure 6b). It is worth mentioning that such nonlinearity is typical for the adaptive reorganizations of the metabolism. Nonlinear changes had been repeatedly observed earlier when examining the variations in the RNA and protein synthesis rate, as well as in the oxygen uptake rate in cells of some mollusks undergoing acclimation to water salinity changes (Kharazova and Berger, 1974; Lukanin, 1979; Berger, 1986). Similar dynamics reflects the staging of the adaptation processes and a delay of feedback in the corresponding regulatory mechanisms (Grodins, 1963).

A comparison of the results obtained for the salts content variations in the mantle cavity liquid (Figure 5) and for the Hsp70 content dynamics in the gill epithelium cells (Figure 6b) allowed us to conclude that the induction of the Hsp70 family proteins detected in the mussels on the fifth day of exposure to 10‰-salinity represents, in fact, a response of the cells and their chaperone system to the salinity level of 15 ± 1.6‰ (Figure 5), this level being at the extreme point of the mussels 100% activity range (Table 1). The effect of the 10‰ salinity, which for *M. edulis*, that is, for its organs and cells, is more stressful than a salinity of 15‰, could be observed on the ninth day of acclimation only, when the salinity of the mussels' internal medium became equal to that of the surrounding water. This stressor factor also led to an increase in the Hsp70 level as compared to the control (Figure 6b, *lanes* C and 9). Therefore, for a proper comparison of the Hsp70 content on the 5th and 9th days of acclimation to 10‰ salinity, shorter intervals between the experimental points should apparently be chosen both for the analysis of the salt content in the mantle cavity liquid and the analysis of the Hsp70 levels in the gill epithelium of the experimental mussels.

Both in case of the acclimation to a salinity within the tolerance range and to a lower salinity, we observed an increase in the Hsp70 content in the mussels after the adaptation, as compared to the control mollusks. Thus, we may conclude that the stress protein of 70kDa participates in the process of acclimation and contributes to salinity adaptation of the White Sea M. edulis.

It is very important to emphasise that it is the salinity changes not in the surrounding, but in the internal medium of the mollusks, that the *M. edulis* respond to with an induction of the stress proteins synthesis. These changes, in turn, are an indirect response to the external stressor, at least when the isolation reflex is provoked by a salinity change, as was the case when the salt content was decreased to 10‰ in the present study. This idea is further supported by the results obtained in the experiments on the isolated gills, where expression of the 70 kDa stress proteins was induced as early as after a day of the mollusks exposure to a 10‰ salinity level (Podlipaeva et al., 2016). In fact, this induction may even occur earlier, as we may expect based on the data obtained by A. Kharazova (1999) for the isolated mussel gills, and as it was shown in this work (Figures 3, 4, 6).

Thus, when studying the dynamics of the Hsp70 expression in the mollusks gills in course of the mollusks acclimation to an environmental salinity beyond their salinity

tolerance range, data on the changes in the salts content in their mantle cavity liquid should always be taken into account. Depending on how remote the values of the experiment points are from the tolerance range limits, it will take the mussels a different time to equalise the salt content in the environment and that in their internal medium. This should also be kept in mind when setting time frames of the analysis.

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Chapter 29

NOVEL FIBRINOGENOLYTIC METALLOPROTEASE FROM THE ANTARCTIC SCALLOP (*ADAMUSSIUM COLBECKI*)

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ABSTRACT

Novel fibrinogenolytic metalloprotease from Antarctic scallop *Adamussium colbecki* was purified and characterized in terms of substrate specificity and enzyme kinetics. A three-step chromatographic procedure which included affinity chromatography on SBTI Sepharose 4B and Blue Sepharose 6FF columns as well as size exclusion chromatography on Superdex 200 PG column was used for purification of the fibrinogenolytic enzyme. The enzyme had an apparent molecular weight of 40 kDa, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The Aα- and the Bβ-chains of fibrinogen were completely cleaved by the enzyme within 2 and 4 h, respectively. However, the γ-chain was much more resistant to digestion by the enzyme. The fibrinogenolytic enzyme also exhibited activity toward fibrin, cleaving the α- and β-chains of fibrin, but did not effectively digest the γ-γ polymer. The fibrinogenolytic enzyme showed detectable amidolytic activity on chromogenic substrates for activated protein C and factor XIa (S_{2366}), thrombin (S_{2238}), factor Xa (S_{2222}), and plasmin (S_{2251}). Among the various divalent ions tested, Zn^{2+} and Cu^{2+} showed strong inhibitory effects on enzyme activity. The activity of enzyme was unaffected by Ca^{2+} and slightly activated by Mg^{2+}. The optimal pH was found to be 10.0 when S_{2238} was used as a substrate. The enzyme activity was also inhibited by treatment with EDTA but not with PMSF suggesting that the fibrinogenolytic enzyme was a metalloprotease and not a serine protease. Activity of the fibrinogenolytic enzyme was inhibited by cysteine, indicating the significance of SH-groups for the structural and functional enzyme integrity. The

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fibrinogenolytic enzyme significantly prolonged activated partial thromboplastin time, prothrombin time and thrombin clotting time and inhibited ADP-induced platelet aggregation in platelet-rich plasma. The enzymatic parameters K_M, k_{cat} and k_{cat}/K_M were estimated to be 0.023 mM, 0.25 sec^{-1} and 10.8 $mM^{-1}sec^{-1}$, respectively, when S_{2238} was used as a substrate.

INTRODUCTION

Hemostasis is the physiological process that involves in maintaining of blood fluidity and prevention of blood loss following injury through the formation of blood clots. Various systems work in strong coordination to provide the delicate balance between all of the compounds. A disruption of this balance may induce hypercoagulation or hypocoagulation state resulting in thrombosis or hemorrhage, respectively. At the present time, cardiovascular diseases (CVDs) such as acute myocardial infarction, ischemic heart diseases, and stroke are the leading cause of the human mortality worldwide. Intravascular thrombosis due to abnormal fibrin accumulation in the blood vessels or a fibrin clot adhering to the unbroken vessel walls of the endoepithelium is one of the main causes of CVDs. Fibrinogen as one of the key molecule of blood clotting system plays an essential role in many physiological processes, including blood clotting, fibrinolysis, wound healing, inflammation, matrix and cellular interactions et al. But high plasma fibrinogen levels are associated with an increased risk for coronary heart diseases, myocardial infarction and peripheral arterial diseases. Fibrinogen is related to both preclinical atherosclerosis and to most of the cardiovascular risk factors. Anticoagulant, antithrombotic and thrombolytic agents have been extensively used in the therapeutic treatment of hemostasis disorders. Up to date, a lot of effective thrombolytic agents have been isolated and well-studied from plants (latex, fruits, and seeds) and animals (bee, snake, scorpion and spider venoms). Despite their wide spread use, most of them have some serious shortcomings, including limited efficacy, short plasma half-life, large therapeutic dose or allergic response, and are also relatively expensive. Therefore, there is a strong rationale for searching new molecules with reduced side risk for their use in human therapeutics. Recognition that clot fibrin formation stronger depends on present in bloodstream intact fibrinogen or its early high-molecular degradation products led to the creation of alternative enzyme-mediated approaches based on direct action on fibrinogen. In this case, fibrinogenolytic enzymes have been a topic under debate in several studies for its clinical relevance in the treatment of thrombotic diseases. These enzymes act directly on fibrinogen molecule, do not require any other additional factors for the manifestation of activity and therefore may be potentially promising as preventive means for the development of complications caused by the activation of pro-coagulating level as well as in treatment of diseases associated with excessive thrombosis.

Extremely promising objects for the search of new compounds with unique properties are marine organisms. Among the products of their life activities were found a number of compounds that have antibacterial, antifungal, antiviral, anti-inflammatory, immunostimulating, anti-tumor, anti-apoptotic effects [1, 2, 3]. However, in spite of such a structural and functional diversity of metabolites, only a few works are devoted to the study of the influence of compounds from marine hydrobionts on hemostasis. For example,

peptides with significant anticoagulant activities were isolated from *L. aspera* [4], *U. unicinctus* [5], *M. edulis* [6], *S. broughtonii* [7].

The current paper described the purification of the fibrinogenolytic enzyme from the crude tissue extract of *Adamussium colbecki* as well as some results of biochemical characterization of this enzyme.

CONCLUSION

The specimens of *A. colbecki* (n = 35) about 7-8 cm long were sampled near the island Galindez (geographical coordinates - 65°15' south latitude, 64°15' west longitude) of Argentine Islands archipelago. After collection the scallops were immediately frozen in liquid nitrogen to prevent enzyme deterioration and stored at -80^{0}C. Shells were removed, tissues were rapidly cut into small pieces, weighed, and then were powdered by a blender with addition of liquid nitrogen. The powder (1:2, w/v) was suspended in the extraction buffer 0.1 M Na-phosphate (pH 7.4), containing 0.15 M NaCl, 0.15 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100 and stirred continuously at 4^{0}C for 1 h. Afterwards, the sample was centrifuged at 10 000 g for 60 min at 4^{0}C to remove the tissue debris. The supernatant was collected and lyophilized.

Purification of the fibrinogenolytic enzyme from *A. colbecki* employed a three-step procedure including two-step of affinity chromatography and one step of size exclusion chromatography. The progress of purification was followed by determining the following for each fraction: general proteolytic activity on casein, activity on chromogenic substrates pyroGlu-Pro-Arg-*p*NA, *N*-α-benzoyl-DL-Arg-*p*NA and fibrinogenolytic activity. The lyophilized-crude tissue extract powder of *A. colbecki* (50 mg/mL) was dissolved in 5 mL 10 mM Tris-HCl (pH 8.0) and allowed to stand for 30 min at 4^{0}C. After centrifugation at 10 000 g for 5 min the supernatant was used for purification procedure. In step 1, an affinity chromatography, SBTI-sepharose 4B column was pre-equilibrated with 10 mM Tris-HCl (pH 8.0) containing 5 mM $CaCl_2$. The supernatant was loaded on column at flow rate of 3 mL/min. Elution was carried out with the 50 mM glycin-HCl (pH 3.0) containing 5 мМ $CaCl_2$ and 1M NaCl at the same flow rate. The unbound fraction was collected and analyzed for the presence of activity to casein, chromogenic substrates and fibrinogen. In step 2, an affinity chromatography, Blue Sepharose 6 FF column was pre-equilibrated with 10 mM Tris-HCl (pH 8.0). The unbound fraction was loaded on Blue Sepharose 6 FF column at flow rate of 1 mL/min, the bound proteins were eluted from the affinity matrix by washing with the same buffer but containing 1M NaCl at a flow rate of 1 mL/min. After removing of NaCl and concentrated the obtained fraction was dissolved in a small volume of 10 mM Tris-HCl (pH 8.0), containing 0.13 M NaCl and subjected for next step of chromatography - size exclusion chromatography. HiLoad 16/60 Superdex 200 PG column was pre-equilibrated with 10 mM Tris-HCl (pH 8.0), containing 0.13 M NaCl. The fraction was loaded and collected at a flow rate of 0.5 mL/min. Elution was carried out with the same buffer. As result, nine fractions were obtained (Figure 1).

Despite the presence of caseinolytic activity in fractions 2-8, the fibrinogenolytic activity was found only in fractions 6 and 7. These fractions were further analyzed by SDS-PAGE,

according to that the purified enzyme appeared as a single protein band with molecular weight of 40 kDa (Figure 2).

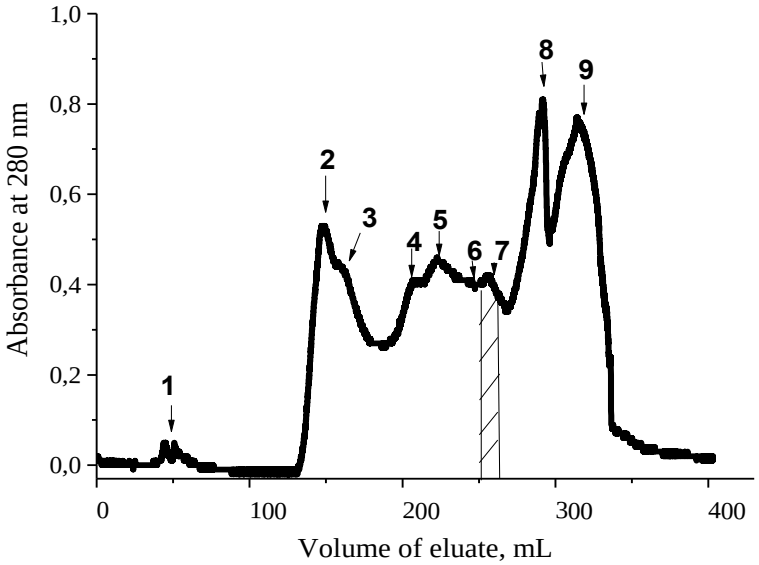

Figure 1. The elution profile of the fibrinogenolytic enzyme purification by size exclusion chromatography on HiLoad 16/60 Superdex 200 PG column: 1-9 – fraction number. Shaded zone contains the fibrinogenolytic enzyme.

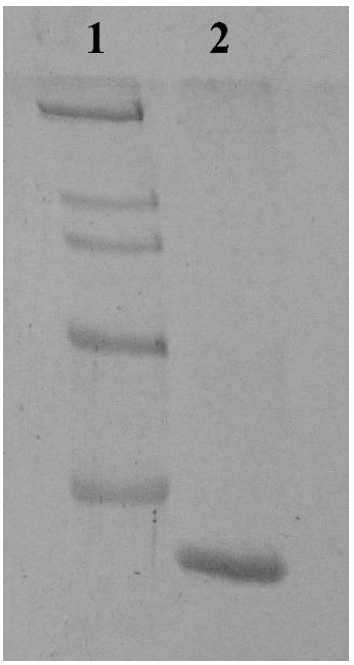

Figure 2. SDS-PAGE of the fibrinogenolytic enzyme. SDS-PAGE was carried out in 10% gel. Lane 1-molecular weight markers: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa). Lane 2 – the purified fibrinogenolytic enzyme.

Based on this result, the fractions 6 and 7 were pooled, lyophilized and further investigated as the fibrinogenolytic enzyme. The molecular weight of the purified enzyme was about 40 kDa which correlated with the clear area presented at substrate gel electrophoregram (Figure 3).

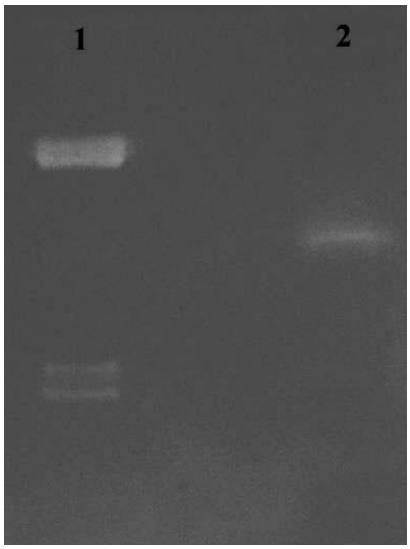

Figure 3. Substrate SDS-PAGE of the fibrinogenolytic enzyme. Activity was assayed by using fibrinogen (1 mg/mL) as substrate in 10% SDS-PAGE. Clear area in the gel indicated region of enzymatic activity. Lane 1 - molecular weight markers: plasmin (86 kDa), mii-plasmin (36 kDa). Lane 2 – the purified fibrinogenolytic enzyme.

Table 1 summarized the purification procedure for the fibrinogenolytic enzyme. The final step resulted in an overall yield of 4.95% and the specific activity of the purified enzyme was 35.5 Units·mg^{-1}, corresponding to a purification factor of 9.8.

It is well known that blood coagulation is brought about by enzymatic cascade consisting of intrinsic and extrinsic pathways which join at the step of factor IX or factor X activation, and then the coagulation process eventually terminates in fibrinogenesis [8]. To clarify the site(s) of action of the fibrinogenolytic enzyme, the effects of the enzyme on the clotting activities of human plasma were examined as the measurement of clotting times in three different tests such as activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT).

Table 1. Purification of the fibrinogenolytic enzyme from the crude tissue extract of *A. colbecki*

Step	Total protein, mg	Specific activity, units·mg^{-1}	Total activity, units	Purification, fold	Yield, %
The crude tissue extract	22.2	3.6	79.9	1.0	100.0
SBTISepharose 4B	20.0	4.97	99.4	1.4	90.0
Blue Sepharose 6 FF	4.4	24.7	108.8	6.8	19.8
Superdex 200 PG	1.1	35.5	28.0	9.8	4.9

Table 2. Effect of the fibrynogenolytic enzyme on plasma clotting time in chronometric tests

	APTT, s	PT, s	TT, s
Control	18.0 ± 0.5	7.2 ± 0.3	28.7 ± 0.7
The fibrinogenolytic enzyme at concentration of 40 μg/mL	The excess of interval measurements	16.4 ± 0,7*	The excess of interval measurements
The fibrinogenolytic enzyme at concentration of 20 μg/mL	68.8 ± 0.8*	9.2 ± 0.3*	The excess of interval measurements
The fibrinogenolytic enzyme at concentration of 10 μg/mL	18.8 ± 0.4	7.0 ± 0.4	The excess of interval measurements

One-way analysis of variance (ANOVA) was used to compare the means among different groups. Differences were considered to be statistically significant when $p < 0.05$ in comparison with control value. Data were reported as mean ± standard deviation (SD) ($n = 5$).

As shown in Table 2, the fibrinogenolytic enzyme prolonged APTT and PT in a dose-dependent manner. By comparing the results of both tests, the most logical assumption is that the effect of the fibrinogenolytic enzyme is realized at the stage of the general path, in particular, the transformation of fibrinogen into fibrin. Therefore, the thrombin time (TT) was determined further, which allowed to assess the final stage of the coagulation process, namely the formation of fibrin. The indicators of thrombin time directly depend on the concentration of fibrinogen, its properties, the presence of abnormal forms, and fibrin stabilization, so the using of this test allows us to speak, first of all, about the functional integrity of the fibrinogen. Our studies have shown a significant prolongation of thrombin time during the action of the fibrinogenolytic enzyme in all investigated concentrations (10, 20, 40 μg/mL). At the same time for all concentrations there was an excess of the interval of measurements. Obtained results may suggest a significant fibrinogen deficiency and the development of a state of deep hypofibrinogenemia. Taking into account the ability of the fibrinogenolytic enzyme to cleave the fibrinogen, it can be explained by the presence of fibrinogen with impaired polymerization capacity.

The substrate specificity of the fibrinogenolytic enzyme was assessed by using chromogenic *p*-nitroanilide substrates, which are widely used to study the properties of hemostasis enzymes. This includes H-D-Phe-Pip-Arg-*p*NA (S_{2238}), pyroGlu-Pro-Arg-*p*NA (S_{2366}), Bz-Ile-Glu(γ-OR)-Gly-Arg-*p*NA (S_{2222}), and H-D-Val-Leu-Lys-*p*NA (S_{2251}). *N*-α-benzoyl-DL-Arg-*p*NA (BA*p*NA) was also used. Nevertheless being metalloprotease the investigated enzyme was able to hydrolyze substrates specific for serine proteases which could indicate the broad substrate specificity of the fibrinogenolytic enzyme. In accordance with the obtained results (Table 3), the fibrinogenolytic enzyme from *A. colbecki* exhibited the activity against all tested substrates, but the effectiveness of the hydrolysis of the substrates contained at the P_1-position of arginine residue was significantly higher than that of the substrate, which in this position had lysine residue. The fibrinogenolytic enzyme exhibited a higher activity for the substrates pyroGlu-Pro-Arg-*p*NA and H-D-Phe-Pip-Arg-*p*NA. The activity on Bz-Ile-Glu(γ-OR)-Gly-Arg-*p*NA which is very sensitive to trypsin, and the activity on H-D-Val-Leu-Lys-*p*NA were in 3.4 and 3 times lower comparing to the activity toward pyroGlu-Pro-Arg-*p*NA. It should be noted that the fibrinogenolytic enzyme did not hydrolyze BA*p*NA – specific substrate for trypsin. This observation can serve as an indirect

confirmation that the fibrinogenolytic enzyme from *A. colbecki* did not belong to trypsin family.

Table 3. Activity of the fibrinogenolytic enzyme from *A. colbecki* towards chromogenic substrates

Substrate	Enzyme activity, µmol *p*NA·min^{-1}·mg protein^{-1}
pyroGlu-Pro-Arg-*p*NA (S_{2366})	3.0 ± 0.063
H-D-Phe-Pip-Arg-*p*NA (S_{2238})	2.63 ± 0.025
Bz-IIe-Glu(γ-OR)-Gly-Arg-*p*NA (S_{2222})	0.94 ± 0.047
H-D-Val-Leu-Lys-*p*NA (S_{2251})	0.88 ± 0.035
N-α-benzoyl-DL-Arg-*p*NA (BA*p*NA)	-

Data were reported as mean±standard deviation (SD) (n = 5).

Table 4. Effect of protease inhibitors on activity of the fibrinogenolytic enzyme from *A. colbecki*

	Concentration of inhibitors, mM	Enzyme activity, µmol *p*NA·min^{-1}·mg protein^{-1}	% inhibition
Basal activity	-	2.63 ± 0.05	-
EDTA	2	1.2 ± 0.04 *	55
	5	0.75 ± 0.02 *	72
PMSF	2	2.58 ± 0.03	2
	5	2.7 ± 0.03	-

One-way analysis of variance (ANOVA) was used to compare the means among different groups. Differences were considered to be statistically significant when $p < 0.05$ in comparison with basal activity. Data were reported as mean ± standard deviation (SD) (n = 5).

Table 5. Effect of divalent metals ions, cysteine and β-mercaptoethanol on activity of the fibrinogenolytic enzyme from *A. colbecki*

	Concentration, mM	Enzyme activity, µmol*p*NA·min^{-1}·mg protein^{-1}
Basal activity	-	2.9 ± 0.3
Ca^{2+}	2	2.85 ± 0.21
	5	3.0 ± 0.33
Mg^{2+}	2	3.4 ± 0.34
	5	3.9 ± 0.33*
Zn^{2+}	2	2.1 ± 0.45*
	5	1.75 ± 0.15*
Cu^{2+}	2	1.5 ± 0.2*
	5	0.2 ± 0.08*
cysteine	2	2.61 ± 0.12*
	5	2.17 ± 0.1*
β-mercaptoethanol	5	-

One-way analysis of variance (ANOVA) was used to compare the means among different groups. Differences were considered to be statistically significant when $p < 0.05$ in comparison with basal activity. Data were reported as mean ± standard deviation (SD) (n = 5).

For clarifying the nature of the purified enzyme the effect of main class-specific inhibitors like EDTA and PMSF on the enzyme activity was assessed. The enzyme activity was significantly inhibited by metal-chelating agent EDTA, indicating that it was a metalloprotease (Table 4).

Thus, treatment with EDTA at concentration 2 and 5 mM decreased the enzyme activity by 55% and 75%. In contrast, PMSF, an inhibitor of serine proteases, had no effect on activity of the fibrinogenolytic enzyme. These observations indicated that the fibrinogenolytic enzyme from *A. colbecki* was not serine protease, but confirmed that it was metalloprotease.

In order to determine the sensitivity of the fibrinogenolytic enzyme to the action of natural inhibitors, we have incubated the investigated enzyme with blood plasma, which contains endogenous protease inhibitors. Pre-incubation of the fibrinogenolytic enzyme with blood plasma resulted in significant inhibition of enzyme activity evaluated by using chromogenic substrate pyroGlu-Pro-Arg-*p*NA. Thus, at the concentration of 10 μg/mL, there was an inhibition of enzyme activity in 8 times. Increasing the concentration of the enzyme to 80 μg/mL was accompanied by a somewhat less pronounced, but still significant inhibition of enzymatic activity - under these conditions there was an inhibition of the enzyme activity in 4.6 times. Our results are consistent with the literature on the activity of α-fibrinogenases in terms of their incubation with plasma [9]. It well-known fact, that α-fibrinogenases are markedly inhibited by plasma protease inhibitors. On the other hand, β-fibrinogenases are less suppressed by plasma inhibitors. Since the fibrinogenolytic enzyme is metalloproteases, it is unlikely that the inhibition of the activity of the enzyme in the conditions of our experiment may be due to the effect of serine proteases inhibitors (serpins) which effectively control the activity of the most enzymes of the hemostasis system and present in plasma in sufficient concentrations. The exception may be α2-macroglobulin, which is a universal inhibitor of plasma proteases, regardless of their class. The effect of divalent metals ions on enzyme activity was also investigated. Metals are known to serve as important cofactors for the catalytic activity of several enzymes, they mediate efficient substrate binding and take part in protein folding. Among the major metals ions that are required for enzymatic reactions are Zn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}. It is likely that Ca^{2+} and Mg^{2+} are quite essential for the stabilization of enzyme structure, while Zn^{2+} may actually participate in catalysis. The effect of some metals ions, at a concentration of 2 and 5 mM, on the activity of *A. colbecki* fibrinogenolytic enzyme was examined. According to obtained result (Table 5), Ca^{2+} did not affect activity of the fibrinogenolytic enzyme, whereas Mg^{2+} increased activity by 17 % and 34 % at concentration of 2 and 5 mM Mg^{2+}, respectively. The increase of the enzyme activity may be due the link substrate and enzyme by this ion. It was found that the activity of the fibrinogenolytic enzyme was inhibited by Zn^{2+} and Cu^{2+}. The activity of the fibrinogenolytic enzyme from *A. colbecki* was inhibited by 28 % and 40% after pre-incubation with Zn^{2+} at 2 mM and 5 mM concentration, respectively. Similar result regarding the effect of Zn^{2+}on activity of the fibrinogenolytic enzyme was detected in [10, 11]. Cu^{2+} was found to be more effective inhibitor of the enzyme activity.

The activity of the investigated enzyme was abolished by the reducing agent β-mercaptoethanol, indicating that disulfide bonds may be fundamental to the structural and

functional integrity of *A. colbecki* fibrinogenolytic enzyme. There are some reports highlighting the significance of disulfide bridges for enzymatic activity of snake venom metalloproteases [12]. The addition of cysteine caused the inhibition of enzyme activity by 10% and 25% at the concentration of cysteine, respectively, 2 mM and 5 mM. Such results allowed to assume the importance of free SH-groups for the manifestation of the enzymatic. In general, our data on inhibition of cysteine exposure are consistent with the data presented in the literature [13], where the authors also found significant inhibition of the activity of fibrinenolytic metalloproteinases when they were incubated with cysteine.

The pH influence on the activity of the purified enzyme was monitored from pH 2.0 to 13.0 using pyroGlu-Pro-Arg-*p*NA as substrate. The pH at which enzyme had maximum activity was 10.0 (Figure 4). Therefore, the fibrinogenolytic enzyme was regarded as a basic protease. This result was similar to other fibrinogenolytic proteases described in the literature, since most of the fibrinogenolytic enzymes have optimum pH at neutral and alkaline values [14]. The increase of pH values to 11.0 and 12.0 led to a slight inhibition of enzyme activity. Under these conditions, the enzyme retained 96% and 90% activity, respectively. However, at pH 13.0, the activity of the enzyme was half of the maximum. The enzyme activity decreased rapidly at levels below pH 6.0 and at pH 4.0 and 5.0 was about 9.5% of its initial activity.

To evaluate the catalytic characteristics of the fibrinogenolytic enzyme, the main kinetic parameters were estimated. The determination was performed using a chromogenic substrate H-D-Phe-Pip-Arg-*p*NA in respect of which the fibrinogenolytic enzyme showed one of the highest activity. For the study, the substrate was used in the range of final concentrations from 0.01 to 0.3 mM. Kinetic constants K_M and k_{cat} for the fibrinogenolytic enzyme were calculated from a Lineweaver-Burk double-reciprocal plot (Figure 5) and shown in Table 6.

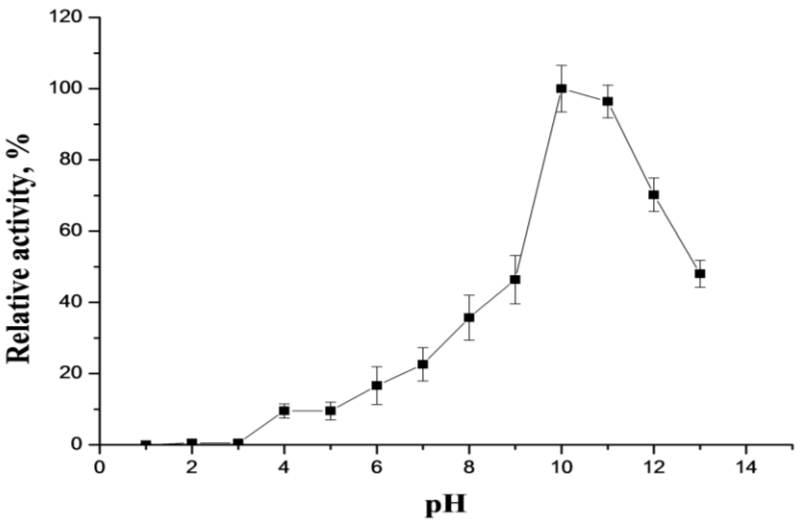

Figure 4. Effect pH on activity of the fibrinogenolytic enzyme. The relative activity was measured in a series of buffers using H-D-Phe-Pip-Arg-*p*NA as substrate at 37^0C and varying pH from 2.0 to 13.0. Percentage of enzyme activity was estimated considering 100% the highest activity detected in this assay.

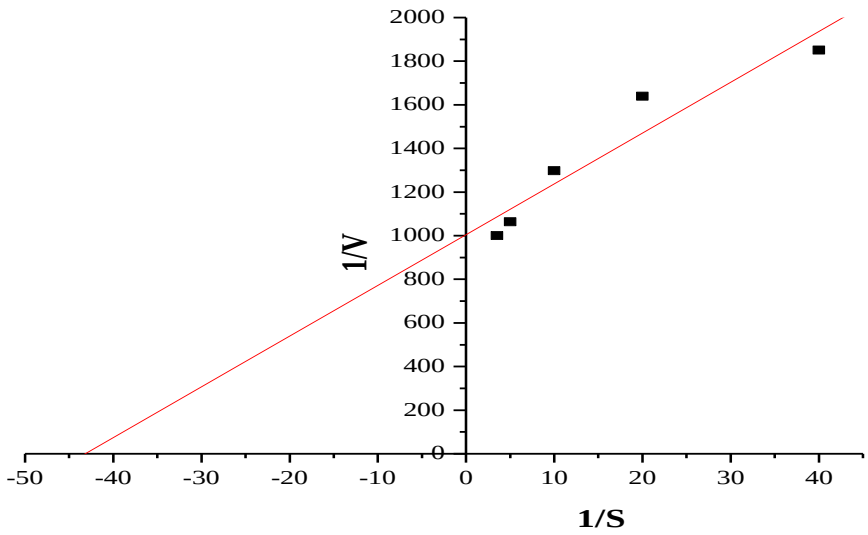

Figure 5. Lineweaver–Burk plot (double reciprocal) for the fibrinogenolytic enzyme kinetics. Kinetic constants were calculated at pH 10.0 and 37^0C by varying H-D-Phe-Pip-Arg-*p*NA substrate concentration between 0.01 and 0.3 mM.

Table 6. Kinetic parameters for hydrolysis of chromogenic substrate H-D-Phe-Pip-Arg-*p*NA by the fibrinogenolytic enzyme (M ± m, n = 5)

V_{max}, μM·s-1	K_M, mM	k_{cat}, s^{-1}	k_{cat}/K_M, $mM^{-1}·s^{-1}$
0.99 ± 0.04	0.023 ± 0.001	0.25 ± 0.012	10.8 ± 0.45

Data were reported as mean ± standard deviation (SD) (n = 5).

According to the calculations, the maximum velocity of the enzymatic reaction was 0.99 $\mu M·s^{-1}$. The concentration of the substrate at which the velocity of the enzymatic reaction is equal to half of the maximum for the fibrinogenolytic enzyme from *A. colbecki* was 0.023 mM. Comparing the values of K_M for the studied enzyme and K_M for fibrinogenolytic enzymes, isolated from the snake venom, we can note that the fibrinogenolytic enzyme from *A. colbecki* had a higher affinity for the H-D-Phe-Pip-Arg-*p*NA. Thus, the affinity of the fibrinogenolytic enzyme to this substrate was 2 and 20 times higher when compared with the K_M for the fibrinogenolytic enzymes from *T. jerdonii* and *T. stejnegeri* [15]. Given that H-D-Phe-Pip-Arg-*p*NA is a synthetic substrate widely used in the study of thrombin and thrombin-like enzymes, it was interesting to compare the value of K_M for the fibrinogenolytic enzyme from *A. colbecki* with the value for thrombin. It should be noted that we did not calculated K_M for thrombin, but used the data of Chromogenix catalog [16]. The K_M value for the studied fibrinogenolytic enzyme was 2.3 times higher, and therefore its affinity for the thrombin substrate was as many times as low as compared with the corresponding value for thrombin. The parameter describing the efficiency of catalysis was catalytic efficiency k_{cat}/K_M. As can be seen from Table 6, the catalytic efficiency of H-D-Phe-Pip-Arg-*p*NA hydrolysis by the fibrinogenolytic enzyme was 10.8 $mM^{-1}·s^{-1}$.

An important feature of fibrinogenolytic enzymes is their specificity with respect to fibrinogen chains. Serine proteases cleave fibrinogen from the N-terminal end of the A- or B-chains, causing the release, respectively, of the fibrinopeptide A or B. Metaloproteases, on the

contrary, are characterized by the specificity to the C-terminal end of the Aα-chain of fibrinogen and do not act on the N-terminal end of molecule. All fibrinogenolytic enzymes can be classified as being either α- or β-fibrinogenases according to their proteolytic preference toward the fibrinogen chains. The α-fibrinogenases preferentially attack the Aα-chains of fibrinogen and are metalloproteases, whereas β-fibrinogenases cleave the Bβ-chains and are serine proteases [17]. Identification as either a α- or β-fibrinogenase is not absolute since there is significant degradation of the alternate chain with increasing time of incubation. These enzymes are directly acting endoproteases which do not require any other factors for their activity. Although fibrinogenases can cleave fibrinogen actively, they do not induce the release of fibrinopeptides A or/and B. In contrast to thrombin, they degrade the chains of fibrinogen with formation of truncated fibrinogen which has reduced clotting ability.

Therefore, in order to assess the nature of obtained fibrinogenolytic enzyme we analyzed its specificity towards the chains of fibrinogen. Fibrinogenolytic degradation assay was carried out using fibrinogen solution mixed with the fibrinogenolytic enzyme from *A. colbecki* at a ratio 100:1. At different time intervals (0, 1, 2, 3, 4, 6 and 24 h), the reaction mixture was removed and SDS-PAG electrophoresis was performed. As can be seen in Figure 6 reduced fibrinogen was separated into the Aα-, Bβ- and γ-chains, and showed three bands on the electrophoregram.

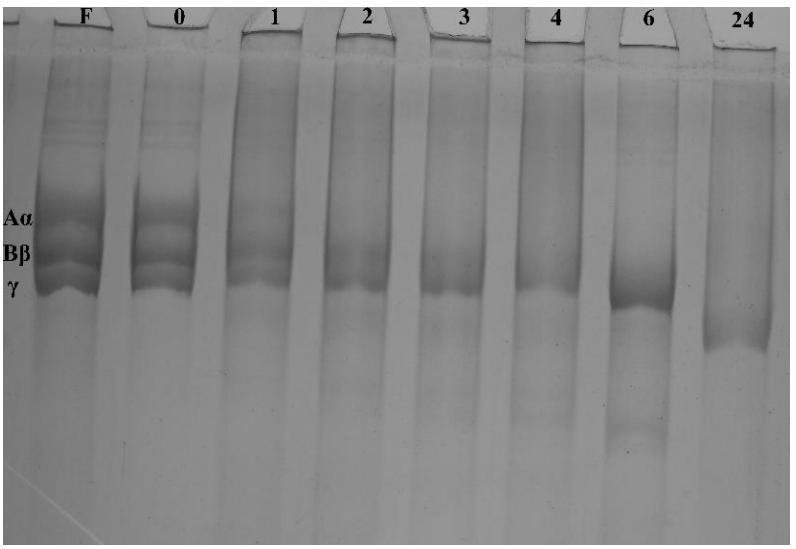

Figure 6. SDS-PAGE analysis of fibrinogen after incubation with the fibrinogenolytic enzyme. SDS-PAGE was carried out in 10% gel. Lane F - control of fibrinogen sample without incubation with the fibrinogenolytic enzyme. Lanes 0–24 - fibrinogen samples after incubation at 37^0C with the fibrinogenolytic enzyme for 0, 1, 2, 3, 4, 6 and 24 h, respectively.

When fibrinogen was incubated with the fibrinogenolytic enzyme, the Aα-chain started to disappear after 1 h of incubation and essentially disappeared within 2 h. Fibrinogen Aα-chains are almost twice as long as the Bβ- and γ-chains, and their C-terminal domain (αC-domain), which protrude from the fibrinogen molecule, is more susceptible to proteolysis by fibrinogenolytic enzymes. The C-domain of fibrinogen plays an exclusively important role in lateral aggregation and cross-linking of protofibrils during the formation of a fibrin clot [18].

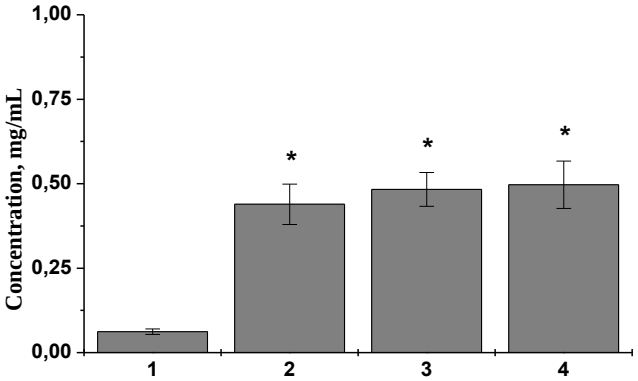

Figure 7. Concentration of functionally inactive fibrinogen. 1- control. 2, 3, 4 - samples after incubation with the fibrinogenolytic enzyme for 1 h (2), 3 h (3), and 6 h (4). *$p < 0.05$ significantly different from basal activity.

Moreover, C-domain mediates the interactions with some plasma proteins, in particular, this domain contains binding sites for fibronectin, FXIII, plasminogen, and tissue plasminogen activator. When linked to the C-domain of fibrinogen, the plasma proteins are included in the fibrin clot. Fibrinogen, nicked in its αC-domain, forms a loose fibrin clot that is more prone to lysis. The band corresponding to the Bβ-chain of fibrinogen disappeared in about 4 h of incubation with the fibrinogenolytic enzyme. Such results do not contradict the literature, because, as already noted, despite the expressed specificity to one of the fibrinogen chains, fibrinogenolytic enzymes are also able to hydrolyze another chain of fibrinogen under continuous incubation. Degradation of the γ-chain occurred after 6 h of incubation. Most of the γ-chain was digested after 24 h of incubation. The degradation of the γ-chain by the fibrinogenolytic enzymes is a rare phenomenon. To date, in literature presented a few work on fibrinogenases capable of hydrolyze the γ-chain. Thus, enzymes isolated from the snakes venom of *A. acutus, B. moojeni, B. isabelae* [19, 20] possesses the ability to cleave the γ-chain of the fibrinogen, and its complete degradation was observed at relatively early terms of incubation (up to 8 h), and as well as after 18 h of incubation. Afterward, the fibrinogenolytic enzyme completely digested all fibrinogen chains in 24 h. Hydrolysis of fibrinogen by the fibrinogenolytic enzyme resulted in the appearance of low molecular weight fragments. The major degradation product was a fragment about 40 kDa. In addition, some amounts of 30 and 35 kDa polypeptides appeared after 24 h incubation. Thus, on the basis of obtained results, we could suggest that the fibrinogenolytic enzyme from *A. colbecki* belonged to the α-fibrinogenase. It is important to emphasize that the fibrinogenolytic enzyme treatment did not result in clotting of fibrinogen per se, despite the fact it could degrade fibrinogen; therefore, it is unlikely that this enzyme produces fibrinopeptides A and/or B, which are required for the initiation of fibrin polymerization. The ability of *A. colbecki* fibrinogenolytic enzyme to cleave fibrinogen without the initiation of the polymerization process, may serve as a confirmation of the belonging of the enzyme to fibrinogenases. Our observations also indicated that the fibrinogenolytic enzyme could not clot plasma by itself, suggesting that this enzyme was unable to activate prothrombin into thrombin, as do other α-fibrinogenases from snake venom [21].

The conversion of fibrinogen into fibrin plays an important role in coagulation and hemostasis. The fibrinogen cleavage due to the action of fibrinogenolytic enzymes impairs

the ability of fibrinogen molecules to polymerize and it directly depends on the depth of hydrolysis of the fibrinogen and the nature of the fibrinogenolytic enzyme. Taking into account the ability of the fibrinogenolytic enzyme to cleave the fibrinogen, we determined the concentration of fibrinogen lacking ability to form fibrin monomers after incubation with the fibrinogenolytic enzyme. For this purpose, fibrinogen was incubated with the fibrinogenolytic enzyme for 1, 3 and 6 h prior to the addition of thrombin. Obtained fibrin clots were dissolved in 0.125% acetic acid and fibrinogen concentration was determined by optical absorption at 280 nm. The concentration of the fibrinogen lacking ability to polymerization was calculated as the difference between the total concentration of fibrinogen (0.5 mg/mL) and the concentration of fibrinogen in the acetic lysates of the fibrin clots. In accordance with the obtained results (Figure 7), the incubation of fibrinogen with the fibrinogenolytic enzyme was accompanied by the accumulation of functionally inactive fibrinogen, the concentration of which increased depending on the time of incubation. The concentration of functionally inactive fibrinogen in the control sample was 0.062 ± 0.008 mg/mL, which corresponded to 12% from the total content. After 1 h incubation, the concentration of functionally inactive fibrinogen increased in 7 times and amounted to 0.439 ± 0.06 mg/mL. The extension of the incubation time to 3 hours resulted in the further accumulation of fibrinogen cleavage products, the concentration of which was already 0.483 ± 0.08 mg/mL. Our findings of changes in the concentration of fibrinogen were confirmed by a visual assessment of the fibrin clot, that formed after the addition of thrombin - the size of the clot decreased with incubation time and the addition of thrombin after 6 h of incubation with the enzyme did not cause the formation of a fibrin clot at all, which may serve as a confirmation of the lack of a functionally active fibrinogen.

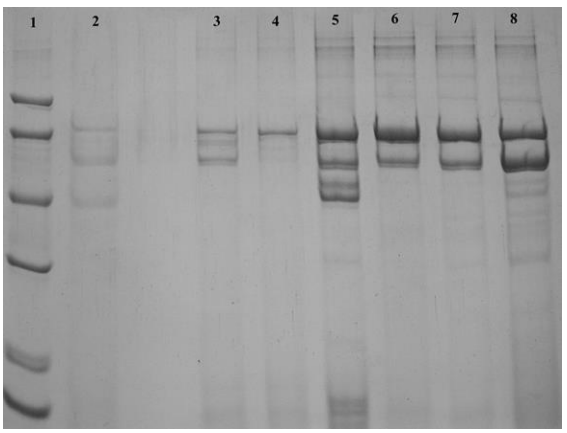

Figure 8. SDS-PAGE analysis of incubation medium (3-5) or fibrin clots (6-8) after incubation with the fibrinogenolytic enzyme. SDS-PAGE was carried out in 10% gel. Lane 1 - molecular weight standards: phosphorylase b (97 kDa), serum albbumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Lane 2 - control of fibrin sample without incubation with the fibrinogenolytic enzyme. Lanes 3–6 - samples after incubation with the fibrinogenolytic enzyme for 1 h. Lanes 4–7 - samples after incubation with the fibrinogenolytic enzyme for 6 h. Lanes 5–8 - samples after incubation with the fibrinogenolytic enzyme for 24 h.

The resultant fibrin monomers spontaneously polymerize to form fibril strands that undergo linear extension, branching and lateral association, leading to the formation of a 3D network of fibrin. A number of fibrinogenolytic enzymes, in particular those which are

metalloproteases, along with the ability to cleave the fibrinogen also have fibrinolytic activity. To determine whether the fibrinogenolytic enzyme exhibits fibrinolytic activity, fibrin clots are incubated with the fibrinogenolytic enzyme for 1, 6 and 24 h. The reaction was stopped by addition of SDS-PAGE sample buffer in the presence of β-mercaptoethanol and immediately subjected to 95^0C for 5 min. The hydrolysis pattern of fibrin clot as well as incubation medium was analyzed by SDS-PAGE (Figure 8).

Degradation of fibrin clot by the fibrinogenolytic enzyme resulted in cleavage of α- and β-chains. Thus, bands corresponding to these chains of fibrin were appeared in incubation medium upon incubation for 1 and 6 h. We found that with increasing time of incubation to 24 h, bands related to γ-chains were also detected, confirming the presence of fibrinolytic activity. We also revealed the presence of a number of protein bands with molecular weights above 100 kDa. In addition, the fragments with molecular weights of 11 and 15 kDa were also identified. Electrophoretic analysis of samples of fibrin clots after their incubation with the enzyme showed the presence of clearly expressed protein bands with molecular weights of 67 and 55 kDa in all investigated time intervals. Given the molecular weight and the correspondence to the fibrin chains in the control sample, we can say that these bands are α- and β-chains of fibrin. The absence of the band that corresponds to the γ-chain can be partially explained by the formation of γ-γ-polymers. It is known that the covalent firmware of fibrin monomers is carried out with the participation of a XIIIa factor that catalyzes the formation of a specific bond between the ε-amino groups of lysine residues and the γ-carboxyl groups of glutamine residues of the corresponding chains. Despite the fact that we did not use factor XIIIa to initiate the formation of a fibrin clot its certain amount is always present in preparations of fibrinogen and can cause the firmware of chains. Comparison of the electrophoretic profile of the samples obtained after incubation of the fibrin clot with the fibrinogenolytic enzyme from *A. colbecki* during different time intervals suggested a slight hydrolytic activity of the enzyme in relation to α-α-polymers of fibrin. The intensity of the band corresponding to α-α-polymers (Figure 8) was somewhat reduced with an extension of the incubation time up to 6 h, but the complete cleavage was not observed even after 24 h of incubation. A certain confirmation of fibrinolytic activity of the investigated enzyme was the accumulation of a number of fibrin degradation products that are visualized in the region between 45 and 31 kDa. Despite the ability of the fibrinogenolytic enzyme under long-term incubation with fibrinogen, cleave its γ-chain, the enzyme was not hydrolysed γ-γ-polymers of fibrin, as evidenced by the presence on the electrophoregram of bands with a molecular weight of about 90 kDa. Similar results were obtained in works [22, 23, 24], where the authors showed that the fibrinogenolytic metalloproteases preferentially cleave the the Aα- and Bβ-chains of fibrinogen, however, they cannot exhibit any effect on the γ-chains of fibrinogen and γ-γ polymer of fibrin. According to literature, fibrinolytic activity is predominantly characteristic of metalloproteases, whereas serine enzymes from snake venoms, as a rule, do not possess such activity. To sum up, *A. colbecki* fibrinogenolytic enzyme has higher affinity to fibrinogen than to fibrin.

Several lines of evidence support the fact that metalloprotease from snake venom often cause the development of hemorrhagic syndrome [25]. Hemorrhagic activity is predominantly characteristic of fibrinogenolytic enzymes belonging to the class of metalloproteases and related not so much to the presence of a catalytic or metal-protease domain in the enzyme molecule, as with the presence of disintegrin (for PII class metalloproteases) or cysteine-rich (for PII and PIII class metalloproteases) domains [26]. These domains contain the SECD

sequence required for binding to collagen and other proteins of the extracellular matrix [27, 28]. The hemorrhagic activity of fibrinogenolytic metaloproteases depends on their ability to hydrolyse proteins of basement membrane and extracellular matrix. Hemodynamic forces, imposed by blood streaming through the vessel, ultimately disrupt the damaged vessels. Since *A. colbecki* fibrinogenolytic enzyme was metalloprotease, and due to its molecular weight (about 40 kDa) it may contain a disintegrin domain, therefore we tested whether this enzyme can cleave collagen, which is an important component of the basement membranes and the extracellular matrix. The degree of hydrolysis of collagen samples was evaluated by electrophoresis in 8% SDS-PAAG after incubation of the fibrinogenolytic enzyme with collagen for 1, 2, 3, 4, 6 and 24 h. According to obtained results (Figure 9), the studied fibrinogenolytic enzyme did not possess the ability to cleave the collagen, since in the control sample and samples after incubation with the enzyme the collagen remained intact throughout the entire incubation period.

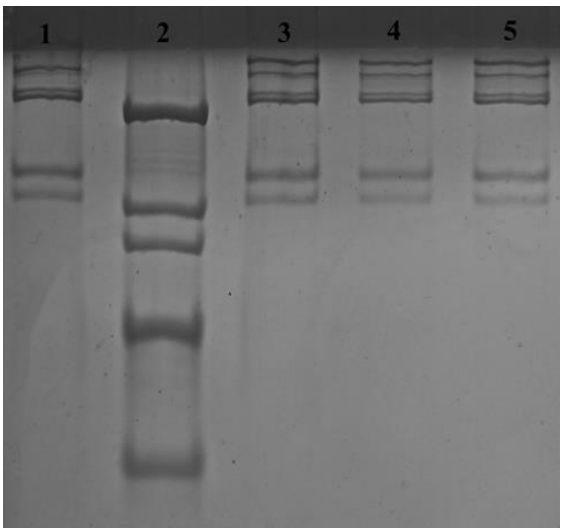

Figure 9. SDS-PAGE analysis of collagen after incubation with the fibrinogenolytic enzyme. SDS-PAGE was carried out in 18% gel. Lane 1 - control of collagen sample without incubation the fibrinogenolytic enzyme. Lane 2 - molecular weight markers: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa). Lanes 3-5 - collagen samples after incubation at 37°C with the fibrinogenolytic enzyme for 1, 2, 3 and 24 h, respectively.

Under physiological conditions, the platelets are inactive and circulate in the bloodstream as separate cells that have limited ability to interact with both the endothelium of the vascular wall and other blood cells, including with each other. Expression of the adhesive properties of platelets occurs in response to the action of a number of the stimulating agents, in particular, ADP. It is well-known that for platelet aggregation, one of the necessary conditions is the presence of fibrinogen, which, through the formation of fibrinogen bridges, provides the connection of individual platelets to a fluff platelet during the first phase of aggregation. Therefore, the fibrinogen is considered to be a mandatory cofactor for platelet aggregation. Taking into account the ability of the fibrinogenolytic enzyme from A. colbecki to cleave the fibrinogen, further we investigated whether the enzyme could influence on ADP-induced platelet aggregation. The purified enzyme was assayed for platelet aggregation inhibitory

effect using platelet-rich plasma, which allowed to assess whether the effect of the enzyme due to cleave of fibrinogen. The results of the studies convincingly indicate the ability of the enzyme to inhibit ADP-induced platelet aggregation (Figure 10).

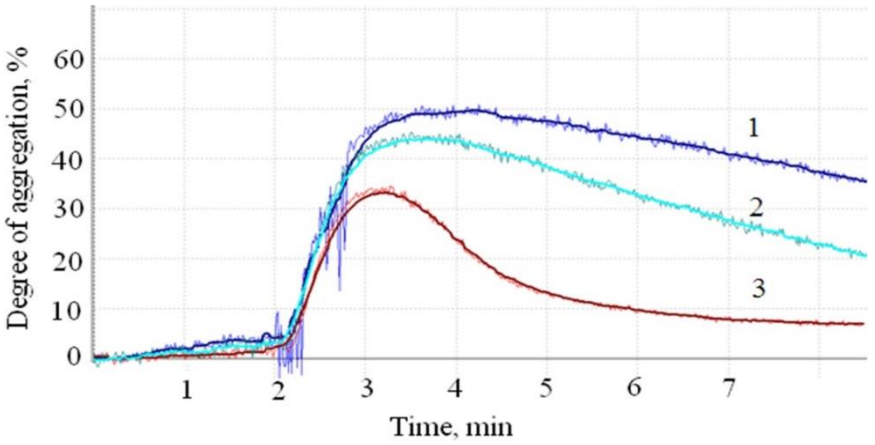

Figure 10. Effect of the fibrinogenolytic enzyme on ADP-induced platelet aggregation: 1 – control. 2 – 6.25 μg/mL. 3 – 12.5 μg/mL.

The fibrinogenolytic enzyme mediated a dose-dependent inhibition of platelet aggregation. Thus, when the enzyme was used at concentrations of 12.5 μg/mL and 6.25 μg/mL, only 8% and 28% aggregation was observed compared to 41% which was recorded in control sample. Obtained results are fully consistent with the data presented in the literature, according to which the ability to suppress platelet aggregation is predominantly characteristic of α-fibrinogenases, whereas for the β-fibrinogenase, this effect is not typical. A similar effect has been shown for α-fibrinogenolytic enzymes from the snake venom [29, 30]. The inhibitory effect of α-fibrinogenase depends on the concentration of the enzyme and the time of incubation, which may indirectly indicate that their action is associated with the manifestation of enzyme activity. Since fibrinogen plays an important role in platelet aggregation, we supposed that the fibrinogenoloytic enzyme inhibited aggregation by destroying intact fibrinogen to prevent fibrinogen from combining with fibrinogen receptor (GPIIb-IIIa) on platelet membrane.

In conclusion, in view of the specificity towards chains of fibrinogen and effects of inhibitors, the fibrinogenolytic enzyme from *A. colbecki* can be classified as a metaldependent α-fibrinogenase with platelet aggregation inhibitory activity. This enzyme due to its effects on hemostasis may be used as a biological tool to explore many facets of hemostasis.

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Chapter 30

INVESTIGATION OF MARINE BIOTOXINS AND HUMAN TOXICITY

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ABSTRACT

The marine biotoxins are correlated with a proliferation of harmful algal blooms (HABs) and can accumulate in the seafood products, with severe consequences both on the fishery industry and consumer health. Recently, an increase of HABs occurred worldwide due to climate change or anthropogenic activities such as discharge of nutrients from domestic and industrial waste, shellfish translocation and global shipping. Different types of food poisoning are described, i.e., Amnesic Shellfish Poisoning (ASP), Diarrhetic Shellfish Poisoning (DSP), Neurologic Shellfish Poisoning (NSP), Paralytic Shellfish Poisoning, (PSP) and Ciguatera Fish Poisoning (CFP). The symptoms are dependent on the specific biotoxin, and maximum levels have been established for some of them in the global legislation. Moreover, acute reference doses are reported for some marine biotoxins, whereas tolerable daily intakes cannot be determined due to the absence of appropriate toxicological data. The real incidence of poisoning events is often underestimated because the symptoms are similar to viral or bacterial infections as well as allergic reactions. In addition, the lack of knowledge aiming at the diagnosis and treatment of human patients can represent a serious problem for communities and educational programs are considered efficient tools to prevent these events. The majority of human intoxications are related to the consumption of live bivalve molluscs, which are filter feeders and concentrate the marine biotoxins produced by toxic phytoplankton species, but these substances may increase at higher trophic levels in the food chain and therefore also other marine organisms can be involved in foodborne outbreaks. Most marine biotoxins are not destroyed by food technologies like freezing or cooking, and their presence cannot be perceived because they give no specific taste, flavor or smell to food. The monitoring plans are generally performed to assess the risk of seafood

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contamination, and *in vivo* assays and chemical analysis are the most frequently used tests. In addition, other rapid methods have been developed, such as ELISA tests, receptor binding or antibody-based lateral flow assays and *in vitro* tests.

In this chapter, the authors described the main foodborne illnesses linked to the marine biotoxins, their toxicity, detection methods and prevention strategies. In particular, they reported the results of the monitoring analyses carried out in the samples of *Mytilus galloprovincialis* collected along the Central Adriatic Coast, Italy.

ABBREVIATIONS

ASP	Amnesic Shellfish Poisoning
ARfD	Acute Reference Dose
AZA	Azaspiracid
BTX	Brevetoxin
CFP	Ciguatera Fish Poisoning
CTX	Ciguatoxin
DSP	Diarrheic Shellfish Poisoning
DA	Domoic Acid
EFSA	European Food Safety Authority
HAB	Harmful Algal Bloom
LC-MS/MS	Liquid Chromatography-Mass Spectrometry
LOQ	Limit of Quantitation
MBA	Mouse Bioassay
NSP	Neurotoxic Shellfish Poisoning
OA	Okadaic Acid
PSP	Paralytic Shellfish Poisoning
PTX	Pectenotoxin
STX	Saxitoxin
TEF	Toxicity Equivalency Factor
UHPLC-HRMS	Ultra-High Performance Liquid Chromatography-High-Resolution Mass Spectrometry
YTX	Yessotoxin

IMPACT OF THE HARMFUL ALGAL BLOOMS IN THE MARINE ECOSYSTEMS

The harmful algal blooms are natural phenomena developing mainly near coastal waters due to nutrient composition changes (excess of nitrogen and phosphorus) and anthropogenic activities such as discharges from domestic, industrial and agricultural sources, fertilizers usage, aquaculture farms and marine traffic (Narale and Anil, 2017; Park et al., 2017). Moreover, the increase of HABs is also the consequence of climate change (alterations of temperature, ocean acidification, stratification) that influences the marine planktonic systems (Faggio et al., 2011). The global warming is one of the main causes of increase in frequency and severity of HABs, favouring the growth of toxic dinoflagellates of genera *Alexandrium*

and *Dinophysis* in both the Atlantic and Pacific Oceans (Gobler et al., 2017) and diatom *Pseudo-nitzschia* spp. (Zhu et al., 2017).

The HABs can have negative effects on the marine ecosystems through two ways of action. The first one is due to the sheer number of microalgae cells that create a barrier between the surface and deeper waters, while the second type is linked to the production of marine biotoxins and their metabolites. The exposure of the marine organisms can be attributed to the environmental conditions during the algal blooms and their potential contact with the toxins. The last event can be significantly reduced by the physical and chemical properties of seawater, especially when the volume of water is large and dilutes its concentration (Lopes et al., 2013).

The bioaccumulation of the marine biotoxins is a dynamic process because they are produced by different phytoplankton species (Table 1) and transferred to the marine organisms through the food chain. It is well known that the marine biotoxins accumulate above all in the bivalve molluscs as filter-feeding organisms, but they can also be found at higher trophic levels through the bioaccumulation in fish, marine mammals, seabirds and humans (Orellana et al., 2017). Several benthic species such as gastropods, bivalves and echinoderms have been described as new vectors of some marine biotoxins (Silva et al., 2013). Turner (2014) described that the HABs can be concentrated in some upper-trophic level pelagic consumers such as fish and marine mammals. Costa et al. (2010) also reported that fish can accumulate the marine biotoxins without signs of toxicity or transfer them up to piscivorous predators such as marine mammals. AZA have been found in shellfish and crabs mainly in Europe (Álvarez et al., 2010), whereas PSP toxins accumulated in Atlantic horse mackerel over a bloom of *Gymnodinium catenatum* (Lage and Costa, 2013).

The main economic losses due to the HABs and marine biotoxins in shellfish are related to the closure of coastal fisheries, implications on international trade, reduction of job in seafood industries and recreational activities (McPartlin et al., 2016). Furthermore, the marine biotoxins are toxic also for animals belonging to the marine food web (Franchini et al., 2010). Therefore, the HABs have adverse impact on the aquatic ecosystems, fisheries and public health due to the production of the marine biotoxins that can be classified on the basis of their toxic effects or their chemical structure and solubility in solvents (Gobler et al., 2017).

Table 1. Origin and classification of the marine biotoxins affecting human health

HAB taxa	Marine biotoxin	Human toxic syndrome
Pseudo-nitzschia spp.	Domoic acid	Amnesic Shellfish Poisoning
Gambierdiscus spp.	Ciguatoxin	Ciguatera Fish Poisoning
Dinophysis spp. *Prorocentrum* spp. *Gonyaulax spinifera* *Azadinium spinosum*	Okadaic acid Pectenotoxin Yessotoxin Azaspiracid	Diarrheic Shellfish Poisoning
Karenia brevis	Brevetoxin	Neurotoxic Shellfish Poisoning
Alexandrium spp.	Saxitoxin	Paralytic Shellfish Poisoning

The European legislation set the maximum levels in the live bivalve molluscs by Regulations (EC) No 853/2004 and (EU) No 786/2013. These regulatory limits correspond to:

- 160 μg OA/AZA/PTX equivalent/kg;
- 800 μg/kg for STX;
- 3.75 mg YTX equivalent/kg;
- 20 mg/kg for DA.

The Codex Committee on Fish and Fishery Products developed the standard for live and raw bivalve molluscs for 5 toxin groups, with the same above reported values for STX, OA, DA and AZA, and 200 mouse units/or equivalent/kg for BTX (Botana et al., 2017).

The consumption of the contaminated seafood generally causes acute and short-term events in humans, therefore ARfD have been established by EFSA for the marine biotoxins, corresponding to the concentration per kg of shellfish meat (400 g) that can be safely consumed. In some cases, the ARfD are lower than the existing limits to ensure food safety. The lowering of the maximum limits could have implications for the shellfish industries, but besides the regulatory limits the monitoring programs are necessary to prevent outbreaks due to the marine biotoxins (Nicolas et al., 2017).

Some marine biotoxins are characterized by the presence of analogues in the same group that can have dissimilar toxic potencies. In that case, a potential approach aiming at defining and applying TEFs could be essential for the monitoring and control of the regulatory limits set for the related compounds (Botana et al., 2017).

Another important aspect is related to the non-bivalve organisms that can be toxin vectors, such as echinoderms, tunicates and marine gastropods as well as crustaceans, as reported in human poisoning incidents occurred worldwide. Therefore, their relevance or potential threat for the public health should also be considered in the monitoring and regulatory surveillance plans (Costa et al., 2017).

HUMAN TOXICITY OF THE MARINE BIOTOXINS

The marine biotoxins need to be monitored in the fishery products because they cause food poisoning outbreaks in humans. The symptomatology varies from some gastrointestinal (nausea, vomiting, diarrhea or abdominal cramps) or neurological (confusion, lethargy, disorientation, paresthesia) signs up to a permanent neuropathy or death (Botana et al., 2017; Visciano et al., 2016).

The marine biotoxins can be classified on the basis of the adverse effects observed after the consumption of the contaminated seafood. Most of them are well known, even if there is a great number of recent studies reporting emerging toxicity mechanisms and some organ/system damages.

DA is the main toxin responsible of ASP, characterized by neurological symptoms such as disorientation, confusion, hallucinations and memory loss. It is structurally related to the kainic acid, an analogue to the neuro-transmitter L-glutamate, and can cause the apoptotic and necrotic neuronal cell death. Further studies reported the enhancement of its neurotoxicity due to the involvement of glial cells, with inhibition of glutamate uptake as secondary effect of the glutamate-receptor activation, intracellular acidification or free-radical formation (Ross et

al., 2000). The interaction between the glia and neurons is particularly important during the brain development and neonates have been shown to be more sensitive than adults (Hogberg and Bal-Price, 2011). However, Crespo et al. (2015) reported that the affected people showed other symptoms than neurological, such as unstable blood pressure and arrhythmias or gastrointestinal disorders.

Among the marine biotoxins belonging to the DSP group, OA and its analogues are responsible of some gastrointestinal disorders, i.e., nausea and severe diarrhea (García et al., 2016), whereas AZA, PTX and YTX show still unclear effects in humans (Ferron et al., 2016). In particular, the OA group toxins have citotoxic effects on the intestinal cell lines through a disruption of the cell cycle, apoptosis, inflammatory response and DNA damage (Ferron et al., 2014). Moreover, other toxicological studies reported also neurotoxicity, immunotoxicity, genotoxicity and carcinogenicity (Valdiglesias et al., 2013). A recent study (Espiña et al., 2010) demonstrated that the methyl ester derivative of OA could be considered more toxic for the metabolism of primary cultured hepatocytes.

The AZA group toxins represent an emerging human health risk. Besides gastrointestinal symptoms with a severe and protracted diarrhea, they can affect also other organs and have been shown to be cytotoxic, carcinogen in mice and teratogen to developing fish (Twiner et al., 2012a). AZA1 has been described as lung tumors inducer (Ito et al., 2002), whereas AZA2 showed acute arrhythmogenic potential *in vivo* (Ferreiro et al., 2014a) and *in vitro* chronic effects on a specific cardiac potassium channel (Ferreiro et al., 2014b). In particular, an open-state hERG (human *ether-a-go-go*-related gene) blocking activity has been reported for AZA1, AZA2 and AZA3 by Twiner et al. (2012b). The alteration of hERG function is linked to the ventricular repolarization disorders (Ferreiro et al., 2014b) and the electrophysiological changes at cellular and tissue levels triggering a form of arrhythmia with the QT interval prolongation of electrocardiogram (Ferreiro et al., 2014a).

The adverse effects of the PTX and YTX groups are still unclear even if PTX have been shown to induce an actin depolarization with following disruption of the cytoskeleton (Espiña and Rubiolo, 2008). YTX and its analogues show cardiotoxicity with a mitochondrial damage in the cardiomyocytes after repeated exposures (Ferreiro et al., 2016), but also some changes of the intracellular calcium and cyclic AMP levels and alteration of adhesion molecules like E-cadherin (Tubaro et al., 2010). The selective disruption of E-cadherin system can cause a potential loss of its tumor-suppressing activity (Schirone et al., 2013). Ferreiro et al. (2016) reported a marked bradycardia and hypotension associated to the ultrastructural cardiac damage due to repeated exposures to YTX. Other *in vitro* studies (Fernandez-Araujo et al., 2015) showed some histopathological alterations and cytotoxicity in many cell lines after the acute exposure to this marine biotoxin. Recently Ferreiro et al. (2017) studied the subacute immunotoxicity in rats by evaluating some plasmatic inflammatory cytokine biomarkers and alterations in spleen and thymus. This study suggested that the exposures to low amounts of YTX could affect the health of the consumers especially if immuno-compromised.

STX, BTX and CTX are different neurotoxins causing a nervous and muscular deficit (Cocilova et al., 2017). STX is one of the most potent natural neurotoxins that cause a severe illness in humans known as PSP. Further 57 analogues have been described since its discovery in 1957, when it was isolated from the butter clam *Saxidomus giganteus* (O'Neill et al., 2016). The members of this group can be found both in the marine environments, during HAB events characterized by the proliferation of dinoflagellates of the genera *Alexandrium*, *Gymnodinium* and *Pyrodinium*, and in the freshwater environments, where they are produced

by prokaryotic cyanobacteria, resulting in the contamination of drinking and recreational water resources (Wiese et al., 2010). The mechanism of action of STX is linked to the interaction with the voltage-gated sodium channels, and the consequent inhibition of ion conduction across the plasmatic membrane of the neurons and muscle cells (Cusick and Sayler, 2013). Among the PSP symptoms there are muscle weakness, tingling or numbness around mouth and limbs, paraesthesia, incoordination, headache, shortness of breath, dysarthria, dysphagia, hypotension and potential fatality due to the respiratory paralysis (O'Neill et al., 2016; Wharton et al., 2017).

BTX induces a syndrome known as NSP with severe gastrointestinal disorders, reversal of temperature sensation and paresthesia (James et al., 2010). BTX has been also associated with marine mammal deaths and mass mortalities in manatees, seabirds and fish. The human exposure can occur through the consumption of contaminated molluscs as well as by aerosols during the red tide events. In the last case, a respiratory symptomatology can be observed (Chen et al., 2017) showing more severe symptoms in asthmatics (Fleming et al., 2009). It has been reported that BTX binds to and opens voltage gated sodium channels in the excitable cells, causing a continuous depolarization of the excitable membranes. So, the neuronal, muscular and cardiac systems can be affected, with ataxia, muscle twitching, from partial to the complete paralysis and potential long-term behavioral changes (Cocilova et al., 2017). At low concentrations, BTX can cause the depression of the pulmonary and cardiac function, inflammation (Baden et al., 2005) and immune function suppression (Perrault et al., 2016). A recent study (Chen et al., 2017) showed the inhibition of the thioredoxin-reductase system in mammalians, but this effect could be supposed also in humans because this system is present in almost all living organisms.

CFP is the most common non-bacterial cause of human disease due to the consumption of fish containing relatively high concentrations of CTX. This is a group of lipophylic neurotoxins produced by dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa* (Kibler et al., 2017). These dinoflagellates can be consumed by herbivorous and omnivorous fishes and bioaccumulated in the food web in piscivores (Catania et al., 2017). Even if the occurrence of ciguateric fish is more frequent in the tropical and sub-tropical areas, a spread of dinoflagellates into the temperate climate zones has been observed in recent years and CFP has become a global human health concern (Mattei et al., 2014). The disease presents several symptoms, that can be gastrointestinal (diarrhea and/or vomiting) and neurologic (estremity paresthesias, weakness and fatigue). While the gastrointestinal disorders can be resolved within 48 h, the others can persist for several weeks and some patients can develop a chronic form characterized by fatigue and long-term disability (Lopez et al., 2016).

METHODS OF DETECTION OF THE MARINE BIOTOXINS

The main assay used worldwide for the detection of the marine biotoxins is the MBA where mice are intraperitoneally injected with a sample extract and the toxic effects, above all lethality, are observed to reveal their presence. However, this test presents some ethical problems and can give also false-negative or false-positive results (EFSA, 2009). While the false-negative results can cause some dangerous problems to the human health, the false-positive results can involve economic loss for the shellfish industry.

MBA is also limited because it is poor sensitive and does not provide information as to the exact toxin present in the sample (McPartlin et al., 2016). In recent years, Regulation (EU) No 15/2011 substituted the MBA with a validated technique of LC-MS/MS as the reference method for the detection of the lipophilic toxins, to be used as matter of routine, whereas the biological methods should be applied only during the periodic monitoring of the production areas for detecting new or unknown marine biotoxins.

However, LC-MS/MS methods can present some difficulties due to the lack of the certified standards and/or reference materials for many toxins. For instance, only 20–30 certified reference standards are currently available, even if 24 STX analogues, 13 OA-ester derivatives, 90 YTX analogues, 15 BTX analogues and around 30 AZA analogues have been described. For these reasons, many countries prefer to adopt other assays such as *in vitro* tests able to detect both known and new emerging marine biotoxins (Bodero et al., 2017). The methods of detection currently in force according to the European legislation are often expensive and time consuming, so that other faster techniques such as the use of biosensors, that target both marine biotoxins and the toxin-producing micro-algae, could be used for the implementation of their monitoring programs. These assays are based on the naturally high binding affinity of the marine biotoxins to their cellular target, e.g., voltage-gated sodium channel receptors for PSP, BTX, CTX, glutamate receptor for DA, or protein phosphatase for OA (McPartlin et al., 2016).

Another interesting aspect to be considered during the monitoring regards the significant amounts of algal biotoxins dissolved in the seawater when toxic species are present (MacKenzie et al., 2010), even if the current regulation fixed maximum levels only in seafood but not in seawater. MacKenzie et al. (2004) first demonstrated that the passive adsorption of dissolved toxins from seawater, coupled to sensitive analytical technologies such as LC-MS/MS and ELISA assays, provided a simple means of monitoring that simulated a biotoxin contamination of the filter feeding bivalves.

Many recent studies (Blay et al., 2011; Domènech et al., 2014; García-Altares et al., 2014) demonstrated that the UHPLC-HRMS is an excellent tool to detect the marine biotoxins in algae and seafood. These techniques could be particularly useful to assess a potential exposure to a multi-toxin mixture. As a future perspective, a confirmatory identification of potential toxins by studying their fragmentation spectra (using new tools such as hybrid quadrupole Q-ExactiveTM Orbitrap-MS) is designated (Orellana et al., 2014, 2015).

THE MONITORING PROGRAMS IN MOLLUSCS FROM THE ADRIATIC SEA, ITALY

In recent years, the increase of HABs has led to the need of new strategies aiming at the monitoring of the marine biotoxins in coastal waters, that vary among the geographical locations and HAB types (Asnaghi et al., 2017). According to the food legislation currently in force in the European Union, sampling plans are periodically applied in the production areas of the live bivalve molluscs. The frequency is generally to be weekly during the periods at which the harvesting is allowed, but it can be reduced if a risk assessment on the marine

biotoxins and/or toxin-producing plankton occurrence suggests a very low risk of intoxication, as reported in Regulation (EC) No 854/2004.

In our study (Schirone et al., 2011) a total of 415 specimens of *Mytilus galloprovincialis* were collected from some shellfish aquaculture plans located along the coasts of the Central Adriatic Sea (Abruzzo and Molise regions) during the monitoring programmes of the years 2006-2009 (Figure 1). The MBA was applied on extract from whole body or edible part of the molluscs to investigate the presence of OA, DTX, PTX, AZA and YTX, whereas DA was determined by HPLC-UV. No biotoxins were detected and these results demonstrated a good condition of the marine area as well as the safety of the bivalve molluscs.

The detection of YTX in specimens of *M. galloprovincialis* was performed by an *in vitro* functional assay based on the fragmentation of the cell-adhesion molecule E-cadherin in cultured cells. The samples were collected from three regions (Abruzzo, Molise and Emilia Romagna) along the Central Adriatic coasts and grouped in two aliquots in a sufficient number to obtain pools of 100 g whole flesh. All samples were analyzed in duplicate. The mean values of YTX in samples coming from Abruzzo and Molise regions ranged from 0.20 to 0.67 mg of YTX equivalent/kg, whereas in those collected along the coasts of Emilia Romagna they were higher up to 1.80 mg of YTX equivalent/kg (Schirone et al., 2013).

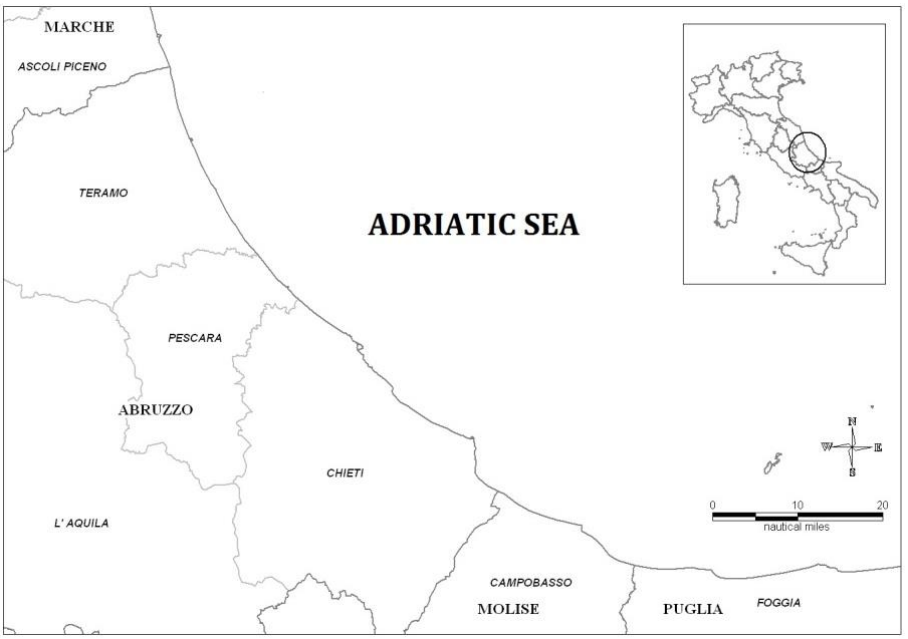

Figure 1. Sampling area (Abruzzo and Molise regions) of specimens of *Mytilus galloprovincialis*.

Samples of *M. galloprovincialis* collected from several shellfish aquacultural plans (n = 15) located along the coasts of Abruzzo, Molise and Emilia Romagna regions, were analyzed for YTX content by three different methods, i.e., MBA, the *in vitro* functional test described by Schirone et al. (2013), and a LC-MS/MS method. The results obtained by MBA showed that the specimens coming from Abruzzo and Molise regions were all negative, whereas all the samples coming from Emilia Romagna region caused the death of mice, except for one that could be considered negative because only one of the three mice died after 16 h. The

concentrations of the investigated marine biotoxins were detected by the *in vitro* functional assay and ranged from a minimum of 0.24 mg to a maximum of 1.63 mg of YTX equivalent/kg, with the highest values in the specimens derived from Emilia Romagna region. The samples analyzed by LC-MS/MS showed YTX concentrations only in the molluscs coming from Emilia Romagna region, whereas in all the other samples the values were below the LOQ. More in detail, the major detected analogues were carboxy homo YTX (ranging from 0.227 mg to 1.934 mg of YTX equivalent/kg), followed by homo YTX (ranging from 0.239 mg to 1.023 mg of YTX equivalent/kg). These results seemed to confirm the eutrophic phenomena that have been frequently reported in the Northern Adriatic Sea, and could have an economic impact on shellfish industry considering that this area covered about 90% of the Italian mussel production (Visciano et al., 2013).

Also other authors (Ciminiello et al., 2010) found YTX concentrations ranging from 0.001 to 0.032 μg/g in samples of *M. galloprovincialis* coming from Emilia Romagna coasts. These values could vary due to different reasons such as mussel metabolisms, their physiological conditions, water temperature, season and presence of phytoplankton species (Amzil et al., 2008). Bacchiocchi et al. (2015) found levels of OA, YTX and AZA in *M. galloprovincialis* collected along the North-Central Adriatic Sea of Marche region. The highest concentrations were detected always in autumn-winter periods probably due to the increased inflow of freshwater from the Po river.

CONCLUSION

Several food poisoning outbreaks associated with the consumption of seafood contaminated with marine biotoxins have been reported worldwide and therefore a regular and periodic monitoring of the marine waters especially during well-defined periods, i.e., when the environmental conditions are favorable to HABs, could serve to prevent these events. The presence of the marine biotoxins should be also investigated in molluscs and other potential vectors above mentioned, because most of them cannot be destroyed by freezing or cooking and the contaminated food generally do not present any alteration or specific taste capable to alert the consumers for this health hazard. A recent study (Rodríguez et al., 2016) demonstrated that DTX3 was destroyed by heat under steaming conditions (i.e., 2 min at 105°C and 3 min at 100°C) depending on the matrix environment. Moreover, a 50% decrease in total toxicity was reported for OA and DTX2 after sterilization at 121°C for 20 min.

There is often an underestimation of the real incidence of fish/shellfish poisoning due to the marine biotoxins, especially because the symptoms are similar to allergic reactions and viral or bacterial infections (Iwamoto et al., 2010). Besides the official LC-MS/MS methods used for the detection and the quantification of the marine biotoxins, sensitive and high throughput effect-based *in vitro* assays should be developed and validated to allow the identification of unknown toxins that could cause new adverse effects in consumers.

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Chapter 31

NATURAL PRODUCTS FROM MARINE SPONGES: CURRENT STATUS AND FUTURE POTENTIAL FOR NOVEL DRUGS

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ABSTRACT

Marine sponges (Phylum Porifera) have consistently been a prolific source of new marine natural products with unprecedented chemical scaffolds and potential pharmaceutical applications. Other than their chemical diversity, these compounds possess remarkable bioactivities. This great potential has aroused applications of sponge natural products as therapeutics against important diseases, such as cancer, a range of viral diseases, malaria, and inflammations and at present, a number of promising compounds are in clinical and preclinical trials. Although the molecular mode of action of most of these metabolites is still unclear, for a substantial number of compounds the mechanisms by which they interfere with the pathogenesis of a wide range of diseases have been reported. Research on marine sponges continues in the hope that drugs of the future will be culled from different parts of the oceans. Though research on marine sponge-derived bioactive compounds are increasingly promising in drug discovery, multiple technical issues are yet to be addressed prior to turning the wide-scale

bioprospecting of marine sponges into a reality. This chapter focuses on some novel compounds having antioxidant, antimicrobial, anti-inflammatory, anticancer and other bioactive properties identified and isolated from marine sponges, which could serve as leads for drug discovery. We also discuss the factors that contribute to new discoveries and the challenges associated with translating marine sponge-derived compounds into clinical trials. In addition, providing an outlook into the future, we examine the advances that may further expand the promise of drug discovery from marine sponges.

Keywords: bioactivity, drug discovery, natural products, sponges

1. INTRODUCTION

Nature represents an infinite reservoir of novel chemotypes and pharmacophores, as well as blueprints for drug development against a multitude of disease indications including cancer, diabetes and infectious diseases. Throughout history natural products (NPs) have been the cornerstone of traditional system of healing worldwide. Although the use of bioactive NPs as herbal drug preparations dates back hundreds, even thousands, of years ago, their application as isolated and characterized compounds to modern drug discovery and development started only in the 19th century (Veereshen, 2012). Examples of early identified NPs are undoubtedly the isolation of morphine from *Papaver somniferum* poppies, first reported in 1803, and the discovery of the first antibiotic penicillin from the fungus *Penicillium notatum* in 1929 by Flemming (Carter, 2011). To date approximately 60% of the drugs currently on the market are of natural origin (Newman and Cragg, 2012). Although the pharmaceutical industry turned to combinatorial chemistry and high throughput screening in the 1980s, NPs have again attracted the attention of drug hunters in recent years because they have proven to be a more promising source of drugs than combinatorial synthesized chemicals (Baker et al. 2007, Corson and Crews, 2007, Harvey, 2007, Hostettmann, 2007, Li and Vederas, 2009, Kumar and Walmann, 2009). Today, with a movement into a modern era of biotechnology and automation, natural product-based compounds have an immense impact on modern medicine with a number of top selling drugs such as vincristine from *Vinca rosea* and Taxol from *Taxus brevifolia* (Newman and Cragg, 2012). The natural product-based drug discovery depends largely on the continuous supply of novel natural agents, especially those with novel scaffolds. Since natural building blocks and biosynthetic strategies are rather limited, natural products are highly redundant. Therefore, although the unexplored natural product universe is still ample, it is not an easy task to find novel agents from nature (Li and Vederas, 2009). Accordingly, research must continue to progress to improve existing therapies and to develop novel cures. Since ancient civilizations, research has essentially focused on edible and natural sources such as fruits, vegetables and plants, mainly because these specimens are easily available and folk traditions have described beneficial effects from their use. However, in contrast to these described terrestrial sources of drug molecules, a completely unexplored potential source of bioactive natural agents is represented by the sea (Schumacher et al. 2011, Beesoo et al. 2014). Although oceans have attracted the attention of researchers since the 1950s with the discovery of the *Cryptotheca crypta* sponge-derived anti-cancer spongothymidine and anti-viral spongouridine, the technical difficulties of collecting marine organisms together with the poor knowledge of their habitat and identification of the

true sources of the bioactive agents have posed relevant obstacles. Nevertheless, the implementation of modern scuba diving tools such as remotely operated vehicles and the development of instruments for the isolation of NPs from marine organisms have rejuvenated research in the arena of marine natural product (Gerwick and Moore, 2012).

2. MARINE NATURAL PRODUCT

The world's ocean, covers 70% of the earth's surface and 95% of its tropical biosphere is home to nearly one million multi-cellular (plants and animals) and one billion unicellular organisms surpassing that of the terrestrial diversity (Minh et al. 2005, Burgess, 2012, Romano et al. 2017). Living in such a highly competitive environment, marine organisms have evolved biochemical and physiological mechanisms that include the production of secondary metabolites in direct response to environmental stressors and thus act as protective and/or adaptive agents. Such responses include up-regulation of toxic compounds as protection, or in response to damage or predator attack; adaption to heat, mechanical deformation, salinity, pH, or cold; aggression (stinging) or other environmental stressors such as UV irradiation, overgrowth, competition for mates, tissue damage, local lack of essential nutrients (Minh et al. 2005, Monaco and Quinlan, 2014). A number of sessile benthic organisms including Porifera, Cnidaria, Bryozoa, and Tunicata produce a myriad of secondary metabolites as their defense mechanism. As these organisms typically lack the physical attributes to escape (fins, legs), fight (claws, spines), or protect (shells), it is of little wonder that they have experienced both the selection pressure and time to evolve chemicals to enhance survival (Capon, 2010). These chemical adaptations generally take the form of structurally unique "marine natural product" (MNPs) that involve well known chemical classes as terpenoids, alkaloids, polypeptides, peptides, shikimic acid derivatives, sugars, steroids, and a multitude of mixed biogenesis metabolites (Firn and Jones, 2003). In addition, and unique to the marine environment, these metabolites possess a diverse mix of novel chemical features endowed with an amalgam of bioactivities such as anti-tumor, anti-microtubule, anti-proliferative, photoprotective, antibiotic and anti-infective (Vinothkumar and Parameswaran, 2013, Beesoo et al. 2014). So far 300 patents have been issued on marine natural products, many of which have shown potentials as pharmaceuticals, cosmetics, dietary supplements, and agrochemicals (Sukarmi and Sabdono, 2008). In view of the great chemical diversity, one is tempted to suggest that further marine bioprospecting may lead to novel therapeutics for the management of health diseases. Nevertheless, the marine habitat is still poorly explored. It is estimated that, in spite of 250 years of taxonomic classification and over 1.2 million species already catalogued in a central database, some 91% of species in the ocean still await description (Mora et al. 2011).

Although this "silent world" has a much richer biodiversity than that of the terrestrial areas, efforts to exploit marine organisms for drug discovery is still at a relatively early stage. Nevertheless, extensive research in the past 30-40 years on MNPs have been very productive and has led to the discovery of many potently active agents worthy of further clinical applications. Few marine plants, animals and microbes have already yielded more than 22,000 novel natural products (compared to 131,000 from terrestrial sources) (Shumacher et al. 2011). A large proportion of these natural products have been extracted from marine

invertebrates including cone snails, soft corals, sponges, sea squirts, marine worms, bryozoans, sea slugs and other marine organisms which not only produce a great number of MNPs currently known but also show the largest diversity of natural products (Bharate et al. 2013). Their low effective dosage, better selectivity against target malignant tissues and relative non-vulnerability to resistance development as compared to compounds of terrestrial origin, render them useful target molecules. According to US National Cancer Institute estimates, more than 1% show antitumour properties as against only 0.01% amongst their terrestrial counterparts (Beesoo et al. 2014).

A cursory review of the literature reveals that marine sponges (Porifera) are the richest sources of novel marine natural products. (Sipkema et al. 2005, Perdicaris et al. 2012, Leal et al. 2012, Mehbub et al. 2014). In 2012, Leal et al. (2012) compiled scientific studies about existing MNPs and classified them according to phyla of their invertebrate source. They found that approximately 80% were into the phyla Porifera (47.1%) and Cnidaria (33.5%). The rest of them were in the phyla Echinodermata (7.4%), Chordata (6.0%), and Mollusca (5.0%). This makes sponges the most prolific marine producers of compounds. Consequently, there is a renewed interest in exploring the marine sponges, with the aim of identifying novel chemical entities as sources for new lead compounds. This chapter gives a general overview of the global marine pharmaceutical pipeline drug, focusing particularly on some of the most interesting novel secondary metabolites/extracts having antioxidant, antitumour, anti-microbial, anti-viral, anti-coagulants and other bioactive properties identified and isolated from marine sponges, which could represent potential lead compounds for drug discovery. The factors that contribute to new discoveries and the challenges associated with translating sponge-derived compounds into clinical trials are also discussed. In addition, providing an outlook into the future, the advances that may further expand the promise of drug discovery from marine sponges are also examined.

3. MILESTONES IN RESEARCH ON MARINE NATURAL PRODUCTS

Throughout history, the use of marine organisms as drug source has played a minor part in the development of traditional medicines and is not as heavily documented as terrestrial organisms. While the terrestrial environment boasts a rich historical association with human therapeutics, evidence of MNPs derived therapeutical products is rather scarce. Historical records show that human beings have been aware of the venomous nature of some sea creatures for at least 4000 years (Colwell, 2002). The few historical references include the use of seaweed extracts by the San Blas Indians of Panama and ancient Chinese people for pain relief, abscesses and cancer. During the Roman times, physicians recommended the use of marine sponges against sunstrokes, wounds, fractures, infectious diseases and testicular tumors and even as implants after breast operations (Sipkema, 2004). Ancient maritime people, notably the Chinese and Japanese, used to eat a variety of iodine-rich seaweeds that undoubtedly accounted for their low incidence of goiter (Thakur et al. 2005). Coral reef products have also been traditionally used for treating various disorders in Taiwan, Japan, China and India. In the 19th and early 20th centuries, cod liver oil was used as a nutritional supplement (Colwell, 2002). However, only in the middle part of the 20th century did scientists begin to systematically probe the oceans for medicines. In the early 1950s, Ross

Nigreli of the Osborn Laboratories of the New York aquarium extracted a toxin from cuvierian organs of the Bahamian sea cucumber, *Actynopyga agassizi*. This toxin was named "Holothurin", and showed some antitumour activities in mice (Nigreli et al. 1967). These few anecdotal references aside, the marine world has been largely regarded as a source of food and a medium of transport, instead of a source of healing.

4. CURRENT MARINE PHARMACEUTICAL PIPELINE

The website "*The Global Marine Pharmaceuticals Pipeline*", maintained by Alejandro M. S. Mayer of Midwestern University, IL, USA, tracks marine-derived drugs and gives summarizing information about licensed drugs and those in clinical trials (Mayer, 2016). Presently, there are seven therapeutic agents (four anti-cancer, one anti-viral, one pain control, and one for hypertriglyceridemia) that derive from the marine environment (Figure 1). Of these, two are the actual chemical structure as isolated, and five others are synthetic agents that capture their chemical idea from a marine product. In addition, a further 13 agents are in phase I, II, or III clinical trials. Table 1 summarizes the collected sources of organisms that have yielded these agents and reveals that sponges, molluscs, and tunicates are the richest collected sources of bioactive MNPs. However, as noted above, the collected source has often times been shown or is strongly suspected of harboring or feeding upon microorganisms that are the actual producers of the bioactive agent.

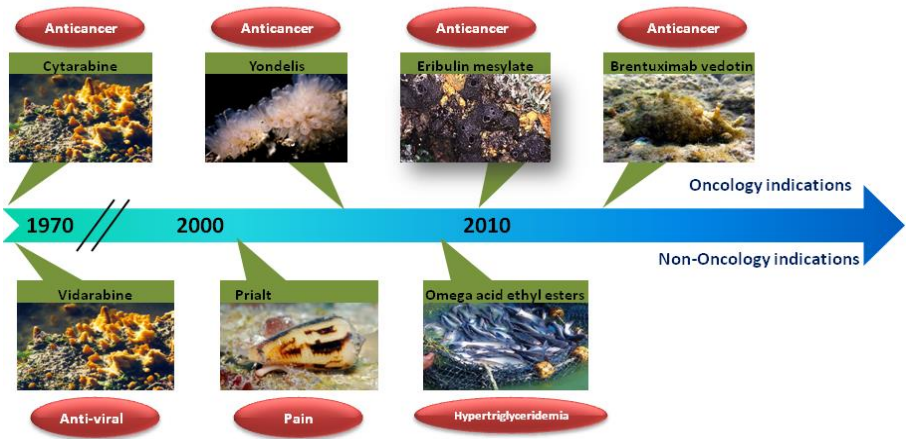

Figure 1. Clinical development timeline for marine drugs on the market.

An early finding in the marine drug discovery field was Cytarabine (Ara-C) and vidarabine (Ara-A), the first FDA-approved marine-derived drugs. These are synthetic pyrimidine and purine nucleosides, respectively, derived from nucleosides originally isolated from the Caribbean sponge *Cryptotheca crypta*. Cytarabine gained FDA approval in 1969 as an anticancer drug, whereas vidarabine was endorsed in 1976 as an anti-viral agent (Mayer et al. 2010) and is currently being used in the form of eye drops for the treatment of acute kerato conjunctivitis, recurrent epithelial keratitis caused by herpes simplex type 1 and 2. However, in the USA, its approval has been discontinued since June 2001 due to its restricted therapeutic window (Mayer et al. 2010, Anjum et al. 2016). Crucially, more than 40 years

after its approval, cytarabine, is still in the forefront of cancer drug treatment. By 2007, the global revenues of cytarabine and vidarabine were estimated at $93 M each (Martins et al. 2014). More than 20 years after the entry of cytarabine into the market, ziconotide (commercial name Prialt®) was granted FDA and EMEA approval in 2004 and 2005 respectively, for the management of severe chronic pain associated with cancer, AIDS and neuropathies. Ziconotide was synthesized in 1987 (Molinski et al. 2009) after its equivalent ω-conotoxin, a naturally occurring peptide, isolated from the venom of the marine gastropod *Conus magus.* In 2010 Prialt® sales reached $6.1 M (Elan, 2010) and its development prompted the investigation on other *Conus* peptides, several of them having reached human clinical trials (Olivera, 2006). On the other hand, Trabectedin (ET-743 Yondelis®), is the first marine anticancer agent to obtain approval by the European Union (EU) for the treatment of soft tissue sarcoma and relapsed ovarian cancer, and is currently awaiting Food and Drug Administration (FDA) approval. Trabectedin is a marine alkaloid isolated from the tunicate *Ecteinascidia turbinata* and was registered as anticancer agent in 2007 (Mayer et al. 2010, Beesoo et al. 2014, Beedessee et al. 2015). Eribulin mesylate (Halaven™) is the third anticancer agent from the marine biome, which gained FDA approval in November 2010 for metastatic breast cancer. It is a structurally simplified macrocyclic ketone analogue of the potent cytotoxic compound halichondrin B, which was initially isolated from the marine sponge *Halichondria okadai* in 1986 (Mayer et al. 2010, Beesoo et al. 2014, Beedessee et al. 2015). The most recent addition to the arsenal of approved anticancer agents from the marine environment is brentuximab vedotin (SGN-35), an antibody chimerized through the addition of a dipeptide This agent is used for the treatment of Hodgkin's lymphoma and anaplastic large cell lymphoma (Katz et al. 2011). This agent is used for the treatment of Hodgkin's lymphoma and anaplastic large cell lymphoma (Katz et al. 2011). On their way to the pharmacological market, there are 13 other marine-derived drugs in clinical trials including cytotoxic compounds such as hemiasterlins, plitidepsin, bryostatin 1, salinosporomide A, pseudopterosins, etc. In addition, there is a continuous supply of marine compounds in the preclinical pipeline, which is continuously feeding the global marine clinical pipeline (Beesoo et al. 2014). Table 1 highlights the evolution of this pipeline to date (Blunt et al. 2010, 2011, Mayer, 2016).

5. SPONGES (PORIFERA) AS A SOURCE OF DRUG TREASURE TROVE

Sponges (phylum Porifera) are primitive invertebrate metazoans that are found in all parts of the world's oceans. They have consistently been the richest source of new natural products reported in the marine natural products literature since the early 1970s, and the majority of the novel natural product chemotypes reported from sponges have no close analogs in terrestrial plant and microbial natural products or in natural products isolated from other marine organisms (Anderson, 2017). The biological effects of new metabolites from sponges have been reported in a number of scientific research papers. Highly cited reviews have been published in the last decades on the sponge-derived chemicals with bioactive properties which have the potential to be used for future pharmaceutical applications (Higa et al. 1994, Haefner, 2003, Sipkema et al. 2005). Scientists have been investigating the ecological role of sponge metabolites and the reasons behind sponges producing so many bioactive components that can be potentially useful to treat a great range of human diseases.

Table 1. Seven marine natural products and thirteen marine natural products inspired compounds that are FDA-approved agents or in clinical trial with details of their collected source, predicted biosynthetic source, molecular target, and disease treated (adapted from Mayer et al., 2016)

Clinical Status	Compound Name	Natural Product or Derivative	Collected source organism	Predicted Biosynthetic source	Biosynthetic Class of Agent	Molecular target	Disease area
FDA approved	Cytarabine (Ara-C)	Derivative	Sponge	Bacterium	Nucleoside	DNA polymerase	Cancer
	Vidarabine (Ara-A)	Derivative	Sponge	Bacterium	Nucleoside	Viral DNA polymerase I	Anti-viral
	Ziconotide	Natural Product	Cone snail	Mollusk	Cysteine knot peptide	N-Type Ca channel	Pain
	Eribulin mesylate (E789)	Derivative	Sponge	Bacterium	Complex polyketide	Microtubules	Cancer
	Omega 3 acid Ethyl esters	Derivative	Fish	Microalgae	Omega 3 fatty acids	Triglyceride synthesizing enzymes	Hypertrigly-ceridemia
	Trabectedin (ET-743) (EU registered only)	Natural product	Tunicate	Bacterium	NPRS derived alkaloid	Minor groove of DNA	Cancer
	Brentuximab vedotin	Derivative	Mollusk	Cyanobacterium	Antibody drug conjugate (MM auristatin E) Linear NRPS/PKS	CD30 and microtubules	Cancer
Phase III	Plitidepsin (Aplidine)	Natural product	Tunicate	Bacterium	Cyclic depsipeptide	Rac1 and JNK activation	Cancer
	DMXBA (GTS-21)	Derivative	Worm	Worm?	Alkaloid	α7 Nicotinic acetylcholine receptor	Cognition schizophrenia
	Plinabulin	Derivative	Fungus	Fungus	Diketo-piperazine	Microtubules and JNK stress protein	Cancer
	Tetrodoxin	Natural product	Putterfish	Putterfish	Guanidium alkaloid	Sodium channel	Pain
	Squalamide	Natural product	Dogfish shark	Dogfish shark	Aminosterol	Growth factors of neovas-cularisations	Neo-vascular diseases
Phase II	Elisidepsin	Derivative	Mollusk	Bacterium	Cyclic depsipeptide	Plasma membrane fluidity	Cancer
	Zalypsis (PM00104)	Derivative	Nudibranch	Bacterium	Alkaloid	DNA binding	Cancer
	Glembatumumabvedotin (CDX-011)	Derivative	Mollusk	Cyanobacterium	Antibody drug conjugate (MM auristatin E) Linear NRPS/PKS	Glycoprotein NMB & microtubules	Cancer

Table 1. (Continued)

Clinical Status	Compound Name	Natural Product or Derivative	Collected source organism	Predicted Biosynthetic source	Biosynthetic Class of Agent	Molecular target	Disease area
Phase I	Marizomib (Salinosporamide A; NPI-0052)	Natural product	Bacterium	Bacterium	NRPS with b-lactone & g-lactam	20S proteosome	Cancer
	Trabectedin analog (PM01183)	Derivate	Tunicate	Bacterium	NRPS Alkaloid	Minor groove of DNA, Nucleotide Excision Repair	Cancer
	SGS-75	Derivative	Mollusk	Cyanobacterium	Antibody drug conjugate (MM auristatin F) – Linear NRPS/PKS	CD70 and microtubules	Cancer
	SGS-75	Derivative	Mollusk	Cyanobacterium	Antibody drug conjugate (MM auristatin E-linear NRPS/PKS	ASG-5 and microtubule	Cancer
	Hemiasterlin derivative (E7974	Derivative	Sponge	Bacterium	Modified linear tripeptide (NRPS-PKS)	Microtubules	Cancer
	Bryostatin 1	Natural product	Bryozoan	Bacterium	Polyketide	Protein kinase C	Cancer Alzheimer's
	Pseudopterosins	Natural product	Soft coral	Bacterium	Diterpene glycoside	Eicosanoid metabolism	Wound healing

Additional perspectives on clinical trial agents that were derived or inspired by marine natural products can be found in Mayer et al. (2016).

Marine sponges are an important component of benthic communities throughout the world, regarding its biomass as well as their potential to influence benthic or pelagic processes (Higa et al. 1994, Haefner, 2003, Sipkema et al. 2005). They are among the oldest multi-cellular animals (Metazoa) and show relatively little differentiation and tissue coordination (Bergquist, 1978, Simpson, 1984, Leys and Meech, 2006). More than 8,000 species of sponges have been described; they inhabit a wide variety of marine and freshwater ecosystems and are found throughout tropical, temperate and Polar Regions (Proksch, 1994). They are sessile invertebrates with a wide variety of colours, shapes, and consistencies. Sponges consist of small pores, called ostia. Ostia draw water into them, and circulate it throughout their body by the action of cells called choanocytes. These choanocyte cells contain whiplike structures called flagella that move around and push water through the sponge. As water is drawn in and out, food and oxygen are brought to the sponge and waste and carbon dioxide is removed. The presence and abundance of spicules in marine sponge is variable: some species have dense or fused siliceous skeletons and thus a hard consistency, while other species have few or no spicules, thus lacking physical defenses (Figure 2) (Pawlik et al. 1995). Instead, they have evolved to develop chemical defenses against environmental stress factors such as predation, overgrowth by fouling organisms or competition for space (Rohde and Schupp, 2011, Pawlik, 2011). Studies showed that the highest concentration of toxic sponge metabolites are found in habitats such as coral reefs that are characterized by intense competition and feeding pressure from carnivorous fish. The adaptive significance of sponge's chemical constituents is derived from the observation that their chemical defences are highly effective against most species of fish, shell-less gastropods, and nudibranchs that feed on sponges (Perdicaris et al., 2013).

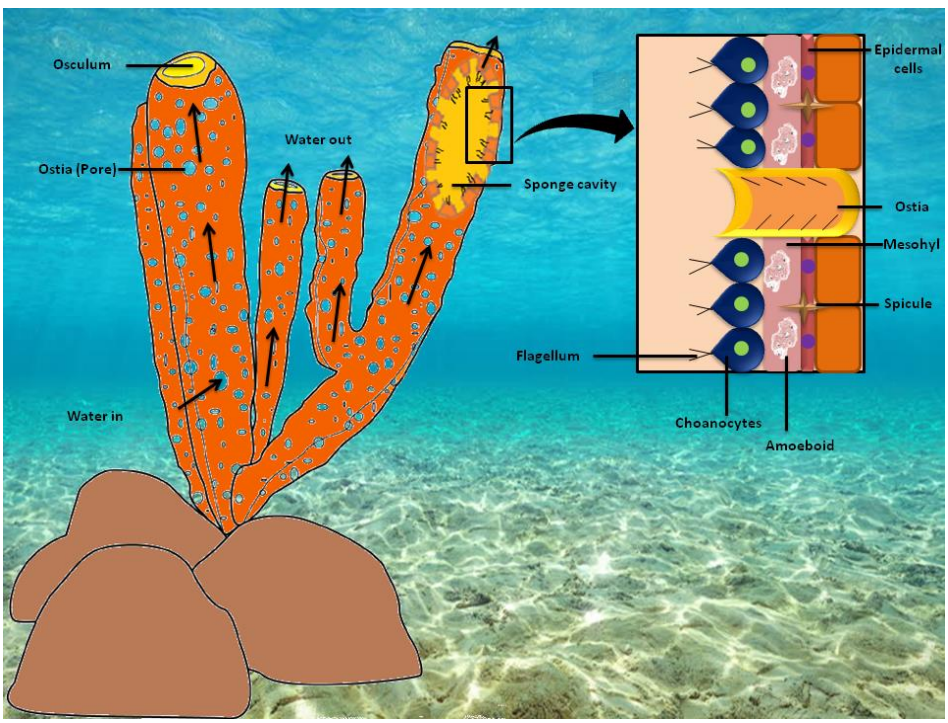

Figure 2. Sponge anatomy.

By producing different types of toxins or malevolent tastes and odors, sponges protect themselves against predators or inhibit coral overgrowth. In addition, sponges are one of the most efficient sessile filter feeders: they can filter up to 24 $m^3 \cdot kg^{-1}$ day^{-1} (Hentschel et al. 2002). Bacterial numbers in sponge tissue often exceed those of the surrounding seawater by two to three orders of magnitude as the sponge mesohyl provides a unique ecological niche for particular bacterial species. In many cases, sponge mesohyl harbours the bacterial symbionts (30%–60%) (Selvin et al. 2007). Bacteria provide their hosts with products of their metabolism, thereby granting the sponge access to bacteria-specific traits such as autotrophy, nitrogen fixation and nitrification (Wilkinson and Fay, 1979). Other examples show that sponge-associated bacteria can process metabolic waste compounds, stabilize the sponge skeleton and provide protection against UV radiation (Lee et al. 2001, Diaz and Rutzler, 2001, Thoms et al. 2003). The most prominent example of sponge bacterial symbiosis, however, is the involvement of bacteria in the production of bioactive metabolites (Thoms and Schupp, 2005) that have a role in defense (Proksch, 1994). Many of these compounds are very potent because the diluting effect of the ocean drives the construction of molecules that are highly active and stable in saline conditions (Abad et al. 2011).

These highly intensive, constant interactions with the environment have given sponges a unique biochemistry to produce a high diversity of metabolites that can either help them survive or prompt them to evolve. In an ecological context, sponges have developed special mechanisms to protect themselves from pathogenic bacteria, viruses, parasites, fungus and other predators that include both chemical defense mechanisms and physiological responses. Chemical defense mechanisms help to protect sponges against certain deleterious bacteria (Paul et al. 2001, Mahon et al. 2003, Paul et al. 2006). In this way, sponges provide novel leads against viral, fungal and parasitic diseases (Unson et al. 1994).

6. MARINE SPONGES AND NATURAL CHEMICAL COMPONENTS

Among all the marine organisms investigated, marine sponges (Porifera) are recognized as the richest sources of novel MNPs, with about 4851 compounds to date, contributing to nearly 30% of all marine natural products discovered so far. It should be noted that of these, 1499 new compounds were isolated in the five years from 2008 to 2012 (Blunt et al. 2010, 2011, 2012, 2013, 2014). This makes sponges the most prolific marine producers of compounds with more than 200 new compounds reported each year for the last decade (Laport et al. 2009). The chemical diversity of sponge natural products is remarkable, including unusual nucleosides, bioactive terpenes, sterols, cyclic peptides, alkaloids, fatty acids, peroxides, and amino acid derivatives (which are frequently halogenated) (Blunt et al. 2008). With this myriad of MNP available, numerous studies have revealed a broad spectrum of biological activities for these compounds, including anti-cancer, anti-viral, anti-bacterial, anti-fungal, anti-protozoal, anthelmintic, anti-inflammatory, immunosuppressive, neuro-suppressive, neuro-protective, antifouling and a range of other bioactivities (Blunt et al. 2005). Studies showed that different components affect the targeted disease by different mechanisms (e.g., microtubule stabilization or interaction with DNA to combat tumors). Natural chemical products that can act as inhibitors of transcription factors may be effective against both malignant neoplasms and viral diseases. Most bioactive metabolites from

sponges proved to be inhibitors of certain enzymes, which often mediate or produce mediators of intracellular or intercellular messengers involved in the pathogenesis of a disease (Sagar et al. 2010, Villa and Gerwick, 2010, Frota et al. 2012). Therefore, bioactive natural products isolated from marine sponge extracts have a solid track record as inspiration for drug discovery and the drug lead potential of this largely unexplored chemical diversity pool has been the primary motivation for our ongoing focus on sponge natural products.

7. PHARMACOLOGICAL POTENCY OF MARINE SPONGES

The scientific literature records numerous examples of bioactive extracts/compounds from marine sponges that were assessed for their anti-oxidant anti-inflammatory, anti-tumour, immune-suppressive, neuro-suppressive, anti-microbial and anti-viral properties. Therefore, this section focuses on the discoveries of sponge derived natural products which displayed interesting pharmacological potential both *in vitro* and *in vivo* as well as their underlying mechanism of action by which they interpose at different points during human pathogenesis. Our objectives are to highlight the compounds by disease type, their mode of action and the greatest potential to drive towards the development of clinically useful drugs.

7.1. Anti-Oxidant Activity

Antioxidants play an important role in the protection of human body against damage by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Govindarajen et al. 2005). Antioxidant system includes enzymatic and non-enzymatic components. Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are the major antioxidant enzymes whereas non antioxidant antioxidants consist of endogenous components (uric acid, reduced glutathione and albumin) in addition to dietary antioxidants such as carotenoids, flavonoids, ascorbic acid and α tocopherol (Rietveld and Wiseman, 2003). Highly reactive free radicals and oxygen species including singlet oxygen, hydrogen peroxide, superoxide, nitric oxide anion radical and hydroxyl radical are present in biological systems from a wide variety of reactions. These free radicals are known to oxidize nucleic acids, proteins, lipids, or DNA resulting in oxidative stress, a condition implicated in the pathogenesis of various diseases such as atherosclerosis, ischemic heart disease, cancer, Alzheimer's disease, Parkinson's disease and in the aging process (Shahbudin et al. 2011). Therefore, exogenous antioxidants may have positive effects in the removal or suppression of the generation of ROS/RNS. Based on that, many studies have been driven towards the assessment of the antioxidant properties of natural products through both chemical (*in vitro*) and biological (*in vivo*) methods.

Despite the large number of antioxidants revealed, the search for new chemical entities with antioxidant activity from the marine biome still remains a burgeoning field. Marine organisms particularly micro/macro algae have been widely studied as rich sources of polyphenols, astaxanthin, fucoxanthin, phlorotannins and fucoidans with antioxidant potentials (Lordan et al. 2011). While marine antioxidants are primarily from algae, very few studies have focused on marine animals. Similar to various algal species, marine sponges

particularly those inhabiting the intertidal zones are exposed to stressful vagaries of nature. To fight the stress posed by the high level of ROS, which is triggered through a combination of photosynthesis, symbiont oxygen production, intense light intensities and UV radiation, marine sponges are known to produce numerous antioxidant compounds (Brouwer et al. 2004, Almeida et al. 2005, Jiang et al. 2009). As such, microorganisms living as exobionts and endobionts in the tissue of such organisms are implicated in combating the oxidative stress that causes damage to host cells and tissue (Velho-Pereira et al. 2015).

In recent years, the antioxidant activity of marine sponge derived extracts has garnered enormous attention in food industries as they can be used as functional ingredients in food formulations to promote consumer health and improve shelf life of food products. A number of novel metabolites derived from marine sponges, such as indole derivatives, aromatic alkaloids, aromatic polyketides, and phenolic compounds have exhibited strong antioxidant potential compared to vitamin E and ascorbic acid (Kim et al. 2012). Aromatic polyketides isolated from the marine sponge-derived fungus *Aspergillus versicolor* have shown significantly higher antioxidant capacity than that of butylated hydroxytoluene (BHT) (Li et al. 2009). Another scientific study reported that marine sponge-derived yeasts are capable of producing anti-oxidative indole derivatives which can scavenge free radicals and ROS. These findings were very attractive since these sponge microbes are culturable, and there is a possibility of reproducing the compound in large scale (Sugiyama et al. 2009). Extracts from the sponge *Smenospongia* sp, and *Stylissa* sp collected from Red Sea at Egyptian coasts also showed appreciable radical scavenging activity against DPPH and INOS radicals. Subsequent chemical characterizations of these sponge extracts revealed the presence of numerous antioxidative compounds including di-isobutyl phthalate, di-n-butyl phthalate, linoleic acid, β-sitosterol, and cholesterol from the *Smenospongia* sp while *Stylissa* sp. produced bis-[2-ethyl]-hexyl-phthylester and triglyceride fatty acid ester (Shabaan et al. 2012). Furthermore, an *ex vivo* study on albino rats revealed that phenolic rich ethyl acetate extracts from the sponges *Rhabdastrella globostellata* and *Spirastrella inconstans* increased the level of almost all antioxidant related enzymes including glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase, accelerating the removal of ROS (Chairman et al. 2012). Hence, these data argue that the marine fauna can be important sources of anti-oxidative agents with potential application as pharmaceutical or nutraceutical agents for prevention and control of degenerative diseases.

7.2. Anti-Bacterial Activity

The optimism of the 1950s and 1960s of a world without infections is gradually being replaced by an era of pessimism characterized by widespread emergence of anti-bacterial resistance (Raghunath, 2008). Even today, infectious diseases including HIV/AIDS, tuberculosis, and malaria continue to elude the prospect of morbidity and mortality worldwide killing around 700, 000 people annually (O'Neill, 2016). Of all the major infectious diseases, bacterial infections present a major threat to human health. Tuberculosis caused by the bacteria *Mycobacterium tuberculosis*, for instance, ranks among the world's leading causes of death (Mohajan, 2015). *Streptococcus* (Group B *Streptococcus*), another bacterium, continues to be a frequent cause of life-threatening infection during the first three months of life (Edwards and Baker, 2001). Food borne and waterborne bacteria such as *Salmonella* and

Campylobacter are responsible for the increase in diarrheal disease (Fletcher et al. 2013). Meanwhile, during the last decade, scientists discovered many new organisms and new strains of many familiar bacteria, such as *Escherichia coli*. Diseases from these emerging bacterial strains present a clear challenge to biomedical researchers. The complexity of this challenge is becoming even clearer as researchers begin to appreciate the many mechanisms associated with antibiotic resistance (NIAD, 2012). Resistance is often a result of expression of efflux pumps, modification of drug targets, modulation of transcription factors, and biofilm formation. Moreover, gene transfer among different strains of bacteria, and even between different species of bacteria, is now understood to be a common means whereby these organisms acquire resistance to antibiotics (Laport, 2009).

The importance of drug-resistant bacterial infection has produced an imperative requirement for the quick and sustained development of new antibiotics classes, which may keep pace with the varying face of bacterial antibiotic vulnerability. Continuous development of new antimicrobial drugs with multiple targets and potentials is expected to efficiently combat Multi Drug Resistant (MDR) microorganisms (Anjum et al. 2016). Several studies have focused on the mechanisms by which natural compounds, which attack the microorganisms' cell walls and cell membranes, resulting in release of various intracellular constituents (example; ribose and Na glutamate) (Sabir, 2007, Hasan, 2012, Rahman, 2014). Another mode of action is by interfering with the electron transport, nutrient uptake, protein, nucleic acid synthesis and enzyme activity (Bajpai et al. 2007) (Figure 3). Synergism of drug therapy and natural products may also give optimum results in the fight against existing and emerging infectious diseases. Regarding the application of natural products on bacterial infections, besides their direct antimicrobial activity, natural extracts have also been studied as antimicrobial drug resistance modifiers (Beesoo et al. 2017).

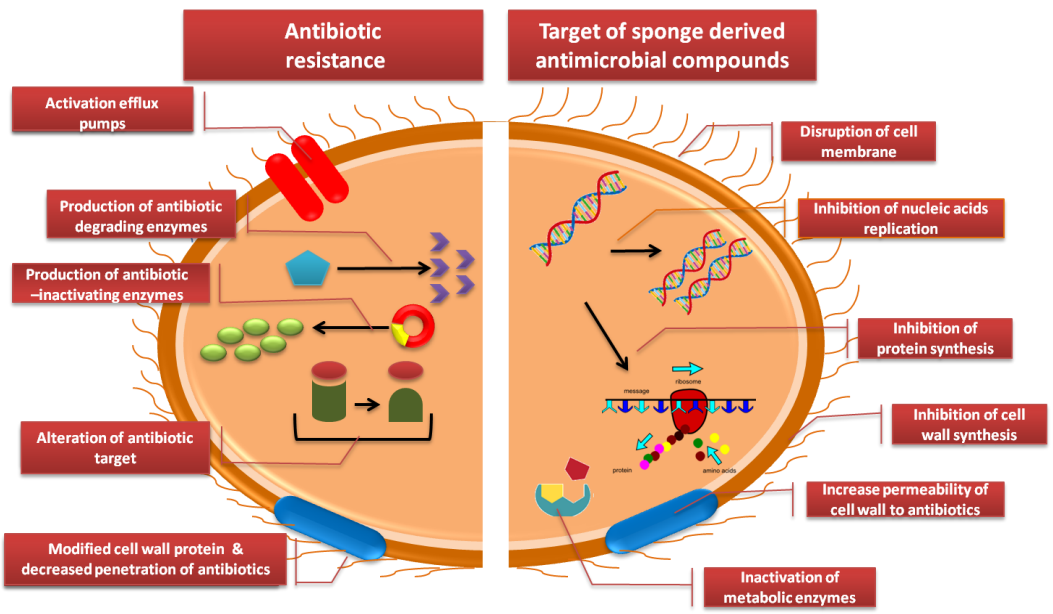

Figure 3. General mechanisms of antibiotic resistance and target of sponge derived anti-bacterial compounds.

Table 2. Selected examples of antimicrobial compounds isolated from marine sponges

Compound	Class	Species	Action spectrum	Reference
Anti-bacterial activity				
Axinellamines B–D	Alkaloids	*Axinella* sp/*Halichondria* sp	*H. pylori*	Urban et al. 1999
Petrosamine B	Alkaloids	*Oceania* sp	*H. pylori*	Carroll et al. 2005
6-hydroxymanzamine E	Alkaloids	*Acanthostrongylophora* sp	*M. tuberculosis*	Rao et al. 2004
Arenosclerins A–C	Alkaloids	*Arenosclera brasiliensis*	*S. aureus* (MRSA strain), *P.aeruginosa* (antibiotic resistant strain)	Torres et al. 2002
Agelasine D	Purine derivative	*Agelas* sp	Gram-positive and Gram-negative bacteria, *M. tuberculosis*	Vik et al. 2006
Latrunculins	Macrolides	*Negombata magnifica*	*S. aureus* and *B. cereus*	El Sayed et al. 2008
Psammaplin A	Bromotyrosine-derived	*Psammaplysilla* sp	*S. aureus* (MRSA strain)	Nicolaou et al.,2001
Topsentiasterol sulfates A-E	Sulphated sterol	*Topsentia* sp	*E. coli* and *P. aeruginosa*	Fusetani et al. 1994
Cribrostatin 3	Alkaloid	*Cribrochalina* sp	*N. gonorrheae* (antibiotic-resistant strain)	Pettit et al. 2000
(S)-(+)-curcuphenol	Sesquiterpene	*Myrmekioderma styx*	*M. tuberculosis*	Gul et al. 2007
Manoalide	sesterterpenoid	*Luffariella variabilis*	*S. aureus*	Da Silva and Scheuer, 1980
Exiguaquinol	Pentacyclic hydroquinone	*Neopetrosia exigua*	*Helicobacter pylori*	Leone et al. 2008
Fulvynes A-I	Polyoxygenated acetylene	*Haliclona fulva*	Chloramphenicol resistant *Bacillus subtilis*	Nuzzo et al. 2012
Manadodioxan E	Polyketide endoperoxide	*Plakortis bergquistae*	*Bacillus cereus*	Gushiken et al. 2015
Gelliusterol E	Steroid	*Callyspongia aff. implexa*	*Chlamydia trachomatis*	Abdelmohsen et al. 2015
Dysidavarone A	Sesquiterpene quinone	*Dysidea avara*	*Methicillin-resistant S. aureus*	Schmalzbauer et al. 2013
Axistatins	Pyrimidine diterpenes	*Agelas axifera*	*N. gonorrhoeae*	Pettit et al. 2013
CvL	Lectin	*Cliona varians*	*B. subtilis, S. aureus*	Moura et al. 2006
Jasplakinolide	Macrocyclic depsipeptide	*Jaspis* sp	*C. albicans*	Crews et al. 1986, Zabriskie et al. 1986
Callipeltins J, K	Peptides	*Latrunculia* sp	*C. albicans*	Abad et al. 2008, Grkovic et al. 2014
YM 202204	Lacotone	*Phoma sp associated fungus*	*C. albicans*	Nagai et al. 2002
Theonellamide G	Bicyclic glycopeptide	*Theonella swinhoei*	Amphotericin-B-resistant strains of *C. albicans*	Youssef et al. 2014
Ptilomycalin A	Polycyclic guanidine alkaloid	*Ptilocaulis spiculifer*	*C. albicans*	Dalisay et al. 2011
Fascaplysin	Bis (indole) alkaloid	*Fascaplysinopsis sp*	*S. cerevisiae*	Mcinnes, 2008
Haliclona diamine	Alkaloid	Haliclona sp.	*C. albicans*	Wattanadilok et al. 2007
Aurantoside	Polyketide	*Silquariaspongia japonica*	A. fumigates	Donia and Hamann, 2003

Compound	Class	Species	Action spectrum	Reference
Anti-bacterial activity				
Manzamine A	Alkaloid	*Acanthostrongylophora sp*	*C. neoformans*	Yousaf et al. 2004
Agelasines & agelasimines	Purine derivatives	*Agelas* sp	*C. krusei*	Vik et el. 2007
Axistatins	Pyrimidine diterpenes	*Agelas axifera*	*C. neoformans*	Pettit et al. 2013
Eurysterols A-B	Sterols	*Euryspongia* sp	*C. albicans*	Boonlarppradap et al. 2007
Naamine A	Alkaloid	*Leucetta cf. chagosensis*	*C. neoformans*	Dunbar et al. 2000
Secomanoalide	Sesterterpenoid	*Luffariella variabilis*	*C. glabrata*, *C. krusei* and *C. albicans*	Zhou et al. 2006
Discobahamin A-B	Peptides	*Discodermia* sp	*C. albicans*	Gunasekera et al. 1994
Anti-viral activity				
Ara-A	Nucleoside	*Cryptotethya crypta*	HSV-1, HSV-2, VZV	Bergman and Feeney, 1951
Avarol	Sesquiterpene hydroquinone	*Dysidea avara*	HIV-1	Muller et al. 1987
Papuamides A–D	Cyclic depsipeptides	*Theonella* sp.	HIV-1	Ford et al. 1999
Manzamines	Alkaloids	*Indo pacific sponge*	HIV-1	Yousaf et al. 2004
Haplosamates A and B	Sulfamated steroid	*Xestospongia sp/Haplosclerida sp*	HIV-1	Qureshi and Faulkner, 1999
Dragmacidin F	Alkaloid	*Halicortex* sp	HSV-1	Cutignano et al. 2000
Mycalamide A-B	Nucleosides	*Mycale* sp.	A59 coronavirus, HSV-1	Perry et al. 1990
4-methylaaptamine	Alkaloid	*Aaptos aaptos*	HSV-1	Souza et al. 2007
Variolin B	Pyridopyrrolopyramidine alkaloid	*Kirckpatrickia varialosa*	HSV-1	Perry et al. 1994
Anti-malarial activity				
Manzamine A, 8-hydroxy manzamine	Alkaloid	*Haliclona sp* *Cymbastela hooperi* *Diacarnus levii*	*P. falciparum*	Ang et al. 2001, Konig et al. 1996, D'Ambrosio et al. 1998
Kalihinol A	Isonitril containing kalinane diterpenoid	*Acanthella sp*	*P. falciparum*	Miyaoka et al. 1998
Axinonitrile 3	Sesquiterpenoid isocyanide	*Acanthela klethra*	*P. falciparum*	Angerhofer et al. 1992
Tsitsikammamine C	Bispyrroloiminoquinone alkaloids	*Zyzzya* sp	*Chloroquinone sensitive and resistant P. falciparum*	Davis et al. 2012

Contributing to the global search for new antimicrobials to combat antibiotic-resistant strains of pathogenic bacteria, marine ecological niches have been described recently as "particularly promising" (Fischbach and Walsh, 2009). Many new molecules with antibiotic properties are discovered every year, but in marine sponges their ubiquity is remarkable. Their complexity enables them to interact with multiple molecular targets thus conveying accrued difficulty for target microorganisms to develop resistance due to multiple response sites (Lagunin et al. 2016). So far a medley of studies on screenings of marine sponges for anti-bacterial activity has led to the isolation and characterization of a wide range of active compounds, including some promising therapeutic leads (Munro et al. 1999, Berlinck et al. 2004, Mayer and Hanann, 2004, Sipkema et al. 2005). An early preliminary anti-bacterial screening by Marihno and his group revealed that 10 out of 12 sponge species screened exhibited anti-bacterial activity with a large action spectrum over Gram-positive *cocci* (Marinho et al. 2006). This is in congruence with most of the available reports on anti-bacterial property of sponges that revealed that Gram-positive bacteria are more susceptible to sponge extracts as compared to Gram negative ones (Liu et al. 2004). For example, extracts derived from 28 marine sponge species collected along the French coast had high anti-bacterial activity against Gram positive bacteria (77%) than Gram negative bacteria (53%) (Lippert et al. 2003). Similarly, McCaffrey and Endean (1985) also showed that Gram-positive bacteria were more sensitive to sponge extracts of subtropical and tropical species than Gram-negative.

The importance of marine sponges as a source of novel alkaloids, in particular those with anti-bacterial activity, has largely been recognized. The axinellamines B-D from an Australian marine sponge, *Axinella* sp, had bactericidal activity against *Helicobacter pylori*, a Gram-negative bacterium associated with peptic ulcer and gastric cancer (Urban et al., 1999). Another compound active against *H. pylori*, petrosamine B, an inhibitor of the enzyme aspartyl semialdehyde dehydrogenase, was isolated from an *Oceanapia* species (Gulf of Carpentaria, Australia) (Caroll et al. 2005). In bacteria, this enzyme is involved in the production of 25% of all amino acid residues that are required for protein synthesis (Hadfield et al., 2005). Inhibition of enzyme aspartyl semialdehyde dehydrogenase is thus considered to be an important target for the development of anti-bacterial agents. Noteworthy were reports of three novel manzamine-type alkaloids, 12,3,4-oxamanzamine E, 8-hydroxymanzamine J, and 6-hydroxymanzamine E, which were isolated from an *Acanthostrongylophora* species from Indonesia. All three manzamines were active against *Mycobacterium tuberculosis* with minimum inhibitory concentration (MIC) < 12.5 μg/ml, with 6- hydroxymanzamine E having the most potent antituberculosis activity (MIC = 0.4 μg/ml) (Rao et al. 2004). The added value of some new sponge-derived antibiotics was also shown by the inhibitory effect of arenosclerins A–C from *Arenosclera brasiliensis* on 12 antibiotic- resistant bacteria isolated from a hospital (Torres et al. 2002). Likewise, Vik and colleagues (2006) also have shown that purine derivatives including Agelasine D isolated from marine sponges of the genus *Agelas* display a broad spectrum of anti-bacterial activities including effect on *M. tuberculosis*, Gram-positive and Gram-negative bacteria. Leone et al. (2008) isolated exiguaquinol from the sponge *N. exigua* which inhibited the bacterial enzymes *Helicobacter pylori* (Glutamate racemase that inter converts L- and D- glucose) needed for the construction of bacterial cell walls. Renieramycin J, tetrahydroisoquinoline alkaloid and araguspongine M, both of which showed strong anti-fungal activities were also isolated from the same species from Japanese waters. A *Synechococcus*-like cyanobacterium has been detected in the tissue

of *Neopetrosia exigua* by visible and fluorescent microscopic observations (Oku et al. 2003). Hence these compounds or their precursors may be produced by these symbiotic microorganisms. It has been widely reported that synergism of drug therapy and natural products may also give optimum results in the fight against existing and emerging infectious diseases. Recently our group showed that ethyl acetate fraction from the Mauritian *N. exigua* displayed interesting antibiotic potentiating activity against a panel of Gram positive and Gram negative bacterial isolates (Beesoo et al. 2017). Till date marine sponges have produced up to 800 antibiotic substances (Torres et al. 2002) but no sponge derived anti-bacterial product has yet entered the global pharmacological market. However, many of them are currently under preclinical investigation as potential anti-bacterial therapeutics (Table 2).

7.3. Anti-Fungal Activity

The incidence of fungal infections has increased rapidly over the past decades, in part as a result of increasing number of immunocompromised patients and invasive procedures (Sydnor and Perl, 2011). Several fungal species that often cause human infections include *Candida albicans*, *Candida glabrata*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (Shapiro et al. 2011). Fungicides that are currently used are less diverse than antimicrobials, and the use of many of them is restricted because of toxic effects to humans, animals, and plants (Sipkema et al. 2005). Therefore, the lack of some new anti-fungal agents and the emergence of resistance to anti-fungal therapy, leads to greater research and interest of optimal and new anti-fungal agents. Screening for anti-fungals is often focused on finding compounds active against *Candida albicans*, the prominent agent for candidiasis. Invasive candidiasis is accounted as the most common nosocomial fungal infection resulting in an average mortality rate between 25%–38% (Dimopoulos et al. 2008).

Jasplakinolide is the first example of cyclodepsipeptide 19-membered macrocyclic depsipeptide isolated from the sponge *Jaspis* sp which has a selective *in vitro* anti-fungal activity with MIC of 25 μg/ml against *C. albicans* while *in vivo* topical activity of a 2% solution against *Candida* vaginal infection in mice (Crews et al. 1986, Zabriskie et al. 1986). Callipeltins are another group of anti-fungal peptides isolated from the marine sponge *Latrunculia* sp. collected off Vanuatu islands. Callipeltins (F-I) inhibit the growth of *C. albicans*, with MICs of 0.1-0.15 μg/ml (Sepe et al. 2006). The peptides callipeltin K (Grkovic et al., 2014) and J (Abad et al. 2008), which were isolated from the sponge *Latrunculia* sp., from Emae, Vanuatu, South Pacific, inhibited the growth of *C. albicans* (ATCC 24433), with MIC values of 0.15 and 0.07 μg/ml (D'Auria et al. 2007). Anti-fungal activity was also detected from the sponge-associated fungus *Phoma* sp. Q60596. The sponge-derived fungus produced a new lactone compound, YM-202204 (Nagai et al. 2002), which was effective against *C. albicans* (MIC: 6.25 μg/ml), along with *Cryptococcus neoformans* (MIC: 1.56 μg/ml), *Saccharomyces cerevisiae* (MIC: 1.56 μg/ml) and *Aspergillus fumigatus* (MIC: 12.5 μg/ml). Furthermore, Nagai et al. (2002) showed that YM-202204 was able to block the glycophosphatidylinositol (GPI) anchor, an important structure for protein attachment in the membrane of eukaryotic cells and one of the targets in developing anti-fungal drugs (McLellan et al. 2012, Butts et al., 2012). Theonellamide G is a bicyclic glycopeptide isolated from the polar fraction of the organic extract of the Red Sea sponge *Theonella swinhoei*. The latter exhibited anti-fungal activity against wild and

amphotericin-B-resistant strains of *C. albicans*, with MIC values of 7.84 and 3.49 μg/ml, respectively (Youssef et al. 2014). Nishimura and his colleagues previously revealed that the anti-fungal theonellamides represent a new class of sterol-binding molecules that induce membrane damage and activate Rho1-mediated 1,3-*β*-*d*-glucan synthesis (Nishimura et al. 2010). As part of a continuing investigation of new manzamine-related alkaloids with activity against infectious disease, new manzamine homologues were isolated and characterized from *Acanthostrongylophora* sp. The manzamines: neokauluamine, manzamine A, 8-hydroxymanzamine A, 6-deoxymanzamine and manzamine F showed potent activity against *C. neoformans*. Oral and intravenous pharmacokinetic studies of manzamine A in rats indicated that the substance has low metabolic clearance, a reasonably long pharmacokinetic half-life, and good absolute oral bioavailability of 20.6%, which supports the value of this substance as a potential lead for further preclinical assessment and possible drug development (Yousaf et al. 2004). Many sponge derived anti-fungals are currently being investigated for further clinical trials and drug development (Table 2). However, most of them have proved to be cytotoxic. Consequently, they have not been considered promising anti-fungal agents for clinical development. Therefore, in many cases, the assessment of whether anti-fungal activity outweighs cytotoxic effects is required, followed by rational modifications to improve the therapeutic index for these molecules (Donia and Hamann, 2003).

7.4. Anti-Viral Activity

Viral infections play an important role in human diseases, and recent outbreaks in the advent of globalization and ease of travel have underscored their prevention as a critical issue in safeguarding public health. Several hard-to-cure diseases and complex syndromes including Alzheimer's disease, type 1 diabetes, and hepatocellular carcinoma have been associated with viral infections (Hober et al. 2012, Ball et al. 2013, Morgan et al. 2013). Despite the progress made in immunization and drug development, many viruses lack preventive vaccines and efficient anti-viral therapies, which are often beset by the generation of viral escape mutants. Examples include the recent emergence of dengue virus, influenza virus, measles virus, severe acute respiratory syndrome (SARS) virus, and West Nile virus outbreaks (Christou, 2011, Cascio et al. 2011, Grais et al. 2011). Thus, identifying novel anti-viral drugs is of critical importance and natural products are an excellent source for such discoveries.

The officially clinically approved anti-viral drug armamentarium contains approximately 40 lead compounds (Da Silva et al. 2006). Some new approaches are being employed to introduce novel anti-viral agents from the marine biome. In an overview of 132 natural products from marine sources obtained during the period 2002-2011, which exhibited anti-HIV activity, it was reported that sponges contributed more than half of all anti-HIV natural products from marine organisms (Zhou et al. 2013). Among the most interesting anti-viral sponge metabolites, manzamines (Yousaf et al. 2004) Papuamides C and D (Ford et al. 1999), haplosamates A and B (Qureshi and Faulkner, 1999), and avarol (Muller et al. 1987) represent promising potential and future anti-viral drugs. Avarol, isolated from sponge *Dysidea avara* is one of the few compounds for which the mechanism by which it inhibits progression of HIV infection has been elucidated to quite some extent. *In vitro* and *in vivo* experiments show that it increases humoral immune response (Muller et al. 1987).

Mechanistically, avarol inhibits HIV by (almost completely) blocking the synthesis of the natural UAG suppressor glutamine transfer tRNA. Synthesis of this tRNA is upregulated after viral infection, and it is important for the synthesis of a viral protease, which is necessary for viral proliferation (Muller and Schroder, 1991). Moreover, the derivatives of avarol such as 6′-hydroxy avarol and 3′-hydroxy avarone were noted as very strong inhibitors of HIV reverse transcriptase. This enzyme has a key role in the early stages of HIV infection and is a specific target for anti-viral drugs (Loya and Hizi, 1990). Manzamine A, 8-hydroxymanzamine A, 6-deoxymanzamine X, and neokauluamine are manzamine-related alkaloids isolated from an Indo-Pacific sponge. These bioactive agents showed significant anti-HIV-1 activity with EC_{50} of 4.2, 0.59, 1.6, and 2.3 M, respectively (Yousaf et al. 2004). Several of these compounds have a great potential for drug development (Table 2). Among these compounds, preclinical assessments were started for avarol and manzamine A. Overall, the most important anti-viral discovery from marine source reported so far is the nucleoside Ara A (vidarabine) which was originally isolated from *Cryptotethya crypta* sponge and first synthesized in 1960. Adenine arabinoside is rapidly converted into adenine arabinoside triphosphate, which inhibits viral DNA polymerase and DNA synthesis of herpes, vaccinia and varicella zoster viruses. Vidarabine (Vira-A) received FDA approval in 1976, however, its marketing status is currently listed as discontinued by the FDA in the US market which was possibly associated with the lower therapeutic window of vidarabine relative to newer anti-viral compounds currently on the market (Mayer et al. 2010).

7.5. Anti-Malarial Activity

Malaria is one of the most prevalent parasitic diseases in the world. According to the report, there were 212 million new cases of malaria worldwide in 2015 (range 148–304 million). The WHO African Region accounted for most global cases of malaria (90%), followed by the South-East Asia Region (7%) and the Eastern Mediterranean Region (2%) (WHO, 2015). The disease is caused by several protozoans belonging to the genus *Plasmodium* (*P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae*), with *P. falciparum* being the parasite responsible for most severe diseases and most fatal cases. The global importance of this disease, current limitations of vector control and increasing resistance among *Plasmodium* strains towards anti-malarial drugs such as chloroquinone, pyrimethamine, and sulfadoxine, makes the development of anti-malarial therapeutics the main strategy of malaria control (Shrikant and Bhimanna, 2016).

During last decades, a few anti-malarial compounds have been isolated from sponges (Table 2). Kalihinol A from *Acanthella* sp. (Miyaoka et al. 1998) and a number of terpenoid isocyanates, isothiocyanates, and isonitriles from *Cymbastela hooperi* (Konig et al. 1996) display selective *in vitro* anti-malarial activity against *P. falciparum*. Also a number of free carboxylic acids from *Diacarnus levii* were used as precursors to yield new cyclic norditerpene peroxides after esterification. These epidioxy-substituted norditerpenes and norsesterterpenes displayed selective activity against both chloroquine-sensitive and chloroquine-resistant *P. falciparum* strains (D'Ambrosio et al. 1998). The manzamines, which are the most promising marine anti-malarial compound, have been discovered in a number of sponges (Sakai et al. 1986, Yousaf et al. 2002). Manzamine A, and its 8-hydoxy derivative, were found to potently inhibit the growth of *P. falciparum* both *in vitro* (IC_{50} = 5.0 ng/ml) and

in vivo (a parasitemia suppression of the same order of magnitude of that of artemisinin). It has been suggested that the anti-malarial effect of manzamine A is due to an enhanced immune response. Unfortunately, its therapeutic index is somewhat narrow (gastrointestinal distress) and further studies are needed to improve this value.

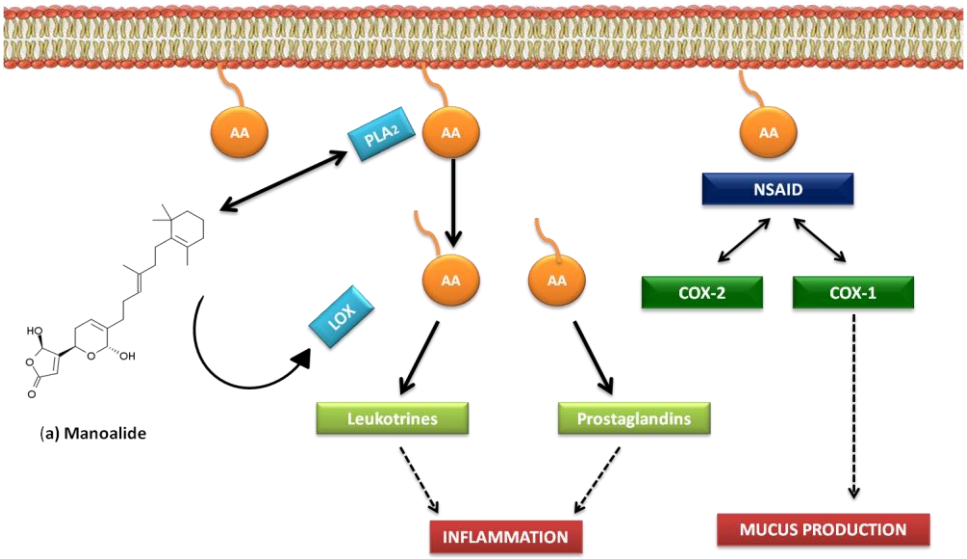

Figure. 4. Diagrammatic process of inflammatory cascade inside the cell. Phospholipase A2 (PLA2) catalyzes the release of membrane-bound arachidonic acid (AA) to free arachidonic acid. Arachidonic acid is then converted to leukotrienes and prostaglandins by lipoxygenase (LOX) and cyclooxygenase-2 (COX-2), respectively. Sponge derived anti-inflammatory compounds such as manoalide (a) are mainly inhibitors of PLA2 or LOX, while nonsteroidal anti-inflammatory drugs (NSAID) inhibit COX-2, but also the constitutive COX-1 (Adapted from Sipkema et al. 2005).

7.6. Marine Invertebrates with Anti-Inflammatory Properties

Inflammation is responsible for many widespread diseases including rheumatoid arthritis, osteoarthritis, atherosclerosis, diabetes, neuro-degeneration, allergy, infection and cancer. Multiple signaling pathways form a network of pro-inflammatory, immune-modulatory and pro-resolving cascades, which define the physiological and pathophysiological aspects of inflammation. Hence, it becomes evident that, an interference with multiple targets is superior to targeting a single key factor regarding drug efficiency, side-effects and adverse compensatory mechanisms involved in inflammation related diseases (Koeberlie and Andwerz, 2014). Accumulating epidemiological and clinical study indicates that various bioactive MNPs suppress pro-inflammatory pathways by inhibiting the expression of pro-inflammatory cytokines (tumor necrosis factor-α (TNF-α), intercellular adhesion molecule expression and pro-inflammatory mediators (inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2), master transcription factors (nuclear factor-κB), reactive oxygen species (ROS) and by improving the antioxidant activity (Pan, 2011, Debnath, 2013, Senthikumar and Kim, 2013).

Manoalide (Figure 4(a)), previously described as an important antimicrobial agent (Da Silva and Scheuer, 1980) has been studied extensively for its anti-inflammatory properties (Bennet et al. 1987). Its anti-inflammatory action is based on the irreversible inhibition of the release of arachidonic acid from membrane phospholipids by preventing the enzyme phospholipase A2 from binding to the membranes (Glaser et al. 1989). A rise in the intracellular arachidonic acid concentration would lead to upregulation of the synthesis of inflammation mediators as prostaglandins and leukotrienes (Figure 4). Phospholipase A2 inhibition has been recorded for many sesterterpenes from sponges of the order Dictyoceratida (Jacobs et al., 1994). The mechanism by which they affect the inflammation process is different from commonly used nonsteroidal anti-inflammatory drugs. Only a few sponge-derived terpenoids have been found to inhibit lipoxygenase, another enzyme that is involved in the inflammatory response (Carroll et al. 2001). Indole alkaloids including aplysinopsin-type compounds (Aoki et al. 2001), manzamines (El Sayed et al., 2008), and carteramine A (Koyabashi et al. 2007) isolated from various sponge species have also been reported to have anti-inflammatory activities. In an early study, Kossuga et al. (2008) showed that the polyketide plakortide P isolated from the Brazilian sponge *P. angulospiculatus*, potently decreased the release of thromboxane B2 from activated rat brain microglia. Moreover, a novel dimeric oroidin derivative carteramine A in the marine sponge *Stylissa carteri*, also inhibited neutrophil chemotaxis (Koyabashi et al. 2007). The anti-inflammatory sponge products are selective inhibitors of specific enzymes of a range of diseases, like psoriasis or rheumatic arthritis. The currently used nonsteroidal anti-inflammatory drugs often fail to control the disease and present important side effects such as risk of gastrointestinal bleeding and renal complications (De Rosa, 2002). These are caused by unselective inhibition of cyclooxygenases, some of which are also involved in the promotion of the production of the natural mucus that protects the gastrointestinal tract (Bjarnason et al. 1993).

7.7. Anti-Coagulating Activity

Some of the very common blood-related diseases such as thrombosis and atherosclerosis are unquestionably the main cause of death in the world. For example, the World Health Organization stated that in 2015 alone, approximately 15 million people have died from ischemic heart disease and stroke - the two top causes of mortality reported for that year (WHO, 2015). Based on these numbers, it is evident that there is a high demand for anticoagulant/antithrombotic agents in the global pharmaceutical market. The mechanism of blood coagulation is managed by a complex photolytic cascade that leads to the production of fibrin. Fibrin, a major component responsible for blood coagulation has been generated by the peptide cleaving of fibrinogen by thrombin (Kołodziejczyk and Ponczek, 2013). Cyclotheonamide A, derived from the sponge *Theonella* sp (Maryanoff et al. 1993) is an unusual class of Serine protease (an enzyme responsible for the conversion of fibrinogen into fibrin) inhibitor and is a drug of choice for thrombosis (Maryanoff et al. 1993, Schaschke and Sommerhoff, 2010). Eryloside F derived from *Eryltus formosus* sp. was found to be a potent Thrombin-receptor antagonist (Kalinin et al. 2012) (Figure 5). Thrombin receptor plays a central role not only in thrombosis but also the main agent to cause atherosclerosis (Chackalamannil, 2001, Ikenaga et al. 2016). Atherosclerosis is a disease in which plaque (fats, cholesterol, and calcium, etc.) builds up layer by layer inside the arteries and resulting

by narrowing of the arteries, causing a barrier to blood circulation leading to serious problems including heart attack, stroke or maybe death (Zapolska-Downar et al. 2001, Ikenaga et al. 2016).

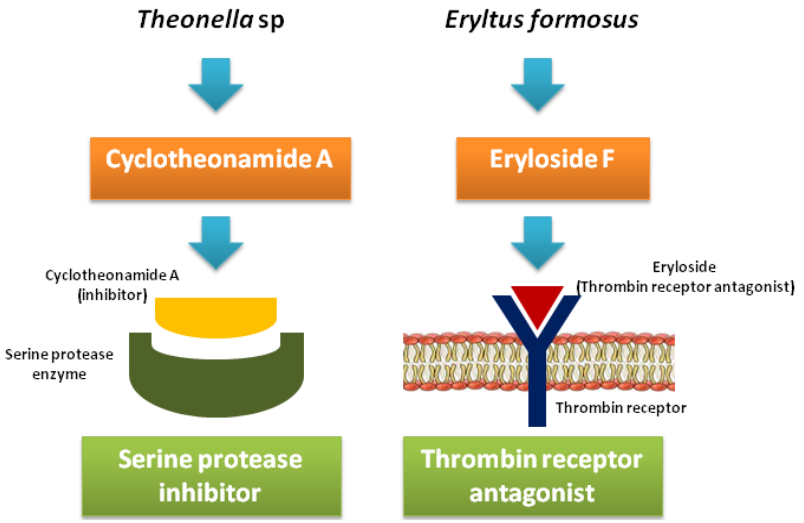

Figure 5. Molecular targets of sponge derived anticoagulant compounds.

Table 3. Selected examples of anti-inflammatory compounds isolated from marine sponges

Compound	Class	Species	Action spectrum	Reference
Manoalide	Cyclohexane sesterpenoid	*Luffariella varabilis*	Phospholipase A_2 inhibitor	Bennet et al. 1987
Carteramine A	Alkaloids	*Stylissa carteri*	Phospholipase A_2 inhibitor	Koyabashi et al. 2007
Plakortide P	Polyketide	*Plakortis angulospiculatus*	Phospholipase A_2 inhibitor	Kossuga et al. 2008
Dysidotronic acid	Dimane sesquiterpenoid	*Dsyidea* sp.	Phospholipase A_2 inhibitor	Giannini et al. 2000
Spongidines A-D	Pyridinium alkaloid	*Spongia* sp.	Phospholipase A_2 inhibitor	Marino et al. 2000
Topsentin	Bis indole alkaloid	*Topesentia genitrix*	Phospholipase A_2 inhibitor	Jacobs et al. 1994
Scalaradial	Scalarene sesterpene	*Cacospongia scalaris*	Phospholipase A_2 inhibitor	De Carvalho and Jacobs, 1991
Jaspaquimol	Diterpene benzenoid	*Jaspis splendens*	Lipoxygenase inhibitor	Carroll et al. 2001
Subersic acid	Diterpene benzenoid	*Suberea* sp.	Lipoxygenase inhibitor	Carroll et al. 2001

7.8. Marine Sponges with Anti-Tumour Properties

Cancer figures among the high ranking causes of deaths worldwide, accounting for approximately 8.8 million global deaths in 2015 (GBD, 2015). However, projections based on the GLOBOCAN 2012 estimates predict a substantive increase to 19.3 million new cancer

cases per year by 2025, due to growth and ageing of the global population (GLOBOCAN, 2012). This no doubt translates into one of the largest epidemic in human history. Although advances in the field of chemo-preventive and therapeutic medicine have been made regularly over the last ten years, the search for novel anticancer treatments continues. Despite the efforts of numerous researchers worldwide to ameliorate the dismal outcomes of cancer, it still continues to be huge burden on mankind (Shumacher et al. 2011). Cancer is a multi-factorial disease that arises as a consequence of alterations in many physiological processes. Recently, hallmarks of cancer were suggested that include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis, along with two emerging hallmarks including reprogramming energy metabolism and escaping immune destruction. In addition, tumors exhibit another dimension of complexity; they contain a repertoire of recruited, ostensibly normal cells to create the "tumor microenvironment" and contribute to the acquisition of hallmark traits (Hanahan and Weinburg, 2011). Understanding these concepts will provide new insights into the development of novel effective therapies to treat human cancer.

Marine chemotherapy is well recognized nowadays and profound development has been achieved by researchers to deal with different molecular pathways of tumors (Shwartsmann et al. 2011). During the search for novel and natural anticancer compounds, crude extracts and pure isolated compounds from marine sponges have been the subject of several investigations. An important feature of sponge- derived compounds is their ability to induce apoptosis by interacting with different molecular targets that are implicated in carcinogenesis. Cytoskeletal elements, nuclear factor-kappa B (NF-kB), hypoxia-inducible factor-1 (HIF-1), breast cancer resistance protein (BCRP), nuclear receptors, P-glycoproteins (P-gp), topoisomerases, matrix metalloproteinases (MMPs) and protein kinase C (PKC) are some examples of targets that have been identified to be modulated by sponge-derived compounds (Sipkema et al. 2005). Till date, more than 2500 cytotoxic compounds isolated from sponges have been reported, several of which are now in clinical trials against various cancers. A review Beesoo et al. (2014) collected the most important marine natural compounds which were already available in the pharmacological market or undergoing clinical trials (I, II, III) for anticancer activity. Among the compounds from sponges were the following:

Cytarabine

The first successful sponge-derived pharmaceutical drugs were the nucleosides spongothymidine and spongouridine which were isolated from the sponge *Cryptotheca crypta* (Newman et al. 2012). Derivative of these nucleosides, Ara-C also known as 1-beta-D-Arabinofuranosylcytosine or cytarabine is documented as the first marine derived anticancer agent that is currently being used for the treatment of leukemia and approved by the Food and Drug Administration (FDA, MD, USA) in 1969. Cytarabine (Figure 6(i)) is an S-phase specific anti-metabolite cytotoxic agent, which when converted intracellularly to cytosine arabinoside triphosphate competes with the physiologic substrate deoxycitidine triphosphate, thus resulting in both inhibition of DNA polymerase and DNA synthesis. At present it is being screened for the treatment of acute myeloid neoplasms in combination with Daunoribicin and other anticancer drugs (Mayer et al. 2010).

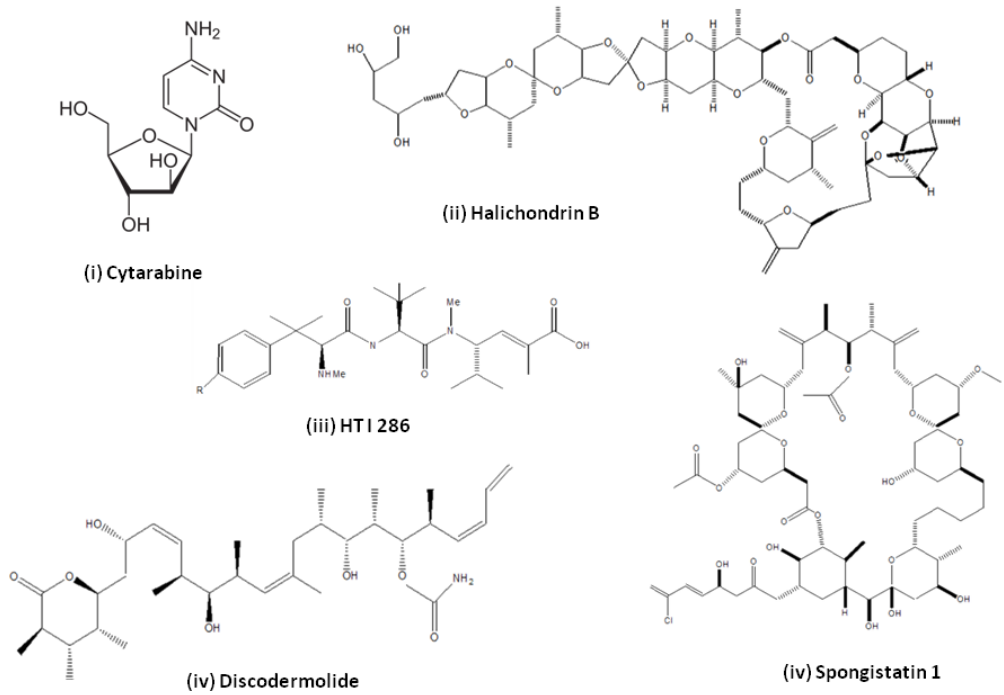

Figure 6. Chemical structures of marine natural products/derivatives thereof isolated from sponges approved for use by the FDA or EMEA and currently in the clinical and preclinical pipeline in the area of cancer.

Halichondrin B

The halichondrins are a family of polyether macrolides that were originally isolated in small quantities from the rare Japanese sponge *Halichondria okadai* (Uemura et al. 1985). Many studies have reported the cell growth inhibitory activity of Halichondrins at nanomolar concentrations (1×10^{-9} M) (Pettit et al. 1991). Among the Halichondrin family, Halichondrin B (Figure 6(ii)) was identified as the most potent congener which exhibited high *in vitro* IC_{50} value of 0.3 nM against L1210 Leukemia and remarkable *in vivo* activities against various human solid tumour xenografts, including LOX melanoma, KM20L colon, FEMX melanoma and OVCAR-3 ovarian tumours. Following the discovery of potent anticancer activity of Halichondrin B, it was tested and compared with other known antimitotic and anticancer agents using the United States (US) National Cancer Institute's (NCI) 60 cell line screen (Bai et al. 1991, Towle et al. 2001). While anti-proliferative patterns of Halichondrin B were found similar to those of other antitubulin drugs, its biochemical mechanism interaction with tubulin was unique (Bai et al. 1991). Differential cytotoxicity data indicated that halichondrin B binds tubulin at the vinca peptide binding site (Bai et al. 1991, Dabydeen, 2006). Induction of mitotic arrest by Halichondrin B leads to multisite phosphorylation and inactivation of anti-apoptotic protein Bcl-2, cytochrome c release from mitochondria, proteolytic activation of caspase-3 and -9, and cleavage of the caspase-3 substrate poly(ADP-ribose) polymerase (PARP) eventually stimulating cell death via apoptosis (Kuznetsov et al. 2004) (Figure 7). Currently, the synthetic derivative of this compound namely Eribulin is in phase II trials for non small lung cancer, pancreatic, prostate, head and neck cancer and bladder and ovarian and related gynaecological tumours and Phase III clinical trial as second line therapy for the

treatment of advance breast cancer. The trials are taking place across the world under the sponsorship of Eisai (Mayer et al. 2010).

Hemiasterlins

Hemiasterlins comprise a small family of naturally occurring tripeptides containing three highly modified amino acids which were originally isolated from the marine sponge *Hemiasterella minor* by Kashman and co workers in 1994 (Talpir et al. 1994) and subsequently from other unrelated sponges such as *Cymbastella* sp., *Siphonochalina* sp., and *Auletta* sp.(Coleman et al. 1995). All hemiasterlins have shown cytotoxicity in the nanomolar range (concentration $1x10^{-9}$ M) against a variety of cultured human and murine cell lines. The related isomers hemiasterlin A and B, as reported by Anderson and co workers are the most potent members in the hemiasterlin group (Coleman et al. 1995). Their potent cytotoxicities are due to induction of mitotic arrest in metaphase with similar dynamics to those of known tubulin binders such as paclitaxel or vinblastine (Anderson et al. 1997). The antimitotic activity of hemiasterlin is mediated via a tubulin-based mechanism that leads to tumor cell apoptosis**.** It binds to the *Vinca*-peptide site in tubulin and induces G (2)-M arrest, caspase-3 activation and poly ADP ribose polymerase cleavage (Figure 7), which are typical biochemical markers of apoptosis (Kuznetsov et al. 2009). Extremely limited quantities of hemiasterlins hampered development of this series of compounds, particularly in animal models where limited testing has been reported (Coleman et al. 1995). The optimal analog was considered to be HTI-286 (Figure 6 (iii)) that retains potency in cellular models resistant to several chemotherapeutics, including taxanes and *Vinca* alkaloids (Coleman et al. 1995). Preclinical studies has shown that HTI-286 causes tumour regression and growth inhibition of human xenografts in mice. Even cell lines expressing P-glycoprotein or resistant to paclitaxel were shown to be sensitive to HTI-286 inhibition, but required higher doses than non-resistant cell lines (Loganzo, 2003). An open-label Phase I clinical trial was completed in patients with advanced solid tumours; however there were no objective responses and common toxicities observed included neutropaenia, nausea, alopecia and pain (Ratain, 2003). Phase II trials have been halted. Nevertheless, there is still interest in view of recent results including high antitumour activity in androgen-dependent and androgen-independent mouse models of refractory prostate cancer and in a newly established *in vitro* taxane resistant prostate PC-3 cell lines (Ratain, 2003).

Discodermolide

Discodermolide is a marine sponge derived polyketide lactone, first isolated from the deep water sponge *Discodermia dissoluta* (De Souza, 2004). Crude extracts of the sponge showed strong potency against the murine P388 lymphocytic leukemia cell line and bioassay guided fractionation resulted in the purification of discodermolide. Discodermolide (Figure 6 (iv)) acts as an immunosuppressant and induces G2/M Phase cell cycle arrest and apoptosis in various tumor cell lines. Investigations into the mechanism of cycle-arrest by discodermolide competitively bind to tubulin and stabilize microtubules with another tubulin binding agent, paclitaxel. Fortunately discodermolides bind with a higher affinity for tubulin and have effects even in paclitaxel-resistant cell lines. Despite their competitive behaviour, discodermolides and paclitaxel show synergistic effects (Martello et al. 2000). This contradictory effect may be caused by overlapping rather than identical binding sites. Although both compounds bind to the taxane binding pocket, taxol interacts with the M-loop

and discodermolide orients itself away in the direction of S1-S2 loop. Furthermore, the two compounds seem to show a complementary stabilising effect on microtubules (Khrapunovich-Baine et al. 2004). Discodermolide treatment causes a late activation of caspase 3 and caspase 8 as well as a cleavage of PARP in NSLCS cells (Figure 7). Treatment with discodermolide also leads to an efflux of cytochrome c from the mitochondria. Despite these findings, neither overexpression of Bcl-2 nor FADD-negative cells or inhibition of caspases could prevent cells from undergoing apoptosis (Broket et al. 2002). However, although discodermolide has shown more potent effects in tumour cells than paclitaxel and has also shown promising effects in murine models, the pharmaceutical company Novartis has withdrawn it from Phase I trials due to cytotoxicity problems (Molinski et al. 2009). However potentials remain for its use in combination drug therapy.

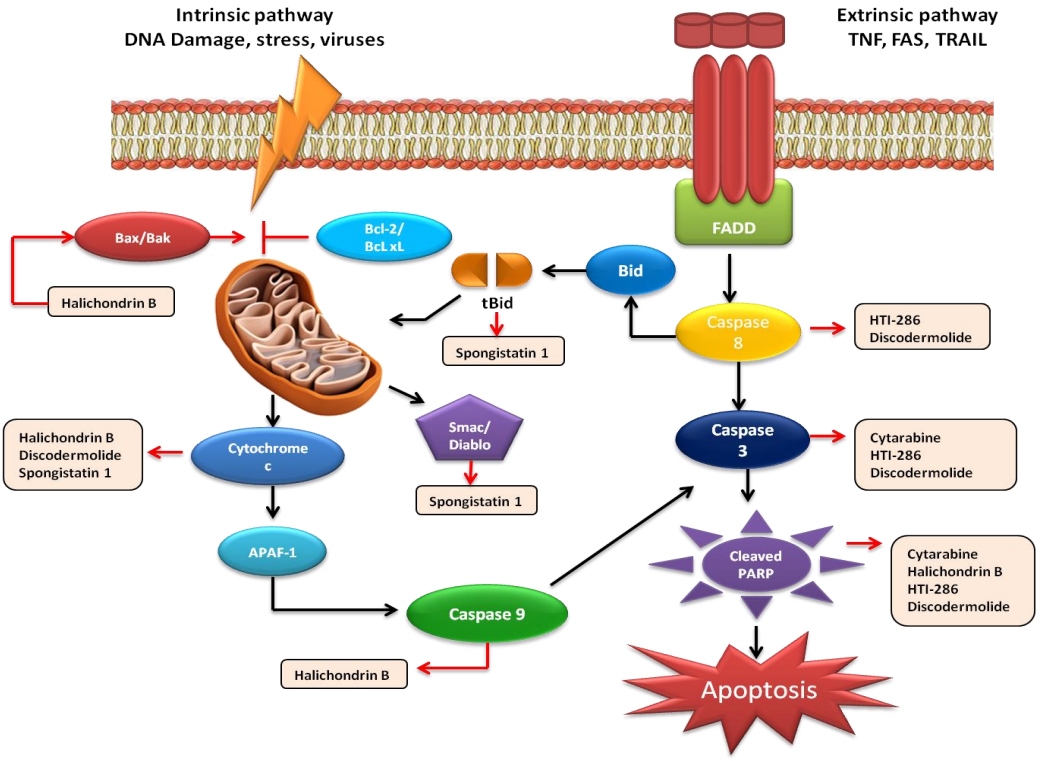

Figure 7. Interaction of marine sponge derived compounds on apoptotic signaling molecules. Regulation of this pathway with marine compounds; arrow indicates activation and blocked arrow indicates inhibition of the molecules.

Spongistatin 1

The spongistatins comprise an important family of marine macrolides that display extraordinarily antitumour activities against a variety of human cancer cell lines. These unique marine compounds were isolated from the sponge *Spirasterella sp.* (Pettit et al. 1993). The spongistatin family includes 9 macrolides all of which possess remarkable cytotoxic activity against the US National Cancer Institute's panel of sixty human cancer cell lines.

Table 4. Current status of sponge-derived anti-tumour products in the drug development process

Clinical Status	Compound Name	Natural Product/ Derivative	Collected Source Organism	Class of Agent	Molecular Target	References
FDA Approved	Cytarabine	Derivative (Arabinosyl Cytosine)	*Tethya crypta*	Nucleoside	DNA polymerase	Mayer et al. 2016
	Eribulin Mesylate (E7389)	Derivative (Halichondrin B)	*Halichondria okadai*	Macrolide	Tubulin	Mayer et al. 2016
Phase I	Hemiasterlin (HTI-286)	Derivative (Hemiasterlin)	*Agelas mauritianus*	Tripeptide	Tubulin	Mayer et al. 2016
*Phase I	PM060184	Derivative	*Lithoplocamia lithistoides*	Polyketides	Minor groove of DNA	Mayer et al. 2016
	Discodermolide	Natural product	*Discodermia dissolute*	Polyketide lactone	Tubulin	Mayer et al. 2010
Preclinical	Peloruside A	Natural product	*Mycale hentscheli*	Macrolide	Tubulin	Ganguly et al. 2015
	Salicylihalamides A and B	Natural product	*Haliclona* sp.	Macrolide	Vo-ATPase	Erickson et al. 1997
	Variolins	Natural product	*Kirkpatrickia Variolosa*	Alkaloid	Cdk	Walker et al. 2009
	Dictyodendrins	Natural product	*Dictyodendrilla Verongiformis*	Alkaloid	Telomerase	Zhang et al. 2017
	Spongistatin 1	Natural product	*Spirasterella* sp.	Macrolide	Tubulin	Pettit et al. 1993
	NVP-LAQ824	Derivative (Psammaplin)	*Psammaplysilla* sp.	Phenolic	HDAC/DNMT	Shim et al. 2004

* Discontinued from further development.

Spongistatin 1 (Figure 6(v)) is known as the most cytotoxic member of the spongistatin family and has an average IC_{50} value of 2.5-3.5 x 10^{-1} against a subset of highly chemoresistant tumor types (Bai et al. 1993, Pettit et al. 1993). Spongistatin 1 shows interesting apoptotic features in various tumour cells. In leukemic cell lines it triggers caspase dependent apoptosis through the release of cytochrome c, Smac/Diablo and Omi/HtrA2 (Figure 3) from the mitochondria into the cytosol. Spongistatin 1 also leads to the degradation of the anti-apoptotic X-linked inhibitor of apoptosis protein (XIAP) thus positioning itself as a promising drug for the treatment of chemoresistance due to overexpression of XIAP (Schyschka et al. 2008). Moreover spongistatin 1 induces apoptosis more efficiently in human primary leukemic cells of children suffering acute leukemia at low nanomolar concentrations than clinically applied conventional drugs used in micromolar concentrations (Schyschka et al. 2008). Besides leukemic cells, spongistatin showed promising apoptotic potential in mammary cancer cells including the treatment-resistant cell line MCF-7 lacking caspase 3. Spongistatin-1 -induced cell death mainly caspase independent, involves the pro-apoptotic proteins AIF and endonuclease G. Both proteins translocate from mitochondria to the nucleus and contribute to spongistatin-1 mediated apoptosis as shown via gene silencing (Figure 7). Second, spongistatin acts as a tubulin depolymerising agent and is able to free the pro apoptotic Bcl-2 family member Bim from its sequestration both by microtubular complex and by the anti-apoptotic protein Mcl-1 (Schneiders et al. 2009). Silencing of Bim by siRNA leads to a diminished translocation of AIF and endonuclease G to the nucleus and subsequently reduces rate of apoptosis. By using spongistatin as a chemical tool, Bim has been suggested to be an important factor upstream of mitochondria by executing a central role in the caspase-independent apoptotic signalling pathway induced by spongistatin 1 (Schneiders et al. 2009). These different apoptotic features indicate that the apoptosis signalling is cell line specific.

The robustness of the global marine pharmaceutical pipeline in the area of cancer is evident by two FDA approved compounds isolated from marine sponges namely cytarabine and eribulin mesylate and many others such as hemiasterlin, discodermolide and spongistatin 1 (Table 4) being currently investigated under different phases of the clinical trial as the next possible clinical candidates.

8. FUTURE PROSPECTS OF SPONGE-DERIVED NATURAL PRODUCTS IN DRUG DISCOVERY

The above examples illustrate the intense excitement which surrounds the achievement particularly in the area of infectious diseases and cancer research from marine sponges. A substantial number of bioactive lead compounds isolated from sponges are progressing through the development process, primarily with indications in cancer. Sponge chemicals span a wide range of chemical classes (e.g., terpenoids, alkaloids, peptides and polyketides) with an equally variety of biotechnologically relevant properties (e.g., anti-oxidant, anti-bacterial, anti-fungal, anti-viral and anti-tumour). There are now some significant reports of activities from a particular class of metabolites, the manzamines from marine sponges and/or associated bacteria as potential drugs or leads to drugs that might be effective against HIV, tuberculosis, malaria, and other infective agents. Cytarabine, halichondrin B, hemiasterlins

and discodermolide isolated from different sponge species are all examples of interesting sponge anticancer agents of potential relevance in cancer therapy.

These promising sponge (or microbial symbiont) -derived compounds in advanced stages of clinical trials emphasize the potential of sponges as auspicious source for drugs against various diseases. However, compared to the vast number of over 4,000 compounds isolated from sponges during the last three decades, the number of sponge derived drugs that have so far entered the market is surprisingly small. There are three major reasons for this phenomenon: one is the extremely long time frame involved in the process of drug development. For example, the development of the famous anticancer drug Taxol® from its initial description in the yew tree (Wall et al. 1995) to its approval as a commercial pharmaceutical took over 20 years. Thus, since many interesting sponge-derived substances were initially reported in the 1980s and early 1990s, there is hope that in not too far future the number of commercially available "marine drugs" will considerably increase. The second reason is that Big Pharma programs mostly avoid looking at marine invertebrate natural products as potential lead compounds because of the legal and logistical difficulties encountered in accessing the source organisms in the ocean waters of foreign countries, combined with the challenges associated with solving the compound supply issues involved in turning a structurally complex invertebrate natural product into a commercial drug. Conversely, the lack of Big Pharma interest in marine invertebrate natural products as a source of chemical diversity for their drug discovery efforts has made the examination of marine invertebrate natural products as potential cell biology tools and drug leads a very attractive area for academic research. University researchers do not face the same timeline pressures found in industry and bioactive natural product discovery projects are excellent interdisciplinary training platforms for the next generation of drug discovery scientists. Academic drug discovery research on bioactive marine invertebrate natural products is complementary to Big Pharma drug discovery efforts because it facilitates the exploration of an important drug discovery chemical diversity resource that would not otherwise be exploited (Koehn et al. 2005, Andersen et al. 2017).

The third and major reason is the unfortunate fact that most pharmaceutically interesting compounds found in sponges or microbial symbionts are available only in minute amounts from their natural sources. To develop *in vitro* screening assays, small amounts are needed; however, in preclinical studies, hundreds of grams to kilograms are often required for testing purposes. Perhaps this is the most important factor challenging in the development of products from the sea. Indeed, in order to obtain 300 mg of halicondrins, close to 1 metric ton (wet weight) of the sponge (*Lissodendoryx* sp) has to be collected and extracted. (Proksch et al. 2002, Minh et al. 2005). Consequently, the application in clinical is extremely difficult because of the low productivity of these compounds. Clearly, natural exploitation of these compounds cannot be reasonably envisaged without causing the destruction of marine ecosystems. Synthesis of marine bioactive compounds remains therefore the alternate method. However, due to the high complexity of certain structures their synthesis, in many cases, is not feasible. One successful example of the synthetic production of a marine-product drug in unlimited quantities is the conus toxin ziconotide, because of its peptide nature (Mayer and Gustafson, 2000). Another alternative to exploit natural products is marine culture. The aquaculture of the source organisms, including sponges, tunicates, bryozoans, with the aim of securing a steady supply of drug product, has progressed notably in cancer applications. For example, by culturing *B. Neritica*, American groups have obtained large amounts of

bryostatin 1 and the latter was produced at reasonable cost (Mendola, 2000). On the other hand, the majority of pharmaceutically interesting marine organisms, especially sponges, cannot be cultured under artificial conditions. Better understanding of living conditions in natural environment is necessary to develop alternative cultivation methods and to maintain the metabolite production over a long time. Culture of sponge cells and, more likely, primmorphs can become a future source of metabolites, cell and primmorph cultures are not feasible at present for producing large amounts of biomass (Belarbi et al. 2003).

Another limiting factor in the use marine sponges in the discovery of novel drug leads is the potential presence of toxins from the organisms or of environmental origin and the presence of inorganic salts. These species may compromise the use of raw extracts for *in vitro* screening purposes. Often, the production of toxins by a sponge is an indicator that this organism is a candidate source of bioactive compounds. Therefore, an effort should be made to characterize the possible contaminants (inorganic salts and toxins) in order to make marine extracts compatible with in vitro testing. Many analytical techniques are currently available for the analysis, isolation, characterization and separation of active compounds in marine extracts (Grienke et al. 2014). Moreover, analysis of drug development programs has shown that only 5-10% of experimental drugs with new chemical scaffolds hitting new cellular targets that enter phase I clinical trials will succeed, so the failure rate is high (Kola and Ladis, 2004, Schumacher et al. 2016). The major causes of failure are lack of efficacy and/or toxicity, as was described above for HTI-286. There is a constant effort to find new ways to increase the success rate for new drug candidates, but simple solutions to reducing the high rate of attrition are elusive. Ideally, marine natural product lead compounds must have biological activity that addresses an unmet medical need by hitting a new molecular target, or represent a completely new approach to modulating an already highly validated target. Therefore, robust pipelines of new drug candidates are essential and marine/sponge natural products can make meaningful contributions to these pipelines.

A long-standing and perplexing question in marine natural products chemistry has been the identification of the metabolite producing organism or potentially metabolite biotransforming organism, in systems involving an invertebrate host and symbiotic microorganisms. Many studies have successfully provided data to support the involvement of microorganisms including actinomycetes, cyanobacteria, microalgae such as dinoflagellates, and others in the biosynthesis of natural products isolated from invertebrates. For instance, the structural study on ET-743 has led to the identification of a congener named safracin B from *P.f luorescens*. Also Symbostatin 1, a similar skeleton to dolastatin 10 was isolated from the green alga *Symploca hydnoides* (Minh et al. 2005). In a sense, this is quite fortunate, for the biosynthetic pathways that code for natural compounds in prokaryotes are better understood and more amenable for study than those in eukaryotes. Progress in understanding how these biosynthetic pathways operate at the genetic and biochemical levels is opening new doors for harnessing this potential, and the future looks very optimistic for realizing tangible benefits from such efforts, such as new designer molecules with improved biological and pharmaceutical properties (Beesoo et al. 2014).

CONCLUSION

This chapter summarizes the current pipeline of marine sponge natural products that are currently being evaluated in clinical and pre-clinical trials as well as it provides a view into the promise that these compounds pose to improve the diversity of our pharmacopeia to treat a wide variety of human diseases. Presently, two FDA approved drugs from marine sponges namely the anticancer citarabine and anti-viral vidarabine are on the market while many are rowing the different phases of the clinical trial. Clearly, marine sponges will play an important part in the future control of the global disease burden. Although substantial progress has been made in identifying novel drug leads from sponge's resources, a major frontier will be the application of metagenomic and genomic tools which will allow identification of the true sources of bioactive compounds. Fast evolving technologies and the increasing knowledge of molecular processes and mechanisms at the base of secondary metabolite production will continue to improve possibilities of mining for novel natural products from marine sponges and their exploitation for clinical applications.

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Chapter 32

BLACK VULTURES (*CORAGYPS ATRATUS*) FORAGING ON OLIVE RIDLEY (*LEPIDOCHELYS OLIVACEA*) SEA TURTLE EGGS AND HATCHLINGS

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ABSTRACT

The incubation period, emergence, and dash of freshly emerged hatchlings to the sea is a critical period in the life cycle of sea turtles, although it is short compared to their life span. Predators can claim up to 100% of sea turtle eggs or hatchlings, depending upon the site, time of year, or location. We examined the foraging behavior of black vultures (*Coragyps atratus*) on an *arribada* beach of the olive ridley (*Lepidochelys olivacea*) sea turtle at Ostional, Guanacaste, Costa Rica. We address the following: 1) What methods of foraging do vultures use? 2) How successful are they in obtaining food? and 3) What factors affect their foraging, and we speculate on the possible effects of vultures on olive ridley reproductive success. The number of black vultures feeding on a 1500 m beach averaged 643 $\pm$ 104 (max of 1243). Vultures foraged by hunting (nests with eggs or embryos, emerging or crawling hatchlings), social parasitism (using other birds or dogs to locate foraging opportunities), parasitism (eating eggs or hatchlings at nests other vultures found first), piracy, and scavenging of washed up adults, eggs, and dead hatchlings. Foraging opportunities included finding nests at eroded stream banks, nests uncovered by tides or wind, nests dug up by dogs, eggs thrown up by digging female turtles, and hatchlings emerging on their own. Overall, almost half the vultures gathered around exposed nests ate eggs/embryos (mean number of vultures was 10.6), while 64% obtained hatchlings at nests with emerging hatchlings. Both the number of vultures, and

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the percent feeding, varied by foraging opportunity, with the highest percent feeding on and below vertical banks of eroded streams, and the lowest percent feeding with dogs. Interference competition with wood storks (*Mycteria americana*) resulted in significant decreases in the number of foraging vultures within 5 m of an exposed nest, and a significantly lower percent were able to feed, when storks were present at nests exposed at stream banks and by tidal action, and when hatchlings were emerging. 82% of the variance in the number of vultures feeding was explained by feeding location (foraging opportunity), number of storks, number of vultures present, and time of day. Although the turtles are supporting a healthy population of black vultures, most of the vulture food source on the beach are eggs that would not otherwise hatch, including inviable eggs, developing embryos and nearly-developed hatchlings that were exposed by stream bank collapse, tidal erosion, dogs or Wood Storks, digging female turtles during *arribadas*, and human egg-harvesters.

Keywords: reptilia, testudines, cheloniidae, *Lepidochelys olivacea*, olive ridley sea turtles, hatchlings, marine debris, foraging, predation, Costa Rica; conservation

All seven species of sea turtles are listed on the International Union for Conservation of Nature (IUCN) Red List of Threatened Animals (IUCN 2011). The threats facing sea turtles are many, both on land and at sea, including habitat loss, predation, and human exploitation (Plotkin 2007). The olive ridley (*Lepidochelys olivacea*), the most numerous sea turtle species, has a pantropical distribution (NRC 1990). It exhibits two breeding strategies: solitary nesting, and *arribadas*, in which up to hundreds of thousands of turtles nest on a single beach over the course of a few nights (Pritchard 2007).

Predator swamping is the hypothesis proposed to explain the latter phenomena. The superabundance of prey in a short period of time makes it impossible for predators to take them all (Gochfeld 1980; Ims 1990). In olive ridleys, the mass laying and hatching results in the emergence of large numbers of hatchlings at one time (over a period of a week), and predators may become sated, allowing some hatchlings to safely reach the sea. When millions of hatchlings crawl to the sea, terrestrial predators (including nocturnal ones) can consume only a small proportion of the young (Cornelius et al. 1991). Thus, the evolution of *arribada* behavior is ascribed to the predator-satiation hypothesis (Eckrich and Owens 1995).

However, an *arribada* has drawbacks. Predators may be recruited in large numbers, and the mass nesting facilitates the harvest of adults and eggs. In some places, the harvest of eggs has led directly to declines and elimination of *arribada*-nesting populations (Campbell 2007a,b; Cornelius et al. 2007), and *arribadas* are rare among turtles. *Arribadas* can attract a range of predators, both on land and at sea. At sea, hatchlings are vulnerable to fish and to avian predators, such as frigatebirds (Witherington and Salmon 1992; Lagarde et al. 2001). On land, nests, eggs, and hatchlings are vulnerable to reptilian, avian, and mammalian predators, the latter mainly at night (Santidrian et al. 2010).

Most hatchlings emerge at night (Drake and Spotila 2002). Predation rates on sea turtles nests can be as high as 95% (Engeman et al. 2003), and in some cases, 100% of hatchlings observed diurnally were taken by predators (Lagarde et al. 2001). In Costa Rica, Fowler (1979) reported that feral dogs, raccoons (*Procyon lotor*) coatis (*Nasua narica*), black vulture (*Coragyps atratus*) and turkey vultures (*Cathartes aura*) preyed on the eggs and hatchlings of sea turtles. Vultures also prey on hatchling sea turtles in other places, such as Trinidad (Safina

2006). Although mammalian and avian predation on turtle eggs and hatchlings is well-known, studies normally concentrate on the adult sea turtles, and do not specifically examine the behavior of the birds or mammals, their foraging strategies, and the potential positive effects of their foraging on sea turtle eggs and hatchlings.

In this paper we examine the foraging behavior of black vultures when feeding on olive ridley sea turtle (*Lepidochelys olivacea*) eggs and hatchlings at Ostional, Guanacaste, Costa Rica. Our objectives were to 1) describe the foraging behavior of black vultures on sea turtle eggs and hatchlings, 2) describe their foraging success using different types of foraging opportunities, and 3) describe the factors affecting foraging success. We were particularly interested in the potential effects of vulture foraging on nests and hatchlings, and in the abundance and behavior of vultures on the *arribada* beach. While the presence of vultures, and their scavenging and predation on eggs and hatchling sea turtles in Costa Rica was well known (Fowler 1979; Valverde et al. 2007), the number of vultures present on the beaches, their foraging behavior, and their effect on eggs and hatchling sea turtles at Ostional had not been examined.

METHODS

Study Species. - Black vultures range from northeastern and southern United States through most of the neotropics to northern Argentina. Their main foods are dead animals, including refuse. They locate food mainly by soaring and watching for other vultures. Although their food occurs sporadically, landfills and turtle beaches like Ostional provide some guarantee of food. Wood storks (*Mycteria americana*) were also present regularly on the beach, and also foraged on sea turtle eggs and hatchlings. The other diurnal predators at the Ostional beach were turkey vultures (*Cathartes aura*), crested caracaras *(Caracara cheriway)*, and great-tailed grackles (*Quiscalus mexicanus*). Although turkey vultures and caracaras preyed on turtle eggs or young, there numbers were small and their influence on black vultures probably negligible. All observations reported below are for black vultures, other birds are discussed in detail elsewhere (Burger and Gochfeld, 2014a).

Olive ridleys nest both solitarily and in *arribadas* that occur approximately monthly on traditional beaches in Central and South America, Africa, India, and Australia (Plotkin and Bernardo 2003). Even on *arribada* beaches, solitary turtles come up to nest in between *arribadas*. During a study from 2006 to 2010 at Ostional, the largest *arribada* occurred in October 2008, when an estimated 476,550 female turtles nested (Valverde et al. 2012; (Figure 1). *Arribadas* at Ostional occur in every month, although those early in the year are the smallest (Pritchard 2007). Ostional *Arribadas* ranged from 3,564 to 476,550 egg-laying females, with the largest occurring in the rainy season (August to December). The olive ridley *arribadas* at Ostional are the second largest nesting assemblages of sea turtles in the world (Valverde et al. 2012). The mass nesting of turtles has provided the opportunity for harvesting of turtles and eggs, much of it illegal (Cambpell 1998; Campbell et al. 2007a; Bernado and Plotkin, 2007). However, egg harvesting is legal at Ostional, and is allowed for the first 2.5 days of an *arribada.* People harvested an average of 21.2% of the clutches laid during their study, and there is some suggestion that this nesting assemblage has decreased over the last two decades (Valverde et al. 2012).

Figure 1. The Ostional (Costa Rica) arribada of Olive Ridley Sea Turtles in November 2012. Shown are 125 turtles.

Study Area. - The *arribada* beach at Ostional National Wildlife Refuge (Guanacaste, Costa Rica, lat 9.993913 N, long 85.700403 W) is 7 km long, with a variable width (typically less than 100m). Permanent markers established every 50 m allowed us to accurately census vultures along the beach and associated trees and shrubs at the edge of the upper beach. Windrows of logs and driftwood of varying sizes litter the beach. Ostional village (population of 450) has established the Association for the Integral Development of Ostional (Asociación de Desarrollo Integral de Ostional= ADIO) that legally has authority to harvest olive ridley sea turtle eggs from the beaches (Valverde et al. 2012) (Figure 2a and 2b).

Behavioral Observations. - Observations on foraging in vultures were made from October 19 to November 8, 2012, supplemented with data from refuge personnel and on-site collaborators. Our observations were obtained during a one month cycle at the height of the rainy season. The November 2012 *arribada* was considered the largest of the year, with over 110,000 females nesting the first night (7-8 November). We collected data on 1) number of vultures, 2) foraging methods, and 3) factors affecting foraging behavior and success. In addition, observations were made of the protective measures of the Association for the Integral Development of Ostional (ADIO) with regard to influence on vulture predation. Observations were made from 04:30 AM until 17:30 PM daily, with periodic observations at night. Vultures arrived from their nocturnal roost around 5:00 AM and were flying to roost by 17:00 PM.

Figure 2a and 2b. The people of Ostional have CITES permits to harvest turtle eggs, and protect the beaches, emerging hatchlings, and nesting turtles from predators, poachers, and beach debris.

We conducted censuses of the number of vultures present on the beach, and in the shrubs/trees at the top of the beach, in the morning and afternoon every day to determine the species and numbers that were present and actively engaged in hunting or foraging on eggs, nests, or hatchlings. We also recorded the number of dogs (using photographs for individual recognition) that dug down to nests, ate eggs or hatchlings, and then abandoned the nests to vultures and other birds. Data recorded included: date, time of day, transect segment (pole number), and number of vultures by location (beach, trees/shrubs at top edge of beach). During the peak hatchling period (6 days), vultures and other birds were scared from the beach from 05:00 AM to 07:30 AM by members of ADIO. Thus, vulture numbers were analyzed only for censuses where there were no people keeping them from the beach. Vultures began returning to the beaches within minutes of the departure of beach protectors, but during that six day period, their numbers never reached the high numbers recorded before the peak hatchling period.

Behavioral observations on predation of nests, eggs, and non-emerged and emerged hatchlings were made by walking the same transects in the morning and afternoon each day. We recorded the following information for all nests where vultures were foraging: date, time of day, transect segment, depth the nest was open (sand depth), type (eggs, hatchlings with or without residual yolk sac), the number of birds within 3 m, number of birds eating/30 sec, number of chases (or defensive behaviors)/30 sec, and handling times when vultures ate hatchlings (time from when it picked up the hatchling until it finished eating). We also recorded the number of wood storks, caracaras, grackles and other birds present along transects (Burger and Gochfeld, unpubl data). The present ms concentrates on the behavior of foraging black vultures.

Linear regression procedures were used to determine the variables that contributed most strongly to explaining variations in the foraging behavior of vultures (PROC GLM, SAS 2005). We used a stepwise model that adds the variable that contributes the most to the R^2, continuing until all significant variables are added. Thus, variables that vary co-linearly are entered only if they add independently to explaining the variation. We used Kruskal Wallis tests to determine significant differences among variables, and Contingency X^2 tests for small samples sizes. Levels of $p < 0.05$ were considered significant.

RESULTS

Vulture Numbers. - Vultures normally arrived at the beach well before sunrise, when light levels were very low (around 05:00AM). While some vultures immediately started foraging at the tideline, others flew to trees and shrubs at the top edge of the beach. The average number of vultures present on the beach was 643 $\pm$ 104, with a maximum of 1243.

Foraging Behavior. – Vultures foraged using a number of methods, including hunting on foot or from the air, social parasitism (looking down the beach for groups of vultures that might indicate a prey source), parasitism (eating eggs or hatchlings at nests found by other vultures), kleptoparasitism (piracy from other vultures), and scavenging (Table 1, Figure 3). They flew, walked, or hopped over the beach looking for exposed eggs or hatchlings, flew over the beach looking for live hatchlings emerging or crawling toward the sea, took eggs or hatchlings exposed by people, other birds, or female turtles in full *arribada*, stole eggs or hatchlings from other vultures (piracy), and searched for the presence of a group of vultures or a digging dog that indicated an exposed nest (social parasitism). Thus, in most cases vultures were searching for place-based prey (e.g., eggs and hatchlings at nests, surrounded by groups of other vultures).

Figure 3. Black vultures dig up nests that are near the surface or are exposed by high tides or dogs.

Table 1. Foraging strategies of black vultures on olive ridley sea turtles in Ostional, Costa Rica. ** = eggs or hatchlings would not make it because of exposures not due to the vultures

Strategy	Description	Physical or biological conditions	Target
Hunting	Searching for hatchlings on the sand surface or in the shallow surf	Hatchlings in the nest prior to moving (20-200 second period) and moving over the beach and into the surf	Hatchlings[a]
	Finding nests, eggs or hatchlings exposed on or near the surface	Heavy rains increased stream flow, causing banks to collapse, exposing nests. When nests are exposed, some eggs fall down the sand cliff and the Vultures pick them off the stream edge or water. Vultures scramble for footholds on slanted banks to reach the exposed nests.	**Nests, **eggs, **hatchlings
Social parasitism	Searching for the presence of other Vultures as an indication of prey	Vultures course over the beach, or sit on trees and logs, watching for other Vultures that appear to be feeding, and join them. Only hatchlings found this way may have been viable.	**Eggs, **hatchlings
	Finding nests, eggs or hatchlings exposed on shallow tidal banks	High or severe wave action, rush up the creeks increasing the erosion of the banks, exposing nests	**Eggs, **hatchlings
Parasitism	Taking eggs or hatchlings exposed by other organisms	Dogs dig up nests that they find either exposed from high tides or erosion along storm banks, or that they dig up (presumably from the odors of hatching eggs). When the dogs leave, the Vultures move in and eat what remains, or dig more	**Eggs, **hatchlings
	Taking eggs or hatchlings exposed by people	People sometimes dig up nests and Vultures can steal eggs or hatchlings when they are not looking. When people dig up hatchlings to take them safely to the surf, Vultures watch and may dig up the area later. Even in the surf hatchlings are vulnerable, often for several minutes until finally able to swim out through a wave. When scientists dig up unhatched nests to determine the cause of failure, Vultures crowd around to eat the eggs, embryos or dead hatchlings.	**Eggs, **hatchlings
	Taking eggs or hatchlings exposed during an *arribada*	Turtles digging their own nests often dig up the nests of other turtles, allowing the Vultures to eat these eggs or dead hatchlings	**Eggs, **hatchlings dead embryos
Klepto-parasitism or piracy	Stealing eggs or hatchlings from other birds (Vultures, grackles, Wood Storks)	Presence of other birds trying to eat eggs or hatchlings. This often happens when hatchlings are emerging and a large group of Vultures at present in close proximity at a nest.	**Eggs, **Hatchlings
Scavenge	Fly low over the beach, surf and exposed rocks	Opportunistic feeding on dead turtles washed up on the beach, and hatchling or eggs that have been exposed or are floating in water.	**Adults washed up on the beach, **eggs, **hatchlings, partial carcasses
	Walk over the beach	While the turtles are nesting, vultures walk gingerly among them, looking for eggs of previous or current *arribada* that were excavated by digging females	**Eggs
	Walk over the beach	After the turtles have nested, during and after the townspeople harvest eggs and discard spoiled eggs or those partly developed	**Eggs, **embryos

a. This form of predation is partly eliminated by the ADIO because from 05:00AM to 07:30AM or 08:00AM they station people every 50 m to scare or chase Vultures away, effectively removing them from the beach during this time.

The rain-swollen stream cuts were a dynamic system eroded by the continuous pressure of flowing water. The sides were 2-4 m high with 60 to 80 degree slopes. Groups of vultures were lined up at the top of the cut, or in the streambed, waiting for a subsidence event to expose a nest. Anywhere from 10 to 40 vultures would congregate in a frenzied effort to maintain a footing on the steep slope and snatch exposed eggs. Often groups of eggs would roll to the bottom of the slope to be opened and sucked dry by the vultures (Figure 4).

Figure 4. Erosion of stream banks sometimes exposes eggs that are eaten by vultures or wood storks. These are eggs that would never hatch.

Figure 5. (Continued).

Figure 5a and 5b. During the arribada the vultures try to eat eggs thrown up by digging turtles, but only at the edge of the mass of turtles.

The vultures switched foraging strategies depending upon prey available (eggs in nests, embryos in nests, hatchlings emerging). When no hatchlings were emerging, the vultures coursed low over the dunes, searching for exposed nests or eggs, and for groups of vultures that indicated an exposed nest. Once hatchlings began to emerge from nests, vultures switched to solitary hunting in the early morning when they first arrived. When light levels were very low, vultures arrived in small groups, landed at the south end of the *arribada* beach, and followed two strategies: they hopped rapidly on the upper beach, moving in groups down the beach searching for hatchlings still crawling toward the sea, or they hopped or flew low over the surf searching for hatchlings being buffeted by the waves (some hatchlings required several waves before they were carried out to sea). When the beach and surf had been searched, they switched to looking for dogs or groups of vultures that indicated an exposed nest.

While female turtles were digging during an *arribada*, significant numbers of eggs from earlier nests were thrown up by their back flippers while digging. Such nests were from females that nested in the same *arribada*, or in a previous one. Vultures ate these eggs or embryos while the turtles were still on the beach, or after they departed. This occurred only near the top edge of the beach where turtle density was low. The vultures were wary of the digging turtles, kept an eye on them, and ran or flew back when the turtles moved (Figure 5a and 5b). Few turtles were accompanied by vultures, and in the mid-beach there was very little room between turtles. This contrasted with vulture behavior in the two days before the full *arribada*, when fewer than 200 turtles nested, and some were still completing nesting at 05:00AM. Then groups of vultures foraged around each female, waiting for eggs to be thrown up by the digging activity.

Additionally, legal egg harvesting by the local residents resulted in eggs (rotten, not viable) and developing embryos (up to nearly fully formed hatchlings) being left on the sand

surface as they harvested only the freshly-laid eggs, but sometimes encountered previously laid clutches. During the early morning harvest, egg-harvesters, nesting turtles, and vultures were all on the beach. When the harvesters moved on up the beach, they left the few turtles behind, providing foraging opportunities for the vultures.

Eating hatchlings. – When a vulture discovered a nest with newly emerging hatchlings, there was a frenzy of activity as other vultures flew in and began seizing other hatchlings. Within 15 sec, the initial vulture was joined by half dozen others, or more. Foraging on hatchlings differed from eating eggs because the vultures either swallowed the hatchling whole, removed parts to eat, or removed parts and then swallowed the rest of the hatchling. Further, vultures tried to pirate hatchlings from one other. Total handling times ranged from 9 sec to swallow one, to 409 sec to fail. Although some swallowed a hatchling quickly, most did not. Attempting to eat a hatchling often involved pulling, dropping, defending, dismembering, disgorging, swallowing, and sometimes abandoning it completely. A solitary vulture dismembered a hatchling, removing the head, and often much of the viscera, by holding the turtle body with its foot, and tugging upward vigorously and repeatedly. When we tried this feat, it required great strength, and often we could not dislodge a leg. Any vulture with a hatchling tried to retain control of the hatchling, while trying to remove the legs or swallow it whole.

When hatchlings were abundant (peak of emergence), defense may have been more costly than finding another hatchling and vultures that lost a hatchling to a pirate just looked for another rather than fight for it. Some vultures appeared satiated, and merely pulled off the head and legs to eat, and then reached for another hatchling, or stopped feeding altogether. During this time, we examined 100 dropped hatchlings, and found that: 2 were intact, 7 had the head and viscera missing, 58 had both foreflippers missing, 18 had one foreflipper missing, 7 had hind flippers missing, and 5 were open carapaces only.

We timed 171 hatchling captures by black vultures, but only 127 were complete. It was difficult to follow complete sequences (N = 16), and some were abandoned almost immediately (N = 28). The mean handling time for 49 adults when they were not interrupted was 49 $\pm$ 5.2 sec. However, when an adult vulture was interrupted by pirates (N = 58), the mean time for the hatchling to be swallowed was 92.3 $\pm$ 7.7 sec ($X^2 = 18.3$, $P < 0.001$). This involved up to 5 separate chases, by up to 5 different vulture pirates. There were only 20 juvenile vultures that ate hatchlings without interruption, or with one or two apparent parents guarding them, the mean time to swallow was 74.9 $\pm$ 12.7 sec, longer than required by adults ($P = 0.08$). It is likely that the one or two vultures near the juveniles were parents because they did not attempt to pirate from the juveniles, and did not allow other vultures near, Otherwise, juveniles did not pick up hatchlings first, but tried to pirate from others.

Foraging Opportunities. – While the vultures generally foraged by hunting, parasitism, or scavenging, there were many foraging opportunities on the Ostional beach. Although sometimes vultures foraged by searching for solitary hatchlings crawling toward the sea, or battling the surf, they usually searched for exposed nests or emerging hatchlings at nests, depending upon the point in the turtle nesting cycle. We identified six major types of foraging opportunities: 1) nests exposed by tidal action, 2) nests exposed by erosion of stream banks (shallow banks or vertical cliffs), 3) nests exposed by dogs digging up nests, and abandoning them before all eggs were predated (dog dug up, post-dog), 4) nests exposed on the surface, either by previous tides or winds removing sand, 5) emerging hatchlings and those crawling to the sea, and 6) eggs exposed by female ridleys digging up the eggs of previous nesters.

Table 2. Foraging behavior of black vultures when feeding on nests (eggs, embryos) on olive ridley hatchlings emerging on the surface or crawling to the sea (Ostional, Costa Rica, 2012)

	Overall			Nests (eggs/embryos)			Hatchlings (on surface)			X^2(p)
Number of observations	860			675			185			
Foraging depth (cm)	4.5	±	0.16	5.41	±	0.18	0.97	±	0.07	229 (<0.0001)
Number of vultures present	12.2	±	0.25	11.1	±	0.3	16.2	±	0.33	115 (<0.0001)
Number of vultures feeding	6.4	±	0.20	4.2	±	0.15	14.4	±	0.35	347 (<0.0001)
Number of vulture chases	2.16	±	0.07	2.3	±	0.09	1.6	±	0.1	11.1 (0.0009)
Percent of vultures feeding	54	±	1.0	45	±	1.0	89	±	1.0	219 (<0.0001)

Each of these opportunities attracted other vultures and other species of birds, which resulted in competition for the eggs or hatchlings. Further, these foraging opportunities were not equally available. Vulture foraged using these opportunities as follows (N = 858 nests): tidally-exposed nests (28%), stream-exposed nests (26%), emerging hatchlings (22%), dog-exposed nests (16%), surface (exposure cause unclear, 5%), digging female-exposed nests (4%). The latter was relatively low during the *arribada* because the vultures seemed unwilling to walk among the very dense nesting ridleys, seemingly because the turtles simply continued walking and would have walked over them if they didn't move, and they flew back to avoid the flinging sand when ridleys covered their nests.

Factors Affecting Foraging Behavior and Success. - Overall, there was an average of 12 vultures present within 5 m of an exposed nest (with eggs, hatchlings, or emerging hatchlings) being preyed upon, and half were actively feeding (e.g., able to get an egg or hatchling) within 30 sec (Table 2). In that time period, there was an average of only two vulture-vulture chases. The number of vultures eating was significantly correlated with the number of vultures within 5 m ($r = 0.48$, $P < 0.0001$), and negatively correlated with the number of dogs present or chasing vultures ($r = -0.29$ and $r = -0.26$, $P < 0.0001$), number of storks present and eating ($r = -0.24$, $P < 0.0001$ for both), and number of stork chases of vultures ($r = -0.23$, $P < 0.0001$).

Table 3. Models explaining variations in feeding behavior of black vultures feeding on eggs and hatchlings of Olive Ridley sea turtles at Ostional, Costa Rica. NS = not significant

	Number of Vultures Present	Number of Vultures Feeding	Percent of Vultures Eating
Model			
F (p)	86.8 (<0.0001)	327 (<0.0001)	75.7 (<0.0001)
df	12	12	12
r^2	0.55	0.82	0.52
Factors entering (F, p)			
Feeding Location	19.6 (<0.0001)	233 (<0.0001)	83.4 (<0.0001)
Depth of nest [a]	NS	NS	NS
Time	3.5 (0.06)	5.1 (0.02)	5.9 (0.02)
Number of Storks	10.3 (0.001)	123 (<0.0001)	60.0 (<0.0001)
Number of Vultures	-----	247 (<0.0001)	117 (<0.0001)
Number of Vultures feeding	247 (<0.0001)	-----	-----
Number of chases	159 (<0.0001)	6.1 (0.01)	5.3 (0.02)

a. Where predation occurred.

Between 47% (number of vultures present within 5 m of an exposed nest) and 82% (number of vultures feeding) of the variation was explained mainly by feeding location (feeding opportunity), number of storks, and number of vultures present (Table 3). The number of vultures present was largely explained by the number of vultures feeding (e.g., when it appeared that most vultures were feeding, other vultures were rapidly attracted to the nest), and the number of storks present. Thus, foraging at exposed nests became frenetic when more vultures were present, and more were feeding, still more vultures arrived and attempted to feed, resulting in threat displays, chases, and actual fights. The percent of vultures feeding (of the vultures that were present) was also dependent upon the feeding location, and the number of vultures and storks that were present.

These relationships are clearer from figure 6, where the number of vultures present, the mean number of vultures feeding, and the mean percent of vultures feeding is examined by foraging opportunity. In this figure we divided nests exposed by stream erosion into two types: cliff bank and stream bank. Both types were by streams, but cliff banks were steep cuts that were nearly vertical and stream banks had a shallower incline. On the vertical cliff banks, the eggs (and embryos) that were exposed often fell directly down a couple of meters to the bottom of the bank. Thus, vultures could eat both from the nest itself (which required rapid beating of wings to maintain their position on the steep cliff), and from the eggs dropped at the bottom. Thus, the number of vultures feeding, and the percent feeding was very high. In contrast, feeding from a stream bank meant that fewer vultures had access to space where the eggs were exposed. When dogs were actively digging up nests, some dogs defended these nests, and chased any vultures that came too close. Other dogs were more tolerant. Although not enumerated, dogs typically ate 2 – 5 hatchlings, and then retired for several minutes before resuming their digging. Vultures would take advantage of these interludes, and although vultures are poor diggers they occasionally captured hatchlings in this manner. There were fewer vultures that stayed, and far fewer were successful in obtaining eggs when dogs were present, although once dogs left vultures could obtain some eggs or hatchlings. Dog predation was easy to identify because they made large holes that were often a half meter wide and a third of a meter deep, with dirt piles scattered on one side. Dogs were mainly able to locate nests with hatchlings (either with remaining egg yolk or ready to emerge). For example, 66% of the nests dogs located had hatchlings, and only 34% had eggs. In contrast, only 20% of nest nests exposed along stream banks, and 10% of the nests exposed by tides contained hatchlings.

The number of vultures feeding decreased significantly with depth of the nest being predated, and was lowest in the middle of the day (Figure 7). When there were few vultures feeding, there was significantly less aggression, however, when over 10 vultures were feeding, the number of chases declined with increases in the number of vultures (Figure 7).

Feeding on emerging hatchlings (compared to eggs and hatchlings still in nests) provided a different situation in that they were both place-based (some emerging from the nest), and mobile (often scattered down the beach). Thus, once a nest with emerging hatchlings was dispersed, vultures descended in groups, numbers were high, and success was high because of the spatial dispersion, and the time required to eat a hatchling (30 sec to 4 min). Solitary vultures that captured hatchlings pulled off the legs, and the head, and then swallowed the rest. Once a vulture obtained a hatchling, it walked or flew a short distance away to eat it without being disturbed, allowing other vultures to move in and capture their own hatchling at the nest.

There were several significant differences between vultures feeding at nests with intact eggs, and those feeding on hatchlings, including foraging depth, number of vultures present, number of vultures feeding, and percent of vultures foraging but not number of chases (Table 1). Partly this is a result of the scattering of the hatchlings from the nests; eggs were all in the nest, whereas nests discovered in the early morning (under low light conditions) often had hatchlings strewn down the beach as they crawled to the sea. It took many minutes for a hatched clutch of turtles to completely emerge, and the earliest would reach the sea before the last had poked their heads out of the sand. This allowed vultures to scatter down the beach, picking them off. Most nests hatching in the early morning (05:00-07:30AM) were attended by a person, either from the ADIO, the refuge, or a tourist. Unattended hatchlings were all taken by vultures, storks, or grackles, and even when two of us attended a nest to time and protect the hatchlings, bold vultures or grackles succeeded in capturing a few.

Wood storks had a negative effect on the number of vultures feeding (successfully capturing an egg from a nest or an emerging hatchling), and on the percent of vultures feeding under many foraging opportunities (Figure 6). Wood storks were generally less aggressive, and moved in less rapidly to a dense group of feeding vultures, but when they did move in, they displaced the vultures, due mainly to their longer bill and larger size (wood stork length = 115 cm ; black vultures = 65 cm). Although the storks were less aggressive, they did chase vultures that came too close, with the effect that vultures then stayed away from them. On the other hand, storks were more wary of humans and did not make or tolerate an approach closer than about 20 m.

There were significant differences in the mean number of vultures foraging, and in the percent of vultures that were feeding for those feeding on nests at stream banks, on nests exposed by tides, and on hatchlings (Figure 6). In all cases, fewer vultures could feed when storks were present, and a significantly lower percentage of the vultures present were able to feed in the presence of storks compared to when no storks were present. The greatest difference, in both number and percent of vultures feeding, was when vultures fed on hatchlings. When no storks were present, 90% of the vultures that were present obtained a hatchling during a 30 sec observation period, but when storks were present, it dropped to 60% (Figure 6). Further, it appears that vultures moved away from foraging situations with storks because the storks dominated any nests where hatchlings were emerging. Because of their long bill, storks probed deeper to obtain eggs or hatchlings in the sand and capture hatchlings that had not reached the surface. As we approached, the storks would abandon this effort and walk away; the vultures would crowd in, but were unable to reach hatchlings due to their much shorter bills.

We also computed models to examine the number of vultures feeding on emerging and crawling hatchlings, compared to the number of vultures feeding on eggs, embryos, or sub-hatchlings (those nearly ready to emerge, but still below the sand surface, usually with some egg yolk attached). The former are viable offspring that had the potential to reach the sea successfully if not for vulture predation, while the latter did not (as the forces of nature had already exposed the nests, preventing their further development). Over 70% of the variation in the number of vultures feeding at nests (eggs/embryos) and on emerging hatchlings was explained by the number of storks and the number of vultures that were present, as well as feeding location, number of chases, and time of day for feeding at nests (Table 4). That is, when they fed on hatchlings that were moving away from the nest, only the number of storks

and vultures explained how many vultures were eating. In contrast, when they foraged on eggs and developing embryos at nests (place-based), feeding location, time of day, and aggression explained differences in the number of vultures that could feed.

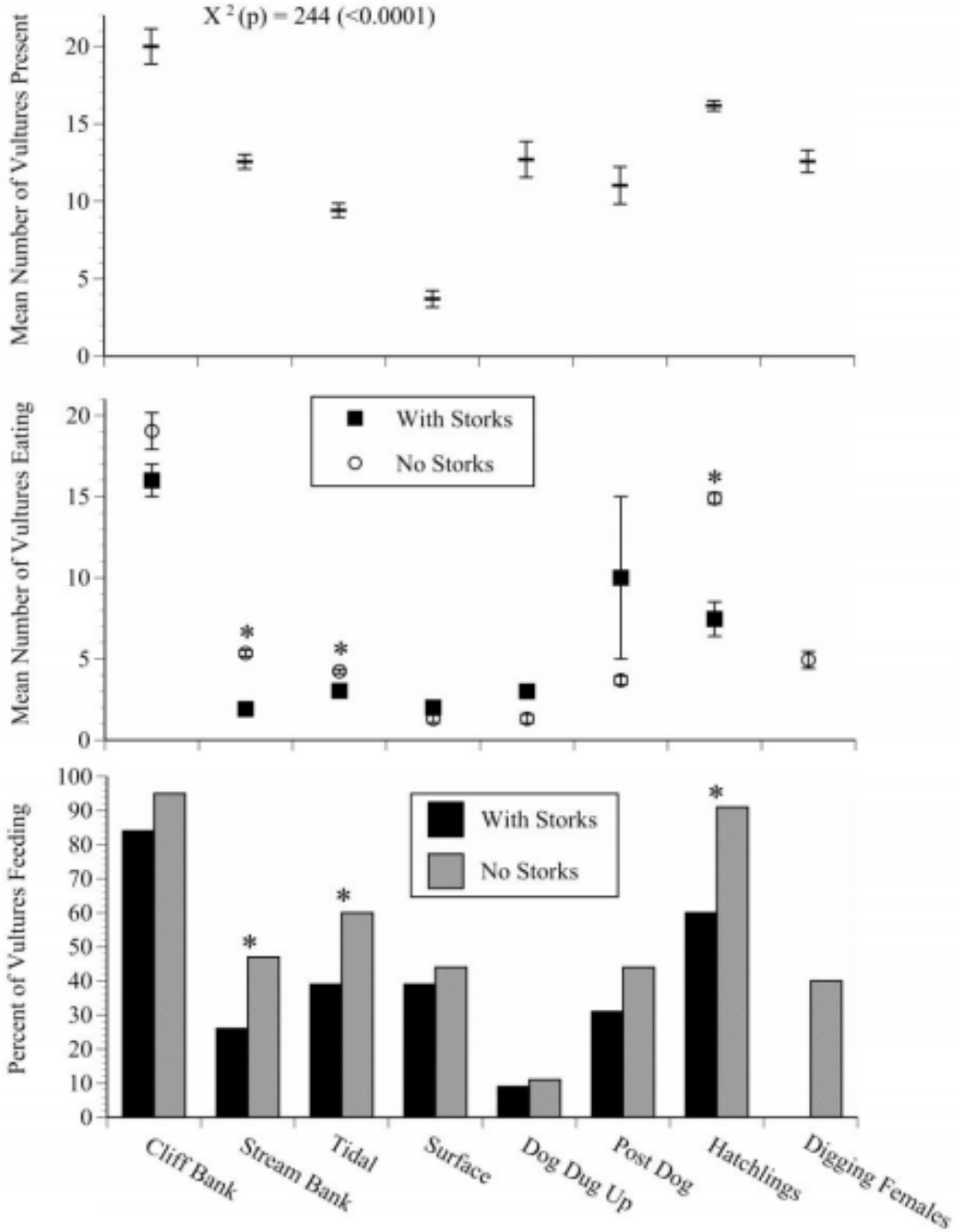

Figure 6. The relationships between the number of vultures present and foraging opportunity at Ostional.

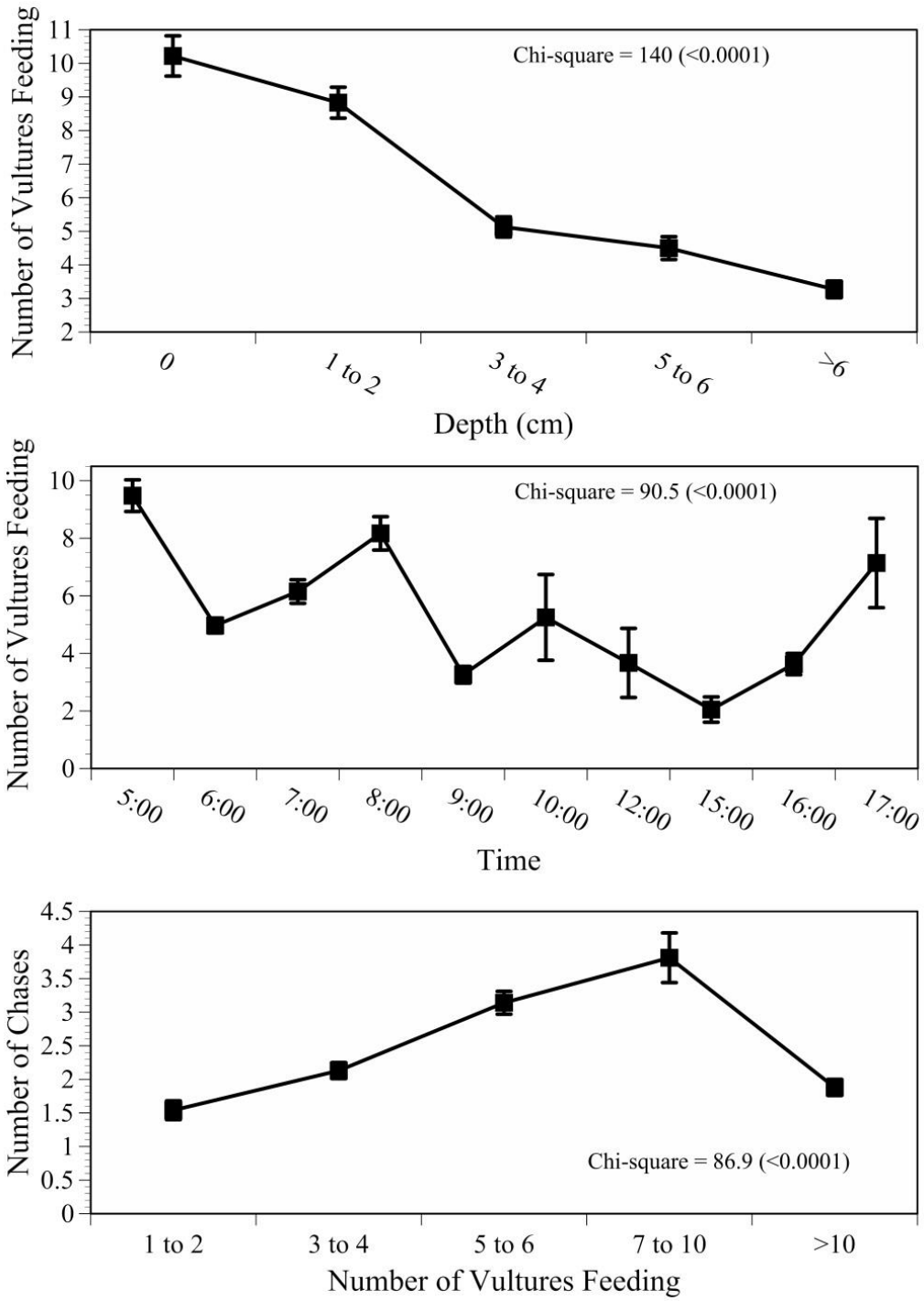

Figure 7. Number of Black Vulture feeding as a function of nest depth and time of day, as well as the number of chases by vultures as a function of the number of vultures present at a nest being preyed upon.

Table 4. Models explaining variations in behavior of black vultures feeding on olive ridley hatchlings at Ostional, Costa Rica. NS = not significant

	Number of Vultures Eating when feeding on hatchlings	Number of Vultures Eating when feeding on nests with eggs or embryos
Model		
F (p)	108 (<0.0001)	155 (<0.0001)
df	5	12
r^2	0.75	0.74
Factors entering (F, p)		
Egg or Developed Hatchling	-----	NS
Feeding Location	-----	164 (<0.0001)
Depth of nest [a]	NS	NS
Time	NS	17.8 (<0.0001)
Number of Storks	9.0 (0.003)	68.3 (<0.0001)
Number of Vultures	385 (<0.0001)	134 (<0.0001)
Number of chases	NS	19.8 (<0.0001)

NS = not significant.

DISCUSSION

Vulture Populations – At Ostional, black vultures have a variety of foraging strategies, and a range of foraging opportunities provided by environmental and social factors (Figure 8). These interact to provide continuous foraging opportunities that supported an average population of over 600 vultures foraging on the beach in the morning and late afternoon. The maximum number of vultures present was 1243 along the 1500 m section of beach that we surveyed immediately in front of and north of Ostional village. Even so, the actual local population of vultures available to exploit the turtles could have been much larger. We regularly surveyed only half of the beach that was available, and once vultures were satiated, some may have left to roost away from the beach.

Vulture populations would be expected to decrease during the vulture breeding season, when black vultures are breeding (half would be incubating at any one time). Further, vulture populations may change during migration periods when more birds move about Costa Rica, or when other food sources draw them from the beaches. Vultures in Costa Rica move to Panama in the winter (GRIN 2013).

Foraging Methods and Strategies - Environmental forces provided opportunities by exposing nests from stream bank erosion, tidal erosion, and wind erosion. Social factors provided opportunities because of the presence of other vultures and birds that indicated exposed or open nests, the presence of dogs that provided these opportunities, and the presence of people who exposed nests or scattered rotten eggs or developing embryos following egg harvesting. The presence of other predators provided additional opportunities for piracy, but also resulted in competition for eggs or hatchlings with vultures and other species (especially wood storks, Burger and Gochfeld, 2013, 2014a,b).

Physical forces, such as rain, wind, and tidal erosion provided the vultures with most of their foraging opportunities during the nesting cycle when hatchlings were not emerging. Stream and tidal erosion accounted for half of the total nests (whether they had eggs or

emerging hatchlings) that were destroyed by black vultures. In our study, more nests were preyed upon as a result of stream erosion than tidal erosion, but over the course of the year, tidal erosion may be a stronger driver because it occurs all year, and stream erosion occurs mainly during the heavy rainy season when large volumes of water result in the stream changing course nearly daily (and thus eroding one bank daily). Additional stream erosion occurred during our study when children played at stream bank edges, pushing the sand down to reveal nests, with the eggs falling down to the stream bed.

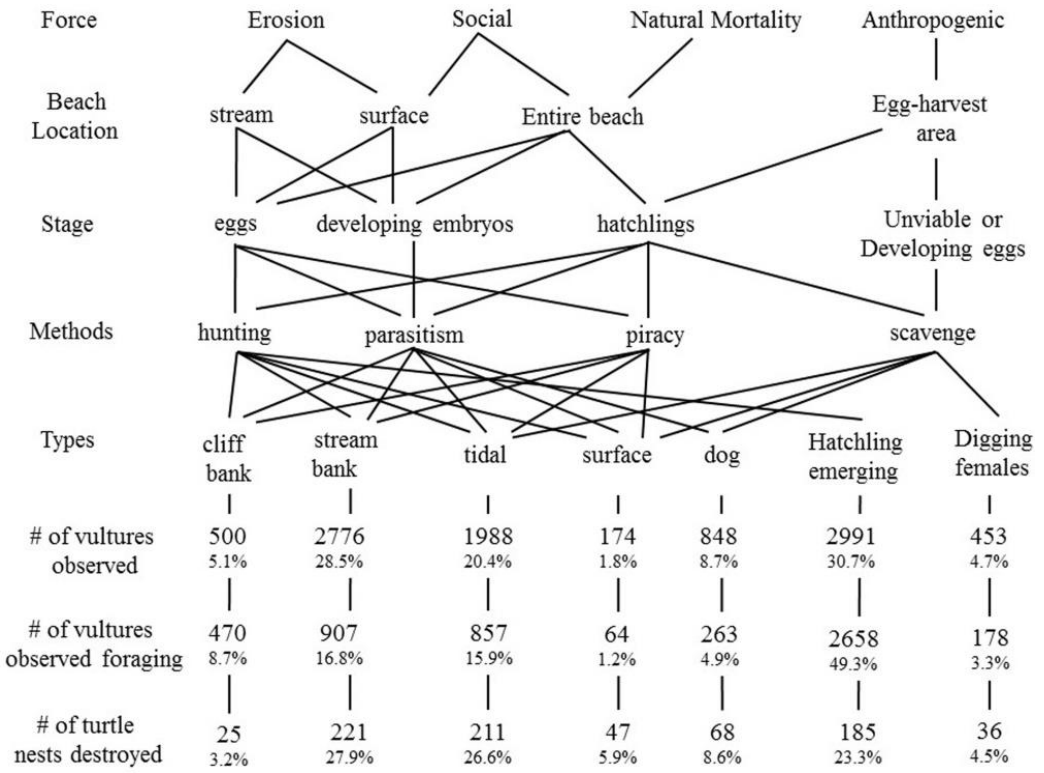

Figure 8. Vulture foraging opportunities provided by environmental and social factors. This is a model of Black Vulture foraging behavior, with data showing the relationships. Shown are the forces that affect the availability of foraging opportunities, the vulnerable turtle stages, methods of vulture predation, and the frequency of foraging types with the effects on the Olive Ridley sea turtles.

Tidal erosion would likewise vary during the month and the year, depending upon tide height, tidal force, and whether storms increased tide strength. Since females turtles choose where they nest, those nesting farther up the beach can decrease the chance that their nest experiences tidal erosion and decrease the likelihood that their clutch will be disturbed. However, nesting higher on the beach has costs in that hatchlings have farther to go to reach the water (which is likely not a problem for hatchlings emerging under darkness). Nesting later in the *arribada* also decreases the risk of their clutch being dug up by other females.

Most hatchling sea turtles emerge at night, and thus avoid most avian predators; Glen et al. (2006) found that 93% of green turtles (*Chelonia mydas*) emerged at night (Glen et al. 2006). The presence of emerging hatchlings provides the second greatest foraging opportunity because at the height of hatching, many emerged in the early morning after dawn (Figure 9).

The number of emergences declined during the morning. During the peak of hatching the early morning hatchers were protected by people on the beach, but after 08:00AM, the beach was largely abandoned, and on hot sunny days the sand heated quickly, reaching 50ºC, causing thermal stress and death to the late hatchers. Although there is nocturnal predation from mammals, owls, and yellow-crowned night herons (*Nyctassa violacea*), the risk for a hatchling at night is much lower than for those emerging in daylight. Thus, some of the offspring of those females survived to reach the sea. Except for the occasional nest dug up by a dog during the night, there was no evidence of nocturnal mammalian predation.

Figure 9. Hatchlings emerging from the nest in the early morning.

In other nests, however, the first hatchlings to emerge did so when there was light and most of these nests were detected by vultures, even before hatchlings had moved more than a few cm from the nest. The number of vultures that were coursing over the beach searching for nests was sufficiently high that nests were quickly noticed, vultures descended, and picked up those crawling to the sea or just emerging from the nest. Thus, hatchlings emerging in the early morning are exposed to intense vulture predation, and increasing sand temperatures. At higher temperatures, hatchlings overheat (Moran et al. 1999; Glen et al. 2006), and at Ostional, some died from the high temperatures. Hatchlings crawling to the sea over debris face additional stresses, as hatchlings can become ensnarled, increasing the time to crawl to the sea and exposing them to greater predation (Triessnig et al. 2012). Vultures at Ostional often patrolled debris piles searching for hatchlings caught there, and people seeking to help hatchlings survive, search debris piles as well. On most of the beach, debris was removed before the peak of hatchling, although those emerging in the first two days faced intense black vulture predation.

Further, hatchlings that emerged in the early morning when it was raining all made it to the sea because vultures flew from the beach to roost (often away from the beach) during heavy rains. Since it was the rainy season, on several rainy mornings many hatchlings reached

the sea safely. Although predation on hatchlings, particularly where large numbers emerge at once has been noted, predation rates on nests of olive ridleys vary. In India, nest predation was as high as 83% on sporadic (solitary) nests, but it was as low as 2.6% at *arribada* nests in some years (Tripathy and Rajasekhar 2009).

Competition and Social Parasitism. – Any specific vulture is not feeding in isolation, but is feeding within a context of many other vultures that are foraging on the beach, as well as other birds, dogs, and humans. The presence of other vultures has both positive and negative effects. Vultures watched other vultures far down the beach, and flew to places where other vultures were feeding, hoping to obtain food (social parasitism). Arriving at a nest where a group of other vultures were already present worked to varying degrees. In general, more vultures feed when more are present, but this is partly a result of two factors: 1) vultures continue to arrive and remain if they are obtaining food, and 2) foraging success differs under different foraging opportunities. For example, at cliff banks, the vertical aspect means that a vulture or two can try to feed at the nest itself, but many more forage on the stream bed where the eggs have fallen to the ground (thus more vultures are successful), When foraging on emerging hatchlings, each vulture that has captured a hatchling has to move away and spend time eating it, allowing others to move in. Social parasitism (searching for other birds that appear to be foraging at some distance away) worked quite well for scavenging on carcasses of adult ridleys washed up on the shore. Such carcasses were large, and they often were broken in many pieces, allowing many vultures to actively feed at once.

Another advantage of foraging with other vultures, although it was less apparent at Ostional, is predator warning (Coulson 2001). If vultures had predators, they would be vulnerable while foraging on the ground. At Ostional, the only threats to foraging vultures were dogs and people that chased them. Thus, the advantages of many vultures feeding on the beach was parasitism (taking eggs or hatchlings exposed by other vultures), social parasitism (location a food source found by other vultures, and flying to that location), and piracy (actually stealing eggs or hatchlings from others). The main disadvantage is competition, and the chance that another vulture will steal your prey.

At Ostional, wood storks provided the most competition because of their larger size and longer bill, allowing them to probe deep in the sand (Coulter et al. 1999; Burger and Gochfeld, 2013) (Figure 10). Whenever wood storks came to a nest located by vultures, they moved in and displaced the vultures. The mean number of vultures able to eat, and the percent of vultures feeding, decreased significantly at the most common foraging situations (stream bank erosion, tidal erosion, hatchlings emerging, Figure 6). Further, because storks have longer and thinner bills, they were able to probe down well below the sand surface to extract eggs or hatchlings that were unavailable to the vultures. In the case of nests exposed by tides or stream erosion, but with hatchlings ready to emerge from the nest in the next night or two, the storks were obtaining viable offspring. There were far fewer storks on the beach (usual flock of about 30), and they remained in areas far from the village of Ostional, Their effect on foraging vultures, however, was far greater than their numbers suggest.

At Ostional, the behavior of humans also greatly influenced vulture foraging. During the peak of hatchling emergence (6 days, from 05:00AM to 07:30AM), the beach was extensively monitored by ADIO members that scared off all the vultures. This allowed foraging by vultures only before 05:00AM (on clear days a few vultures arrived before that) and after 07:30AM (when some hatchlings continued to emerge, particularly on rainy days. Conversely, we also observed that vultures and grackles were present in beach segments

where we were studying hatchling behavior. Vultures braved our presence to snatch hatchlings we were watching, even within 5 m, and it appeared they quickly learned to associate our presence with food. Even during the morning period when vultures were being chased by ADIO members, the vultures appeared disproportionately in our segment, to snatch hatchlings either from the nest or wave front. Hatchlings encountering their first wave were often driven tumbling back up the sand, where they are readily captured by predators.

Figure 10. Wood storks often forage on eggs in nests that are exposed by the rising tides when females dig nests that are too close to the water's edge.

Finally, the foraging observations and interactions described in this paper apply to the time we made observations – that is, during the rainy season, the period of a large *arribada*. At other times of the year, the relative important of different forces (erosion, social factors) and of different types of foraging opportunities would differ. However, the foraging behavior and competition would not, and the vultures would make use of whatever foraging opportunities were available. Moreover, avian predation pressure will be influenced by the breeding season of the relevant species.

Effect of Vulture Foraging on Ridley Sea Turtle Success - The overall effect of black vultures foraging on olive ridley sea turtles at Ostional is a function of: 1) total number of turtles nesting and the availability of nests, 2) the strength of the different physical drivers (wind, rain, and tides), 3) total number of vultures and other predators present and their foraging interactions, 4) the presence of other predators (wood storks, dogs, other birds or mammals), and 5) human intervention. All of these factors vary over the year, many vary over a given month, and still others vary over the day. The number of nests and emerging hatchlings varies by month, the rains and tides vary monthly and daily, and the number of predators varies monthly and daily.

Overall, vultures foraging at Ostional do so within a context of environmental and social forces making eggs, developing embryos and emerging hatchlings available on several beach locations . This allows vultures to engage in several different foraging methods, scavenging

on eggs, developing embryos, and adult carcasses, parastizing or pirating from other vulture (or other birds), and actively hunting for emerging hatchlings. The relative number of vultures present in the different situation, the number of vultures able to obtain food, and the number of turtle nests destroyed varied among the types of foraging opportunities (Figure 8).

The number of nesting ridleys at Ostional varies from over 3,000 to over 450,000 nesting females in a single *arribada*. Thus, the number of nesting ridleys is gigantic compared to the number of vultures foraging on the beach. This is thus a classic case of predator swamping (Ims 1990, Cornelius et al. 2007; Colbert et al. 2010), whereby so many nests and hatchlings are present, and hatchlings emerge synchronously over a few days, that predators can only eat so many. Further, vultures are only able to feed at nests that are exposed by other forces, and on hatchlings that are emerging or crawling to the sea in daylight. Thus, their main effect is to "clean up" the beach of all the exposed eggs and hatchlings exposed by other forces, or that have died while crawling to the sea. This is a positive benefit as it reduces the chances of increased bacterial and fungal diseases (Trullas and Paladino 2007), thereby decreasing density-dependent mortality (Cornelius et al., 2007; Valverde et al. 2012). Although egg loss and hatching failure are no doubt major constraints for the turtle population, vultures do not contribute to this loss, but "clean up" exposed eggs and sub-mature hatchlings. Vultures do have an impact on mature and viable hatchlings, but only during daylight hours.

Despite all the foraging opportunities, the only viable offspring the vultures take are emerging hatchlings. All the others are eggs and developing embryos (albeit some nearly ready to emerge) that are not viable because they are exposed to the elements (heat, tides) by other forces, and would not develop further. Except on rainy mornings, the vultures (and other birds were able to take almost all of the hatchlings that emerged because so many vultures courses over the beach searching for hatchlings. However, at Ostional, members of ADIO patrolled the beaches and scared away all vultures during the peak egg-laying period. Thus, a high percentage of the hatchlings that emerged in the early morning (and few that emerged much after that time because of the high temperatures) survived. It was only those emerging in the days before and after peak hatching, and after about 07:30AM that were vulnerable to vulture predation.

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Chapter 33

MITIGATION STRATEGIES FOR THE REDUCTION OF SEA TURTLE BYCATCH IN THE MEDITERRANEAN BOTTOM TRAWL FISHERIES

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ABSTRACT

Incidental catch is the major threat to the survival of loggerhead turtles (*Caretta caretta*) in the Mediterranean. In this basin more than 100,000 turtles are estimated to be caught annually due to fishing practices. The mortality due to fishing capture (10-50% depending on fishing gear) can be considered as the main reason for sea turtle decline in this basin. Nevertheless, present knowledge about the interaction of sea turtles with fishing gears and the possible mitigation measures are still insufficiently studied. Successful strategies for sea turtles conservation mainly involve the knowledge of species biology and ecology, the identification of possible areas of interaction between turtles and fishing activities and finally the introduction of mitigation measures, such as gear modification through Bycatch Reducer Devices (BRDs). In this chapter two options adopted in the Adriatic Sea (central Mediterranean Sea) for sea turtle conservation have been described.

The first is a theoretical approach based on developing models that can predict the identification of areas and periods at high risk of trawl-turtle interaction.

Two set of data represented by telemetry data from tagged sea turtles and bottom trawl track from vessel monitoring systems (VMSs) were combined to provide an interaction index enabling prediction of potential trawl-turtle interaction hotspots and periods. The risk-analysis methodology here developed provides key information to

design and implement management strategies, and it might have a worldwide application, considering that Vessel Monitoring System is actually in force in several countries.

Once the areas and periods of possible trawl-turtle interaction have been identified, the effect of a flexible Turtle Excluder Device (TED) on the catching efficiency and performance of a commercial bottom trawl was tested in a gear comparison study in a Mediterranean coastal multispecies bottom trawl fishery.

Considering fishermen reticence to change the gear traditionally used, determining the optimal gear configuration to minimise the loss of commercial catch and avoid turtle captures, was the main issue of the study. Experimental sea cruises showed that the device affected neither technical performances of the gear (horizontal and vertical net opening and door spread) nor fuel consumption during fishing activities. Sorting of the catch into commercial, discard and debris (e.g. rocks, timber, marine litter) fractions demonstrated that TED use did not affect catch efficiency, since the catch of the major commercial species did not exhibit appreciable differences, whereas debris was strongly reduced when the TED was in use. Underwater video camera recordings documented that fish caught in the net swam through the grid and easily reached the codend, missing the TED escape opening. Easy storage and improved catch retention compared with previous devices tested in this area make the flexible TED a practical and valuable solution to reduce turtle bycatch in the coastal Mediterranean demersal fisheries. In conclusion, the synergy of the two approaches described, can be considered as a reliable mitigation strategy in Mediterranean sea turtle conservation.

Keywords: loggerhead turtle, sea turtle bycatch, trawl-turtle interaction, Interaction Index (I.I.), mitigation measures, flexible Turtle Excluder Device (TED), Mediterranean Sea

INTRODUCTION

The loggerhead sea turtle (*Caretta caretta*) is the most abundant turtle species in the Mediterranean Sea. Environmental characteristics (e.g. bathymetry, preys availability, sea water temperature, etc.) determine the distribution and abundance of *C. caretta* in this basin (Casale et al. 2012). Three main ecological phases characterize the life of this turtle: the pelagic phase, when loggerheads feed on pelagic preys; the demersal phase, when they swim close to the bottom to eat benthic species; and finally an intermediate neritic phase, when loggerheads shift from pelagic-oceanic to benthic-neritic foraging habitats (Tomas et al. 2001). Loggerhead turtles exhibit high fidelity to migratory routes, foraging areas and wintering sites. Nesting areas are concentrated especially on the eastern basin (Cyprus, Egypt, Greece, Israel, Italy, Lebanon, Libya, Syria, Turkey and Tunisia) and more than 3000 nests per season are estimated (Margaritoulis et al. 2003).

Nevertheless, recent studies suggest a decline over decadal scales of Mediterranean turtle populations. The main identified threats to sea turtles are degradation of reproductive habitats (Casale and Margaritoulis 2010), intentional killing (Tomás et al. 2008; Casale et al. 2010; Casale 2011), collision with boats, and incidental catch due to professional fishing (Casale and Margaritoulis 2010; Casale 2008, 2011; Casale et al. 2015; Lucchetti and Sala 2010; Lucchetti et al. 2016). Impact of fishing activities is of paramount importance among anthropogenic mortality factors, since the Mediterranean fleets share the same routes of turtles. In particular, fishing fleets of 21 countries regularly fish in this basin and an undefined number of small boats are active in non-EU countries. This results in more than 100,000 sea

turtles incidentally caught each year by a variety of fishing gears: 70,000 by longlines, 40,000 by bottom trawls, and 23,000 by bottom-set nets (Casale 2008, 2011; Lucchetti and Sala 2010) - with a mortality rate from 10 to 50% depending on gear. Thus, different types of fishing gears (towed or passive, on the bottom or in the water column) produce different captures and mortality rates (Gerosa and Casale 1999) and affect different ecological phases of turtle life-cycle. Moreover, bycatch figures are probably underestimated, since catch data are rarely reported by fishermen and information from North African countries is often poor.

The northern Adriatic Sea, the coastal waters of Tunisia and Libya, the areas south of Turkey and the Mediterranean coast of Egypt, where the continental shelf is large and turtles in the demersal phase are commonly found also in winter, are considered as the areas most impacted by bottom trawling (Casale et al. 2012). The northern Adriatic Sea (FAO Geographical Sub-Area 17, GSA 17; Figure 1), with its shallow waters and rich benthic communities, is a major feeding habitat for turtles in the demersal stage, especially for the populations nesting in Greece (Lazar et al. 2004). On the other side, its shallow depth and flat seabed are also considered an ideal fishing ground for bottom-towed gears. More than 1000 bottom trawlers, mainly from Italy and Croatia and to a lesser extent from Slovenia, Montenegro and Albania, usually operate in this area (Lucchetti and Sala 2012). The high density of bottom trawlers and sea turtles suggests that more than 10,000 annual capture events may take place in this area (Casale et al. 2004; Lazar et al. 2004; Lucchetti and Sala 2010). Mortality due to trawling is mainly caused by forced apnoea during towing activity, making towing time a critical factor affecting turtle mortality rates (Henwood and Stuntz 1987).

Present knowledge about the current level of threat affecting the Mediterranean loggerhead populations, its interaction with fishing activities and the use of possible mitigation measures are rispectively unclear and insufficiently studied. Common successful strategies for sea turtles conservation mainly involve the knowledge of species biology and ecology, the identification of possible areas of interaction between turtles and fishing activities and finally the introduction of mitigation measures, such as gear modification through Bycatch Reducer Devices (BRDs). Thanks to reseach activities carried out in TartaLife project (LIFE12 NAT/IT/000937) for sea turtle conservation in Mediterranean, finanched through the LIFE instrument of the European Community, two strategies adopted in the Adriatic Sea (Central Mediterranean Sea) for the reduction of sea turtle bycatch have been described in this chapter. The first is a theoretical approach based on developing models that can predict the identification of areas and periods at high risk of trawl-turtle interaction. In this study two set of data were analysed and combined. One dataset is represented by telemetry data; seven turtles, incidentally caught and subsequently rehabilitated in special rescue centres, were implanted satellite transmitters before their release. The other dataset results from Satellite-based Vessel Monitoring Systems (VMSs), worldwide used by management authorities as a surveillance and enforcement tool (Lee et al. 2010; Russo et al. 2011a; 2013; 2014). In European waters, all the fishing vessels with a Length Overall (LOA) $\geq$ 12 meter has to be equipped with VMSs, as requested by the European Regulation (European Commission 2009). VMS system enables to collect spatial information about fishing activity without any interference from fishermen notifications (Bertrand et al. 2007; Deng et al. 2005). Thanks to VMS data, it was possible to characterize the behaviour of Italian bottom trawl fleet, accounting for about 50% of trawlers exploiting this area. Even though other fishing activities also take place in the area of study, they do not seem to involve

a high risk of turtle bycatch (Lucchetti and Sala 2010). Telemetry data and bottom trawl track from VMSs were combined to provide an interaction index (I.I.) enabling prediction of potential trawl-turtle interaction hotspots and periods.

Once the areas and periods of possible trawl-turtle interaction have been identified, the effect of a new prototype of Turtle Excluder Device (TED) on the catching efficiency and performance of a commercial bottom trawl was tested in a gear comparison study. A TED is a grid-like device that diverts large objects (including turtles) towards an exit positioned before the codend (Mitchell et a1. 1995; Epperly 2003), with the aim of reducing turtle submergence and mortality. Some authors report that the TEDs tested until now are probably not a realistic solution for reducing turtle bycatch in the Mediterranean because they are designed for the shrimp trawl fishery and would exclude larger commercial fish (Laurent and Lescure 1994; Laurent et al. 1996; Casale et a1. 2004). However, Atabey and Taskavak (2001) and Sala et al. 2011 found promising results adopting the typical aluminium *Supershooter* grid since TEDs reduced anthropogenic debris and discards, consequently, sorting operations on board. However, the main trouble derived from the rigid structure of the TED, which often leaded to breaking the TED and the net too. In TartaLife project a new protype of TED, flexible, more lightweight, manageable and resistant, called FLEXGRID, has been experimented in coastal multispecies bottom trawl fishery. Thuis new TED was sufficiently flexible for safe winding around a standard net winch and stiff enough to maintain a rigid configuration during towing. Considering fishermen reticence to change the gear traditionally used, determining the optimal gear configuration to minimise the loss of commercial catch and avoid turtle captures, was the main issue of the study.

METHODS

Study Area

The study area was represented by Central - Northern Adriatic Sea (FAO GSA 17; Figure 1), whose shallow waters (< 100 m) and rich benthic communities are held to provide a major feeding habitat for adult loggerhead turtles.

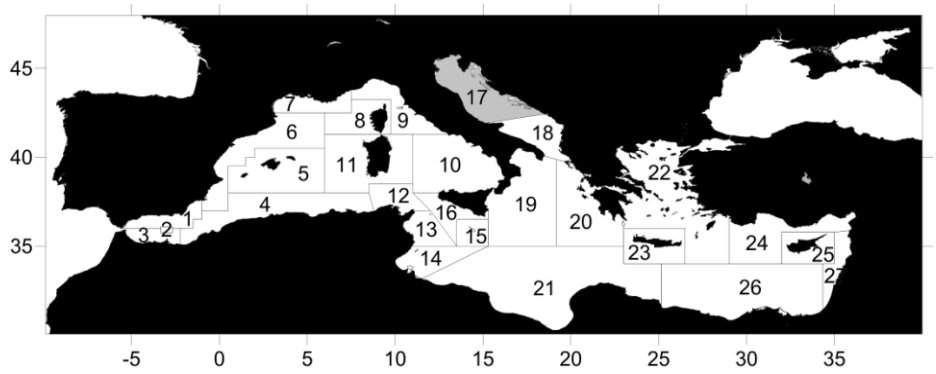

Figure 1. Mediterranean GSAs: in grey, Central - Northern Adriatic Sea (FAO GSA 17) which represented the study area.

Source: Adriatic Sea Turtles Database (2015).

Figure 2. Data on turtles stranded-caught-rescued in the Adriatic Sea in 2013.

Annual turtle bycatch in this area, which mainly involves Italian and Croatian bottom trawlers, has been estimated at more than 6,500 specimens (Casale et al. 2004; Lazar et Tvrtkovic 1995). However, data regarding only stranded-caught-rescued individuals in the Adriatic (Figure 2; Adriatic Sea Turtle Database 2015) reflect a high abundance of turtles in the area, suggesting that bycatch figures may actually be higher.

Assessment of Turtle-Trawl Interaction

Telemetry Data

Seven loggerhead turtles, incidentally caught by bottom trawl in the study area, were tagged with satellite transmitters and released, after rehabilitation in special rescue centres. Telemetry data provided information of the turtle positions and movements from 2006 to 2012. In detail, geographical (latitude and longitude based on the World Geodetic System [WGS-84]) and time (day and hour) coordinates, and location classes (LCs) representing

values grading signal accuracy (Royer and Lutcavage 2008; Casale et al. 2012) were recorded for each individual.

The dataset was analysed by year and season. Seasonal positions were translated into trajectories assuming that movement takes place along the direction that joins two consecutive points. A cell grid of the study area was constructed. To increase space use information, either cells with evidence of turtle presence and those intersected by trajectories were considered as "exploited" cells. Space use intensity was thus assessed (by season) and stratified in space using 5x5 min grid cells by counting the number of detections in each cell. Presence (in percentage) of the turtles monitored by satellite tags in each sub-area for each season. Finally, "presences" were ranked and classified according to a score from one to three: 1 if presence in percentage ranged between 0 and 0.33, 2 if varied between 0.33 and 0.66, 3 between 0.66 and 1.00.

VMS Data

The satellite-based VMS information was available for the whole northern and central Adriatic (FAO GSA 17), but only data for the western (Italian) side were analysed, to prevent that the lack of VMS data for the Croatian trawlers would affect the Interaction Index (I.I.) reliability. The VMS dataset, obtained from the Italian Ministry for Agricultural, Food and Forestry Policies, provided location (latitude and longitude in the WGS-84), course and speed data (km h^{-1}) of Italian commercial fishing vessels at 2 h intervals.

Fishing data were analysed using R-platform VMSbase freeware (www.vmsbase.org). An exhaustive description of VMS data collection and processing is reported in Russo et al. 2011b; 2013; 2014. A fully processed database for each year was thus obtained and the fishing effort was estimated by computing the number of "fishing" points - each representing 10 min of fishing - in each cell at the same spatial and temporal resolution of that used for turtle movement. For each season (*t*) the fishing effort detected in a cell was computed as the mean number of hours estimated over the whole period of time (*T*) and referred to the cell surface:

$$E_{i,t} = \frac{\left[\frac{\sum_1^T E_{i,t}}{T}\right]}{A_i}$$

where $E_{i,t}$ is the effort in cell *i* in season *t*, and *T* is the total number of years. Thus $\left[\frac{\sum_1^T E_{i,t}}{T}\right]$ is the mean effort detected in cell *i* in all years (*T*), and A_i is the area of cell *i*. Finally, the percent seasonal effort was calculated for each cell.

Interaction Index

Satellite data from the tagged turtles were combined with the VMS data of bottom trawlers. The model developed combined spatial and temporal information on the intensity of space use by turtles and on the fishing effort of the Italian trawler fleet in each season. The model was applied to predict the areas where trawl-turtle interactions are more frequent and the risk of bycatch was expressed as an I.I. The data of turtle density on a seasonal base (number of detections per cell) were converted into relative interaction probabilities by

calculating the likelihood that a turtle will occupy i-th cell relative to all cells *n*, using equations modified from Roe *et al.* (2014):

$$P\ (turtles)_{i,t} = x_{i,t}/\mathrm{n}\ (\sum_{i}^{n} x_{i,t})$$

where, $x_{i,t}$ is turtle density in cell *i* in season *t*, and *n* is the total number of cells in the grid.

Similarly, the probability of the fishing effort being carried out in grid cell *i* in *t*-th season relative to all cells *n* is calculated as.

$$P\ (effort)_{i,t} = E_{i,t}/\mathrm{n}\ (\sum_{i}^{n} E_{i,t})$$

Finally, the I.I. in cell *i* during the *t*-th season relative to all cells *n* was computed using the equation:

$$P_{(interaction)_{i,t}} = P_{(turtle)_{i,t}} P_{(effort)_{i,t}} / (\sum P_{(turtle)_t} P_{(effort)_t})$$

where $P_{(turtle)_t}$ is the sum of detections in the *t*-th season and $P_{(effort)_t}$ is the total amount of effort in the *t*-th season.

Flexible Turtle Excluder Device (TED)

In parallel with the prediction of trawl-turtle interaction hot-spots, a study on the performance and catching efficiency of a flexible TED (FLEXGRID) was carried out. This was the first study in the Mediterranean comparing a typical bottom trawl and a trawl equipped with a flexible TED. The choice of this new prototype has been driven by the fact that the models of TEDs tested previously (Sala et al. 2011) have shown some problems relating to the hauling operation logistics and resistance of the aluminium TED.

Trawl Gear and TED Specifications

The gear comparison study was carried out comparing the performance and cacth efficiency of a traditional bottom trawl gear with the same gear rigged with the FLEXGRID. Bottom trawl trials were conducted aboard the Italian Research Vessel “G. Dallaporta” (LOA, 35.30 m; gross tonnage, 285 GT; engine power, 810 kW).

The gear was a typical Italian commercial trawl commonly used in the central Adriatic, made entirely of knotless polyamide (PA) netting (Figure 3A). A 40 mm square mesh codend complying with European Council Regulation 1967/2006 was used in all hauls. Rigging components and trawling speed were those of typical central Adriatic trawl fisheries.

FLEXGRID was made of flexible plastic material and was able to keep a stiff configuration during trawling without hampering hauling operations. The grid was designed according to the technical specifications suggested by Mitchell et al., 1995: height 1130 mm; width 845 mm; circumference 3110 mm; bar diameter 20 mm; space between bars 96 mm (Figure 3B). The grid was mounted on a tubular netting section (Figure 3C) in front of the codend.

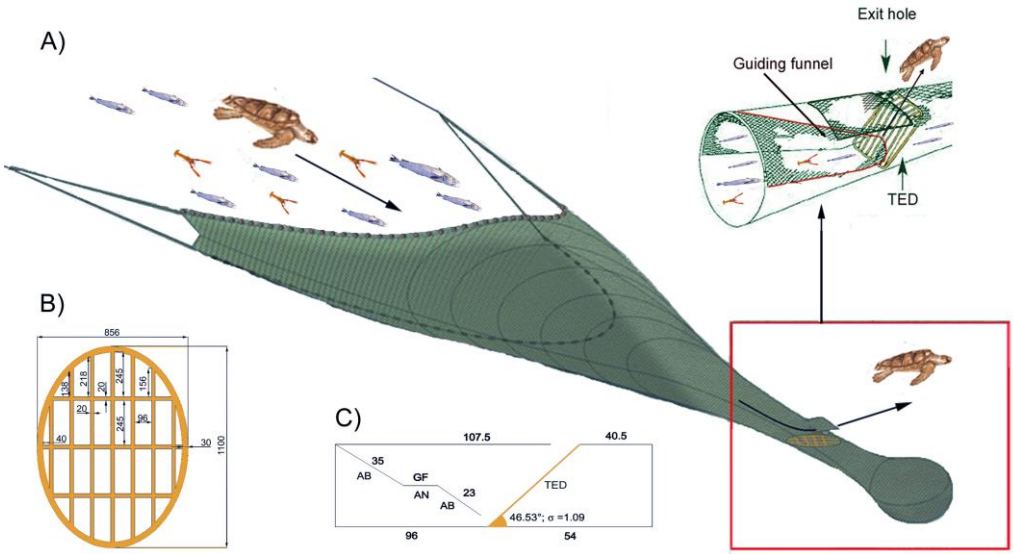

Figure 3. A) Detail of TED functioning and positioning in the trawl. B) Design of TED flexible TED used in the study. Details and size (in mm); C) Technical drawing of TED rigging (lateral view). GF, Guiding Funnel. AB and AN, types of net cuttings (figures indicate number of meshes). The average grid angle recorded during sampling is also reported.

The escape opening was cut on the upper portion of the net, just before the TED and it was covered by a netting panel functioning as an escape hatch both for larger objects (i.e. marine debris) and turtles. In addition, an accelerator funnel installed before the TED, drove the fish down and away from the exit panel and through the TED bars, towards the codend.

Study of Gear and TED Performance

Trawl performance was expressed in horizontal and vertical net opening [HNO and VNO, respectively] and door spread [DS]). To assess the grid's effects on net behaviour the SIMRAD PI50 system (Norway), represented by a PC laptop, hydrophone and acoustic sensors, has been used. To assess grid influence on the net drag and fuel consumption towing force has been measured by 2 TEKKAL (Germany) electronic load cells. Post processing data methodology is reported in detail in Prat et al., 2008 and Sala and Lucchetti, 2011.

The TED angle is a key factor influencing TED efficiency and preventing loss of commercial species during the tow (Mitchell et al., 1995; Eayrs, 2007). An optimal angle is estimated between 40° and 55°; in this regard, the TED angle was continuously monitored by an angle sensor (Star-Oddi DST pitch and roll, Iceland) that collected pitch and roll angle data.

Underwater cameras GoPro *Mod. Black Hero 3* (US) were additionally used to monitor grid position during the haul, grid behaviour with the commercial catch, fish reaction to the TED and fish behaviour inside the net, and grid effectiveness in ejecting debris.

Catch Analysis

The gear comparison study was performed by adopting alternate haul method.

Basically, the net rigged with the TED was the experimental gear and the traditional net was used as “control”. Since experimental hauls were carried in close succession over limited

time periods and the fish population in this area was not expected to vary considerably between hauls, no attached netting bag to the escape opening (to monitor the catch deflected by TED, see Sala et al. 2011) was used. This strategy was chosen to not influence TED behaviour during towing.

Catch was sorted into commercial, discard, and debris (i.e. rocks, timber and marine litter) fractions. Commercial and discard species were classified to the lowest possible taxonomic level, and their biomass was standardized as mean catch per hour ($g \times h^{-1}$). Because the mean of the original (non-transformed) data could be oversensitive to extreme values, and confidence intervals were large, catch rates were transformed using a natural logarithm (Cochran 1977).

One way analysis of variance (ANOVA) was applied to highlight significant differences among categories (commercial, discard, debris and total catch). In case of significant differences, a multivariate multiple permutations test (SIMPER Similarity Percentage analysis, described by Clarke 1993) was used to determine which species were responsible for the differences between the two nets. The analysis was applied to the biomass data and performed using PRIMER (Plymouth Routines in Multivariate Ecological Research) software.

At the end of each haul, the size frequency distributions of the main commercial species were calculated to the nearest 0.5 cm: total length (TL) for fish; mantle length (ML) for cephalopods; and carapace length (CL) for crustaceans.

Random subsamples were taken in case of abundant catches. The length frequency distributions of the main commercial species caught with and without the TED were compared by two samples Kolmogorov-Smirnov test (Sokal and Rohlf 1995), to evidence any possible TED influence on the sizes of fish caught.

RESULTS

Identification of Turtle-Trawl Interaction

Data obtained from satellite tracking were clearly in line with the data available from literature (Lazar et al. 2003; Lazar and Tvrtkovic 1995; 2003; Casale et al. 2004; Casale and Margaritoulis 2010; Vallini et al. 2006), suggesting that the monitored turtles were representative of the Adriatic population.

Telemetry data documented a broad variety of movements, but a seasonal pattern could however be recognised (Figure 4). Turtles seem to winter in the central Adriatic Sea and mainly along the eastern coast, in spring they move from the south Adriatic towards the Po delta, in summer they concentrate to the north of this basin and begin to migrate southward, and in autumn they are scattered throughout the Adriatic with a concentration in the coastal area south of the River Po delta (Figure 4).

Bottom trawl activities were roughly homogenous throughout the year, operating both inshore and offshore (Figure 5). Fishing effort varied according to the seasonality of the main target species. According to VMS data, a high concentration of fishing activities was observed inshore (within 10 miles from the coast) in autumn (Figure 5), after the seasonal fishing ban (i.e. August-September).

The I.I. resulting from the spatio-temporal combination of the two set of data varied within the year depending on seasons and fishing areas (Figure 6). In summer and, more clearly in winter, the I.I. showed a moderate-to-high bycatch risk in some areas, mainly in fishing grounds south of the River Po delta, and a low bycatch risk in the northernmost Adriatic areas. In spring, moderate risk of bycatch was predicted only for fishing grounds in front of the River Po delta. In autumn, a moderate bycatch risk was estimated in the fishing grounds south of the River Po delta; in this case the I.I. demonstrated a very broad area of potential impact, considering the high concentration of vessels inshore. Mean monthly data on stranded turtles, collected between 2003 and 2013 in the coastal area south of the River Po delta (Adriatic Sea Turtle Database 2015), confirmed a high risk of fishery-turtle interactions with a peak in autumn-early winter.

The variability of the I.I. can be explained by taking into account the seasonal migration routes of both turtles and main fisheries target species.

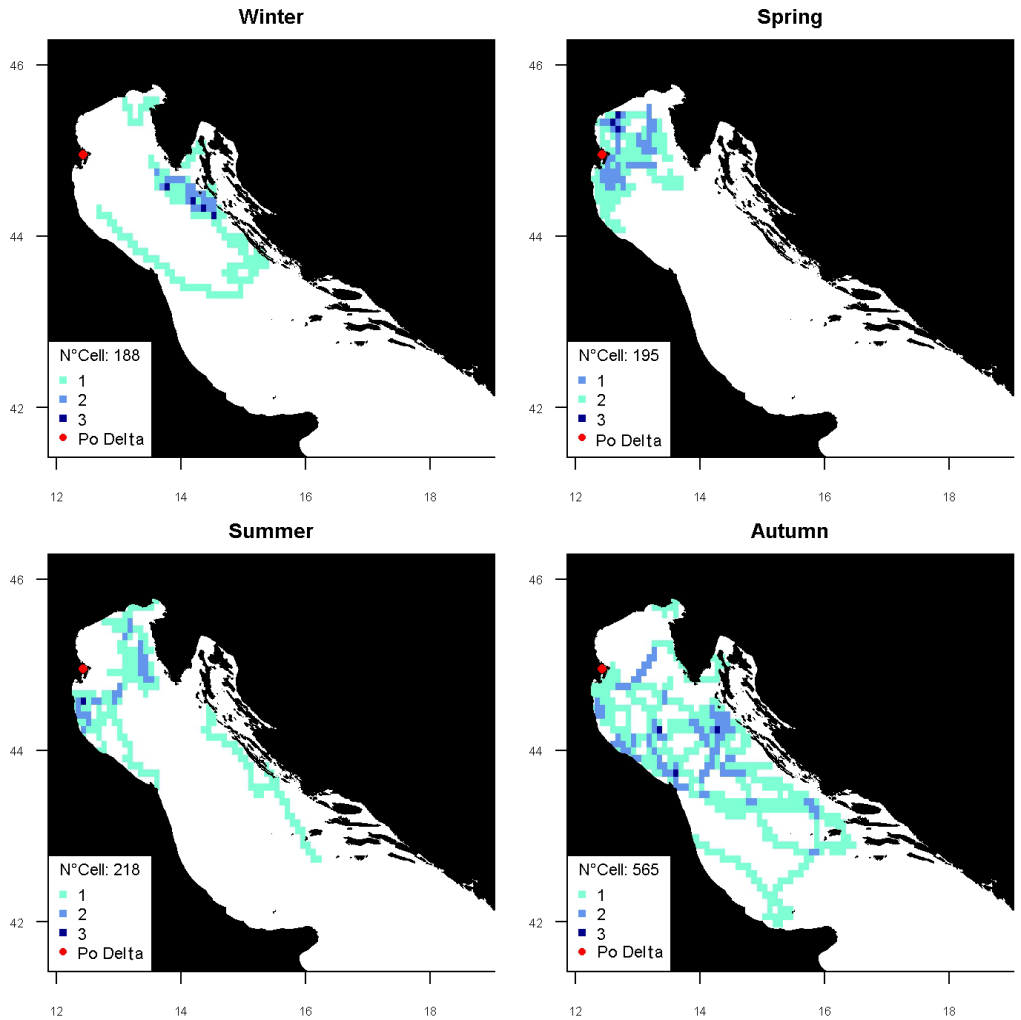

Figure 4. Index of intensity predicting the probability that loggerhead turtles will be swimming in 5x5 min grid cells (central and northern Adriatic). 1, 2 and 3 indicate the intensity of detections in each cell (sum of detections).

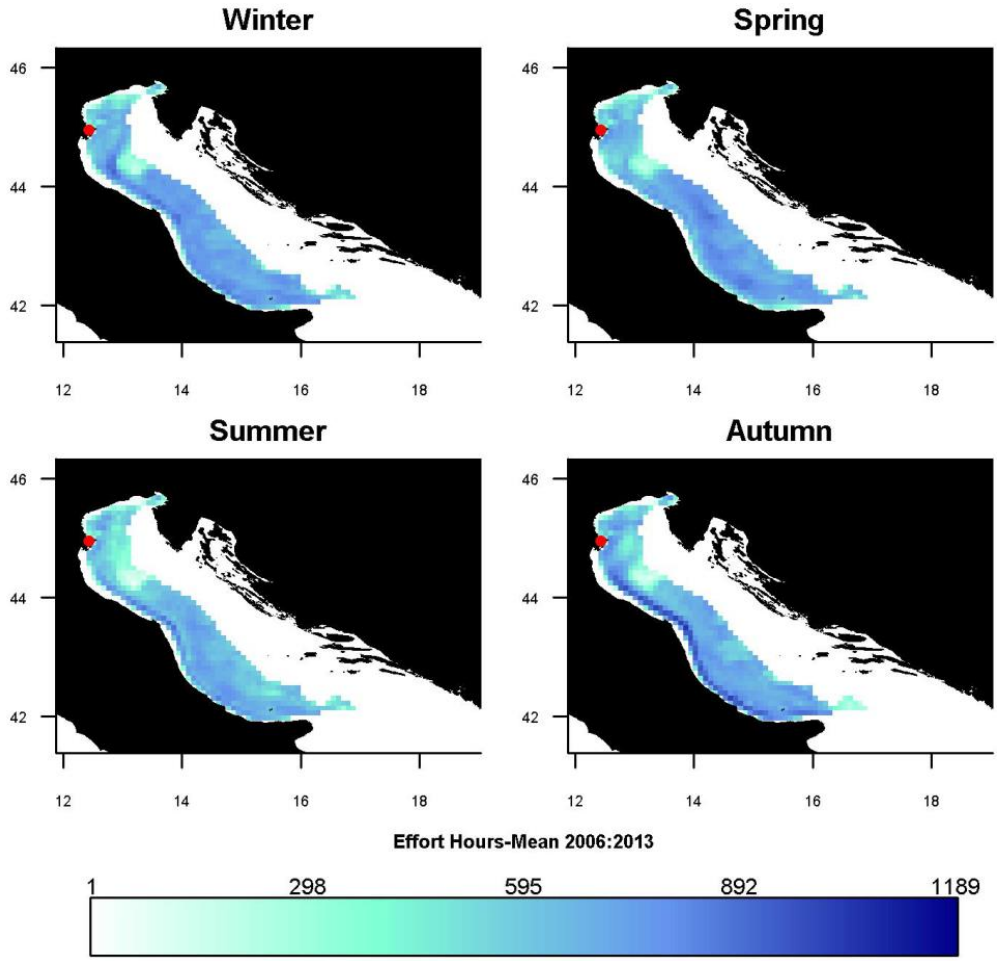

Figure 5. Index reflecting the probability that bottom trawlers will be fishing in each 5x5 min grid cell (central and northern Adriatic). Values are mean hours/cell area of trawling calculated in 2011-2013, stratified by season.

In spring the risk of trawling-turtle interaction in the north Adriatic is high due to the nutrient inputs from the River Po (the primary source of freshwater and nutrients in this basin; Degobbis and Gilmartin 1990) and their effect in terms of increased phytoplankton concentrations (Böhm et al. 2003; Spillman 2007) which result in ideal foraging conditions for turtles due to prey abundance.

Considering that, the main large-scale surface circulation pattern in the Adriatic is cyclonic (Orlic et al. 1992; Artegiani et al. 1997; Poulain 2001), in spring and summer nutrients follow counter-clockwise currents and are more densely concentrated in coastal areas south of the River Po delta, which is a nursery area for most of commercial species targeted by trawlers. In late summer-autumn most commercial species migrate from inshore growing areas to offshore recruitment areas.

Concurrently, in this period, in Italy a one-month fishing ban is established. When fishing resumes, target species have returned to the coastal areas, resulting in a high concentration inshore of fishing effort. A high density of both turtles and trawlers thus occurs in this area in autumn, explaining the high I.I. and risk of bycatch.

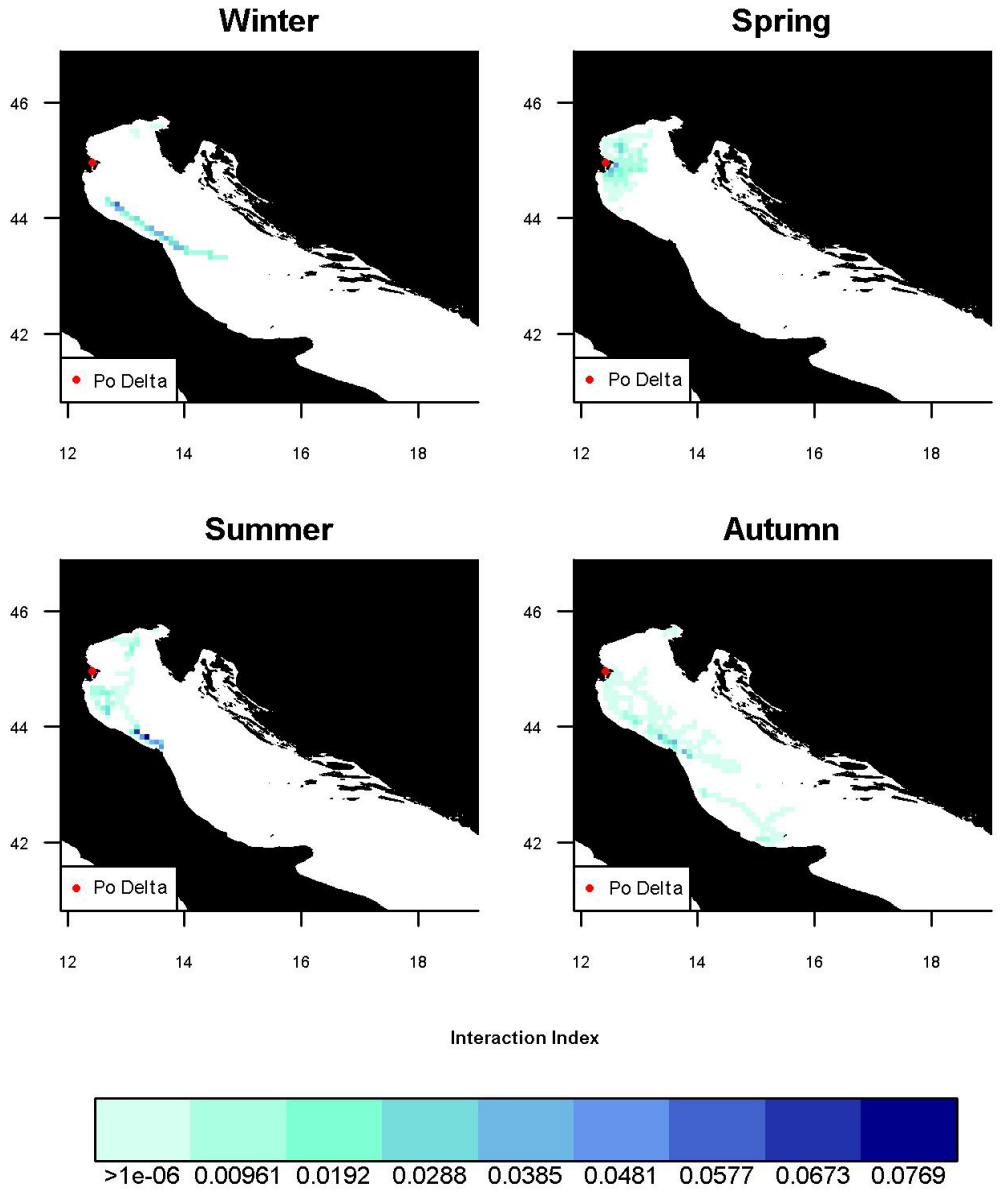

Figure 6. Relative interaction indices of loggerhead turtles and bottom trawlers in 5x5 min cells (central and northern Adriatic). Values derived from the interaction index equation represent the relative bycatch risk for each season and grid cell combination.

This is also confirmed by the records concerning stranded turtles in this area. In winter the main driving factor affecting sea turtle movements is due to sea temperature. The warmer Levantine sea current from the east Mediterranean contribute to increase water temperature in the central and east Adriatic (12-14°C; Russo et al. 2012), offering a warmer wintering habitat compared to the rest of the central-northern Adriatic, where cold winter winds (Bora) sometimes reduce the sea surface temperature to 5-6°C.

This is the reason for the high occurrence of turtles detected by satellite in Croatia in winter. Even though the lack of VMS data for Croatian vessels did not allow calculation of

the I.I. for the east side of the northern-central Adriatic, the likelihood of bycatch might be nonetheless high, especially in the northern Adriatic. Neverthless, this area is less accessible to trawling due to huge number of small islands, and other gears are used in coastal waters (i.e. passive nets and longlines). A high risk of capture also applies to the turtles remaining in the central part of the basin in winter, due to the intense fishing effort, as demonstrated by the high number of turtle stranded in the central Adriatic-west coast in this period.

Flexible Turtle Excluder Device (TED) Performance

During sea trials 25 hauls were performed with TED and 30 with traditional trawl. The main results of the gear comparison study on gear performance and catch efficiency are reported in Table 1.

Gear Behaviour

The technical parameter of the two gears monitored did not show any statistical difference (Table 1) confirming that the TED did not affect bottom trawl behaviour.

In detail, the mean values of the geometric parameters monitored with the acoustic sensors were for traditional gear and TED equipped gear rispectively: HNO = 15.1 ± 0.4 m and 15.4 ± 0.3 m, HVO = 1.2 ± 0.1 m and 1.1±0.1 m, DS = 56.6±1.6 m and 58.8±1.2 m, and TF = 3009 ± 56.7 kgF and 3070 ± 47.2 kgF. The mean TED angle recorded during towing was appreciable (46.5° ± 1.1) and in line with Mitchell et al., 1995 guidelines.

Moreover, the flexible TED did not affect routine onboard procedures or the time required for shooting, confirming its easy use and manageability.

Table 1. Standardized catch (kg·h-1) and gear performance measured in each haul: TED, net equipped with a TED; Tradit, net in a traditional configuration (without TED). Total catch and catch are divided by categories: COM, Commercial; DIS, Discard; DEB, Debris. Gear technical parameters: HNO, Horizontal Net Opening; VNO, Vertical Net Opening; DS, Door Spread; TF, Towing Force

Gear	HNO [m]	VNO [m]	DS [m]	TF [kgf]	COM [kg/h]	DIS [kg/h]	DEB [kg/h]	TOT [kg/h]
TED	15.4±0.3	1.1±0.1	58.8±1.2	3070±47.2	29.7±3.6	17.5±2.2	7.1±2.1	54.3±5.7
Tradit	15.1±0.4	1.2±0.1	56.6±1.6	3009±56.7	20.4±2.6	14.1±2.2	13.8±2.4	48.1±4.2

Catch Efficiency

Commercial catch accounted on average for 42% and 55% of total weight for traditional gear and TED equipped gear respectively (Figure 7). Furthermore, debris was reduced by TED from 29% to 13%, whereas the discard fraction showed similar values (29% without TED and 32% with).

Analysis of dissimilarity (Table 2) showed that most of the catch difference between the two nets was due to horse mackerel (*Trachurus spp*), a gregarious pelagic species whose presence in the fishing grounds varied randomly from one haul to another.

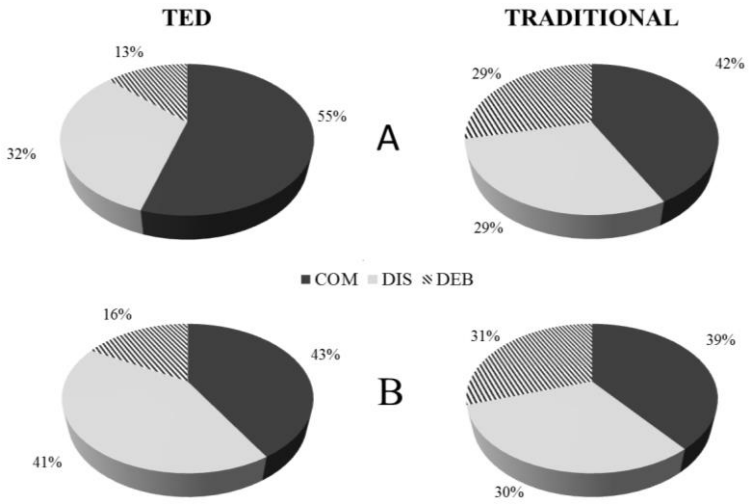

Figure 7. Percentage of the three categories found in the trawl with and without the TED: a) COM, Commercial; DIS, Discard; DEB, Debris; TED, trawl equipped with TED; Tradit, trawl without TED. b) The scenario obtained after exclusion of Trachurus spp from the analysis is also shown.

Table 2. Summary of SIMPER analysis. Average abundances (Avg. ab.), % contribution (Cont. %) and % cumulative contribution (Cum. %) to the average dissimilarity (Avg. Diss.) are given for the most important commercial species within the two nets (TED, net equipped with TED; Tradit, traditional trawl without TED)

Species	Avg. ab. TED	Avg. ab. Tradit	Cont. %	Cum. %	Avg. Diss.
Alloteuthis media	11.57	18.06	2.06	84.88	1.14
Arnoglossus laterna	20.51	18.81	1.67	90.14	0.93
Bolinus brandaris	9.44	15.12	3.97	59.97	2.2
Chelidonichthys lucernus	17.88	18.65	4.35	56	2.41
Diplodus annularis	8.48	0.87	1.75	88.47	0.97
Eutrigla gurnardus	9.87	9.54	2.94	70.2	1.63
Gobius niger	6.57	12.79	2.53	78.29	1.4
Illex coindetii	18.57	10.41	3.75	63.72	2.08
Loligo vulgaris	15.09	11.9	3.54	67.26	1.96
Melicertus kerathurus	18.25	12.91	2.64	75.77	1.46
Merlangius merlangus	7.57	7.75	1.84	86.72	1.02
Merluccius merluccius	38.36	27.01	5.53	32.19	3.06
Mullus barbatus	51.67	34.66	11.42	26.66	6.33
Raja asterias	4.91	6.49	2.08	82.82	1.15
Scomber japonicus	22.01	5.28	4.63	51.65	2.56
Scomber scombrus	24.16	24.26	4.65	47.03	2.57
Sepia officinalis	23.9	22.76	5.1	37.29	2.83
Solea solea	12.46	9.44	2.93	73.13	1.62
Squilla mantis	36.65	39.31	5.09	42.38	2.82
Trachurus spp	78.73	32.68	15.23	15.23	8.44
Trisopterus minutus capelanus	7.23	8.58	2.45	80.74	1.36

Average dissimilarity = 55.37

Its removal from the data analysis showed a similar catching efficiency for the two configurations (Figure 7), respectively 43% and 39% with and without the TED (ca. 18 kg/h in either net), and that only debris was significantly reduced (from 31% to 16%; $p = 0.015$).

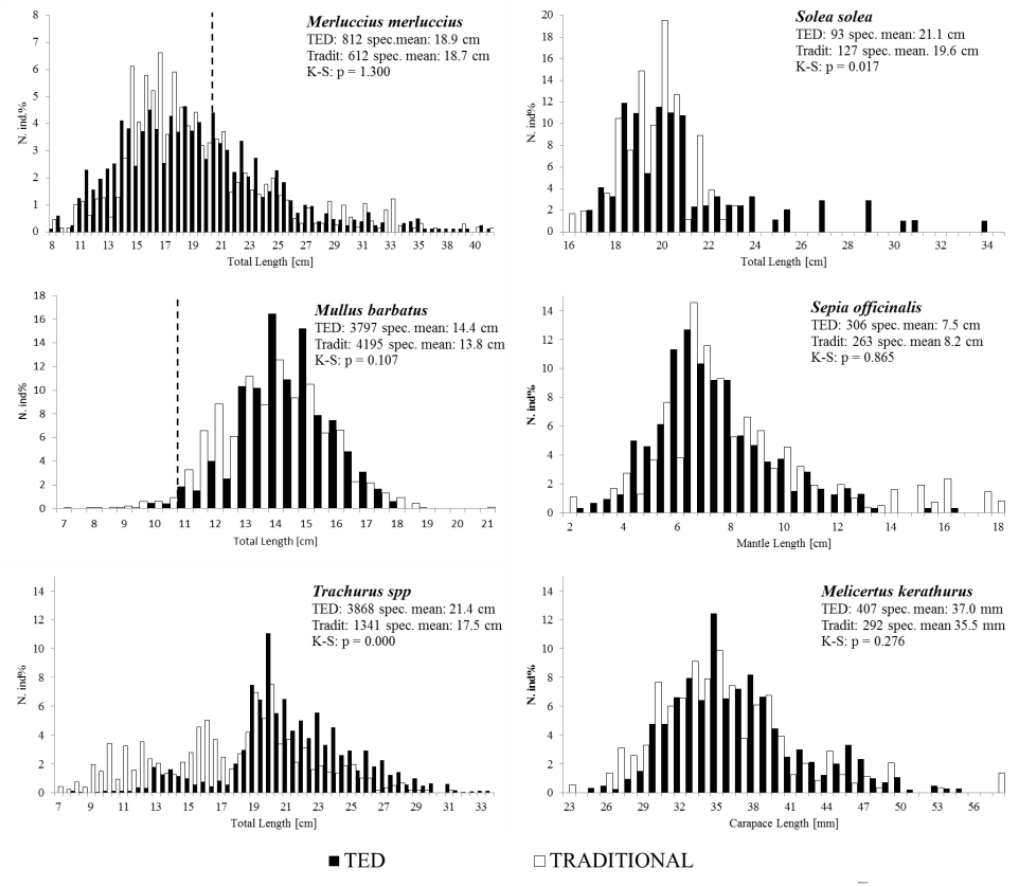

Figure 8. Size frequency distributions of the major commercial species and total length (fish); mantle length (cephalopods); and carapace length (crustaceans). Dotted lines indicate the MCRS (Minimum Conservation Reference Sizes). Spec, specimens. K-S: Kolmogorov-Smirnov test.

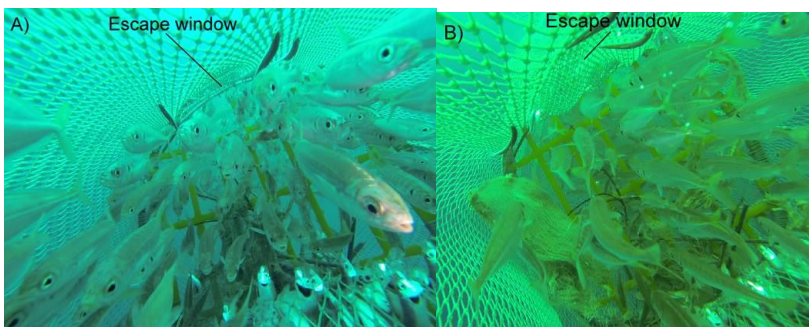

Figure 9. Underwater images of fish behaviour in the TED equipped net: a) fish swam upstream (towards the mouth of the net) during towing, whereas at the end of the haul b), when the vessel speed reduced, they turned towards the codend, swam through the TED bars, and reached the codend, missing the escape opening.

The size frequency distributions of the major commercial species caught by the two nets (Figure 8) showed a significant difference (Kolmogorov-Smirnov test) only for horse mackerel and common sole (*Solea solea*), while the modal class was similar for all species.

The trawl rigged with the TED generally caught commercial species with a greater mean length excepting the cuttlefish (*S. officinalis*). The capture of European hake (*M. merluccius*) and common sole specimens below the Minimum Conservation Reference Sizes (MCRS) were common in both nets. This is manly due to to the low selectivity of Mediterranean trawling.

Finally, the underwater video-recordings allowed to understand the fish behaviour in the TED equipped net. In particular, fish swam upstream (towards the mouth of the net) during towing (Figure 9A), whereas at the end of the haul, when the vessel speed reduced, they turned towards the codend, swam through the TED bars, and reached the codend, missing the escape opening (Figure 9B). Moreover, it was possibile to demonstrate that a large amount of marine litter was deflected by the grid (Figure 10), thus enhancing the quality of fish caught in the codend.

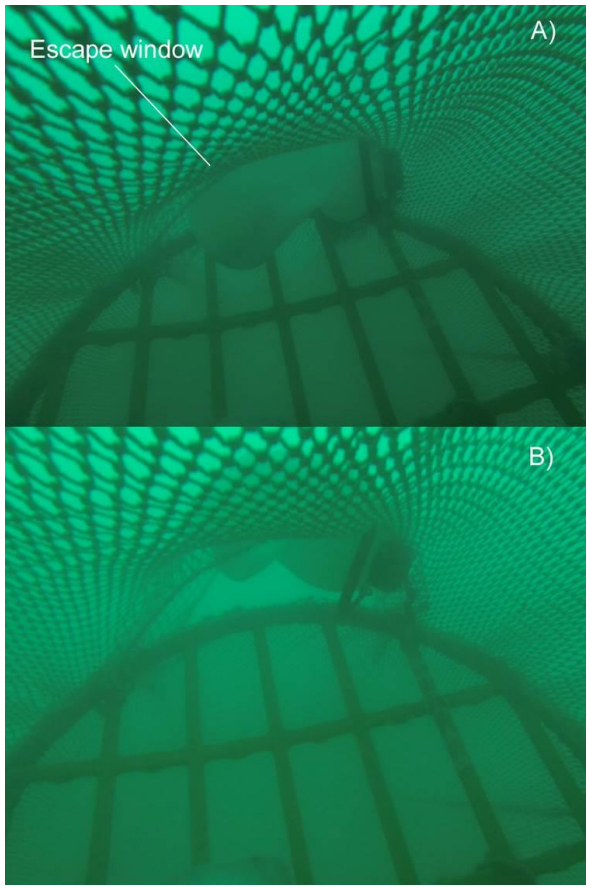

Figure 10. Underwater images of marine litter (in this case a plastic can): a) capture and b) following ejection through the escape opening (TED equipped net).

Video-recordings also enabled to immortalize the capture of three individuals of *C. caretta* (Figure 11): two turtles were released from the net with TED, while one turtle was found in the codend of the traditional gear.

However, the low number of encountered turtles prevented any statistical evaluation of the TED efficacy, but the preliminary results are promising. The exclusion of considerable amounts of marine litter strongly suggests that the flexible TED can be equally effective with turtles, which moreover can actively swim out of the net.

Figure 11. *C. caretta* caught during trawl by TED equipped net. The turtle was successively ejected through the escape opening.

CONCLUSION

According to telemetry data, the seven *C. caretta* individuals resided and spent the whole year in the Adriatic. This result, which is supported by the increased number of turtles stranded in the Adriatic in the last few years, is probably due to the higher sea temperature. A broad variety of movements have been recorded during the year demonstrating seasonal patterns (Casale et al. 2012; Lucchetti et al. 2016).

The analysis of trawl-turtle interactions in the Northern Adriatic (FAO GSA 17) presented herein allowed identification of the areas at highest potential risk of sea turtle bycatch in each season. Even though satellite data were available for seven turtles and detailed VMS data on trawler activity were available only for Italian trawlers, the methodological approach can be further tested in other areas and integrated with new data. Recently, Slovenia and Croatia have also joined the EU and will be required to monitor their vessels by VMS. The additional data will hopefully contribute to draw a more exhaustive picture of turtle movement and distribution patterns in the Adriatic. The present pilot study can be considered as a risk-analysis approach directed at identifying the areas and times of

possible trawl-turtle interactions. By identifying the areas at highest risk of turtle bycatch, the I.I. has the potential to provide key information to design and implement mitigation strategies. The I.I. enabled to predict a high risk of trawl-turtle interaction in coastal areas. In this regard, a new prototype of Bycatch Reduction Device was tested to deal with this problem. Experimental sea cruises showed effective improvements by using the new flexible grid, compared with TEDs developed in previous studies (Lucchetti et al. 2008; Sala et al. 2011). FLEXGRID was able to maintain a rigid configuration during towing and was sufficiently flexible for safe winding around a standard net winch. Therefore, it did not require changes to onboard procedures and did not waste fishermen's time during hauling. Moreover, this device affected neither technical performances of the gear (horizontal and vertical net opening and door spread) nor fuel consumption during fishing activities. Sorting of the catch into commercial, discard and debris (e.g. rocks, timber, marine litter) fractions demonstrated that TED use did not affect catch efficiency, since the catch of the major commercial species did not exhibit appreciable differences, whereas debris was strongly reduced when the TED was in use. Underwater video camera recordings documented that fish caught in the net swam through the grid and easily reached the codend, missing the TED escape opening. Easy storage and improved catch retention compared with previous devices tested in this area make the flexible TED a practical and valuable solution to reduce turtle bycatch in the coastal Mediterranean demersal fisheries. In conclusion, the synergy of the two approaches herein described, can be considered as a reliable mitigation strategy in Mediterranean sea turtle conservation. More in general, identification of bycatch hotspots, coupeld with BRDs implementation and spatio-temporal closure of some areas may contribute to a more effective management of fisheries and to the conservation of endangered species at a global scale.

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Chapter 34

SPATIAL-TEMPORAL DISTRIBUTION OF KEMP'S RIDLEY TURTLES (*LEPIDOCHELYS KEMPI*) AND GREEN TURTLES (*CHELONIA MYDAS*) NESTS IN A BEACH OF THE NORTH OF VERACRUZ, MEXICO

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ABSTRACT

Spatial-temporal distribution of sea turtle nests is a main factor for hatching success. Beaches with good access to the sea, fine sands, adequate humidity and temperature are indicators to carry out oviposition. Many nesting beaches have been modified mainly by human activities and high impact natural phenomena such as hurricanes, causing many sites to show unfavorable conditions for egg laying during the nesting season. This study was performed in Tuxpan, Veracruz, during 2010 and 2011 nesting seasons. This site was chosen because it is one of the few beaches in the State where two sea turtle species nest: Kemp's Ridley Turtles (*Lepidochelys kempii*) and Green Sea Turtles (*Chelonia mydas*). The aim was to determine spatial-temporal conditions for the nesting of the two sea turtle species. We determined the months and sites of highest nesting, monthly number of eggs and distribution of nests in the beach area. The results showed spatial-temporal differences in nesting. There were more Kemp's Ridley Sea Turtle nests on the northern and center portions of the beach, and more Green sea turtle nests in center and southern areas of the beach. Kemp's Ridley Sea Turtle nesting season occurs from April through July, while Green Sea Turtle nesting occurs from May through August. The peak number

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of Kemp's Ridley Turtle nests was in June, and the Green Turtle peak was in the month of July. Both species nest mainly in the zone of the beach that is the nearest place to the vegetation or even inside of it. It is essential to perform long term monitoring of sea turtle nesting populations on center and northern beaches of the Veracruz State, with the purpose of obtaining more information in a regional context.

Keywords: Chelonian, sea turtles, nesting, distribution, abundance, Kemp's Ridley turtles, *Lepidochelys kempii*, Green sea turtles, *Chelonia mydas*

INTRODUCTION

Selection of the nesting site is one of the meaningful stages for oviparous species. In species that do not provide parental care, adequate site selection is vital for embryonic development, and for subsequent hatching success. In sea turtles, the nesting stage is a vulnerable for the deposited eggs and for the female (Márquez-M 2004). The selection of the oviposition site is generally carried out by the female, based on diverse biotic and abiotic factors. Turtles commonly choose sites based on temperature, humidity, access to the sea, and sand grain among others (Bolongaro et al. 2010, Zavaleta-Lizárraga & Morales-Mávil 2013).

Unfortunately, high human activity in beaches due to tourism development, fishing and engineering, amongst other indirect causes, have destroyed or modified the turtles' habitats and their nesting sites (Arias & Vernet 2006, 2007, Ferrer et al. 2007, González & Ugas 2007, Balladares & Mora 2008). This problem is more complex because females come back to spawn to the same place each nesting season. Moreover, once hatchlings mature they may also use the beaches where they hatched (Eckert & Grobois 2001).

For the State of Veracruz, Mexico, nesting beaches have been extremely altered by human activity therefore; there are few beaches with favorable conditions for the spawning of more than one turtle species per nesting season (Montes & Licona 1996). Our study was performed on a beach in the northern area of the State of Veracruz, where at least two different species of turtles nest. We aimed to describe the spatial-temporal condition of the nesting of two turtle species [Kemp's Ridley Turtle (*Lepidochelys kempii*) and Green Turtle (*Chelonia mydas*)], identifying and describing their interaction period.

METHODS

We conducted the study at Villamar beach in Tuxpan, Veracruz, that manages the R-5 Turtle Camp. The beach is located in the extreme north of the State of Veracruz (97° 23' 36"N and 20° 83' 47"W). It has an average altitude of 10 m and has an area of 19 km. It borders to the North with the beaches of the municipality of Tuxpan, to the East with the Gulf of Mexico and to the South with the beaches of the municipality of Cazones (INEGI 2012). It has a warm-humid weather, with an average yearly temperature between 22°C and 26°C. The coldest month is lower than 19°. Rain occurs during the summer and winter. Prevailing winds come from the East during summer and this area is influenced by dry winds from the North in winter. The hurricanes season occurs from July to November, therefore maximum precipitation is recorded during these months. Besides hurricanes, the area receives the

influence of another important climatic force, North winds, which are cold invasions or anti-cyclonic wind masses that come from the U.S. interior (CONAGUA 2012).

The coast is variable and has a rugged profile, with intermittent stretches of sandy beaches that alternate with the rocky coastline. Streams, rivers and runoffs are very common. There are areas where accumulation of logs and garbage are found, abundant mainly during rainfall season and are generally found near the rivers' mouths (INEGI 2008).

Coastal vegetation is mainly composed of grasslands and creeping halophyte plants, which can be found up to the high tide line. In some places pastures have replaced natural vegetation, due to cattle ranching in the area (INEGI 2008). Human settlements are limited. Settlements are formed by families that inhabit low profile constructions, and by some temporary visitors interested in participating in the conservation activities during the spawning months of sea turtles. Agriculture, cattle and fishing for local consumption are the main economic activities of this area. There are some industries located at the Northern area of this beach, such as: Gas Maritime Terminal and a power plant (Tuxpan II 495 MW) (Ortiz-Pérez 2012).

Data Recording

Most species of turtles follow the same behavior pattern during nesting, so that methods for study and management of females at nesting beaches are similar for all species (Choi & Eckert 2009). This study was performed during the nesting seasons 2010 and 2011. Morning and night monitoring were carried out according to the nesting habits of each species. To collect data on Kemp's Ridley Turtle females and nests, monitoring was done during the morning. For Green Turtle females and nests, monitoring occurred at night.

During monitoring we conducted walks along the beachfront, data collection started at the moment in which a nest was located, with the help of a GPS GARMIN model GPSmap 60CSx (precision ±3 meters) the coordinates of each nest were taken. Afterwards, we recorded the distances between the coastline and the nest, and between the nest and the vegetation with a Truper 50 ft fiberglass tape measure. Later, the nest was excavated and the eggs were counted, measured and deposited back in the nest, which was covered again with sand. To mark nest locations, we used wooden stakes and fluorescent - biodegradable flagging tape. We noted the date, species and nest number on the piece of flagging tape with permanent ink.

In order to determine the number of eggs per season, a Mann-Whitney U test was performed, as data were not normally distributed according to the Kolmogorov-Smirnov test.

Nests Distribution per Area

To analyze the distribution of the nests along the beach, the 19 km of the beach that was sampled was divided into three zones. Zones division was done accordingly to the methodology mentioned by Azanza et al. (2003), Ferrer et al. (2007), and Zavaleta-Lizárraga & Morales-Mávil (2013). The three zones were:

- Zone A: Inter-tidal section over the entire coastline, ending in the last tide line.
- Zone B: From the point where the zone A ends, covering all the section of the berms up to the beginning of the dunes.
- Zone C: From the point where the zone B ends, up to the interior of the supra-littoral.

Once the nests were located, their location on the corresponding zone of the beach (A, B or C) was determined using a GPS. Maps were elaborated with the obtained UTM coordinates. Maps show the distribution of the nests for both seasons. For this purpose, satellite images from Google Earth Pro and StitchMaps were used. Later on, these maps were analyzed with ArcGIS 9.3 software

Nests Distribution per Site

Once the distribution of the nests along the 19 km of beach was obtained, it was divided into three similar segments, each of 6.33 km of beach: North area (site 1), Center area (site 2) and South area (site 3).

In order to analyze the distribution of the nests from both species on the beach, a canonical-correlation analysis (CCA) was made with Canoco for Windows 4.51 software. In order to test differences in the distribution of nests from both nesting seasons, a Mann-Whitney analysis was made with SgimaStat 3.1 software.

RESULTS

Nests Number per Month

A total of 178 nests were registered in 2010, 115 were from Kemp's Ridley Turtles and 63 were from Green Turtles. The month with the highest number of Kemp's Ridley Turtle nests was June with 104 nests, and July was the month with the highest number of Green Turtle nests (51). In these two months the nesting of both species overlapped (Figure 1).

For 2011 season, 133 nests were registered, 56 were Kemp's Ridley and 77 were Green Turtle. Kemp's Ridley Turtle showed a nesting peak during the month of April with 34 nests. July was the month with the highest number of Green Turtle nests with 35 nests. During this nesting season the overlapping month was May, with 22 as the highest number of nests of Kemp's Ridley Turtle, while there were only 4 nests of Green Turtle (Figure 2). It was only possible to quantify the number of eggs from this nesting season. The Green Turtle deposited, on average, more eggs per nest than the Kemp's Ridley Turtle. The average number of eggs per nest was similar during the nesting months, both for the Kemp's Ridley Turtle, 89.73 ± 29.1 (Mann-Whitney; $P = 0.921$; $U = 11.000$); and for the Green Turtle, 110.91 ± 19.5 eggs (Kruskal-Wallis; $P = 0.334$; $H = 2.195$).

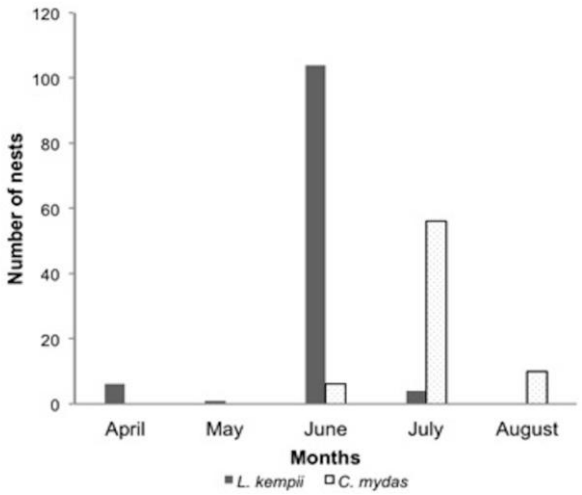

Figure 1. Number of nests by month of Kemp's ridley turtles and Green turtle in 2010 season.

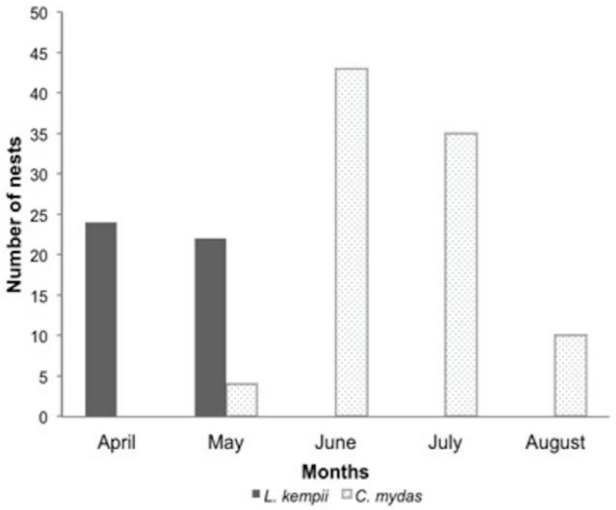

Figure 2. Number of nests by month of Kemp's ridley turtles and Green turtle in 2011 season.

Spatial-Temporal Distribution of Nests

During the nesting season 2010, the total registered number of nests for Kemp's Ridley turtle and Green turtle was 178. The Kemp's Ridley turtle had the highest number of nests in the North-center area of the beach, while the Green Turtle had a higher nesting preference at site 3 (towards the South). During this season, the species with dominant abundance of nests was Kemp's Ridley turtle with 115 nests.

In the 2011 nesting season we registered 133 nests. Kemp's Ridley Turtles had more nests toward site 1 (North of the beach), while Green Turtles had more nests to the south of the beach (site 3). Distribution of the nests was made irregularly, thus nests were presented sometimes in small groups, leaving greater distance between one group and another.

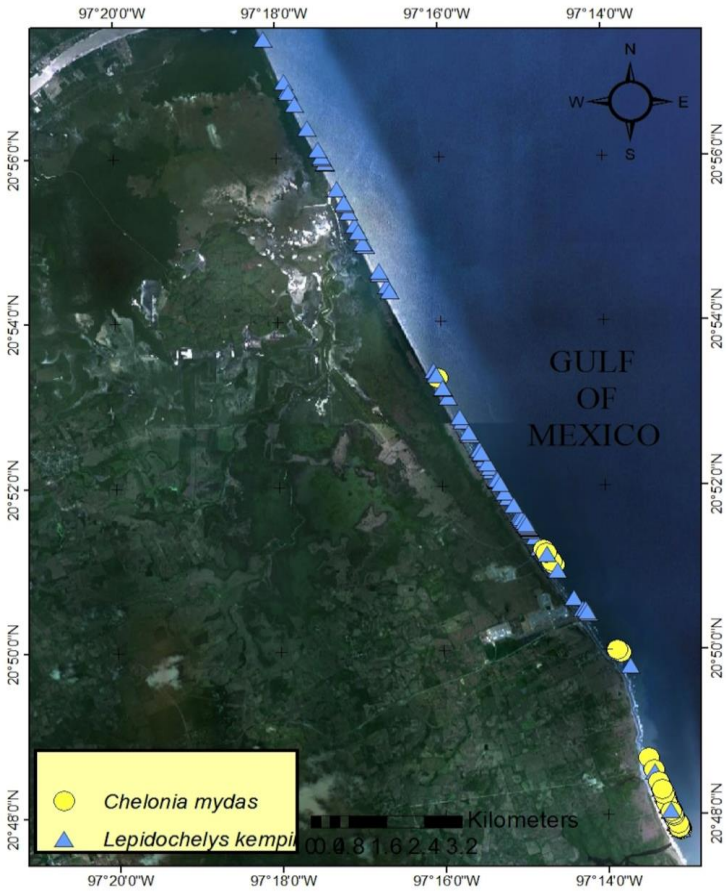

Figure 3. Distribution of nests in three sites of Villamar beach in 2010 season.

Nest Distribution per Zone (A, B and C)

Nests distribution was higher in Zone C for both species and for both seasons: in 2010 season 102 nests Kemp's Ridley Turtle (88.7%) and 60 nests Green Turtle (95.24%) were recorded in Zone C, and the rest in Zone B. During 2011 season, 51 Kemp's Ridley nests were recorded (91.1%) and 61 Green Turtle nests (79.22%) in Zone C, the rest rest of the nests were located in Zone B. Zone A did not have any recordings of nesting for any of the species.

Nests Distribution per Site

For the 2010 nesting season, only Kemp's Ridley nesting was recorded in Site 1, however, it was in Site 2 where the greater nesting for this species occurred, with 58 nests. Green Turtles preferred Site 3 with 57 nests monitored, while Site 2 had the lowest number with only five nests. Site 1 was located toward the North area of the beach. Nests registered during 2010 season belonged only to Kemp's Ridley Turtle (30 nests). The distribution was all over the extension of the beach (Figure 3).

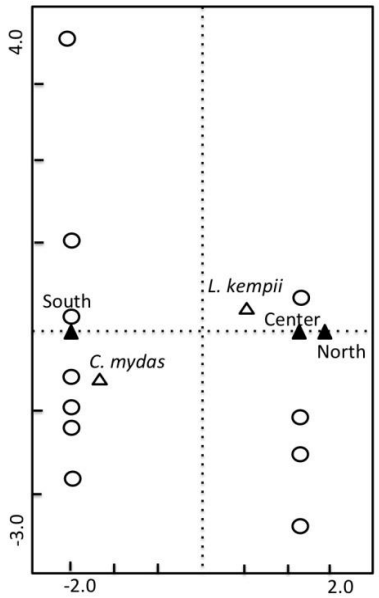

Figure 4. Canonical Correlation Analysis (CCA) of the abundance of nests of *C. mydas* and *L. kempii* in Villamar beach, Tuxpan, Veracruz, in 2010 season (F2, 53 = 123.89, P < 0.001, 100% of the total variance).

In Site 2 (central part of the beach), a greater number of Kemp's Ridley was found, registering a total of 58. In this site nesting from Green Turtle also ocurred, but in low numbers (only five nests). Nests distribution for this site also was found all over the extension of the beach (Figure 4).

In Site 3 (Southern part of the beach) some Kemp's Ridley turtle nests were registered mainly in the coastline with Site 2, even though the greatest record was of Green Turtle nests with 57. Most nests were recorded over the extreme South portion. Therefore, nests distribution on this site was performed in groups toward the ends of the beach, leaving a wide empty space at the center portion (Figure 5).

The canonical-correlation analysis of the three sites during 2010 season, showed a greater relation in nesting of Kemp's Ridley Turtle with Sites 1 (North) and 2 (Center). Nesting of Green Turtle was more related with Site 3 (Southern part) (Figure 6).

During 2011 season, Site 3 was the one that registered the greater number of eggs for both species, with 22 Kemp's Ridley Sea Turtle nests and 67 Green Turtle nests. During this season, the number of Green Turtle nests was greater than the Kemp's Ridley's. Few nests (13) were recorded at Site 1, from which the greater part (11) was Kemp's Ridley and they were mainly distributed in the central section of this beach segment. Only two Green turtle nests were registered toward the Southern part of the segment, which is, the coastline with Site 2.

The number of turtle nests (21) was greater in Site 2 than in Site 1. Thirteen Kemp's Ridley nests and eight Green Turtle nests were recorded. All nests were distributed basically in the central part of the segment toward the South, without showing a marked group along this segment of the beach.

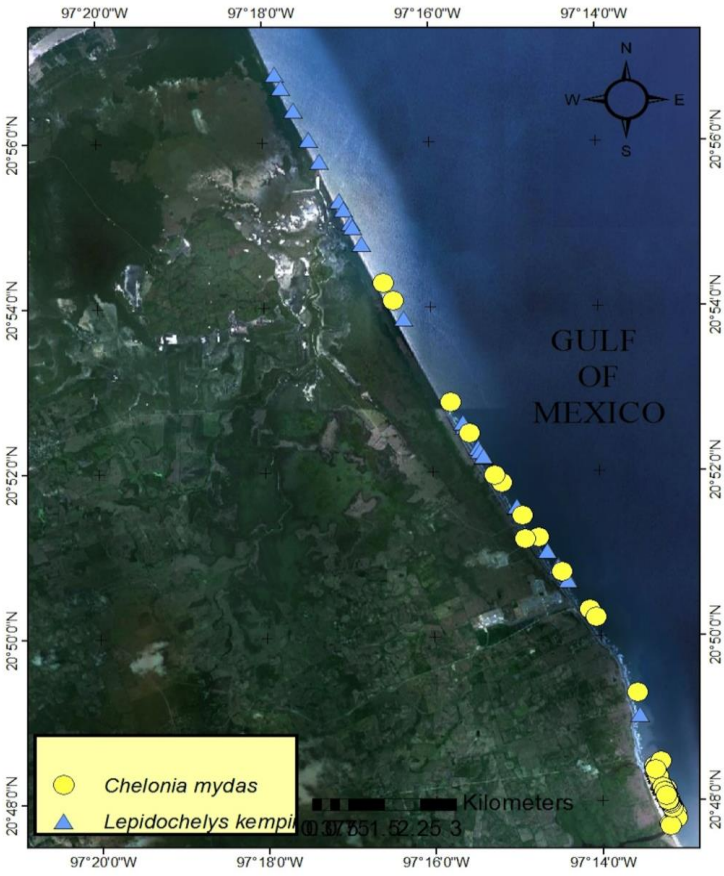

Figure 5. Distribution of nests in three sites of Villamar beach in 2011 season.

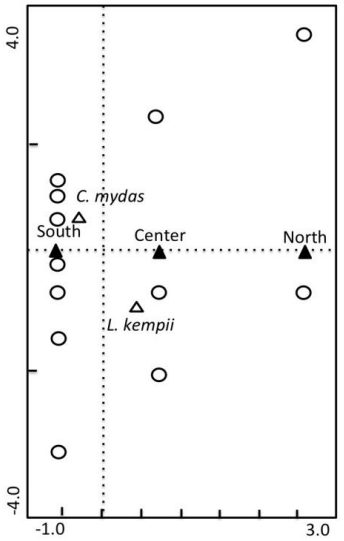

Figure 6. Canonical Correlation Analysis (CCA) of the abundance of nests of *C. mydas* and *L. kempii* in Villamar beach, Tuxpan, Veracruz, in 2010 season ($F_{2, 38} = 16.38$, $P < 0.03$, 100% of the total variance).

Site 3 was the one that showed the greatest number of nests for both species, 22 Kemp's and 67 Green's. It is evident that the greatest part of the nests was grouped to the Southern part of this segment, between the two river mouths located in this Site.

The canonical-correlation analysis for 2011 nesting season showed differences between the sites. Greater relationship in the Green turtle nesting site 3 and Kemp's Ridley with site 2 is seen. Northern area (Site 1) did not have any relevance for nesting of any species during this nesting season.

Considering both seasons (2010 and 2011), nesting of Kemp's Ridley and Green Turtle was similar regarding the number of registered nests (Mann-Whitney: Kemp's, $U = 8.50$, $p = 0.730$; Green, $U = 15.000$, $p = 0.690$). However, the number of Kemp's Ridley Turtle nests was significantly higher than Green Turtle nests in 2010 ($U = 3622.5$; $P < 0.001$), while the number of Green Turtles nests was significantly higher than nests of Kemp's Ridley Turtle during 2011 ($U = 1771.0$; $P < 0.001$).

DISCUSSION

The results show that there are spatial-temporal variations in the nesting of sea turtles in Villamar de Tuxpan, Veracruz. Differences were found in the number of nests registered for Kemp's Ridley and Green Turtles. During the nesting season in 2010, there were more registered nests for Kemp's Ridley Turtle than for the Green Turtle, while in season 2011 the Green Turtle had more registered nests. This annual variation included changes in the nesting peaks. Annual fluctuations are common in sea turtles nesting and have been reported in several species and sites (Meylan & Meylan 2000, Lagueux 2001, and Margaritoulis 2005). For three consecutive years, annual variations in the number of nests have been recorded for the Green Turtle in the central zone of the state of Veracruz, with a spread of 351to 492 nests (Zavaleta-Lizárraga & Morales-Mávil 2013).

The marked variations in the nesting peaks and months are caused by environmental changes in temperature, humidity and wind, which take place differentially each year. These are central factors for the arrival of the Green Turtle, and for the Kemp's Ridley Turtle, which lays its eggs in daylight. Turtles arrive in the beaches and select nesting sites in order to ensure the growth of the embryos (Hawkes et al. 2009). Thus, the nesting month peak variation is connected to the warm season and with specific wind periods, which enhance nesting, oviposition and the embryonic success of the future hatchlings. In other species, such as the Loggerhead Sea Turtle (*Caretta caretta*), Margaritoulis (2005) registered that the months with the highest temperature were July and August and were connected to greater turtle nesting in the island of Zakynthos.

Not every turtle nested every year which may be explained by the annual variations in the climate conditions, and it could reflect in the variations in this study. Variation in nesting over years is a widely documented phenomenon in sea turtles (Meylan & Meylan 2000). Individual sea turtles show annual, biennial, and triennial reproductive periods, or sporadic breeding (Márquez 2003). During the nesting season, the intervals to perform oviposition may vary in different sea turtle species (Lagueux 2001). It is known that the migration cycle of the Green Turtle is biennial, and thus there are high and low nesting seasons, which causes more nesting in one year than in the other (Bowen et al. 1992). Furthermore, inter-annual nesting variation

might be influenced by food availability. Females do not feed before laying their eggs because eggs occupy the major part of their abdominal cavity (Hatase et al. 2004). However, for nesting to succeed, females must load up on nutrients before starting to fast, so that the energy accumulated is sufficient for reproduction. During times of food scarcity, the journey and nesting cannot be accomplished (Hatase et al. 2004).

The number of eggs registered for the two species was different in 2011: Green Turtles laid more eggs (4,247) than Kemp´s Ridley turtles (966). These values are low when compared to the number of eggs registered by García-Romero et al. (2007) during the nesting season in 2001 in 21 turtle centers in Michoacán, Mexico, where 898,134 eggs were registered. This represents over 42,000 eggs per camp, or in other words, ten times than we registered in the present study. Venkatesan et al. (2004) also recorded high nest numbers — 17,964 eggs—for the Green Sea Turtle in the coast of India, during 2000-2002. Although this number was recorded during three years, the annual average is nearly 6,000 eggs, and thus greater than what we recorded. The methods utilized in both studies were similar to ours; however, we could have a more precise appreciation of the extent of differences if we knew the area and distribution of habitat types of the other beaches. Regardless, it is safe to assume that the Villamar has relatively small populations of both species, given that the number of eggs found does not match the quantity found in others.

Regarding the specific nesting zones in the beach, both species preferred nestin in zone C, in the limit or within the dune vegetation area. Surely this behavior allows sea turtles to avoid excessive humidity and nest flooding, and provides better protection from predators. This is the most frequent nesting zone for sea turtles, such as registered for the Hawksbill sea turtle (*Eretmochelys imbricata*) in Barbados (Horrocks & Scott, 1991), and Cuba (Azanza et al. 2003, Ferrer et al. 2007), and the Green turtle in other Mexican beaches (Zavaleta-Lizárraga & Morales-Mávil, 2013).

Despite a clear preference to nest in zone C, nests in zone B were also observed. The nests in this zone are more exposed to predators, and to unfavorable temperature and humidity conditions for the development of embryos, why do females lay their eggs in this zone? There are several explanations. Firstly, nest saturation is possible on the sites with the best conditions, which would force females to use lower quality sites, as has happened in the great arrivals in Tortuguero, Costa Rica (Bjorndal & Bolten 1992). Although due to the low number of nests registered in Villamar, this is not likely. Secondly, the health condition of females is not good enough for them to move and dig for longer time in search for a better site and thus they lay their eggs in zones nearer to the tide line (Moncayo 2014). This is feasible, although blood tests would be needed to verify it. Thirdly, individual differences in corticosterone may compromise the females´ abilities to make good nesting choices, due to stress. Furthermore, female Green Turtles with high levels of corticosterone can make longer movements and greater nesting efforts in search of the best zones to lay their eggs (Ortega-Planell 2013).

Differences with respect to nest distribution of the two species along the coast were registered in this study. The most northern site of the beach (site 1) presented only nests of Kemp's Ridley Turtles during both years. Site 3 presented the highest number of Green Turtle nests during the two nesting seasons. It is likely that the difference in the number of nests along the beach is caused by the presence of reef areas, as well as topographic and humidity differences of the beaches. For example, the greater nesting of Kemp's Ridley in the north side might be connected to the proximity of the reef system Lobos-Tuxpan. This is a

carnivorous species and it most surely finds wide food availability in the reefs, which makes it abundant not only in the region of Tuxpan, but also Rancho Nuevo, Tamaulipas, which is held as its main nesting site (Márquez 2003). The Northern part of the beach can be considered different in terms of humidity conditions, for it is the nearest zone to Tumilco, a wide mangrove area within the RAMSAR site, Tuxpan mangroves and wetlands (Basañez 2005).

The large presence of Kemp's Ridley nests lay towards the central beach, mainly in the zone attached to the north beach. A lower quantity of nests was registered in the rest of the beach, probably because of greater human presence due to Villamar. Villamar has several activities for the residents (fishing, ranching, and tourism), which might deter the turtles from nesting near the settlement. Human settlements also carry artificial lighting, a factor that could affect the females during the oviposition, and later the orientation of the offspring when leaving the nest (Rondón et al. 2009).

On the most southern part of the beach we registered more Green Turtle nests. What are the characteristics of this side of the beach that make it differentiate turtle nesting? To begin with, it is a wider area, farther from the tide line to the dunes, and these present greater vegetation cover. In that sense, dune vegetation has been reported as one of the main nesting sites for the Green Turtle (Ferrer et al. 2007, Azanza et al. 2007, Silveira et al. 2003). While this south beach is not as large as the wetlands of the Ramsar site. It does have good humidity conditions due to the presence of the streams that flow into this zone of the beach (Bouchard & Bjorndal, 2000).

CONCLUSION

The spatial-temporal distribution of Green Turtle and Kemp's Ridley Turtles shows a connection to the conditions of the beach where they nest. The segregation in the nesting of the two species is evident, where Kemp's Ridley has preference for the north side and Green Turtle for the south part. As well, temporal separation is clear, with greater abundance of Kemp's Ridley in April and May, and Green turtle during June and July. The proximity of the nesting sites to zones with infrastructure makes the beach of Villamar a system vulnerable to human disturbance. Performing long-term monitoring upon the nesting populations in the northern and central beaches of the state of Veracruz appears indispensable, so as to get more information in a regional context. This would lead in to a deeper knowledge that will provide a context to plan better studies and conservation strategy proposals in the north of the state.

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Chapter 35

MARINE TURTLES: CONSERVATION STRATEGIES AND FUTURE RESEARCH

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ABSTRACT

Avoiding species extinction will require rapid, greatly intensified efforts to conserve already threatened species and to alleviate pressures on their populations. Modern marine turtles belong to an ancient group of reptiles inhabiting the Earth for over 110 million years, since the Cretaceous. Nowadays, marine turtles comprise seven extant species grouped into two families: Dermochelyidae, with the leatherback (*Dermochelys coriacea*) as the single extant species, and Cheloniidae, with six species: hawksbill (*Eretmochelys imbricata*), Kemp's ridley (*Lepidochelys kempii*), olive ridley (*Lepidochelys olivacea*), loggerhead (*Caretta caretta*), green (*Chelonia mydas*), and flatback (*Natator depressus*) turtles. With the exception of Kemp's ridley, restricted mainly to the North Atlantic and Gulf of Mexico, and the flatback turtle, endemic to the Australian continental shelf, marine turtles are circumglobally distributed. They inhabit nearly all oceans, occupying unique ecological niches, and exhibiting intra-specific variations in population sizes and trends, as well as reproduction and morphology. Six of these species are classified by the World Conservation Union as either critically endangered (hawksbill and Kemp's ridley), endangered (green turtle) or vulnerable (leatherback, loggerhead and olive ridley). Threats vary across regions, but general categories include fisheries bycatch (i.e., incidental capture by marine fisheries operations targeting other species), exploitation of eggs, meat or other turtle products, coastal development, pollution and pathogens, and climate change. A broad range of conservation programmes have been established around the world to face these threats, but with a general bias toward the protection of nesting females and clutches at their reproductive areas. Current and future efforts for

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implementation of effective management and conservation strategies are headed to mitigate the potential impacts of climate change on marine turtle's terrestrial nesting grounds and in-water phases (e.g., loss of nesting beaches, feminization of turtle populations, decreased reproductive success). Although marine turtles are a broadened studied group, there are still many gaps of knowledge on turtle ecology, hatchlings trans-oceanic migrations and natal philopatry, juvenile developmental and foraging habitats, and potential impacts of climate change. Future researches are targeted to further insight all these questions through the integration of innovative technologies such as telemetry, genetic markers, molecular techniques, stable-isotope analysis and habitat/environmental modelling.

1. INTRODUCTION

Modern marine turtles belong to an ancient group of reptiles inhabiting the Earth for over 110 million years, since the Cretaceous (Hirayama 1998). From the Cretaceous, four families of marine turtles diverged: Protostegidae; Toxochelyidae; Dermochelyidae; and Cheloniidae, but only the latter two have survived until present (Spotila 2004). Nowadays, marine turtles comprise seven extant species grouped into two families: Dermochelidae, with the leatherback (*Dermochelys coriacea*) as the single extant species, and Cheloniidae, with six species: hawksbill (*Eretmochelys imbricata*), Kemp's ridley (*Lepidochelys kempii*), olive ridley (*Lepidochelys olivacea*), loggerhead (*Caretta caretta*), green (*Chelonia mydas*), and flatback (*Natator depressus*) turtles (Pritchard 1996). With the exception of Kemp's ridley, restricted mainly to the North Atlantic and Gulf of Mexico, and the flatback turtle, endemic to the Australian continental shelf, marine turtles are circumglobally distributed (Wallace et al. 2011).

Marine turtles depend on both marine and terrestrial habitats for their growth and development, from high energy beaches to benthic reefs, and the open waters of the seas. The seven species have similar life cycles with variations in the duration of phases (Miller 1997). Nesting females are philopatric to natal regions with sexually mature animals returning to their natal beaches to breed and nest, and both males and females can be philopatric to breeding areas adjacent to a nesting beach (FitzSimmons et al. 1997; Velez-Zuazo et al. 2008). However, a certain degree of variations in philopatry among populations and species has been described. In general, female marine turtles typically nest more than once per reproductive season. They do not nest every year and their nesting behaviour is highly stereotypic (Meylan and Meylan 1999). Only females will come ashore to dig a hole into which they deposit between 50-200 soft-shelled eggs, depending on the species (Miller 1997). From six weeks to two months later (depending on the species), hatchlings make their way to the surface of the sand and head to the water. Hatchlings are transported by ocean currents to oceanic habitats, where they live in flotsam, such as Sargassum mats and have an omnivorous diet. Carr (1987) hypothesized that hatchlings spend their first years in oceanic habitats presumably feeding primarily on sea jellies and salps. This time is often referred to as the "lost years"; but a recent study based on satellite telemetry methods has shed light on this period (Mansfield et al. 2014). After this oceanic phase, they return to coastal waters where they forage and continue to mature. Once adult males and females acquire sufficient resources, they migrate to breeding areas to mate. The time it takes to reach sexual maturity (when they are able to reproduce) varies among species, but ranges between approximately

10-30 years. The distance between feeding and breeding areas can be hundreds to tens of thousands kilometres, with turtles performing seasonal migrations moving across large expanses of the marine environment.

Marine turtles have constituted a main source in different civilizations since the Early Bronze age, providing not only foodstuff but also as sources of fat for oil, bones for medicinal purposes and cultural activities such as religious ceremonies and mortuary objects (Frazier 2003; Encyclopædia Britannica 2016). Evidences of the use of turtles (e.g., remains of shell and bones) by Prehistoric human communities have been registered in the Mediterranean and Caribbean Sea, Arabian Peninsula, and Indian Ocean Basin (Mosseri-Marlio 1998; Cooke 2004; Tripathy and Choudhury 2007). The study of archaeological marine resources exploitation in the Caribbean shows that marine turtle's populations decline was already patent in the prehistoric time periods (O'Day 2001). Despite the status of marine turtles populations was far of pristine before the European conquest of the Americas, it is irrefutable that for hundreds of years, marine turtles provided nourishment for European colonists on Caribbean islands (Lefevre 1992). There are many historical sources about the great presence of marine turtles as well as how appreciated their meat and eggs were (Rodríguez Demorizi 1942; Parsons 1962; Jackson 1997). This heavy harvest of turtles supposed loss of widespread nesting areas and reduction in populations through the world (Meylan 1999; McClenacahn et al. 2006; Campbell 2014; Spotila and Santidrian Tomillo 2015). Consumptive use of marine turtles has continued through centuries until modern day. Turtle meat and eggs have constituted an important resource for coastal communities around the world. The adult females slaughter and eggs harvesting as well as its effects on nesting populations has been extensively documented in nesting beaches of the Caribbean Sea (Lagueux and Campbell 2005; Troëng and Rankin 2005), Atlantic coast of Africa (Formia et al. 2003), Mediterranean Sea (Demetropoulos and Hadjichristophorou 1989), Eastern Pacific (Sarti et al. 2007), Western Pacific (Bhaskar 1987; Hitipeuw et al. 2007). Marine turtles have also been exploited for their skin, fat and blood and especially in the case of green and hawksbill turtles for their shell (Groombridge and Luxmoore 1989; Barr 2001). The decline of the marine turtles populations has continued throughout history, with growing human population, habitat degradation, overfishing and changes in models of subsistence, described as the main causes of this decreasing (Bjorndal and Bolten 2003).

Why and how marine turtles progressed from human exploitation to be considered charismatic species highly needed of protection? The conservation status of marine turtles populations took on importance by the 1950s and is closely related to one name, Dr. Archie Carr, who made extraordinary contribution to sea turtle conservation by way of bringing attention to the world's declining turtle populations due to over-exploitation and loss of safe habitat (Spotila 2004). Dr Carr carried out research in all aspects of the biology of marine turtles, he made the first description of life cycles, nesting process and hatchlings behaviour (emergence, sea finding orientation) and turtles migrations (Carr and Ogren 1960; Carr 1961). Besides the importance of his research, he is considered the origin of the international movement for the conservation of marine turtles; the establishment of Sea Turtle Conservancy in Tortuguero in 1959, to study and protect Caribbean green turtles is a milestone in these species conservation and population recovery. Previous to the STC establishment, the green turtle population is believed that was close to extinction since nearly every female turtle arriving to nest in Tortuguero was taken for the export market for turtle soup. A huge number of conservation efforts and research on physiological and ecological

aspects of marine turtles began in the 1960s and 1970s: studies of ecology and migrations of marine turtles (Carr and Caldwell 1956; Carr and Ogren 1960); nesting beaches conservation programs and nest translocation (Lebuff 1974; Schulz 1975; Márquez 1978), species distribution (Hildebrand 1963; Bleakney 1965), marine turtles phylogeny (Gaffney 1975), marine turtles anatomy and physiology (Frair et al. 1972; Greer et al. 1973). In the 80s, significant progress were made in the study of nesting beaches, evaluating some of the most widespread conservation techniques such as the transfer and ex situ eggs incubation beaches, turtles tagging or head starting programs (Klima and McVey 1995). Over the past three decades, a broad range of conservation programs have been established around the world to face the depletion of marine turtle's populations, but with a general bias toward the protection of nesting females and clutches at their reproductive areas (Reina et al. 2002). Current research attention focusing on a wide variety of topics relating to sea turtle biology and ecology, together with the interrelations of sea turtles with the physical and natural environments (Hamman et al. 2010; Abecassis et al. 2013; Griffin et al. 2013; Hawkes et al. 2014; Metcalfe et al. 2015).

In the present chapter it will be described the main achievement, conservation strategies carried out in the different phases of the marine turtles life cycle until now and future research.

2. Conservation Strategies and Future Research on Reproductive Areas

The actions for marine turtle's conservation have its origins in the activities carried out on nesting beaches to protect both nesting females and eggs (Caldwell et al. 1959; Carr et al. 1978; Hirth 1980). Beaches with presence of marine turtle nesting activity are usually surveyed throughout the entire nesting season to record signs of recent marine turtle nesting activity. Research and management needs on the nesting beach, include studies of the adult females, eggs, and hatchlings (Richardson 1999). Added to these data, female tagging has been widely used in nesting beaches, mostly to obtain information on growth, movement, and population dynamics (Chaloupka and Musick 1997). All these studies have provided considerable knowledge about marine turtle's biology that has been critical for marine turtle's conservation and management.

2.1. Population Genetic Structure Phylogeographic Relationships

Identifying individual turtles with metal and/or PIT tags allowed use mark-recapture techniques for estimating turtle's population sizes, describing the re-migration interval and internesting behaviour of adult females (Gerrodette and Taylor 1999; Briseño-Dueñas and Abreu-Grobois 1999). Adult turtles returned to the same beach to lay their eggs, the re-migration periodicity range between two to four years among species and depend on dietary, behavioural and physiological factors (Márquez et al. 1982; Miller et al. 2003; Beggs et al. 2007; Bailey et al. 2008). This information obtained from tag returns was lately confirmed through molecular techniques. Mitochondrial DNA techniques characterize maternal lineages

within species; these analyses of genetic structuring of nesting beaches in relation to other populations allow determine levels of differentiation (Dutton et al. 1999; Shanker et al. 2004; Leroux et al. 2012). First studies on mitochondrial (mt) DNA variation were carried out to assess loggerhead population genetic structure (Bowen et al. 1993), demonstrating the presence of rookery-specific mtDNA haplotypes that distinguish them from turtles of other nesting areas in these species. Bowen and collaborators analyzed mtDNA variation in samples from four nesting beaches in the northwestern Atlantic Ocean and from one nesting beach in the Mediterranean Sea. Significant differences in haplotype frequency between nesting populations in Florida and in Georgia/South Carolina, and between both of these assemblages and the Mediterranean nesting colony, indicated substantial restrictions on contemporary gene flow between regional populations, and therefore a strong tendency for natal homing by females. This has also been confirmed in green turtles (Bowen et al. 1992), hawksbill turtles (Bass et al. 1996) and leatherback turtles (Dutton 1995). Such studies provide exceptional data about genetic differences among regional populations, but later improvements in sequencing technologies have allowed for the analysis of larger mtDNA fragments that have resulted in a better understanding of complexity of marine turtle's local populations and to resolve structure at finer scales (Chan 2005; Velez Zuazo et al. 2008). On the other hand, these improvements have contributed to solve geopolitical management of these endangered species (Mortimer et al. 2007). To refine the understanding about the genetic composition of green turtle populations in the South West Indian Ocean (Bourjea et al. 2015). The use of larger mtDNA fragments has also allowed to track evolutionary and colonisation events of marine turtles. The evolutionary history of Mediterranean loggerhead turtles population has been elucidated through the analyses of mtDNA from dead hatchlings sampled from a selection of rookeries in the Eastern Mediterranean (Clusa et al. 2013). It seems that current genetic structure of loggerhead rookeries in the Mediterranean would be the result of different colonisation events from the Atlantic, combined with local extinctions during cold periods.

2.2. Natal Homing

Molecular studies revealed that hatchlings turtles leave their natal beaches and, once reaching sexual maturity, the female turtles make the long trip back to her natal beach to lay her eggs. This process by which some animals return as adults to their natal areas to reproduce after migrate across vast expanses from their birthplace is known as natal homing or natal philopatry (Bowen and Karl 2007). This mechanism is primarily used by aquatic animals, such as marine turtles and salmon (Lohmann et al. 1999). One idea about how animals accomplish natal homing is that they use the magnetic field of the earth to navigate across the globe (Lohman et al. 2012; Putman et al. 2014). The Earth's magnetic field is a useful navigation tool given its properties of being present on the whole ocean, during the day and night and remains unaffected by weather and season. To achieve their migratory routes, animals integrate two distinct types of information from the geomagnetic field: 1) directional or compass information, which enables an animal to maintain a particular direction such as north or south, and 2) positional or `map' information, which some animals use to steer themselves along migratory pathways or to navigate toward specific target areas (Lohman et al. 2007; Lohman et al. 2008).

Recent studies have gone in depth in the specific use of this process by marine turtles with important implications in conservation. One of these findings is that marine turtles are able to imprint on the unique magnetic field that exists in their natal area and to use this information to return years later (Lohman et al. 2012). Brothers and Lohman (2015) have also confirmed the hypothesis that nest site selection depends at least partly on magnetic signatures consisting of inclination angle, field intensity, or a combination of the two and, as a consequence, variations in the convergence of isolines of inclination and intensity along the nesting coast could result in a concentration increase or concentration decrease of the nest density. However, geomagnetic field intensity is continually changing so this phenomenon of imprint could not explain natal homing by itself. A second hypothesis about turtles imprint on distinctive chemical cues associated with their natal beach has been developed. Turtles might hypothetically use both types of information together. For example, they might first use magnetic information to arrive in the general region of the beach, and then use chemical cues to localize a more specific nesting site (Lohmann et al. 2008; Putman and Lohmann 2008). Although marine turtles can detect chemicals dissolved in water and air (Southwood et al. 2008; Endres et al. 2009), there is still no evidence that support this hypothesis and further research is clearly needed. In addition, the geographic precision of natal homing remains to be determined, many turtle populations has showed absence of precise natal homing, while in other cases a great precision has been described (Lee et al. 2007). Recently an astonishingly event of precise site fidelity of flatback turtle has been recorded in the beach of Mon Repos (Western Australia), where a nesting female tagged for the first time in 1976 was observed again in November 2015 constituting the longest studied marine turtle in the wild. Over these 39 years of adult breeding life, she has been reordered coming ashore to lay eggs on 65 occasions and always on the 1.6km of Mon Repos beach. These findings are key in establish of conservation strategies, for example, if future conservation measures are considering replace a depleted population using animals from other regions, it could be possible that the new arrivals will lack the inherited instructions needed to navigate within and from their transplanted homes. Future lines of research will be targeted long-term studies of chemical and geomagnetic imprinting to try elucidating the mechanisms that underlie natal homing and answer questions about the implications of both geomagnetic imprinting and chemical cues (Lohmann et al. 2013).

2.3. Temperature Dependent Sex Determination

As in a number of reptiles, sexual differentiation in marine turtles depends on the ambient temperature during incubation of the eggs instead of a genetic determination (Pieau 1971; Bull and Vogt 1979). The process is known as temperature-dependent sex determination (TSD) where primary sex ratio is influenced by the temperature experienced by eggs during incubation (Bull 1980). Phenotypic sex in marine turtles is determined by the temperature prevailing in approximately the middle third of the incubation period (Yntema and Mrosovsky 1982). By definition, the pivotal temperature is the temperature at which both sexes are produced in equal proportions (sex ratio= 1:1). The transitional range of temperatures (TRT) is the range of constant temperatures that yields both sexes in variable proportions (Mrosovsky and Pieau 1991). From this TRT, lower temperatures will produce only males and higher temperatures will produce only females.

This knowledge about incubation and effects of temperature has provided considerable progress on eggs management. Since the earliest works carried out in nesting beaches, eggs harvesting has been described as one of the most important threat to many marine turtle populations around the world (Bell et al. 2007; Campbell et al. 2007). Relocation to different sections of the beach or to protected hatcheries has been common management tools in many marine turtle conservation programmes (Pritchard 1995; Abella et al. 2007; Santidrián Tomillo et al. 2009). The incubation of eggs in boxes placed within protective storage was an accepted management practice for many years; however posterior research on incubation temperatures at nesting beaches proved that this measure was ineffective since an abnormally high percentage of male turtles may well have been produced (Whitmore and Dutton 1981; Dutton et al. 1985). The improvement of these ex situ incubation methodologies relies on a number of studies about the effect of temperature on embryonic development that has its origin in the work carried out by Yntema and Mrosovsky (1979) that incubated eggs of loggerhead turtle at different controlled temperatures to determine hatchlings sexual differentiation.

Most of the work on sex ratios of marine turtles has been carried out working with hatchlings; monitoring hatchling sex ratio over time and space and understanding its variation has provided essential information for effective conservation of populations of species with temperature-dependent sex determination. Through laboratory experiments, placing eggs in incubators at different constant temperatures (Godfrey et al. 1999; Mrosovsky et al. 2009), pivotal temperature has been determined for several beaches. The laboratory curves relating incubation temperature to sex ratio are then used to relate field incubation temperature to sex ratios. This information is critical in conservation to avoid produce unnatural skewed sex ratios in those beaches where eggs translocation is still badly needed for nests protection (Abella et al. 2007; Pintus et al. 2009). However, despite the importance of data about pivotal temperature, for most marine turtle populations the pivotal temperature and/or nesting beach temperatures have not been measured; many experts consider collect beach temperature data for nesting sites for all populations a future research priority for marine turtles (Hamann et al. 2010; Fuentes et al. 2011).

For animals that depend on environmental temperatures for sex determination of offspring, there is concern that climate change may put some species at risk altering frequencies of hatchling phenotypes (Hawkes et al. 2007; Hulin et al. 2009). It therefore seems prudent to investigate management techniques with which to mitigate the negative effects of climate change to marine turtles. Baseline information on natural sex ratios in various parts of the world, and on variability in pivotals, is needed before large climate changes occur. Primary sex ratios in nests of all marine turtles species around the world appear to be strongly female biased (Chan and Liew 1995; Wibbels 2003; Glenn and Mrosovsky 2004; Zbinden et al. 2007; Rogers 2013), and there is a widely concern about the effect of increasing temperatures in future climate changes scenarios, in which male production will be severely decreased (Hawkes et al. 2009). Hence, management approaches for future conservation strategies include the protection of beaches that produce male hatchlings, which may be of increased importance (Hawkes et al. 2009). Assuming the fact that artificial shade in hatcheries is a very useful tool to decrease incubation temperature, some experiments have been carried out with the aim of develop ways of experimentally shaded clutches. One example is the use of screening material to manipulate the nesting thermal environment with shade, which seems to be effective in reducing nest temperature,

producing a higher proportion of male hatchlings, without compromising the fitness or hatching success (Patiño et al. 2012). Future changes on precipitation or temperature can have different effects on hatchling output at regional level, thus conservation actions should have into account the heterogeneous effect of climate change (Santidrián Tomillo et al. 2015). Climate change has and will have critical effects on marine turtle populations; however, as the climate changes, biodiversity is beginning to respond, which prompt to think that there are glimmers of hope for their conservation (Morecroft et al. 2012). The threatened Cape Verdean loggerhead population represents this capacity of adaptation to the climate changes in marine turtles; after evaluating some unique features of their habitat use and preferences, researchers conclude that this population should have remarkable resilience to climate change (Abella et al. 2016). Future conservation strategies should consider resilience as a management tool, it is a particularly appealing measure as it is risk averse and seeks to enable current species, communities and systems to persist, rather than to change or move (Lin and Petersen 2013).

It should be consider that the bulk of studies about the effect of climate change on primary sex ratios are mainly focused in direct effects of rising temperatures; however, recent studies show that operational sex ratios (i.e., ratio of total number of males vs females breeding each year) are not as female-biased as previously thought and, and mate choice or competition may play more of a role in marine turtle reproduction (Hays et al. 2010; Wright et al. 2012; Stewart and Dutton 2014). Simultaneously, recent studies highlights the importance of consider other environmental variables such as moisture (sand water content) to predict how sexual differentiation of embryos will be affected under future climate conditions (Santidrián Tomillo et al. 2015; Wyneken and Lolavar 2015). Experiments subjecting loggerhead nests under different hot and wet conditions in the laboratory, and data from nests incubated in years that were wetter than normal, showed that the number of males produced was greater than would be expected based on previous studies of sex ratio and incubation temperature (Wyneken and Lolavar 2015). As a result of all these findings, marine turtle researchers encourage to use more analytical approaches that combine both field and laboratory data and consider both temperature and moisture, for a more accurate set of predictions on hatchling sex ratio biases, but also to anticipate the effects of changing temperature and precipitation on sex ratios. Future research should be also focused on understanding how the primary sex ratio relates to the operational sex ratio and how it varies over an animal's lifetime to better understand population dynamics and assess vulnerability to climate change (Stewart and Dutton 2014).

2.4. Environmental Conditions Affecting Embryonic Development

Effective management of threatened species requires a deep knowledge on population dynamics and demographic parameters; these demographic parameters are all input parameters for population models used to manage the species and promote conservation measures. In marine turtles hatchling production is a critical demographic parameter; thus improving knowledge of embryology and hatchling production should be included as a priority objectives in future nesting beach management.

Temperature is the main environmental factor that affects embryonic development with sex ratio and growth rates of hatchlings influenced by nest temperatures during incubation.

Temperature is decisive not only in sex determination but also affect embryos development, hatching success and hatchlings locomotor abilities (Matsuzawa et al. 2002; Maulany et al. 2012; Read et al. 2013; Pike 2014). Frequently, the reference used in different studies around the world about the range of temperatures that marine turtle embryos can withstand and at which they can successfully hatch, is based on studies dating 30 years (35°C, Ackerman 1997). However, recent studies have addressed that naturally fluctuating thermal regimes can exceed 35°C, and still produce hatchings (Booth and Evans 2011; Wood et al. 2014). Moreover, these fluctuations varying among species and nesting areas (Booth et al. 2013; Read et al. 2013) making essential in future researches determining the lethal temperature limits to embryonic development as well as identifying which marine turtle populations are most at risk from embryonic mortality (Howard et al. 2014). Current discrepancies about marine turtle's potential to cope with climate change highlight the significant gaps in our knowledge of both the direction and the magnitude of responses by marine turtles to climatic factors (Patiño et al. 2014; Wyneken and Lolovar 2015; Santidrián Tomillo et al. 2015). Forthcoming studies on nesting beaches should include studies pointing to better understand the complex outcomes of climate change for marine turtles, in order to highlight the most vulnerable species and populations, and encouraging prioritization of conservation effort to the most threatened systems.

Besides temperature, other environmental factors, such as shading and sun exposure (Standora and Spotila 1985), gas exchange (Hays et al. 2001), partial pressures of oxygen, pO2, and carbon dioxide, pCO2 (Wallace et al. 2004; Garret et al. 2010), and moisture from rainfall (Godfrey et al. 1996; Houghton et al. 2007) influences embryonic development and phenotype in turtles. Variations of these environmental conditions can alter a large variety of hatchling characteristics, including embryonic growth rates (Kuroyanagi and Kamezaki 1993), hatch success, and sex ratios (Bull 1980; Mrosovsky and Yntema 1980; Carthy et al. 2003). Future global warming will also lead environmental changes such as sea level rise, increase oceans acidity, changes in patterns and amounts of precipitation and coastal erosion (Fuentes et al. 2010; Dutton et al. 2015). All of these changes can increase average moisture levels, flooding and erosion of marine turtle nesting beaches (Katselidis et al. 2014) influencing reproductive success of marine turtles. On the other hand, increased exposure to water can be detrimental to the incubating eggs as a strong negative correlation between sand water content and emergence success exists (Patiño-Martinez et al. 2014). Furthermore, an excess of water above a nest can prevent gas exchanges between the air and the nest and asphyxiate the nest (Yalçin-Özdilek et al. 2007).

It is clear that most of future threats for marine turtles in nesting areas are related to potential changes in their reproduction derived from future global warming (Hamman et al. 2010). However, there are other threats that should be keep in mind such as pollutants and its potential effects, which is a key area for future research. There are a wide range of environmental chemical pollutants like metals, organometallics, petroleum products of several types; oil dispersants; polycyclic aromatic hydrocarbons (PAHs); plastics; plasticizers; surfactants; current-use pesticides. A few of these chemical classes have been assessed in marine turtles; most of knowledge about contaminants in marine turtles has been focused in the effects of Persistent Organic Pollutants (POPs). POPs typically are halogenated organic compounds, including a range of substances such as PCBs, DDT, and dioxins characterized for they persist for long periods of time in the environment and can accumulate and pass from one species to the next through the food chain. POPs exhibit high lipid

solubility and it is likely that are transferred from nesting females to eggs and hatchlings during reproduction; but there are scarce data on the toxicological effects of POPs on marine turtle embryonic development and its consequences for hatchling development. There is also currently limited information on POPs in marine turtle populations or the maternal transfer of these chemicals and the effects this may have on the reproductive success of nesting populations (van de Merwe et al. 2009, 2010). More research is required to investigate geographical trends of contaminant concentrations and potential health effects (i.e., abnormalities) caused by these contaminants on marine turtle development (Alava et al. 2011). Many more studies are needed to understand the toxicological effects of not only POPs but all chemical classes in altered embryonic growth, mortality and reduced reproductive success of marine turtles. In tortoisheshells it has been described that some trace elements are passed to eggs directly through the eggshell from contaminated soil (Tryfonas et al. 2006; Yalçin-Özdilek et al. 2011); to study this possibilities in marine turtles eggs could be focused of future research in nesting areas with high levels of plastics accumulation and in those located in touristic areas. Plastic items have become the principal constituent of marine debris, and its presence in nesting beaches alters factors affecting hatching success (e.g., Temperature, moisture). Moreover, turtles are impacted by plastic pollution through entanglement, ingestion, bioaccumulation, and habitats degradation (Gregory 2009; González Carman et al. 2013; Poli et al. 2015). Hence, future actions are required to better understand this issue and its effects on marine turtles, so that appropriate and effective mitigation policies can be developed (Vetger et al. 2014; Nelms et al. 2015).

3. Conservation Strategies and Future Research on Oceanic Phases

3.1. Migratory Routes and Movements

Properly conservation measures for marine turtles require understanding the status and condition of their stocks across all life stages (Heppell et al. 2005); one of the handicaps to establishing conservation strategies for marine turtles is their status of migratory species. As it has been described before, population recruitment begins when hatchlings leave the nesting beach and enter the ocean, where they remain offshore for several years, undergoing long trans-oceanic migrations until they appear as immature turtles in neritic waters (Bjorndal and Bolten 1988). Finally, when turtles reach sexual maturity they migrate among neritic or oceanic foraging, breeding, and nesting grounds (Hopkins-Murphy et al. 2003). Historically, information regarding long range movements has been collected using natural marks and attaching metal or plastic flipper tags or passive integrated transponder (PIT) tags into flipper muscle (Hedrickson 1958; Balazs 1999). The pioneer studies based on tag data provided valuable information on turtle dispersion, nesting frequency and nest site fidelity (Alvarado and Murphy 1999; Schroeder et al. 2003). However they do not provide information on the migratory route followed by the animal, movement patterns and behaviour (e.g., speed, diving). A turning point in the studies of marine turtle's spatial ecology was the use of satellite tracking with an exponential rise in its use in the last decade (Luschi et al. 1996; Godley et al. 2008). Satellite telemetry allows track animals during long periods, provide at-

sea locations and behavioural data throughout complete migratory cycles and have the advantage that the data can be recovered remotely (Costa et al. 2012).

First studies with this tool focussed on tracking post-nesting females tagged on nesting beaches (Balazs et al. 1994; Godley et al. 2003). Advances in satellite telemetry have been crucial to discover and delve into the behaviour of probably the most inaccessible species in its oceanic phase, the leatherback turtle (Eckert 2006). Studies of leatherback turtle's movements are mostly restricted to adults; in general it has been shown that leatherback nesting populations use diverse migratory routes which allow them exploit several foraging grounds (Benson et al. 2007; Eckert et al. 2009; Bailey et al. 2012). Tracking studies of nesting female leatherbacks allowed find out that many females nesting in the Caribbean carried out migrations northward (Ferraroli et al. 2004; Hays et al. 2004); subsequent studies evidenced that temperate shelf and slope waters of the northwest Atlantic support extensive foraging by adult male and female turtles, as well as subadults (James et al. 2005). It has been possible describe the diving behaviour of this species showing the capacity to achieve depths > 1200 m and dive durations greater than one hour (Doyle et al. 2008; López-Mendilaharsu et al. 2008). Satellite tracking studies have also allowed go in deep in the migratory routes and behaviour of the remaining marine turtle's species with significant contributions to the knowledge of their lifecycle stages and conservation (Schofield et al. 2007; Hawkes et al. 2011). Tracking of olive ridley in Australia revealed that the meandering oceanic movements of turtles in the Pacific resulted in distinct foraging areas and, the locations of important foraging areas overlapped with existing trawl fisheries and oil and gas exploration and mining (Whiting et al. 2007). Studies of movements of adult hawksbill turtles in Pacific coasts of El Salvador, Nicaragua and Ecuador exposed a previously unknown life-history paradigm for adult hawksbill turtles; these turtles where located within confined inshore estuarine bays associated with mangrove saltwater forests and shrimp ponds (Gaos et al. 2012) rather than inhabit open-coast coral reef habitats as other adult hawksbill populations (León and Bjorndal 2002). The authors propose the hypothesis that preferred habitat probably represents a relatively recent adaptation and possibly a unique evolutionary trajectory for this population. This unexpected finding highlights the possibility that marine turtles exhibit different life-history strategies to their conspecifics among ocean regions, and the importance of understanding such disparities from an ecological and management perspective. An additional critical knowledge for guiding management decisions is the description of the spatial and temporal habitat use patterns of reproductive adults in the neritic zone (Hawkes et al. 2006). Hence, studies about patterns of movement during the breeding season (internesting periods) have also been carried out (Schofield et al. 2010; Revuelta et al. 2015). For example, the study of movements of the critically endangered kemp's ridley along the Gulf of Mexico, allowed to ascertain the round year residence of males in the adjacent areas to the nesting beach, identify migratory paths and foraging grounds which has been key to develop and implement recovery actions in the area (Shaver et al. 2005). In Central Africa, satellite telemetry was used to assess the efficiency of a National Park in the protection of adult olive ridley nesting females; the results of this study support the proposal of create a transboundary park since only 44.6 percent of high-density areas were found within the current park but the proposed transboundary park would incorporate 97.6 percent of high-density areas (Maxwell et al. 2011).

We are a long way from our goal of developing predictive models of sea turtle distribution patterns in the oceanic zone. Pursuing a better understanding of spatial and

temporal ecology of marine turtles, recent studies have integrated satellite telemetry, remotely sensed environmental data, and habitat/environmental modelling to have broadened knowledge on potential influences of environmental variables such as Sea Surface Temperature, Surface chlorophyll concentration on turtle movements (Seminoff et al. 2008; Dodge et al. 2014). These studies will contribute to understanding changes in behaviour, movements and future aggregations for these species. It is recognised that climate change may alter the spatio-temporal abundance of mobile and migratory animals (Robinson et al. 2009; Kaschner et al. 2011), changing the dynamics of trophic food webs and other inter-specific interactions (Edwards and Richardson 2004; Both et al. 2010). Predictive models will be elementary in describe the impacts of climate change on these globally distributed species; hence, future experimental and ecological focus should be directed to incorporate climate change into species conservation planning (Araújo et al. 2004; Hannah et al. 2002). Furthermore, models might predict available habitat with greater specificity than temperature alone, which is the predominant variable used in bioclimatic envelope modelling, for example, approaches will need to encompass mapping prey distribution (Witt et al. 2010).

The knowledge achieved during decades of study of marine turtle's migrations has had invaluable conservation implications. Fisheries bycatch constitutes one of the greatest threats for marine turtles during their oceanic phases (Lewison and Crowder 2007). The leatherback turtle illustrates again this contribution since dramatic worldwide decline of these species populations is largely due to high levels of bycatch (Lewison et al. 2004a, b). In the Pacific Ocean, leatherback populations have followed dramatic declines and extinctions over the last 30 years (Liew 2011; Tapilatu et al. 2013). The study of oceanic migratory routes of adult leatherback females in the Atlantic and Pacific Oceans, has allowed to identify 'hot spots' where leatherbacks overlap with fisheries, highlighting the areas where conservation efforts should be focused (Ferraroli et al. 2004; Fossette et al. 2014; Roe et al. 2014). Long-line fisheries effort is also a great anthropogenically-derived threat for loggerhead turtles in the Atlantic around West Africa, including the Cape Verde islands, population which is the only substantial rookery in the eastern Atlantic (Marco et al. 2012; Coelho et al. 2013; Melo and Melo 2013). The habitats frequented by these loggerhead turtle are coincident with greatest longline fisheries catch data within the Exclusive economic zones (EEZs) of Western Sahara, Cape Verde and Mauritania, and with all other gear types within the coastal EEZs of Western Sahara, Mauritania, Senegal, Gambia and Guinea-Bissau (Pikesley et al. 2014). In the Mediterranean Sea, interaction with fisheries is the threat with highest impact on loggerhead turtles, being drifting longline and bottom trawling the fisheries that capture most turtles in the region (Nada and Casale 2011; Domenech et al. 2014). Olive ridley turtles seem to be the most abundant species in the Eastern Pacific ranging from Mexico to Chile (Troncoso and Urbina 2007) and incidental capture in trawl fisheries and pelagic longlines is the main mortality source for the species with an estimated caught of 92,300 adult female olive ridley turtles in a period of 1999 to 2010, only by the Costa Rican longline fishery (Dapp et al. 2013). In the Gulf of Mexico, an estimated 2700 Kemp's ridleys (juveniles and adults) died annually from fisheries interactions even after mitigation efforts (Finkbeiner et al. 2011).

Assessing the impact of bycatch on marine turtles is a difficult work since it is significantly under-recorded and far greater than the total level of directed legal take (Humber et al. 2014). This is specially challenging when we refers to artisanal fisheries, which are recognized as a serious threat in areas where marine turtles congregate in high numbers like in foraging grounds (Kelez Sara 2011). It is a priority broadened current knowledge and fill

the gaps about the effects of these small fisheries (Casale 2011). Future studies are highly urged to carry out multinational collaboration for a worldwide assessment of the susceptibility of marine turtles to bycatch; mapping the species spatio-temporal distribution and the extent of interactions with fisheries has been proposed as a key future tool (Fossette et al. 2014). Bycatch of turtles in longline and trawling fisheries results in high rates of post-release mortality that may negatively impact populations. Establishing strategies for mitigate incidental catch is a key conservation measurement to protect specific populations (Gilman et al. 2006). In this connection, a number of operational modifications have been established to modify fishing nets and hooks to reduce turtle's bycatch. Examples of these modifications are increasing gear visibility, incorporating a turtle excluded devices (TEDs), modifying float characteristics (Lucchetti et al. 2008; Gilman et al. 2010). Although there exist data describing the patterns of capture of turtles in various fisheries gear types, knowledge base of post-capture-release survivorship and physiology, both in the short and long term, is very poor. The fate of turtles released alive from fisheries depends on the nature of the interaction and condition at the time of release. A recent study has documented for the first time in marine turtles, the physiological impacts of capture in longline gear on loggerhead turtles in the Mediterranean Sea (Williard et al. 2015). These studies about the effects of bycatch are encouraged to be applicable for other species of marine turtles and regions. On the other hand, there are still places with no data about bycatch levels and mortality rates. Hence, along with the establishment of measures for incidental catch mitigation future research should be focussed on study these unknown areas (Wallace et al. 2013). In other regions the lack of knowledge about what life stages are affected by fisheries have resulted in an inadequate legislation (Stringell et al. 2015), highlighting the importance of streamline the information flow between scientific and policy makers. Incorporating novel techniques already used for the study of bycatch in other marine species such as birds and fishes (Alfonso et al. 2011; Graham and Crawdford 2015), will further improve bycatch mitigation of marine turtles.

3.2. Foraging Grounds

Marine turtles spend the vast majority of their lives in the marine environment; however, yet much less is known about the biology of post-hatchling and early juvenile stages than about the biology of females and hatchlings on the nesting beach (Reina et al. 2002). Hatchlings leave their natal beaches and enter the sea where drift passively with ocean currents which drive their broadscale dispersion into oceanic areas and this likely occurs across the nesting range of marine turtles including the Pacific, Indian, Atlantic and Mediterranean (Lohmann and Lohmann 2003; Boyle et al. 2009; Scott et al. 2014). The juveniles then grow for several years before the ontogenetic behavioural shift from epipelagic to demersal neritic developmental habitats take place (Musick and Limpus 1997; Scott et al. 2012).

The duration of the pelagic state and animal's behaviour during this phase is variable between species with loggerhead turtles spending longer periods as epipelagic within the cheloniid turtles, while for leatherbacks it is hypothesize that hatchlings converge in areas of major upwelling where gelatinous prey are abundant (See Musick and Limpus 1997 for a review). This time lapse of oceanic-stage hatchling has been commonly referred to as the "lost years" due to the lack of knowledge about where these hatchlings go or how they behave

(Miller 1997). The understanding of post-hatchling ecology has primarily been obtained through studies of behaviour and diet and most accepted hypothesis about its behaviour was that they occupy sea surface habitats associate with floating Sargassum communities (Witherington et al. 2012). There is only one recent study that has elucidated the loggerhead oceanic nursery paradigm characterizing neonate oceanic movements and surface habitat use (Mansfield et al. 2014). This study characterize hatchlings loggerhead oceanic movements and surface habitat use, the results show that hatchlings dispersal is not uniformly unidirectional as was previously assumed (Carr 1987); a second important finding is that hatchling actively look for Sargassum habitats that presumably provide favourable foraging and thermal niches. The study opens the opportunity for future studies on hatchlings trans-oceanic migrations of other species of marine turtles. The study of hatchling dispersal will have important direct implications for conservation of this early life stages but also for turtles throughout adulthood since the adult migration routes can be shaped by their past experiences as drifting hatchlings (Hays et al. 2010a; Scott et al. 2014). More quantitative and novel interdisciplinary approaches are necessary to understand the drivers that underpin the movement patterns and foraging habitat selections of adult turtles. Future studies will be improved merging tracking data with oceanographic models, magnetic navigation behaviour and environmental data that have great utility for assessing spatially and temporally relevant dispersal patterns of drifting organisms (Baltazar-Soares et al. 2014). The ontogenic shift from an oceanic to neritic stage has been mainly studied in the case of loggerhead turtles. Studies on this species reveals that this change of zone is probably a period of transition that implies morphological and behaviour changes (Bolten 2003). The neritic juvenile turtles are active and feed primarily on the bottom (epibenthic/demersal) (Bjorndal et al. 2003). Once these large juveniles have entered a benthic life stage, they inhabit coastal waters and make seasonal latitudinal migrations between neritic feeding grounds (Hopkins-Murphy et al. 2003).

One of the main gaps in the understanding of turtle's life cycle was to know which nesting areas contribute to a particular feeding area (Limpus et al. 1992). This lack of knowledge had critical conservation implications since all efforts carried out in a nesting area could be all in vain if these turtles are then harvested in their foraging areas. In the 1990s there were carried out several studies about demographic links between nesting aggregates and feeding aggregates to shed light on the understanding of marine turtle distribution and population structure. With the development of molecular genetic tools (e.g., mitochondrial DNA sequence analyses), the relative contributions of rookeries to mixed stocks of oceanic-stage loggerheads could be evaluated (Bowen et al. 1995). After having been demonstrated the presence of rookery-specific myDNA polymorphism (See above Bowen et al. 1993), the frequency shifts observed between populations were used to indicate the origin of turtles found in distant feeding grounds or in migratory corridors, using a maximum likelihood (ML) algorithm to estimate the relative contribution of breeding stocks to foraging areas (Velez-Zuazo et al. 2008; Jensen et al. 2013). Studies of molecular genetic composition of foraging and developmental grounds for juvenile turtles in different regions and species show that these areas are usually composed of individuals from multiple distant rookeries (Carreras et al. 2011; Anderson et al. 2013). Thus, impacts on such stocks usually affect multiple colonies, which require international efforts for conservation. All these studies have provided evidences regarding migration patterns of marine turtles, offering key information to support the

implementation of mitigation measures (Hart et al. 2012; Dutton et al. 2013; Griffin et al. 2013; Proietti et al. 2014; Prosdocimi et al. 2015).

The advent of molecular techniques has lead important achievements in conservation planning for migratory species such as marine turtles. Genetic analyses have revealed the complex population structure of these species and its importance in defining management units for their conservation at each life stage (Dutton et al. 2014; Phillips et al. 2014). It has been demonstrated that perturbations on pelagic juveniles or subadults would have an impact on nesting colonies, as well as disturbances to adults will have establish impact on corresponding breeding populations (Monzón-Argüello et al. 2010). Knowledge about population structure in different areas have showed the need of preserve the genetic diversity of the marine turtle population; population genetics data have been joined to population abundances and trends, and satellite telemetry data to delimitate Regional Management Units (RMUs) for prioritizing conservation and research across multiple scales (Wallace et al. 2010). RMUs provide a framework for identifying data gaps, assessing high diversity areas for multiple species and genetic stocks, and evaluating conservation status of marine turtles. RMUs allow for identification of geographic barriers to gene flow, and can provide valuable guidance to marine spatial planning initiatives that integrate spatial distributions of protected species and human activities. Despite the advances in the genetic tools (e.g., Bayesian Mixed Stock Analysis (MSA)) there are intrinsic characteristics in the populations structure of marine turtles that should be consider when working with these kind of data. For example, the occurrence of common mtDNA haplotypes that are shared among rookeries may lead to unreliable MSA results with large confidence intervals (Shamblin et al. 2011). Future studies should evaluate the power of specific genetic markers to detect structure in order to correctly identify the appropriate population units to conserve. It is recommended to use fine-scale level through the use of population genetics; large studies comprising vast sampling areas and the use of long segments of mtDNA control region as these highly enhance genetic resolution (LeRoux et al. 2012; Clusa et al. 2014). Additionally, temporal variation in the mtDNA haplotype frequencies of turtles nesting in different years have been observed in some populations (Shamblin et al. 2011) making necessary robust sampling designs to include samples collected across years for the identification of management units. In addition, the employ of longer control region sequences and developments in statistical models for analyzing mixed stock composition will likely result in confidence intervals for the point estimates of rookery contributions to foraging populations (Shamblin et al. 2012, 2014).

Identifying the foraging habitat of marine turtles is vital to understanding the ecology of these species and for their management and conservation. In fact, establishing key foraging areas for marine turtles has been determined as a conservation priority in the 21st century (Hamman et al. 2010). Protection and recovery requires identifying critical at-sea foraging habitats and better understanding the spatial distribution of marine turtles (Blumenthal et al. 2006; Mansfield et al. 2009; Scott et al. 2012). Flipper tagging, satellite telemetry and stable isotope tracking have been key tools for the study and identification of foraging habitats for marine turtle species all around the world. The use of telemetry datasets and modelled movements and behaviour have allowed to discover important habitats for these species (Hays et al. 2002; Hawkes et al. 2011; Hart et al. 2012), to determine distances of foraging grounds from nesting and tagging sites (Troëng et al. 2005; Read et al. 2014), time spent by turtles to cover these distances and fidelity over time to these foraging areas (Esteban et al. 2015) and identify seasonal migrations and home range (Hart et al. 2015; Revuelta et al.

2015). There are numeral examples of application of this knowledge for the development of conservation strategies for marine turtles. The monitoring of adult loggerhead nesting females turtles originating from three separate subpopulations in the Gulf of Mexico revealed the use of a common at-sea foraging area during non-nesting periods, highlighting the importance of protect this area (Hart et al. 2012). In the same region, a different study showed overlapping between areas shared by multiple loggerheads with areas of high fishing effort; this data could contribute to establish efficient conservation management strategies in the area (Hardy et al. 2014).

One of the characteristic of marine turtles foraging grounds is its dynamic condition, which makes difficult distinguish hidden behaviours just analysing satellite tracking data. This hindrance can be enhanced applying modern statistical methods with great potential for modelling population time series (Newman et al. 2006). The State-space models (SSM) refine Argos tracking data by accounting for observation errors and stochasticity in animal movement, incorporate behavioural information estimating the probability that an animal is in a particular discrete behavioural mode, such as transiting or foraging (Jonsen et al. 2003; Patterson et al. 2008; Johnson et al. 2008). The SSM have best recreated habitat use and migration routes of hawksbill turtles nesting on Groote Eylandt, northern Australia (Hoenner et al. 2012); modelling loggerhead turtle movement in the Mediterranean Sea (Eckert et al. 2008) and definition of the different behavioural phases such as migration versus foraging for leatherbacks in the Pacific Ocean (Bailey et al. 2008) and in the northeastern Atlantic (Jonsen et al. 2007).

Molecular and Satellite telemetry studies have also been complemented with stable isotope analyses (Vander Zanden et al. 2015). Stable isotope ratios have been increasingly used as intrinsic markers to trace foraging habits and movements of marine turtle populations (Newsome et al. 2010; Zbinden et al. 2011). The measurement of naturally occurring stable isotopes in animal tissues represents one means of assaying intrinsic markers in animals in order to infer information on their origins. The use of stable isotopes is based on the fact that stable isotope ratios of nitrogen (15N/14N, noted δ15N) and carbon (13C/12C, noted δ13C) concentrations in the consumer tissues reflect those in their resources and those spatial patterns, gradients, or changes in such markers exist in nature (Deniro and Epstein 1978; Hobson 2007). Thus by making isotopic measurements in animal tissues and knowing how stable isotopic patterns in foodwebs change spatially, it is often possible to infer their origins. In the marine environment, stable isotope ratios of nitrogen (δ15N) are typically used to assess trophic level and carbon (δ13C) vary little along the food chain and can offer insight into foraging location (Peterson and Fry 1987; Wallace et al. 2009). Using stable isotopes as dietary tracers has been a key tool for describing foraging variations among individuals (Snover et al. 2010) and between life stages (Arthur et al. 2008), for determining the characteristics of feeding grounds, for determining ontogenetic changes in habitat and diet, and for linking breeding and foraging grounds of marine turtles. Accurate data about marine turtles habitat use can facilitate conservation and resource management efforts (Benson et al. 2011; Block et al. 2011). Stable isotopes analysis supplement the information obtained from satellite tracking about the movements and habitat use by turtles. Studies of tracked juvenile loggerhead turtles in North Carolina, USA showed to have significant variation in movement behaviours (oceanic versus neritic) (McClellan and Read 2007); posterior studies of diet composition determined by stable isotope ratios demonstrated that individuals that use open-ocean habitats have lower stable nitrogen isotope ratios than do those that remain within

neritic areas, indicating a dietary dichotomy (McClellan et al. 2010). Studying isotopic compositions of marine turtle body tissues sampled at nesting beaches showed divergent migratory strategies and within-population segregation of foraging groups of leatherback marine turtles across the Pacific Ocean (Seminoff et al. 2012). In the case of nesting females, foraging ecology can also be characterized using eggshell isotopic signatures (Paddock 2007). Dichotomy in foraging area was also revealed through isotope analysis of egg-yolk and blood for Atlantic leatherback turtles and the results of this work highlighted the urgent need to determine the feeding habitats of the turtle in the Atlantic in order to protect this species from incidental take by commercial fisheries (Caut et al. 2008). These works indicate that egg analysis is a useful and less invasive tool for assessing isotope values in marine turtles. Recent research (Detjen et al. 2015) has demonstrated that it is possible to describe marine turtle migration and habitat use patterns studying isotope signatures in barnacles, an obligate commensals that have been reported widely from marine turtle populations from across the world (Casale et al. 2004; Frick et al. 2010). In future studies, additional methods such as barnacle proxies could therefore complement other methods in understanding regional movement patterns.

Despite the number of studies and techniques applied, connections that exist among rookeries and foraging grounds, location of oceanic 'hotspots' that include key foraging habitats and mechanisms of foraging site selection remain poorly defined. Future steps proposed to improve knowledge about marine turtles foraging habitats include further integration of disparate tracking data-sets at the oceanic scale along with modelling of movements to identify critical at-sea foraging habitats where individuals may be resident during non-nesting periods (Hart et al. 2013). On the other hand, sex-based dispersal remains poorly understood in marine turtles and still studies have been unable to analyse foraging composition by sex (Jensen et al. 2013). Hence, future researches could take into account to increase studies about tracked males in order to know where are their foraging grounds and test if there are different drift scenarios between females and males hatchlings that could result in differences in adult migration routes and, consequently sex differences in foraging location (Poloczanska et al. 2009; Hays et al. 2010). Long-term monitoring of the composition of foraging grounds may provide an effective way of detecting significant population changes as well as identifying female- and/or male-producing rookeries (Pajuelo et al. 2012). Future effects of climate change on shifts on ocean currents that transport juveniles to those foraging grounds (Fuentes et al. 2009) and this in turn might give rise to new post-breeding adult migrations. Future studies should also pay attention to areas where hybridization is a common process (e.g., loggerhead x hawksbill turtles and loggerhead x olive ridley in Brazilian waters) between breeding groups; understanding the distribution, ecology, and migrations of these hybrids is essential for the development of adequate conservation and management plans (Reis et al. 2010; Proietti et al. 2014). Future studies mixing data from stable isotope and skeletochronology can offer insight into foraging location (nearshore vs. offshore) (Wallace et al. 2009; Snover et al. 2010). Skeletochronology identifies significant increases in marine turtles growth rates (as inferred through skeletal growth increment width) that has been proposed to correspond with an ontogenetic change in foraging habitat and/or prey preference (Snover et al. 2010). There are still many questions to answer about marine turtle's behavioral ecology and habitat use that require the future effort of researchers in this field.

CONCLUSION

Marine turtles are a well-studied group, during decades it has been recorded knowledge about their physiology, behaviour, distribution, habitat use, and different characteristics of their lifecycle stages. However there are still many unfilled gaps in our knowledge of marine turtles that require future studies go in deep on. Stunningly, in the last decade there have been discovered new important areas for marine turtles ignored for so long, showing that scientific community's has much to learn about the questions of how many, where and when marine turtles exist. Increase knowledge about the potential effects of future global warming scenarios would have on this species, are considered as a main objective by scientist. The use of more analytical approaches combining both field and laboratory analyses to improve knowledge about environmental conditions affecting embryonic development, and understanding how the primary sex ratio relates to the operational sex ratio, will be some of the study areas to better understand population dynamics and assess vulnerability to climate change. Since marine turtles are migratory species heavily affected by fisheries, it is necessary continuing working on plans to improve knowledge about fisheries effort, improve tools for diminish marine turtle bycatch rates, and identify the most appropriate mitigation measures. The lack of data of artisanal fisheries is an unresolved matter concerning marine turtle's conservation that should be considered as a main objective to solve by researchers and conservationist. There are also many other key questions like hatchling dispersion, sex-based dispersal, genetic relationships between rookeries, composition of foraging grounds, that are still under resolved. The application of state-of-the-art techniques, such as statistical methods with great potential for modelling population time series, longer control region sequences and developments in statistical models for analyzing mixed stock composition in genetic studies, and tracking animal migration with stable isotopes approaches, will yield a wealth of knowledge that will improve our understanding of these species and their habitats, and that will turn into the establishment of better conservation measures.

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Chapter 36

SPIRORCHIIDIOSIS AND OTHER FORMS OF PARASITOSIS IN SEA TURTLES ON THE COAST OF BRAZIL

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ABSTRACT

Spirorchiids (family Spirorchiidae Stunkard 1921) are a group of flukes that inhabit the circulatory system of turtles. The family has approximately 100 species grouped into 19 genera, 10 of which are parasites of sea turtles that have been described for *Chelonia mydas* Linnaeus 1758, *Caretta caretta* Linnaeus 1758, *Eretmochelys imbricata* Linnaeus 1766, *Lepidochelys olivacea* Eschscholtz 1829 and *Chelonia mydas agassizii* Bocourt 1868. Infection by members of the family Spirorchiidae involves egg deposition in the host blood stream, accumulation in tissues, which cause inflammatory reactions and embolisms, leading or contributing to the death of the host. However, little is known regarding this type of parasitosis in sea turtles found on the coast of Brazil. The aim of this chapter was to perform a literature review to provide information on the state of spirorchiidiosis and other forms of parasitosis in sea turtles found along the Brazilian coast. This is pioneering work in the field, as very little information is found on the health status of sea turtles in Brazil, especially with regard to this parasitic disease, which can cause serious injuries in hosts and even death.

Keywords: Brazil, parasites, sea turtles, Spirorchiidae

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INTRODUCTION

Among the seven species of sea turtles in the world, five are found along the coast of Brazil: the leatherback turtle (*Dermochelys coriacea* Linnaeus, 1758), loggerhead turtle (*Caretta caretta* Linnaeus, 1758), olive ridley turtle (*Lepidochelys olivacea* Escholtz, 1829), hawksbill turtle (*Eretmochelys imbricata* Linnaeus 1766) and green turtle (*Chelonia mydas* Linnaeus, 1758) (Marcovaldi & Marcovaldi 1999). Although knowledge on the health status of a population is considered an important conservation tool, very little is known regarding the health problems of sea turtles on the Brazilian coast. Studies on this issue have mainly addressed fibropapillomatosis, which is a virus characterized by tumor masses with a debilitating and even lethal nature (Rossi et al., 2009; Zwarg et al., 2014; Rodenbusch et al., 2014).

As in other hosts, parasites of sea turtles constitute an important ecological-health factor, as these organisms can cause serious illness and even contribute to the death of chelonians (Gordon et al., 1998; Stacy et al., 2010). Sea turtles have a varied parasitic fauna and many families of parasites have been described (Gibson et al., 2002; Jones et al., 2005). Moreover, like other chelonians, sea turtles are hosts of a distinct family of digenetic flukes that inhabit the circulatory system, denominated Spirorchiidae. Although the debilitating and even fatal potential of this form of parasitosis is known, few chelonians are examined for spirorchiids along the coast of Brazil. The specialized literature has reported this parasitosis and associated injuries in sea turtles (Rand & Wiles, 1985; Gordon et al., 1998; Raidal et al., 1998; Santoro et al., 2007; Stacy et al., 2010; Chen et al., 2012), but few data have been reported for Brazil.

The aim of the present study was to perform a literature review on spirorchiidiosis and other forms of parasitosis in sea turtles found along the Brazilian coast.

METHODS

This study was developed using only scientific papers on the helminths identified as well as the injuries caused by spirorchiids in sea turtles from Brazil published between 1919 and 2016. Dissertations, theses and abstracts from conferences were not considered. While the collection and fixation of parasites were not the main focus of this study, the reader can gain a better understanding of these aspects in studies conducted by Snyder & Clopton (2005) and Greiner (2013). Parasite species richness, prevalence, mean infection intensity and mean abundance were reported when described by the authors cited and followed the definition proposed by Bush et al. (1997). For classification, the taxonomic proposal presented by the World Register of Marine Species (WoRMS, 2015) was used.

The Family Spirorchiidae

The family Spirorchiidae Stunkard 1921 includes approximately 100 species distributed among 19 genera. Members of this family are exclusive parasites of terrestrial and aquatic chelonians and use the circulatory system at their habitat (Smith, 1997; Platt, 2002). Among

the 19 genera currently described for the family, ten are restricted to sea turtles. *Hapalotrema* Looss 1899, *Amphiorchis* Price 1934, *Learedius* Price 1934, *Neospirorchis* Price 1934, *Monticellius* Mehra 1939 and *Carettacola* Manter & Larson 1950 are considered cosmopolitan parasites. *Neocaballerotrema* Simha 1977, *Shobanatrema* Simha & Chattopadhyaya 1980, *Cheloneotrema* Simha & Chattopadhyaya 1980 and *Satyanarayanotrema* Simha & Chattopadhyaya 1980 are only reported for India and none has yet had its biological cycle clarified (Platt, 2002). This parasitosis causes serious harm due mainly to the deposition of eggs in host tissues, which can occasionally lead to death (Gordon et al., 1998). Such findings have been described for *C. mydas* (Greiner et al., 1980; Glazebrook et al., 1981; Glazebrook et al., 1989; Gordon et al., 1998; Santoro et al., 2007; Stacy et al., 2010; Chen et al., 2012), *C. caretta* (Wolke et al., 1982; Stacy et al., 2010), *E. imbricata* (Glazebrook et al., 1989; Dutra et al., 2012) and *L. olivacea* (Santoro and Morales, 2007; Jerdy et al., 2016)

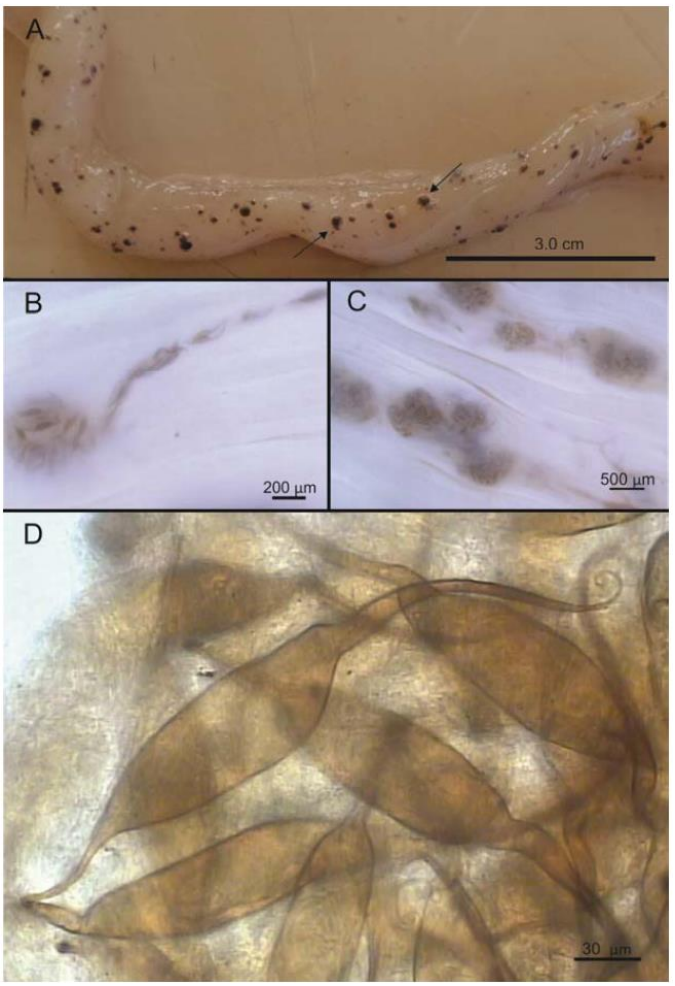

Figure 1. A) Dark nodules in small intestine (arrow), green turtle; B-C) Blood vessel with parasitic embolism composed by spirorchid eggs; D) Eggs of *Learedius learedi* in stereomicroscopy.

The most common macroscopic lesions reported in necropsies are dark nodules measuring approximately 1 to 2 mm in diameter (Figure 1A), which reveal a large amount of fluke eggs when analyzed using stereomicroscopy (Figure 1B,C and D). The histopathology of the nodules reveals chronic inflammation due to the eggs, which, when released into the blood stream, become lodged in different sites of the host organism (Gordon et al., 1998; Santoro et al., 2007; Stacy et al., 2010; Chen et al., 2012). Although a less common finding, adult helminths are also capable of causing inflammatory reactions (Gordon et al., 1998; Santoro et al., 2007). The lesions are granulomatous and contain the parasite eggs, affecting most host organs, such as the central nervous system, cardiopulmonary system, urinary tract, digestive system, salt glands (Glazebrook et al., 1981; Glazebrook et al., 1989; Gordon et al., 1998; Stacy et al., 2010) and gonads. In these lesions of varied intensity and number, lymphocytes and macrophages apparently form the first line of defense against fluke eggs; moreover, macrophages fuse, giving rise to Langhans cells and, at times, foreign-body giant cells (Glazebrook et al., 1981). The presence of eggs in host tissues is irrefutable evidence of parasitosis. Moreover, morphological and morphometric analyses of the eggs contribute to the identification of the genus of the parasite (Wolke et al., 1982).

Spirorchiidiosis in Sea Turtles on the Coast of Brazil

Studies involving the evaluation of cases of spirorchiidiosis remain scarce in Brazil and largely constitute reports conducted with few individuals. The first report of spirorchiidiosis occurred in 2006 (Werneck et al., 2006) and few turtles have been examined for this form of parasitosis whether due to an unawareness of the family or technical problems regarding collection during the necropsy of individual hosts. In recent years, however, a slight increase in the number of reports of lesions caused by spirorchiids along the Brazilian coast has been noted, with descriptions of this form of parasitosis in green turtles (Werneck et al., 2006; Goldberg et al., 2013; Werneck et al., 2015a; Binoti et al., 2016), hawksbill turtles (Werneck et al., 2008a; Dutra et al., 2012) and, more recently, olive ridley turtles (Jerdy et al., 2016).

Spirorchiidiosis in *Chelonia mydas*

The first case of injuries caused by eggs from the family Spirorchiidae in green sea turtles was described by Werneck et al. (2006), who analyzed 11 *C. mydas* juveniles from the coast of the state of São Paulo (southeastern Brazil), six of which (54%) exhibited adult *Learedius learedi* Price, 1934 (Figure 2) in the circulatory system and two exhibited dark nodules measuring 1 to 2 mm containing spirorchiid eggs. Subsequently, Goldberg et al. (2013) reported lesions caused by spirorchiid eggs in the lungs of a specimen of *C. mydas* found in the city of Florianópolis (southern Brazil).

A case of aortic aneurysm and thrombosis caused by *Hapalotrema postorchis* Rao 1976 was reported in a juvenile green sea turtle found on the coast of the state of Espírito Santo (southeastern Brazil). In the same individual, giant cell granulomas due to the parasite eggs were found in the heart, intestines, liver, pancreas, spleen, kidneys and brain (Figure 3). The infection was considered a debilitating factor of the host, but non-fatal. In the same year, Werneck et al. (2015a) reported another case of injuries stemming from the deposition of

spirorchiid eggs in a *C. mydas* juvenile also on the coast of the state of Espírito Santo, with granulomatous reaction in the liver. Analyzing the tissues of 16 green sea turtles found in the same state of southeastern Brazil, Binoti et al. (2016) reported the occurrence of fluke eggs mainly in the spleen and lungs, at times surrounded by giant cells. Less intensive lesions were found in striated muscle tissue, the digestive tract, salt glands, kidneys, heart, pancreas, skin and adrenal glands. Moreover, concomitant infection of parasitic forms and eggs were found in the brain.

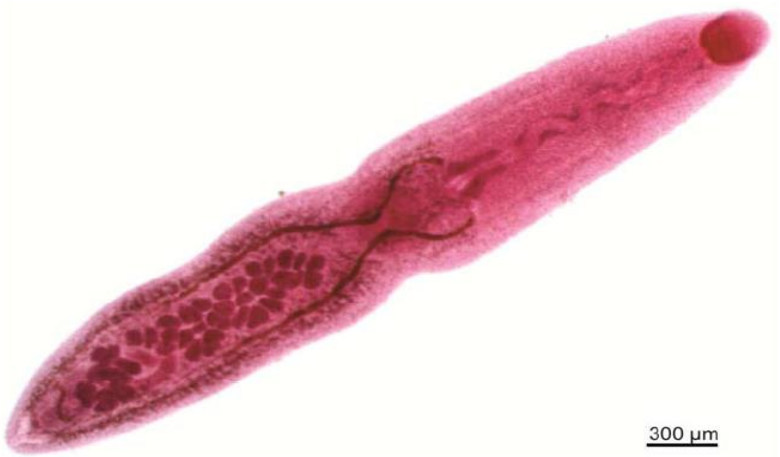

Figure 2. *Learedius learedi* Price, 1934, heart of Green turtle.

Spirorchiidiosis in *Eretmochelys imbricata*

Granulomas due to egg deposition in a hawksbill turtle have been found in the brain, salt glands, pancreas, mesentery as well as the large and small intestines (Werneck et al., 2008a; Dutra et al., 2012; Werneck et al., 2015b). The duodenum exhibited necrosis and lymphoplasmacytic inflammatory infiltrate related to the eggs, whereas the heart and liver exhibited fewer eggs and giant cells were found in the liver and lungs (Dutra et al., 2012).

Spirorchiidiosis in *Lepidochelys olivacea*

The only record of spirorchiidiosis in *L. olivacea* in Brazil was made by Jerdy et al. (2016), who reported the occurrence of lesions in five individuals. The diagnosis was performed by microscopy of giant cell granulomas caused by spirorchiid eggs found in samples obtained from necropsies. The lesions affected the brain, thyroid glands, heart, lungs, spleen, liver and intestines. None of the five turtles had massive infection; all exhibited lesions with few eggs and small granulomas. Giant cells were found in the lesions of the spleen, lungs and intestines. The eggs in the brain, thyroid glands, heart and liver did not cause an apparent inflammatory response. No adult parasite specimens were found and the eggs in all five cases were types 1 and 3 (see Wolke et al., 1982). Type 1 eggs are characteristic of the genera *Hapalotrema*, *Learedius* and *Monticellius*, whereas type 3 eggs

are characteristic of the genus *Neospirorchis*, demonstrating possible infection of the olive ridley turtel by some of these genera.

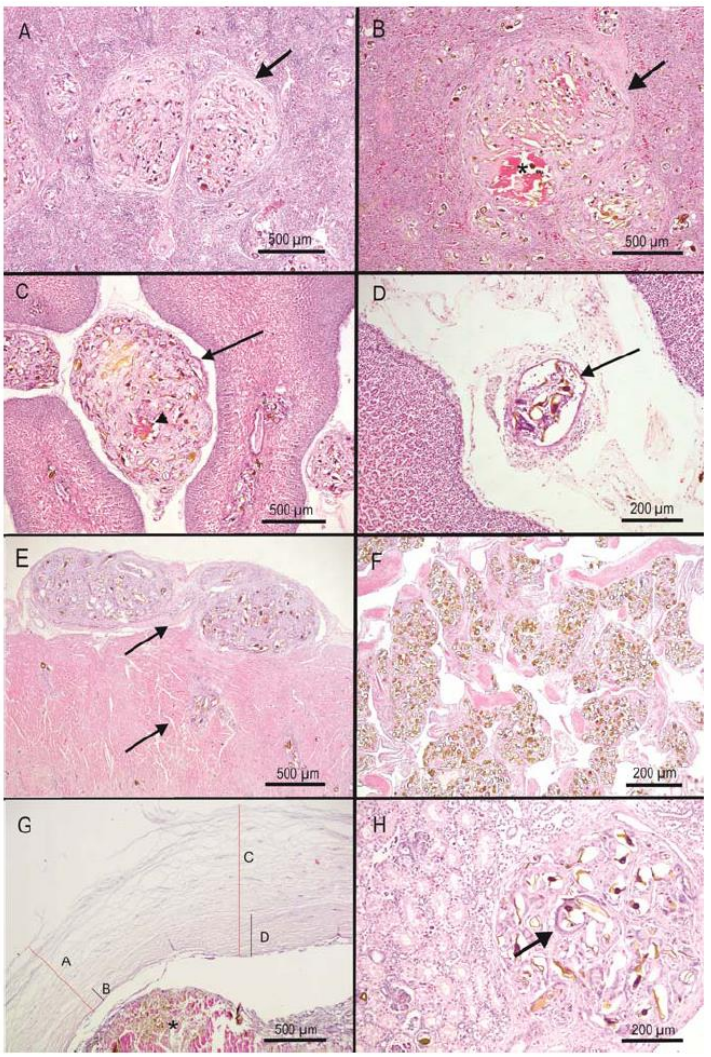

Figure 3. A: Multifocal giant cell granuloma surrounding spirorchid eggs in spleen; B: Granulomatous meningitis with aggregations of spirorchid eggs (Asterisk) and caseous necrosis association, cerebral córtex was compressed (arrow); C: Salt gland with giant cell granuloma and caseous necrosis caused by spirorchid eggs; D: Embolism in salt gland by spirorchid eggs; E: Spirorchid eggs associated with giant cell granuloma in myocardium and epicardium; F –Severe granulomatous pneumonia with gigant cell surrounding inumerous spirorchid eggs; G: Aorta aneurysm associated with spirorchids eggs inside thrombus. Note thickness difference between túnica media (Lines B/D) artery wall layers (A/C); H: Granulomatous nephritis with giant cell surrounding spirorchid eggs.

Parasites in Sea Turtles on the Coast of Brazil

According to Ruiz (1946), the first record of parasites in sea turtles in Brazil was possibly that made by Diesing in 1850, who reported the occurrence of *Monostomu trigonocephalum*

(currently *Cricocephalus albus*) (Digenea: Pronocephalidae) in a sea turtle (likely *C. mydas*). The material was sent to Europe for study.

Although the Brazilian coast is inhabited by five species of sea turtles, studies on the helminth fauna in the individuals found remain scarce, demonstrating a huge gap in knowledge regarding the parasitology of these chelonians. *C. mydas* is the best studied host on the coast of Brazil, followed by *E. imbricata*, *C. caretta*, *D. coriacea* and *L. olivacea*. Most reports are case descriptions in few host individuals, although a gradual increase in studies with a larger number of turtles has been observed.

Parasites in *Chelonia mydas*

Travassos (1918) was the pioneer in studies on these organisms in Brazil. The author reported the occurrence of a nematode from the family Kathlaniidae denominated *Kathlania leptura* (Rudolphi, 1819). The parasite was found in *Testudo mydas* (*C. mydas*), possibly on the coast of the southeastern region of the country.

After the necropsy of a juvenile individual found on the coast of the state of Rio de Janeiro, Freitas & Lent (1938) collected three specimens of an unknown fluke, which was described as *Metacetabulum invaginatum* Freitas & Lent, 1938. The authors created a new family based on this species denominated Metacetabulidae, but the species was subsequently transferred to the family Pronocephalidae. In the same study, the authors described two other parasites based on samples deposited in the helminth collection of the Oswaldo Cruz Institute that had not previously been reported for Brazil: *Orchidasma amphiorchis* Braun 1899 and *Polyangium linguatula* Looss 1899.

Ruiz (1943) identified *Neoctangium travassosi* Ruiz 1943, as a new species of fluke found in a sea turtle rescued on the southern coast of the state of São Paulo (southeastern Brazil). However, the author did not identify the species of sea turtle from which the parasite was collected (*C. mydas*, see Muniz-Pereira et al. 2009).

After a literature review and analysis of material deposited in helminth collections of the Butantã Institute in the state of São Paulo and the Oswaldo Cruz Institute in the state of Rio de Janeiro, Ruiz (1946) presented a revision of the family Pronocephalidae, describing the species encountered in Brazil as well as those described in other regions. Through this bibliographic analysis, the authors presented a reorganization of the family Pronocephalidae, describing 28 genera grouped in seven subfamilies, but only five genera were described for C. *mydas* on the coast of Brazil: *Pronocephalus* Looss, 1899, with the species *Pr. Trigonocephalus* Looss, 1899 and *Pr. minutus* Ruiz, 1946; *Cricocephalus* Looss, 1899, with the species *C. albus* Kuhl et Hasselt, 1822; *Pyelosomum* Looss, 1899, with the species *P. crassum* Loss, 1901; *Pleurogonius* Looss, 1901, with the species *P. longiusculus* Looss, 1901, *P. trigonocephalus* Rudolphi, 1809, *P. linearis* Looss, 1901 and *P. lobatus* Looss, 1901; and *Metacetabulum* Freitas & Lent, 1938, with the species *M. invaginatum*. In the same year, Freitas & Lent (1946) reported the occurrence of *Porrocaecum sulcatum* (Rudolphi, 1819) in a green sea turtle found on the coast of the state of Rio de Janeiro (southeastern Brazil) as well as the description of the diagnosis of chronic and acute gastritis due to a fibrinous, purulent exudate stemming from the action of parasites and their eggs. As far as it is known, this is the first report of lesions caused by helminths in sea turtles on the coast of Brazil.

Vicente and Santos (1968) described *Tonaudia freitasi* Vicente and Santos, 1968 based on "*numerous specimens of nematodes*" collected from the stomach of a *C. mydas* individual from the state of Ceará (northeastern Brazil). Travassos et al. (1969) conducted a study on flukes occurring in Brazil and recorded 14 species found in sea turtles – one in *C. caretta* (*Rhytidodes gelatinosus* Braun, 1899) and 13 in *C. mydas***:** *N. travassosi*, *P. linguatula*, *M. invaginatum*, *O. amphiorchis*, *Rhytidodes gelatinosus* Braun, 1899, *P. lobatus* (synonym of *Glyphicephalus lobatus* Looss, 1901), *Pronocephalus obliquus* Looss, 1901 (synonym of *Pr. trigonocephalus*), *C. albus*, *P. linearis*, *P. longiusculus*, *Pleurogonius trigonocephalus*, *P. crassum* and *Ruicephalus minutus* Ruiz, 1946 as a new denomination for *Pr. minutus*.

After more than three decades, Werneck et al. (2006) analyzed 11 *C. mydas* juveniles from the municipality of Ubatuba on the northern coast of the state of São Paulo (southeastern Brazil). Specimens of *L. learedi* Price were found in six individuals (54.6%). This was the first report of parasites from the family Spirorchiidae in hosts on the coast of Brazil. In the same region, *Monticellius indicum* Mehra, 1939 was found in the heart of two *C. mydas* juveniles (prevalence: 0.83%) among a total of 239 individuals submitted to necropsy in the state of São Paulo between 2005 and 2008 (Werneck et al., 2008b).

The analysis of a *C. mydas* individual from the state of Ceará (northeastern Brazil) revealed the occurrence of *Amphiorchis solus* (Simha & Chattopadhyaya, 1970) Platt, 2002 (Digenea: Spirorchiidae) in the heart of the host (Werneck et al., 2011). This species may be considered one of the rare taxa in the family Spirorchiidae, as only three specimens have been reported to date (Simha & Chattopadhyaya, 1970; Santoro et al., 2006; Werneck et al., 2011).

Werneck & Silva (2013) reported the occurrence of *Amphiorchis indicus* Gupta & Mehrotra, 1981 in six hosts among a total of 348 *C. mydas* individuals found on the southern coast of the state of Rio de Janeiro and northern coast of the state of São Paulo (prevalence: 1.7%). A total of 14 flukes were found in the samples analyzed: liver (n = 6) and small intestine (n = 8). In the same year, Werneck et al. (2013) found *Carettacola stunkardi* (Martin & Bamberger, 1952) in a *C. mydas* juvenile from the coast of the state of Espírito Santo (southeastern Brazil). Also in the same year, the analysis of another *C. mydas* juvenile from the state of Espírito Santo revealed *Rhytidodoides similis* Price, 1939 (Digenea: Rhytidodidae), which was collected from the gall bladder. Moreover, lesions were found in the liver due to parasite eggs from the family Spirorchiidae (Werneck et al., 2015c).Werneck et al. (2015a) also found an aneurism in the aorta of a *C. mydas* juvenile from the state of Espírito Santo due to the occurrence of *H. postorchis* as well as tissue lesions in different organs due to the deposition of eggs from the same parasite One of the broadest-scoped studies on parasites of *C. mydas* in southeastern Brazil was conducted between 2004 and 2011. A total of 136 juveniles from the coast of the states of São Paulo and Rio de Janeiro were evaluated, 90 (prevalence: 66.2%) of which were infected with helminths. The parasite species richness was 4.74, mean intensity was 327 and mean abundance was 216. The researchers found a total of 26 species of flukes belonging to six families (Brachycoeliidae, Cladorchiidae, Microscaphidiidae, Pronocephalidae, Rhytidodidae and Spirorchiidae) as well as the larvae of unidentified nematodes (Werneck & Silva, 2015) (Figure 4A, C, D, E, F).

Analyzing 50 sea turtles from the coast of the state of Espírito Santo, Binoti et al. (2016) found 19 flukes belonging to seven families (Calycodidae, Cladorchiidae, Gongoderidae, Microscaphidiidae, Pronocephalidae, Spirorchiidae and Telorchiidae) as well as lesions caused by parasite eggs in different tissues of the hosts. More recently, Werneck et al. (2016)

reported the occurrence of *Neospirorchis schistosomatoides* Price 1934 in the heart of a juvenile green sea turtle found in the state of Espírito Santo.

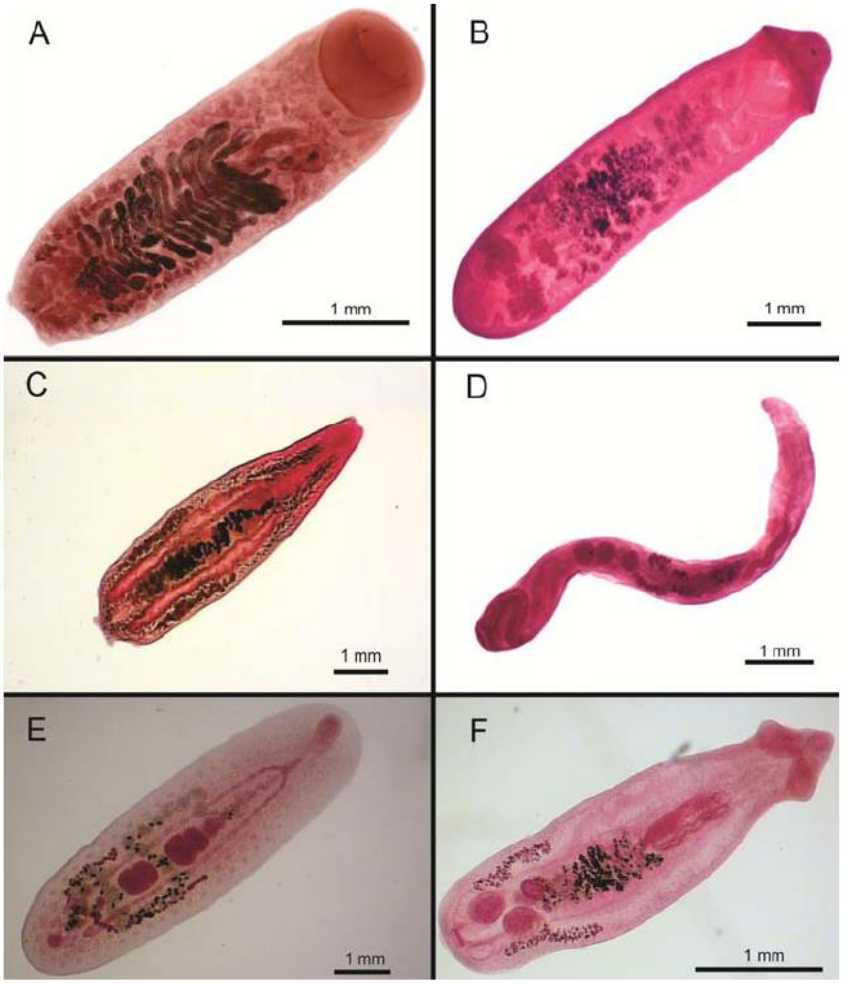

Figure 4. Figures A and C through F parasites from green turtle. Figure B, parasite from loggerhead turtle. A: *Cricocephalus megastomum*; B: *Pyelosomum renicapite*; C: *Neoctangium travassosi*; D: *Metacetabulum invaginatum*; E: *Deuterobaris proteus*; F: *Pronocephalus obliquus*.

Helminths Reported in Green Sea Turtles on the Coast of Brazil

Family Calycodidae

- *Calycodes anthos* (Braun, 1899) Looss, 1901.

Familia Rhytidodidae

- *Rhytidodes gelatinosus* (Rudolphi, 1819) Looss, 1901
- *Rhytidodoides similis* Price, 1939

Family Cladorchiidae

- *Schizamphistomum scleroporum* (Creplin, 1844) Looss, 1912

Family Microscaphidiidae

- *Angiodictyum longum* Blair, 1986
- *Angiodictyum parallelum* (Looss, 1901) Looss, 1902
- *Deuterobaris proteus* (Brandes, 1891) Looss, 1900
- *Microscaphidium reticulare* (Van Beneden 1859) looss, 1900
- *Neoctangium travassosi* Ruiz, 1943
- *Polyangium linguatula* Looss, 1899

Family Pronocephalidae

- *Charaxicephalus robustus* Looss, 1901
- *Cricocephalus albus* Kuhl & Van Hasselt, 1822
- *Cricocephalus megastomum* Looss, 1902
- *Diaschistorchis pandus* (Braun, 1901) Johnstone, 1913
- *Metacetabulum invaginatum* Freitas & Lent 1938
- *Pleurogonius linearis* Looss, 1901
- *Pleurogonius longiusculus* Looss, 1901
- *Pleurogonius trigonocephalus* (Rudolphi, 1809) Looss, 1901.
- *Pleurogoius lobatus* (Looss, 1901).
- *Pronocephalus obliquus* Looss, 1899.
- *Pronocephalus trigonocephalus* Looss, 1899
- *Pyelosomum cochlear* Looss, 1899
- *Pyelosomum crassum* (Looss, 1901) Ruiz, 1946.
- *Rameshwarotrema uterocrescens* Rao, 1975.
- *Ruicephalus minutus* (Ruiz, 1946) Skrjabin, 1955.

Family Gongoderidae

- *Plesiochorus cymbiformis* (Rudolphi, 1819) Looss, 1901.

Family Brachycoeliidae

- *Cymatocarpus solearis* (Braun, 1899) Braun, 1901

Family Telorchiidae

- *Orchidasma amphiorchis* (Braun, 1899) Braun, 1901

Family Spirorchiidae

- *Amphiorchis indicus* Mehrotra, 1973.
- *Amphiorchis solus* (Simha & Chattopadhyaya, 1970) Platt, 2002
- *Carettacola stunkardi* (Martin & Bamberger, 1952)
- *Hapalotrema postorchis* Rao, 1976
- *Learedius learedi* Price, 1934
- *Monticellius indicum* Mehra, 1939
- *Neospirorchis schistosomatoides* Price 1934.

Family Ascarididae Baird, 1853

- *Porrocaecum sulcatum* (Rudolphi, 1819)

Family Kathlaniidae

- *Kathlania leptura* Rudolphi (1819).
- *Tonaudia freitasi* Vicente e Santos, 1968.

Parasites in *Eretmochelys imbricata*

Few *E. imbricata* individuals have been studied on the Brazilian coast. The first description of parasites was performed by Werneck et al. (2008a). The authors reported the occurrence of *Amphiorchis caborojoensis* Fischthal & Acholonu, 1976 and *C. stunkardi* in a juvenile from the northern coast of the state of São Paulo (southeastern Brazil). Werneck & Silva (2012) reported the occurrence of *Styphlotrema solitaria* Looss, 1899 in two juveniles rescued on the coast of the state of São Paulo. In the same year, Dutra et al. (2012) reported *A. caborojoensis* associated with tissue lesions due to the eggs of this parasite in a juvenile beached on the southern coast of the state of São Paulo.

After two years, the first occurrence of *H. postorchis* in an *E. imbricata* from the coast of the state of Espírito Santo (southeastern Brazil) was reported. This parasite had previously only been described in *C. mydas* individuals found in India, the USA, Australia, Costa Rica and Taiwan (Werneck et al., 2014). In 2015, the first occurrence of *M. indicum* was recorded for an *E. imbricata* from the state of Espírito Santo, which had previously been considered exclusive to *C. mydas* and only reported in India, Brazil and Costa Rica (Werneck et al., 2015d). More recently, Werneck et al. (2015b) analyzed 31 *E. imbricata* juveniles from the northeastern and southeastern regions of Brazil and found the following parasites in 17 individuals (prevalence: 54.8%): *Diaschistorchis pandus* (Braun, 1901) Johnstone, 1913, *C. albus*, *M. invaginatum*, *P.s obliquus* (Pronocephalidae), *Cymatocarpus solearis* (Braun, 1899) Braun, 1901 (Brachycoeliidae), *S. solitaria* (Styphlotrematidae), *C. stunkardi*, *A. caborojoensis* (Spirorchiidae) and *O. amphiorchis* (Telorchiidae) as well as *Anisakis* nematode larvae. The parasite species richness was 1.38, mean intensity was 25.1 and mean abundance was 13.8.

Helminths Reported for the Hawksbill Sea Turtle along the Coast of Brazil

Family Pronocephalidae

- *Diaschistorchis pandus* (Braun, 1901) Johnstone, 1913
- *Metacetabulum invaginatum* Freitas & Lent 1938
- *Pronocephalus obliquus* Looss, 1899.

Family Brachycoeliidae

- *Cymatocarpus solearis* (Braun, 1899) Braun, 1901

Family Styphlotrematidae

- *Styphlotrema solitaria* (Looss, 1899) Odhner, 1911

Family Spirorchiidae

- *Amphiorchis caborojoensis* Fischthal & Acholonu, 1976
- *Carettacola stunkardi* (Martin & Bamberger, 1952)
- *Hapalotrema postorchis* Rao, 1976
- *Monticellius indicum* Mehra, 1939

FamilyAnisakidae

- Anisakis larvae

Parasites of *Caretta caretta*

Viana (1924) made the first report of parasites in *C. caretta* on the coast of Brazil. The author offered a broad record of parasite species in different hosts and cited *R. gelatinosus* (family Rhytidodidae) in this host in Brazil. Araújo (1941) reported the occurrence of *Lophotasphis vallei* (Stossich, 1899) in the esophagus and stomach of a *C. caretta* individual from the coast of the state of São Paulo (southeastern Brazil). In the same study, the author cited the occurrence of other helminths in the esophagus and stomach, but did not report the identification of these parasites. Werneck et al. (2008c) analyzed 12 *C. caretta* individuals found on the coast of the state of São Paulo and reported the occurrence of *Sulcascaris sulcata* (Rudolphi, 1819), *K. leptura*, *O. amphiorchis*, *Pyelosomum renicapite* (Leidy, 1856) (Figure 4B) and *C. anthos* in five hosts (prevalence: 41.7%).

Helminths Reported in the Loggerhead Sea Turtle along the Coast of Brazil

Family Aspidogastridae

- *Lophotaspis vallei* (Stossich, 1899)

Family Calycodidae

- *Calycodes anthos* (Braun, 1899) Looss, 1901.

Familia Rhytidodidae

- *Rhytidodes gelatinosus* (Rudolphi, 1819) Looss, 1901

Family Pronocephalidae

- *Pyelosomum renicapite* (Leidy, 1856).

Family Telorchiidae

- *Orchidasma amphiorchis* (Braun, 1899) Braun, 1901

FamilyAnisakidae

- *Sulcascaris sulcata* (Rudolphi, 1819)

Family Kathlaniidae

- *Kathlania leptura* Rudolphi (1819).

Parasites of *Dermochelys coriacea*

Analyses of *D. coriacea* are scarce and only 15 individuals have been studied throughout the entire world. Seven of these individuals were studied in Brazil, with the occurrence of *P. renicapite* found in three of these individuals. This is the only parasite described for *D. coriacea* in Brazil (Werneck et al., 2012).

Parasites of *Lepidochelys olivacea*

There has been only one report of parasites in *L. olivacea* on the coast of Brazil, which was the first occurrence of *Pyelosomum cochlear* Looss, 1899 collected from the urinary bladder of an individual from the state of Rio Grande do Sul (southern Brazil). This species had previously only been reported in *C. mydas* (Werneck et al., 2015e).

Challenges for the Future

As endangered creatures, the study of sea turtles and the parasites found in these chelonians can have legal implications. Moreover, all five species of sea turtles found in Brazil are reported throughout most of the more than 8000 kilometers of coastline. There has been a clear increase in cases of parasitosis in Brazil, especially since the year 2000, when more studies on this topic began to be conducted. There are currently a greater number of rehabilitation centers on the coast of Brazil, which have been monitoring beaches as the result of environmental impact studies regarding maritime activities of oil and gas exploration and production. Such initiatives allow greater access to dead beached sea turtles and those that died at rehabilitation centers, thereby leading to greater knowledge on the helminths that affect these turtles and their impact on this group of hosts.

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Chapter 37

EFFECT OF CLAYS ON REMOVAL OF OKADAIC ACID FROM SEAWATER

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ABSTRACT

The use of clays for mitigating the effects of toxic episodes causing continuous shellfish harvesting closures owing to lipophilic toxins remains unexplored. This strategy is promising for clearance of microalgae through flocculation, but few studies evaluate toxins elimination. This study presents preliminary data on the withdrawal of okadaic acid, testing different clays in seawater, in laboratory conditions. Clays were assayed based on the potential effectiveness in terms of specific surface, particle size and colloidal properties, as well as their local abundance: three kinds of bentonites (one containing sodium bentonite particles <70 µm, other, 83% < 2 µm, and bentonite mainly composed of muscovite, particles >45 µm) and kaolinites of different origins and grain size (69% < 2 µm and 57% < 2 µm). Bentonite (83% < 2 µm) had the best toxin removal ability in our study (68%), while kaolinites with longer decantation times (69% < 2 µm) were the least effective material. The particles with the highest size (bentonite >45 µm) showed a lower efficiency, probably related to the fast decantation process. Altogether, clay applications offer an opportunity for toxin removal that should be explored in depth.

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1. INTRODUCTION

Harmful algal blooms (HABs) pose a serious concern in many coastal zones all over the world, especially those where aquaculture activities are relevant. In the northwest of Spain, Galicia, where the Rías conform a particular habitat supporting aquaculture and fishing activities, toxic episodes are associated to atmospheric and oceanographic interactions, specifically upwelling and downwelling, and transitions between water column stability and vertical mixture. When phytoplanktonic toxin producer species are transported and aggregated influenced by oceanographic conditions, and their trophic needs are covered by the abundance of prey species, massive proliferations of toxic phytoplankton occur [1]. One of the species of concern in Galicia is *Dinophysis* spp., a dinoflagellate responsible for lipophilic toxins production, formerly known as "diarrhetic" toxins, because they trigger the Diarrhetic Shellfish Poisoning (DSP) when contaminated mollusks are consumed. Okadaic acid and dinophysistoxins are the main analogues involved in toxic episodes in Galicia, causing closures in harvesting areas and producing an economical detriment of the bivalve aquaculture sector. Mussels growing in rafts in floating settlements are the most affected species, when they ingest the toxins by filter feeding. Although they do not suffer adverse effects, several weeks are necessary for depuration. At present, this natural elimination of toxins in the marine environment is the only allowed process in shellfish aquaculture.

Closure periods with prohibition of mollusk extraction, have grown in frequency and duration during the last years [2]. Although the spring and the autumn are the main periods of toxic episodes, closures have been extended during 4 consecutive months, or even more, in the worst cases. Estimation of costs associated to the toxic episodes in Galicia ascended to 40 million € in 2010 [3].

Clays have been used in control of harmful algal blooms (HABs) in Asia [4, 5], laboratory and field studies have been performed in several countries over the past 30 years. Some assays reproducing environmental conditions have been performed with the aim of using these kind of materials for mitigation of toxic blooms [6, 7], and clay dispersal was tested in situ in Japan, Korea and Australia [8].

Clearance of microalgae is possible through flocculation processes by use of mineral particles. This mechanism poses a promising strategy for control of the harmful algal blooms and for mitigating the impact on the local resources [9]. Clay dispersal and flocculation has been used in Korea for mitigation of *Cochlodinium polykrikoides* blooms, a dinoflagellate causing fish and shellfish mortality, as a strategy included in the management scheme [8]. Experiments have been done with *Karenia brevis,* a toxic dinoflagellate producing brevetoxins, responsible for mortalities of fish, invertebrates and marine mammals in coastal waters of Florida and Gulf of Mexico [10]; *Prymnesium parvum,* a potent fish killing organism that produces prymnesins, commonly found in low salinity or mesohaline waters [5, 11], and more recently, with paralytic shellfish poisoning causative organisms in the Philippines [12], among others. Nevertheless, few studies have been performed to evaluate the toxins removal when applying these strategies.

Some reports have shown that the cyanobacterial hepatotoxin microcystin-LR can be adsorbed by clay particles, enabling the removal of 81% of this toxin from the water [13], and also the removal of prymnesins [5], and 70% of extra-cellular brevetoxins, from *Karenia brevis,* in seawater [14]. Natural clays and its composites are known to trap pollutants by

taking up cations and anions through ion exchange and/or adsorption, removing different organic and inorganic contaminants from water. Moreover, it has been proved that in many cases clays are better or comparable with commercial filter materials for drinking water, with the advantage of being a low-cost green, nontoxic adsorbent [15]. Some recent studies focus on modified clays or even modified sand [16, 17] and their effects on algal cells and toxins, with the aim of maximizing effectiveness, and to minimize costs and environmental impact. Some examples are assays for removal of microcystins from drinking water using nano-sized montmorillonite K10 [18, 19], nanosilicate platelet derived from natural clay mineral [20], kaolin modified with polyaluminium chloride for removing *Alexandrium tamarense* and paralytic shellfish poisoning toxins [21]. Other recent approach is the development of clay-mimicking materials, such as aminoclays, with algicidal activity [22].

To our knowledge, no studies have been published regarding the effect of natural clays on the okadaic acid group of lipophilic toxins. During proliferations of *Dinophysis*, an important proportion of their toxins are released into the seawater [23]. Mussels ingest toxic phytoplankton and accumulate lipophilic toxins mainly in the digestive gland [24]. Recently, it was described that dissolved azaspiracids are absorbed and metabolized by blue mussels, showing that toxins uptake from the dissolved phase through the gills is a possible route of accumulation [25], although no results on accumulation by the gills have been reported using only dissolved lipophilic toxins. It was suggested that mussels can accumulate diarrhetic toxins dissolved in the seawater, from an experimental study [26], but these results have not been confirmed from field studies. Dissolved toxins may pose a further risk to shellfish farms if mussels are able to take up the toxins in the dissolved form in the same manner as ingesting toxic algae.

This study proposes testing different clays in seawater naturally contaminated with okadaic acid from a toxic episode in the Galician coast and obtain a preliminary evaluation of the possible removal of this toxin from seawater in laboratory conditions, with the aim of providing evidence that further research would be of interest.

Different clays were selected based on the potential effectiveness in terms of specific surface, particle size and colloidal properties, as well as their local abundance. Bentonites have been shown in former studies to be effective against a broad range of algal species, due to their ability to expand in water [5]. Then, three varieties of bentonite were assayed: “Bentonite P” was sodium bentonite, and contained particles <70 μm, “Bentonite C” (83% < 2 μm) and “Bentonite > 45 μm”, the mineral composed mainly by muscovite, with particle size > 45 μm. Other compounds tested in this preliminary study were local clays (“Grove”, 69% < 2 μm, and “Lendo”, 57% < 2 μm) mainly composed by kaolinite.

2. MATERIALS AND METHODS

2.1. Selection of Clays

Clays were provided by two Galician companies: “Caolines de Vimianzo, S.A.” (CAVISA) a company that commercializes kaolins, micas and sands; and “Peloides Termales”, that designs products for balneotherapy and thalassotherapy. Clays were selected for the specific surface and the particle size.

The clay supplied by Peloides Termales, "Bentonite P" was sodium bentonite, and contained particles <70 µm. The studied sample showed a high percentage of phyllosilicates in the total fraction (98%). The clay fraction included the following minerals in decreasing amounts: smectite (56%), sepiolite (29%) and illite (15%) [27, 28]. Sodium bentonite expands when wet, absorbing as much as several times its dry mass in water. It was selected because of its excellent colloidal properties.

The clays supplied by CAVISA were "Bentonite C" (83% < 2 µm), "Bentonite > 45 µm" (mainly muscovite) and kaolinites locally available from the regions Lendo and Grove, near the Galician coast: "Lendo Clay" (57% < 2 µm), "Grove Clay" (69% < 2 µm). Figure 1 shows size distribution (granulometry) of the clays.

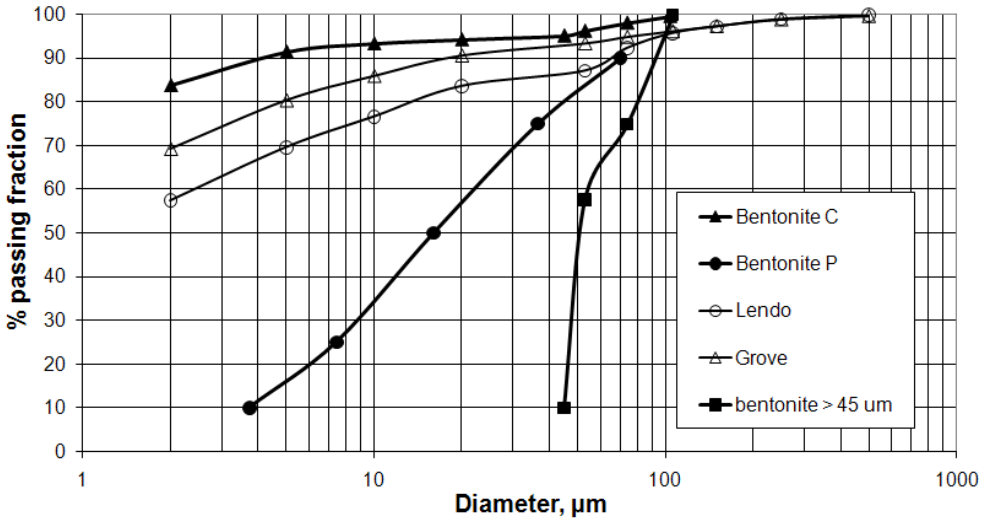

Figure 1. Granulometry of the clays selected in the study. The "passing fraction" is the total percentage of clay that passes through the sieve of a given size (indicated by parameter "diameter").

2.2. Seawater Sampling

Seawater from the Galician coast was sampled during a toxic episode in Ría de Aldán, Galician coast, northwest of Spain (Figure 3) at a depth of about 1 meter, and kept frozen in plastic containers. The species involved in this toxic episode was *Dinophysis acuminata*, registered at densities of 2000-6000 cells/liter in the 10-day period around the sampling date. The harvesting area was closed, with okadaic acid levels higher than the legal limit (>160 µg / kg) in the mollusks. Okadaic acid content was measured using the enzyme-linked immunosorbent assay (ELISA) from Abraxis; the obtained value was 0.37 ± 0.05 ng / mL (n = 11) (mean value ± SEM). These results were obtained before and after freezing, and analyses were done at different times, during a period of several months. Figure 2 shows the distribution of the obtained values of okadaic acid in seawater throughout the study.

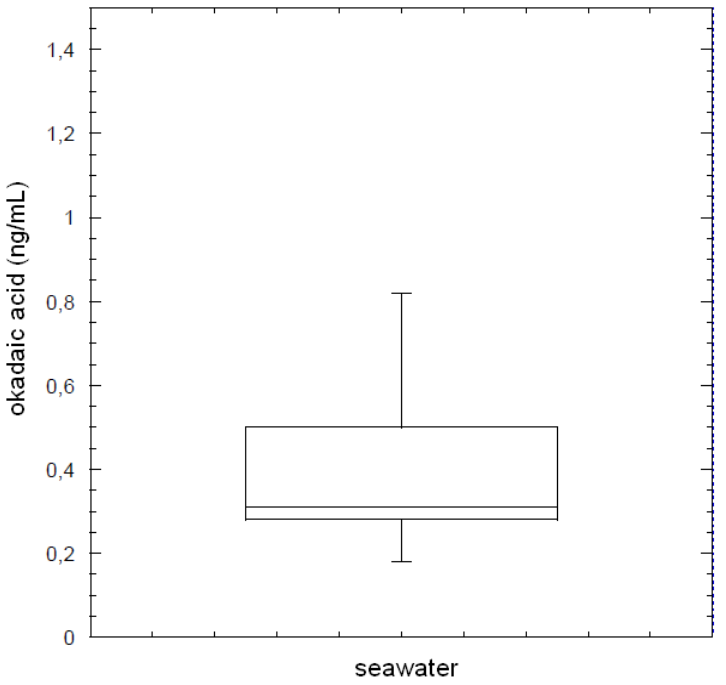

Figure 2. Distribution of values of okadaic acid in seawater during the study.

2.3. Clay Suspensions Preparation

The clays concentrations tested in the present study were in the range from 2.5 to 15.0 g/L. Seawater and clays suspensions were prepared as follows: 50 mL of water were dispensed in test tubes and the corresponding clay was added to achieve 5 different concentrations (0.25, 0.50, 0.75, 1.00 and 1.50 % clay) in the seawater. The mixtures were allowed to settle and separate by gravity. Decantation took place in the tubes, containing each clay/concentration. Seawater subsamples were taken at different sampling times (12, 24, 48 and 72 hours) using a syringe and taking care for not disturbing the sediment. Water samples obtained were analyzed for okadaic acid.

2.4. Okadaic Acid Analysis

Enzyme-Linked Immunosorbent Assay (ELISA) from Abraxis (Warminster, Pennsylvania, USA) was used for the determination of okadaic acid. The test was performed following specifications of the manufacturer. The final color was evaluated using a spectrometer Spectramax M5 microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA) at 450 nm, and the concentrations of the samples were determined by interpolation using the standard curve constructed with each run, covering the range of concentrations from 0.1 to 5.0 ng / mL.

3. RESULTS

First, we tested the effect of the different clays, by preparing suspensions in seawater collected during a toxic episode in Ría de Aldán, Galician coast (Figure 3).

Several clay concentrations were prepared, in test tubes (0.25, 0.50, 0.75, 1.00 and 1.50%) and allowed to stand, until decantation (allowing the mixture to settle and separate by gravity). Seawater was extracted at different sampling times: 12, 24, 48 and 72 hours.

Results obtained after experiments with clays are shown in Figures 4 - 8. Mean value of duplicates obtained in the ELISA test are represented; the coefficients of variation for replicates are <5% in most cases, and <15% in all of them, within the method specifications.

A decrease in the okadaic acid content of the seawater after decantation of clay was registered (Figures 4-8). Figure 4 shows variations in okadaic acid content from seawater after addition of clay "Bentonite P" (<70 μm), results of replicate analyses are shown (mean values ± Standard Error of the Mean (SEM)).

Figure 3. Sampling site for seawater during a toxic episode in Ría de Aldán (Galician Coast, Spain).

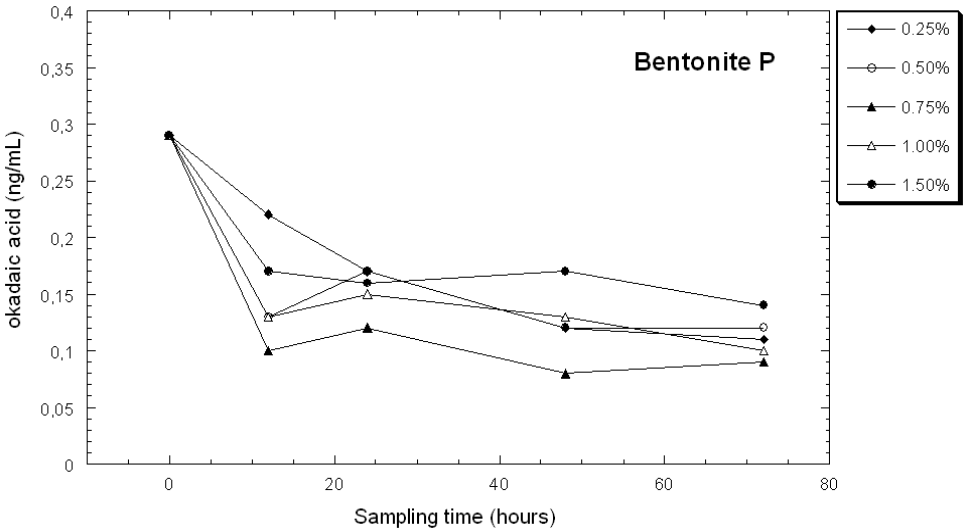

Figure 4. Variations in the okadaic acid content from seawater after addition of clay "Bentonite P" (<70 µm) at different concentrations (0.25 – 1.50%) and sampling times (12 – 72 hours).

The decrease of okadaic acid in the seawater after addition of clay was evident after the first 12 hours with Bentonite P (<70 µm) (Figure 4) and Bentonite C (83% < 2 µm) at all the concentrations tested, obtaining a mean decrease of 68% (8% CV) (Figure 5).

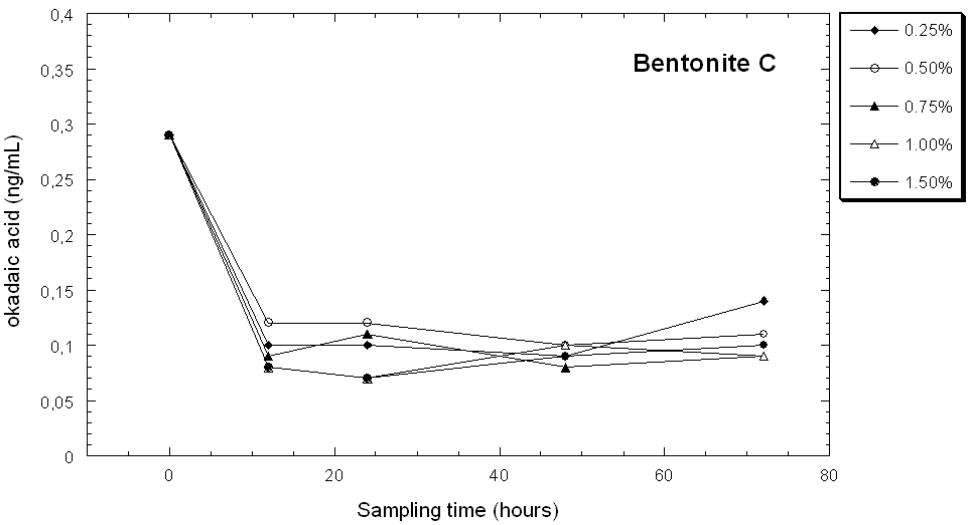

Figure 5. Variations in the okadaic acid content from seawater after addition of clay "Bentonite C" (83% < 2 µm) at different concentrations (0.25 – 1.50%) and sampling times (12 – 72 hours).

With kaolinitic clays from Lendo and Grove, results were also promising, although more variations were observed. At 24 hours, the reduction of okadaic acid was maximum when Lendo clay (57% < 2 µm) (Figure 6) and Grove clay (69% < 2 µm) (Figure 7) were used at all the concentrations tested, 66 and 67% in average, respectively (8 and 13% CV). The increase of okadaic acid observed at 48 h (from 0.1 or < 0.1 ng / mL at 24 h to values of 0.26 -

0.28 ng / mL at 48 h) at the lower concentrations (<0.75%) of Grove clay may be related to the lower particle size of Grove clay (69% < 2 μm), compared to Lendo clay (57% < 2 μm), involving a possible resuspension or not enough "attachment" of the toxin to the deposited clay.

This effect was not observed at higher concentrations of this clay, possibly owing to a more stable settlement of this higher amount of particles and "attached" molecules on the bottom. Higher values than the initial okadaic acid concentration obtained at 12 hours in both clays seemed to be experimental artifacts, probably related with longer decantation times, and hence longer time in suspension in the seawater, possibly causing interference in the ELISA measurement.

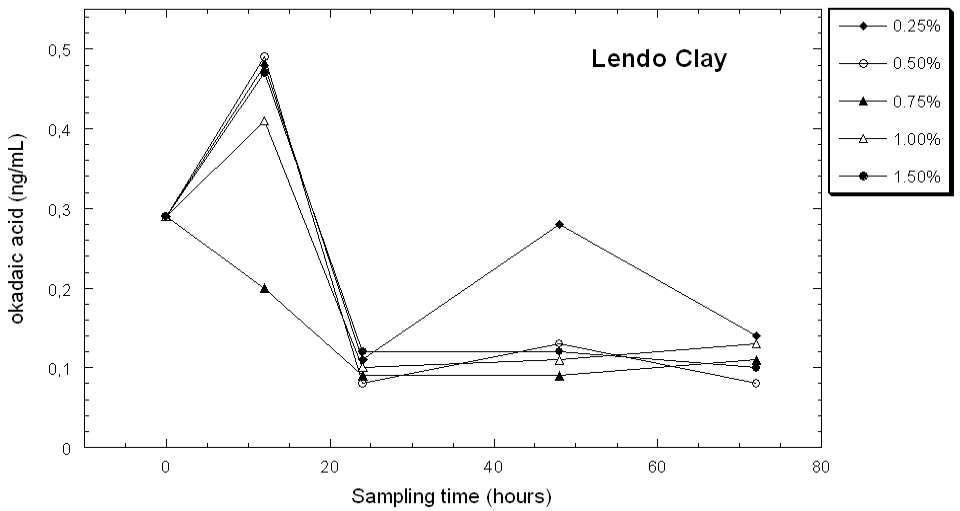

Figure 6. Variations in the okadaic acid content from seawater after addition of kaolinitic "Lendo clay"(57% < 2 μm) at different concentrations (0.25 – 1.50%) and sampling times (12 – 72 hours).

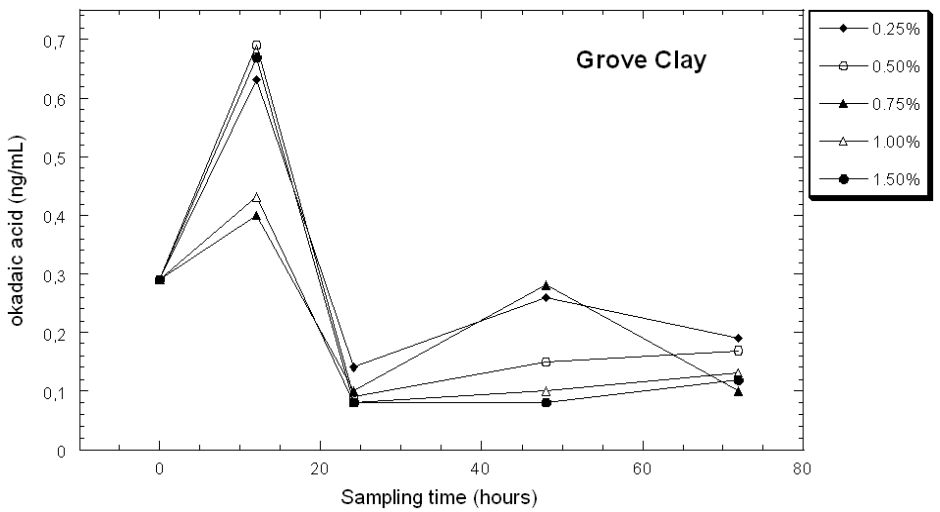

Figure 7. Variations in the okadaic acid content from seawater after addition of kaolinitic "Grove clay"(69% < 2 μm) at different concentrations (0.25 – 1.50%) and sampling times (12 – 72 hours).

Both bentonites (83% < 2 μm and <70 μm, respectively) and kaolinitic Lendo clay (57% < 2 μm) showed a high degree of decantation 2 hours after the suspensions preparation, in the laboratory conditions. By contrast, kaolinitic Grove clay (69% < 2 μm) needed about 22 h for decantation and clarification of the solutions, staying in suspension a longer time than the remaining clays assayed in this study.

The highest particle size material, bentonite >45 μm, also caused a reduction in seawater OA content (Figure 8), but at longer exposure times of the experiment, 48-72 hours, and to a lesser extent, about 43-46%, respectively. Longer time was needed for toxin removal from the seawater, at concentrations over 0.25%. This material was totally decanted after the first 10 minutes of suspension preparation.

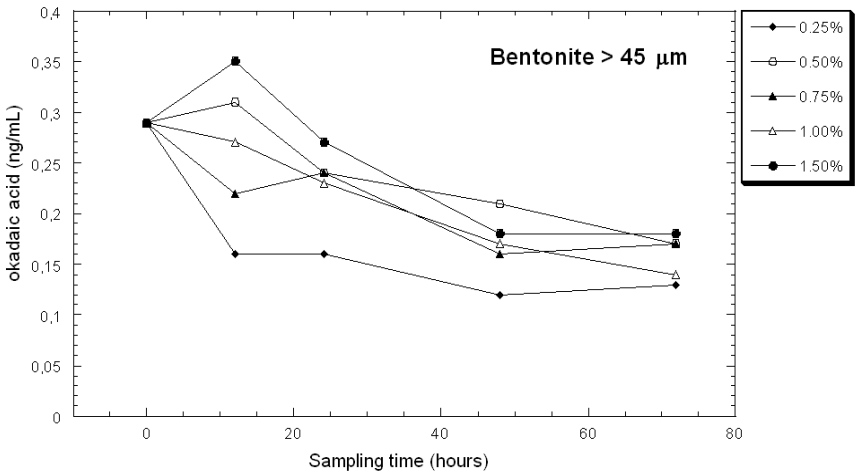

Figure 8. Variations in the okadaic acid content from seawater after addition of clay "Bentonite >45 μm" at different concentrations (0.25 – 1.50%) and sampling times (12 – 72 hours).

In summary, the most promising results of toxin removal from seawater obtained with clays, were obtained at 12 hours after the addition of bentonites C (83% < 2 μm) and P (<70 μm), 24 hours for kaolinitic clays 57% and 69% < 2 μm (Lendo and Grove clays), and 72 hours with bentonite >45 μm. Bentonite C (83% < 2 μm) seems to offer the highest decrease of okadaic acid in seawater.

4. DISCUSSION

Recovery of hydrophobic toxins from seawater by adsorption of toxins onto hydrophobic substances is usually carried out for analyses. It was shown that the finest particles of marine sediment consisting mainly of montmorillonite adsorbed oleic acid (references cited by [13]). Clay properties of fine-grained particles, high surface area and charge suggest that this material could be effective for adsorbing hydrophobic molecules from water, as proposed by others for microcystins and extra cellular brevetoxin molecules [13, 14]. Okadaic acid and dinophysistoxins are fat soluble, have high molecular weights, and belong to a class of compounds called polycyclic ethers. These compounds are similar in chemical structure to other marine toxins such as yessotoxins, brevetoxins, ciguatoxins and palytoxins [29].

Okadaic acid consists structurally in a relatively rigid hydrophobic tail structure connected via a flexible arm to a very rigid spiroketal structure with an acidic group, capable of coordination to the metals and/or the aquo ligands at the active site of the enzyme protein phosphatase-1 [30]. Toxins are associated in most cases with the presence of their producers, inside algal cells, but in some cases, toxins may be present in the water column associated with suspended matter and bottom sediments or water bodies [31]. It is known that microcystins, and especially anatoxin-a, adsorb to fine clay material, and can accumulate in the sediment; a recent study found that okadaic acid was detected at remarkable levels in suspended solids during *Dinophysis* bloom in Alfacs Bay, one of Ebro´s associated marine bays [31]. The present study intends to explore the use of clays for mitigating the effects of the toxic episodes in Galicia, especially those originating from *Dinophysis* sp., dinoflagellates responsible for the production of the formerly known as "Diarrhetic Shellfish Poisoning" toxins, or DSP toxins. The incidence of these lipophilic toxins in the mussel and other bivalve mollusks aquaculture sector is increasing in the last years, globally, possibly influenced by eutrophication, maritime transport and climatic change [2]. Bentonites have been effective in previous studies for a broad range of algal species, due to their ability to expand in water, and to develop strong surface change [5]. As observed by others, bentonite (83% < 2 μm) had the best removal ability in our study (68%). Theoretically, larger particle size would increase the rate of particle collision, while a stronger surface charge would lead to a higher proportion of particle adhesion on contact, and both processes would lead to higher flocculation rates [5]. On the other hand, kaolinite (43% < 2 μm) was the least effective material, only showing a remarkable decrease of okadaic acid at 24 h (58%), but increasing again afterwards. It was described that kaolinites did not swell when wetted, and had a much lower charge density than bentonites [5], so, agreeing with previous studies, they were less effective in toxin removal. Particle concentration is another important factor, as described by others, in flocculation rates, along with particle size and charge [32]. This effect might explain the behavior of the kaolinitic clays locally available from Lendo and Grove, with a high proportion of particles <2 μm, and with better results in okadaic acid removal from seawater at higher concentrations of both clays, especially in Grove clay, with a higher proportion of small particles (69% < 2 μm) than Lendo clay (57% < 2 μm)). It has been suggested that when aggregates are >1 μm, differential sedimentation, i.e., collisions resulting from the interception of smaller particles by larger, more-rapidly sinking particles, is the dominant transport mechanism (reported by [33]). The effectiveness of differential sedimentation may also diminish in time as the aggregates attain a certain size and displace increasing amounts of water during their descent, which can push away smaller particles instead of colliding and attaching with them, phenomenon known as hydrodynamic retardation [33]. This effect would explain the behavior of the clay mainly composed of muscovite, bentonite >45 μm, at concentrations higher than 0.25%, with lower efficiency of toxin removal from the seawater. As expected, practically complete decantation of these particles occurred within the first 10 minutes, after the suspension preparation. Use of clays in the field pose environmental concerns, since the toxic cells and/or toxins sedimentation could lead to toxin accumulation by benthic organisms [34], and also impacts of clay on sessile organisms and other co-occurring species should be evaluated. Nevertheless, the number of studies, although limited, shows only low or no impact on co-occurring species, and a negative effect on bivalve growth [35]. On the other hand, clay treatment would not be recommended if flocculation required several hours to take effect, as no water column would be static for such a length of time in

nature [5]. Then, the materials composed by kaolinite, would not be the most adequate for achieving okadaic acid toxins removal from the water column. Further studies assaying shorter periods of time, lower clay concentration and tests with the causative organisms, *Dinophysis* sp. and *Prorocentrum* sp. would be desirable.

In summary, use of clays as a mitigation strategy for microalgal toxins contamination offered promising results. However, further research on the toxin and/or causative agent removal efficiency and the environmental implications of this strategy should be evaluated.

CONCLUSION

Bentonite (83% < 2 μm) had the best okadaic acid removal ability from seawater in our study (68%). By contrast, kaolinite (43% < 2 μm) was the least effective material, only showing a remarkable decrease of okadaic acid at 24 h (58%), but increasing again afterwards. The other kaolinite clays, from Lendo (57% < 2 μm) and from Grove (69% < 2 μm), also showed promising results, although longer decantation times in Grove clay and kaolinite limit their possible usefulness.

The particles with the highest size (bentonite >45 μm) showed a lower efficiency, probably related to the fast decantation process. Altogether, clay applications offer an opportunity for toxin removal that should be explored in depth.

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Chapter 38

HELMINTH DIVERSITY OF CETACEANS: AN UPDATE

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ABSTRACT

Parasitism is an extraordinary life-strategy that largely influences that of most free-living organisms, including cetaceans. Parasites of cetaceans have a positive intrinsic and instrumental value, but also a potential pathogenic impact on host populations and, therefore, they should be considered in any biodiversity or conservation program. Yet, a starting point for understanding the relationships between cetaceans and their parasites is to have a detailed account on parasite diversity. The knowledge of the parasite fauna of cetaceans goes back to the time when commercial whaling was allowed and material for scientific purposes was available. Over the last decades, however, parasites have been obtained mainly from stranded or by-caught animals, and regular updates of host-parasite lists have been published until the 1990's. In this chapter we provide an updated review of the helminth fauna of cetaceans. The data here presented constitutes a baseline for future diversity surveys and a way to encourage researchers towards a greater awareness about the biological importance of the helminth fauna of cetaceans. Out of the 90 species currently recognized in the order Cetacea, 72 have hitherto been examined for helminths. The family Balaenopteridae among baleen whales and the family Delphinidae among toothed whales harbor the most diverse helminth fauna, which is partly accounted for by a higher sampling effort. In contrast, the helminth fauna of the beaked whales (family Ziphiidae) is the least known since only 9 out of the 22 species in the family have been examined for parasites. Currently, there are 174 helminth species reported in cetaceans, from which nematodes is the most speciose group (62 spp.), followed by digeneans (54 spp.), cestodes (38 spp.) and acanthocephalans (20 spp.). However, 20 (11.5%) of these

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species represent accidental infections that occur because cetaceans and other marine vertebrates (i.e., pinnipeds and marine birds) share common prey. The families Anisakidae, Pseudaliidae and Tetrameridae (Nematoda), Brachycladiidae, Notocotylidae, Heterophyidae and Brauninidae (Digenea), Diphyllobothriidae, Phyllobothriidae and Tetrabothriidae (Cestoda), and Polymorphidae (Acanthocephala) have a major species-representation in the helminth fauna of cetaceans.

PARASITISM IN CETACEANS

Parasitism is an extraordinary and widespread life-strategy because parasites are able to infect almost every living organism, including cetaceans. Previous studies have highlighted the importance of including parasites in biodiversity and conservation programs for their cetacean hosts (Aznar et al. 2010). Like any other organism, parasites have an intrinsic value and should be considered as an integral part of the Biosphere. Parasites also have an instrumental value, and have commonly been used as natural tags for their host populations. Studies concerning cetacean social behaviour (Balbuena and Raga 1994), diet (Blazekovic et al. 2015), distribution (Aznar et al. 1995), stock identity (Marigo et al. 2015) and health status (Aznar et al. 2005) have benefited from the use of parasites as biomarkers. For instance, Blazekovic et al. (2015) found a relationship between the prevalence of three nematode species, i.e., *Anisakis simplex* sensu stricto, *A. physeteris* and *A. pegreffii* and the population structure of four toothed whales in the eastern Adriatic. On the other hand, heavy parasite loads are often detected in apparently healthy cetaceans (Rogan et al. 1997), but some species may have a clear pathogenic impact on cetacean populations. For instance, digeneans of the genus *Nasitrema*, which usually occur in the pterygoid sinuses of some dolphin species, may cause brain injuries (Arbelo et al. 2013). Details about the negative impact of parasites on cetacean populations have been explored in previous studies (see e.g., Aznar et al. 2001a, Raga et al. 2009).

In any event, our understanding of the historical and present-day interactions between cetaceans and their parasites require a detailed and updated account of its biodiversity. Helminths, i.e., acanthocephalans, cestodes, nematodes and digeneans, constitute the most significant portion of the metazoan parasitic fauna of cetaceans (Figure 1). Records of helminths in cetaceans go back to the early 1900's, and efforts for updating data have regularly been made until the 1990's (e.g., Baylis 1932, Price 1932, Delyamure 1955, Tomilin 1967, Yamaguti 1971, Dailey and Brownell 1972, Gibson and Harris 1979, Raga 1994). In this chapter we provide the reader with an updated review of the helminth fauna occurring in cetaceans. This goal is justified for several reasons, including, 1) an increase, over the last two decades, of the sampling effort on poorly known cetaceans and unexplored areas; 2) significant taxonomic changes and re-arrangements of helminth taxa; and 3) an increased use of molecular markers, which have helped to unveil a significant portion of hidden biodiversity (i.e., cryptic species). In the next section, we outline major diversity patterns of the helminth fauna of cetaceans. A host-parasite list is then presented in Tables 1 and 2.

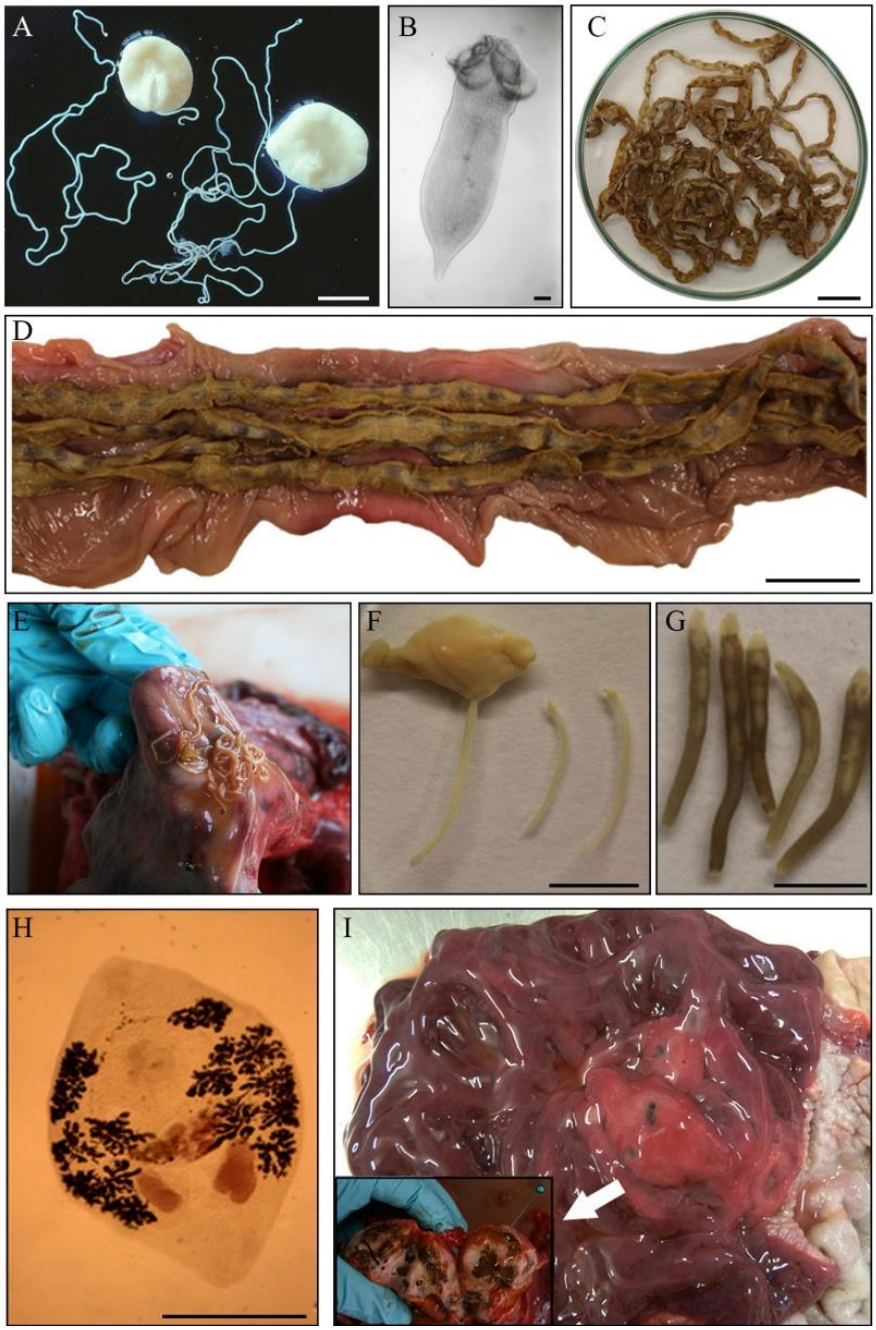

Figure 1. Representatives of helminth species in cetaceans. A) *Monorygma grimaldii* (Cestoda) and its capsule. Scale bar: 1 cm. B) *Scolex pleuronectis* (Cestoda). Scale bar: 0.1 mm. C) and D) *Diphyllobothrium* sp. (Cestoda) in the intestine of a bottlenose dolphin, *Tursiops truncatus*. Scale bars: 2 cm. E) *Anisakis simplex* sensu lato (Nematoda) in the stomach of a striped dolphin, *Stenella coeruleoalba*. F) Two specimens of *Bolbosoma capitatum* (Acanthocephala) and one embedded in host tissue. Scale bar: 1 cm. G) *Oschmarinella rochebruni* (Digenea). Scale bar: 1 cm. H) *Pholeter gastrophilus* (Digenea). Scale bar: 1 cm. I) Cyst of *P. gastrophilus* in the stomach of a striped dolphin. Pictures from the University of Valencia.

HELMINTHS OF CETACEANS

Currently, there are 174 helminth species reported from cetaceans, which can be grouped into 4 major taxa as follows: Acanthocephala (20 spp.), Cestoda (38 spp.), Nematoda (62 spp.) and Digenea (54 spp.) (Table 1). However, a total of 20 species from these taxa represent accidental infections, which occur because cetaceans and other marine vertebrates (e.g., marine birds or pinnipeds) share prey and parasite larvae are exchanged among hosts through the trophic webs. Accidental infections include 7 species of acanthocephalans, 4 of cestodes, 6 of nematodes and 3 of digeneans (Table 1). Most of these taxa are typical from pinnipeds and marine birds. Interestingly, several helminth species that reproduce in cetaceans also have congeneric taxa infecting pinnipeds and/or marine birds, i.e., *Corynosoma* spp. (Acanthocephala), *Tetrabothrius* spp., *Diplogonoporus* spp. and *Diphyllobothrium* spp. (Cestoda), and *Orthosplanchnus* spp. and *Ogmogaster* spp. (Digenea). Also, *Anisakis simplex* sensu lato, which is typical from cetaceans, has also been reported as adult in some pinnipeds (Brattey and Stenson 1993). These observations illustrate the high potential for host-switching events between marine mammals and birds (see Fraija-Fernández et al. 2015 and references therein).

A total of 72 out of 90 species currently recognized within the order Cetacea have ever been examined for helminths. The family Balaenopteridae among baleen whales, and the family Delphinidae among toothed whales, have the most diverse helminth fauna, which is not particularly surprisingly because they concentrate the highest sampling effort (Table 2). The helminth fauna of beaked whales (family Ziphiidae), on the other hand, is very poorly known, and not a single parasitological datum exists for 9 out of the 22 species included in this family.

As noted above, the helminth fauna of cetaceans is, as a whole, highly specific. At a lower taxonomic scale, highly specific taxa have a major representation among digeneans (14 spp. reported in single cetacean species), followed by nematodes (13 spp.), cestodes (8 spp.) and acanthocephalans (3 spp.) (Table 1). Only 19 species (10.9% of the total) has been reported just once. Among the species of Acanthocephala, only species from two genera of the family Polymorphidae, namely *Bolbosoma* and *Corynosoma*, occur in the intestine, and occasionally in the stomach, of cetaceans (Table 2). Three families of cestodes have species infecting cetaceans, namely Tetrabothriidae, Diphyllobothriidae, and Phyllobothriidae, whereas nematodes are represented by taxa of three families, i.e., Pseudaliidae, Anisakidae and Tetrameridae. Finally, the bulk of digeneans reported in cetaceans belong to four families: Brachycladiidae, Notocotylidae, Heterophyidae and Brauninidae (Table 2).

In the Tables 1 and 2 below, information for helminth species, locality and references for each record is organized according to each cetacean species. Cetacean taxonomy follows the lists of species and subspecies of marine mammals made by the Committee on Taxonomy (2014). Only taxonomically accepted parasite name species are included as they were verified with the WoRMS Editorial Board (2015) and the Global Biodiversity Information Facility database (www.gbif.org). Parasite taxa not identified to the species level are not included, except in the cases in which the genus of the named species has not been previously recorded in the cetacean species. References are organized in chronologically order and are identified by numbers in the reference list. The information here presented may constitute a baseline for diversity studies. We encourage researchers to carry out parasite surveys of poorly studied cetacean species and areas, paying special attention to cryptic diversity.

Table 1. List of the helminth species and their families found in cetaceans. Abbreviations: (1) Accidental infections: not adult specimens reported in cetaceans; the species typically infect other vertebrates (i.e., fish, birds, pinnipeds); (2) Specific to a single cetacean species; reported more than once; (3) single records

Acanthocephala		Cestoda			
Species	Family	Species	Family	Species	Family
Bolbosoma balaenae	Polymorphidae	*Diphyllobothrium fuhrmanni*	Diphyllobothriidae	*Strobilocephalus triangularis*	Tetrabothriidae
Bolbosoma brevicolle	Polymorphidae	*Diphyllobothrium lanceolatum*	Diphyllobothriidae	*Tentacularia* sp.[1]	Tentacularidae
Bolbosoma capitatum	Polymorphidae	*Diphyllobothrium latum*[b]	Diphyllobothriidae	*Tetrabothrius affinis*	Tetrabothriidae
Bolbosoma hamiltoni	Polymorphidae	*Diphyllobothrium orcini*[3]	Diphyllobothriidae	*Tetrabothrius arsenyevi*[2]	Tetrabothriidae
Bolbosoma nipponicum	Polymorphidae	*Diphyllobothrium phocarum*[1c]	Diphyllobothriidae	*Tetrabothrius curilensis*[2]	Tetrabothriidae
Bolbosoma tuberculata	Polymorphidae	*Diphyllobothrium polyrugosum*	Diphyllobothriidae	*Tetrabothrius diplosoma*[3]	Tetrabothriidae
Bolbosoma turbinella	Polymorphidae	*Diphyllobothrium stemmacephalum*	Diphyllobothriidae	*Tetrabothrius egregius*[2]	Tetrabothriidae
Bolbosoma vasculosum[a]	Polymorphidae	*Diplogonoporus balaenopterae*	Diphyllobothriidae	*Tetrabothrius forsteri*	Tetrabothriidae
Corynosoma alaskensis[2]	Polymorphidae	*Hexagonoporus calyptocephalus*[2]	Diphyllobothriidae	*Tetrabothrius hobergi*[3]	Tetrabothriidae
Corynosoma australe[1]	Polymorphidae	*Hexagonoporus giganticus*[2]	Diphyllobothriidae	*Tetrabothrius ruudi*	Tetrabothriidae
Corynosoma bullosum[1]	Polymorphidae	*Hexagonoporus physeteris*[3]	Diphyllobothriidae	*Tetrabothrius schaeferi*[2]	Tetrabothriidae
Corynosoma cameroni[a]	Polymorphidae	*Multiductus physeteris*[3]	Diphyllobothriidae	*Tetrabothrius wilsoni*	Tetrabothriidae
Corynosoma cetaceum	Polymorphidae	*Plicobothrium globicephalae*	Diphyllobothriidae	*Trigonocotyle globicephalae*	Tetrabothriidae
Corynosoma curilense[2]	Polymorphidae	*Monorygma grimaldii*[d]	Phyllobothriidae	*Trigonocotyle lintoni*	Tetrabothriidae
Corynosoma wegeneri[1]	Polymorphidae	*Phyllobothrium delphini*[d]	Phyllobothriidae	*Trigonocotyle prudhoei*[e]	Tetrabothriidae
Corynosoma reductum[1]	Polymorphidae	*Phyllobothrium physeteris*[3d]	Phyllobothriidae	*Trigonocotyle sexitesticulae*[3]	Tetrabothriidae
Corynosoma semerme[1]	Polymorphidae	*Scolex pleuronectis*[d]	Phyllobothriidae	*Trigonocotyle spasskyi*[2]	Tetrabothriidae
Corynosoma septentrionalis[2]	Polymorphidae	*Anophryocephalus* sp.[1]	Tetrabothriidae		
Corynosoma strumosum[1]	Polymorphidae	*Priapocephalus eschrichtii*[2]	Tetrabothriidae		
Corynosoma validum[1]	Polymorphidae	*Priapocephalus grandis*	Tetrabothriidae		
		Priapocephalus minor	Tetrabothriidae		
Nematoda					
Anisakis berlandi	Anisakidae	*Procamallanus* sp.[1]	Camallanidae	*Stenurus auditivus*	Pseudaliidae
Anisakis brevispiculata	Anisakidae	*Halocercus brasiliensis*	Pseudaliidae	*Stenurus australis*[2]	Pseudaliidae
Anisakis insignis[2]	Anisakidae	*Halocercus dalli*[2]	Pseudaliidae	*Stenurus globicephalae*	Pseudaliidae
Anisakis nascettii	Anisakidae	*Halocercus delphini*	Pseudaliidae	*Stenurus minor*	Pseudaliidae
Anisakis paggiae	Anisakidae	*Halocercus hyperoodoni*[3]	Pseudaliidae	*Stenurus nanjingensis*[3]	Pseudaliidae
Anisakis pegreffii	Anisakidae	*Halocercus invaginatus*	Pseudaliidae	*Stenurus ovatus*	Pseudaliidae
Anisakis physeteris	Anisakidae	*Halocercus kirbyi*	Pseudaliidae	*Stenurus truei*[3]	Pseudaliidae
Anisakis simplex sensu lato[f]	Anisakidae	*Halocercus kleinenbergi*	Pseudaliidae	*Stenurus yamagutii*[3]	Pseudaliidae
Anisakis simplex sensu stricto	Anisakidae	*Halocercus lagenorhynchi*	Pseudaliidae	*Torynurus convolutus*	Pseudaliidae
Anisakis typica	Anisakidae	*Halocercus monoceris*	Pseudaliidae	*Torynurus dalli*[2]	Pseudaliidae
Anisakis ziphidarum	Anisakidae	*Halocercus pingi*	Pseudaliidae	*Hysterothylacium aduncum*[1]	Rhaphidascarididae
Contracaecum lobulatum[i]	Anisakidae	*Halocercus sunameri*[2]	Pseudaliidae	*Crassicauda anthonyi*	Tetrameridae
Contracaecum multipapillatum[1]	Anisakidae	*Halocercus taurica*	Pseudaliidae	*Crassicauda boopis*	Tetrameridae

Table 1. (Continued)

Species	Family	Species	Family	Species	Family
Goezia sp.[1]	Anisakidae	*Pharurus alatus*	Pseudaliidae	*Crassicauda carbonelli*[2]	Tetrameridae
Phocascaris sp.[1]	Anisakidae	*Pharurus asiaeorientali*[2]	Pseudaliidae	*Crassicauda costata*[3]	Tetrameridae
Pseudoterranova ceticola	Anisakidae	*Pharurus pallasii*[2]	Pseudaliidae	*Crassicauda crassicauda*	Tetrameridae
Pseudoterranova decipiens[1]	Anisakidae	*Pharurus sunameri*[2]	Pseudaliidae	*Crassicauda delamureana*[3]	Tetrameridae
Pseudoterranova kogiae[2]	Anisakidae	*Pseudalius inflexus*	Pseudaliidae	*Crassicauda giliakiana*	Tetrameridae
Terranova sp.[3g]	Anisakidae	*Skrjabinalius cryptocephalus*	Pseudaliidae	*Crassicauda grampicola*	Tetrameridae
Porrocaecum sp.[g]	Ascarididae	*Skrjabinalius guevarai*	Pseudaliidae	*Crassicauda magna*[2]	Tetrameridae
Odontobius ceti	Monhysteridae	*Stenurus arctomarinus*[2]	Pseudaliidae	*Placentonema gigantissimum*[2]	Tetrameridae
		Trematoda			
Brachycladium atlanticum	Brachycladiidae	*Nasitrema gondo*	Brachycladiidae	*Synthesium seymouri*[2]	Brachycladiidae
Brachycladium curilense	Brachycladiidae	*Nasitrema lanceolatum*	Brachycladiidae	*Synthesium subtile*	Brachycladiidae
Brachycladium delphini	Brachycladiidae	*Nasitrema spathulatum*[2]	Brachycladiidae	*Synthesium tursionis*	Brachycladiidae
Brachycladium goliath	Brachycladiidae	*Nasitrema stenosoma*	Brachycladiidae	*Braunina cordiformis*	Brauninidae
Brachycladium nipponicum	Brachycladiidae	*Nasitrema sunameri*[2]	Brachycladiidae	*Galactosomum erinaceum*[h]	Heterophyidae
Brachycladium pacificum	Brachycladiidae	*Odhneriella arctica*[2]	Brachycladiidae	*Pholeter gastrophilus*	Heterophyidae
Brachycladium palliatum	Brachycladiidae	*Odhneriella elongata*	Brachycladiidae	*Ogmogaster antarctica*	Notocotylidae
Brachycladium parvulum[3]	Brachycladiidae	*Orthosplanchnus arcticus*[2]	Brachycladiidae	*Ogmogaster grandis*	Notocotylidae
Brachycladium petrowi	Brachycladiidae	*Orthosplanchnus pygmaeus*[2]	Brachycladiidae	*Ogmogaster pentalineata*[2]	Notocotylidae
Campula folium[2]	Brachycladiidae	*Orthosplanchnus sudarikovi*[2]	Brachycladiidae	*Ogmogaster plicata*	Notocotylidae
Campula gondo	Brachycladiidae	*Oschmarinella albamarina*	Brachycladiidae	*Ogmogaster trilineata*[2]	Notocotylidae
Campula oblonga	Brachycladiidae	*Oschmarinella laevicaecum*	Brachycladiidae	*Amphimerus lancea*[b]	Opisthorchiidae
Cetitrema foliforme[2]	Brachycladiidae	*Oschmarinella macrorchis*[2]	Brachycladiidae	*Delphinicola tenuis*	Opisthorchiidae
Cetitrema meadi[3]	Brachycladiidae	*Oschmarinella rochebruni*	Brachycladiidae	*Opisthorchis tenuicollis*[b]	Opisthorchiidae
Hunterotrema caballeroi[2]	Brachycladiidae	*Oschmarinella sobolevi*	Brachycladiidae		
Hunterotrema macrosoma[3]	Brachycladiidae	*Synthesium delamurei*	Brachycladiidae		
Nasitrema attenuatum	Brachycladiidae	*Synthesium elongatum*	Brachycladiidae		
Nasitrema dalli	Brachycladiidae	*Synthesium mironovi*	Brachycladiidae		
Nasitrema delphini	Brachycladiidae	*Synthesium nipponicum*	Brachycladiidae		
Nasitrema globicephalae	Brachycladiidae	*Synthesium pontoporiae*[2]	Brachycladiidae		

[a.] Described only in cetaceans as immature individuals; adults unknown.

[b.] Probably accidental.

[c.] Synonym: *Pyramicocephalus phocarum.*

[d.] Collective name for larval forms of species of tetraphyllideans whose adult stage infects elasmobrachs; cetaceans act as intermediate hosts for some species.

[e.] Unclear taxonomical status (see Hoberg, 1990).

[f.] Name used for a species complex.

[g.] Synonym of *Pseudoterranova* (?).

[h.] Listed as “indetermined species” by Dailey et al. (2002). Likely accidental.

[i.] Species name not included in the taxonomic database searched.

Table 2. List of helminth taxa reported in cetaceans. Abbreviations: AO, Atlantic Ocean; PO, Pacific Ocean; IO, Indian Ocean; Arc, Arctic; Ant, Antarctic; MS, Mediterranean Sea; BlS, Black Sea; BaS, Baltic Sea; RS, Red Sea; AR, Amazon River; YR, Yangtze River; GR, Ganges River; SA, Region of South Africa; (§) Geographic location not specified by the authors; (*) See Table 1 for specific considerations

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Mysticeti: Family *Balaenidae*											
***Balaena mysticetus* Linnaeus, 1758. Bowhead whale**											
Bolbosoma balaenae	AO	[31, 76, 306]	*Monorygma grimaldii*[*]	§	[17]	*Anisakis simplex* s.l.[*]	§	[137]	*Brachycladium goliath*	AO, PO	[31, 76, 272, 229, 306, 314,]
			Phyllobothrium delphini[*]	AO	[31, 76, 84, 229, 263]	*Odontobius ceti*	§	[274]	*Ogmogaster antarctica*	Arc	[258]
			Phyllobothrium physeteris[*]	§	[31]	*Crassicauda crassicauda*	AO	[76, 270, 306]	*Ogmogaster plicata*	AO, PO	[124, 229, 285, 306]
			Scolex pleuronectis[*]	AO	[306]						
***Eubalaena australis* (Desmoulins, 1822). Southern right whale**											
-	-	-	*Priapocephalus grandis*	Ant, SA	[31]	*Crassicauda costata*[*]	SA	[131]	-	-	-
			Monorygma grimaldii[*]	§	[17]	*Odontobius ceti*	§	[31]			
			Phyllobothrium delphini[*]	§	[17]						
***Eubalaena glacialis* (Müller, 1776). North Atlantic right whale**											
Bolbosoma balaenae	§	[306]	*Diplogonoporus balaenopterae*	§	[306]	*Odontobius ceti*	PO	[262, 274]	*Ogmogaster antarctica*	§	[306]
Bolbosoma brevicolle	AO	[76]	*Monorygma grimaldii*[*]	§	[17]						
Bolbosoma nipponicum	PO	[262, 306]	*Scolex pleuronectis*[*]	§	[306]						
Bolbosoma turbinella	AO, PO	[84, 270, 306]	*Priapocephalus grandis*	§	[31, 76, 84, 306]						
			Tetrabothrius affinis	§	[306]						
			Tetrabothrius ruudi	PO	[78, 306]						

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Family *Balaenopteridae*											
***Balaenoptera acutorostrata* Lacépède, 1804. Common minke whale**											
Bolbosoma balaenae	AO	[31, 76, 115, 306,]	*Diplogonoporus balaenopterae*	PO	[115, 140]	*Anisakis physeteris*	PO	[137, 209]	*Brachycladium goliath*	AO, PO, Ant	[31, 42, 44, 76, 206, 219, 229, 272, 314, 306]
Bolbosoma brevicolle	AO	[31, 76, 84, 306]	*Monorygma grimaldii*[*]	§	[17]	*Anisakis simplex* s.l.[*]	AO	[31, 73, 76, 116, 170, 306]	*Ogmogaster antarctica*	Arc	[239]
Bolbosoma nipponicum	PO	[76, 262, 306, 307, 313]	*Phyllobothrium delphini*[*]	§	[17]	*Anisakis simplex* s.s.	AO	[180, 181]	*Ogmogaster plicata*	AO, PO	[31, 74, 76, 84, 219, 306]
			Tetrabothrius sp.	Ant	[70]	*Pseudoterranova decipiens*[*]	§	[76, 306]			
						Odontobius ceti	§	[274]			
						Hysterothylacium aduncum[*]	Arc	[211]			
						Crassicauda crassicauda	AO	[76, 271, 306]			
***Balaenoptera edeni* Anderson, 1879. Bryde's whale**											
Bolbosoma capitatum	AO	[166]	-	-	-	*Anisakis simplex* s.l.[*]	PO	[115, 201, 306]	*Brachycladium petrowi*	PO	[268, 306]
Bolbosoma tuberculata	IO	[266]				*Crassicauda crassicauda*	AO	[306]	*Cetitrema foliforme*[*]	IO	[267, 306, 314]
Bolbosoma turbinella	PO	[201]							*Ogmogaster grandis*	IO	[306]

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Balaenoptera borealis* Lesson, 1828. Sei whale**											
Bolbosoma balaenae	AO	[31, 76, 306]	*Diphyllobothrium* sp.	PO	[171]	*Anisakis simplex* s.l.*	AO, PO, Ant	[31, 172, 73, 171, 306, 115, 70, 201, 157]	*Brachycladium goliath*	AO, PO, Ant	[42, 206, 31, 76, 172, 289, 314, 171, 306, 272, 70, 200]
Bolbosoma brevicolle	AO	[31, 76, 84, 306]	*Diplogonoporus balaenopterae*	AO, Arc, Ant	[31, 76, 177, 312, 238, 306, 85, 229, 70]	*Crassicauda boopis*	AO, Ant	[14]	*Ogmogaster antarctica*	AO, PO, Arc	[239, 306, 200, 157, 109]
Bolbosoma nipponicum	PO	[76, 306, 313]	*Monorygma grimaldii**	§	[17]	*Crassicauda crassicauda*	AO, Ant	[31, 76, 162, 306, 271, 70, 200]	*Ogmogaster grandis*	Ant	[265, 306]
Bolbosoma tuberculata	Ant	[266, 306]	*Scolex pleuronectis**	Ant	[306]	*Crassicauda delamureana**	Ant	[131]	*Ogmogaster plicata*	AO, PO, Arc	[31, 219, 74, 76, 172, 239, 314, 171, 306, 115]
Bolbosoma turbinella	AO, PO, Ant, SA	[30, 31, 70, 76, 110, 115, 171, 172, 200, 201, 270, 306]	*Priapocephalus grandis*	AO, Ant	[31, 76, 177, 84, 306, 115, 229, 70]	*Odontobius ceti*	PO	[245, 274]			
			Priapocephalus minor	AO	[23, 31, 76, 306, 229]						
			Tetrabothrius affinis	AO, Ant, SA	[31, 76, 306, 115, 229, 70, 157]						
			*Tetrabothrius arsenyevi**	Ant	[76, 312, 306]						
			Tetrabothrius wilsoni	AO, PO, Ant	[156, 31, 177, 115, 63, 229, 70]						

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Balaenoptera musculus* (Linnaeus, 1758). Blue whale**											
Bolbosoma balaenae	AO, PO	[31, 76, 306]	*Diplogonoporus balaenopterae*	AO	[177, 238, 306, 115, 189]	*Anisakis simplex* s.l.*	AO, PO, IO	[31, 189, 76, 73, 306, 115]	*Brachycladium goliath*	AO	[306]
Bolbosoma brevicolle	AO, SA	[31, 76, 96, 306, 115]	*Monorygma grimaldii**	§	[17]	*Pseudoterranova decipiens**	§	[76]	*Ogmogaster antarctica*	Arc	[76, 239, 314, 306]
Bolbosoma hamiltoni	AO	[30, 31, 76, 313, 306, 115]	*Phyllobothrium delphini**	§	[17]	*Crassicauda boopis*	AO	[113, 115, 189]	*Ogmogaster grandis*	AO	[265, 306]
Bolbosoma nipponicum	PO	[78, 306]	*Priapocephalus grandis*	AO, PO, SA	[31, 76, 177, 220, 306, 115, 229]	*Crassicauda crassicauda*	AO, PO	[31, 76, 162, 306, 271, 113, 115]	*Ogmogaster plicata*	AO	[31, 219, 76]
Bolbosoma turbinella	AO, PO	[31, 76, 270, 306]	*Tetrabothrius affinis*	AO, PO	[31, 76, 220, 177, 229, 306, 115]	*Odontobius ceti*	PO	[31, 262]			
			*Tetrabothrius schaeferi**	AO	[189, 177, 306, 115]						
			Tetrabothrius wilsoni	AO	[177, 115]						
***Megaptera novaeangliae* (Borowski, 1781). Humpback whale**											
Bolbosoma balaenae	Ant	[243, 31, 76, 306]	*Diplogonoporus balaenopterae*	PO, Arc	[245, 238, 171, 306]	*Anisakis simplex* s.l.*	§	[73]	*Ogmogaster plicata*	AO	[115]
Bolbosoma nipponicum	PO	[78, 306]	*Monorygma grimaldii**	§	[17]	*Odontobius ceti*	§	[31, 274]			
Bolbosoma turbinella	AO, PO	[31, 76, 270, 306, 115]	*Phyllobothrium delphini**	§	[306]	*Pharurus alatus*	AO	[229]			
						Crassicauda boopis	PO	[31, 76, 162, 270, 306, 115]			
						Crassicauda crassicauda	AO	[76, 285, 306, 271]			

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Balaenoptera physalus* (Linnaeus, 1758). Fin whale**											
Bolbosoma balaenae	AO	[76, 306, 115]	*Diplogonoporus balaenopterae*	Arc	[76, 177, 238, 306, 115]	*Anisakis simplex* s.l.*	AO, PO, Ant	[305, 285, 73, 115, 170, 201]	*Brachycladium goliath*	AO, PO, Ant	[76, 172, 314, 171, 306, 272]
Bolbosoma brevicolle	Ant, SA	[31, 76, 84, 306, 115]	*Monorygma grimaldii**	Ant	[269, 306]	*Odontobius ceti*	§	[31, 274]	*Brachycladium petrowi*	AO	[306]
Bolbosoma hamiltoni	Ant	[31, 76, 54, 313, 306, 115]	*Phyllobothrium delphini**	AO, PO	[172, 285, 171, 306, 170]	*Crassicauda boopis*	AO	[76, 113, 306, 115, 149, 116]	*Ogmogaster antarctica*	AO, PO, Arc, MS, SA	[76, 143, 239, 314, 171, 306, 115, 229, 167, 200, 201]
Bolbosoma nipponicum	PO	[76, 313, 306]	*Priapocephalus grandis*	AO, Ant, SA	[76, 177, 306, 115, 229]	*Crassicauda crassicauda*	AO, Ant, SA	[31, 76, 162, 306, 113, 271, 115, 200]	*Ogmogaster grandis*	Ant	[265, 306]
Bolbosoma turbinella	AO, PO	[76, 270, 306]	*Priapocephalus minor*	AO	[76, 306, 229]				*Ogmogaster plicata*	AO, PO, Arc	[31, 74, 76, 172, 262, 239, 314, 171, 306, 229]
			Tetrabothrius affinis	Ant	[161, 76, 306, 115]				*Ogmogaster trilineata**	PO	[242, 314, 171]
			*Tetrabothrius egregius**	Ant	[276, 306]						
			Tetrabothrius ruudi	Ant	[31, 76, 177, 306, 115]						
Family *Neobalaenidae*											
***Caperea marginata* (Gray, 1846). Pygmy right whale**											
-	-	-	-	-	-	*Anisakis pegreffii*	SA	[181]	-	-	-
Family *Eschrichtiidae*											
***Eschrichtius robustus* (Lilljeborg, 1861). Gray whale**											
Bolbosoma balaenae	PO	[66]	*Monorygma grimaldii**	§	[17]	*Anisakis simplex* s.l.*	PO	[246, 66]	*Brachycladium goliath*	PO	[246]
*Corynosoma semerme**	Arc	[246, 306]	*Phyllobothrium delphini**	§	[17]				*Ogmogaster antarctica*	PO	[246, 65, 171, 66]
*Corynosoma septentrionalis**	Arc	[254, 246, 306]	*Priapocephalus eschrichtii**	Arc	[202, 246, 306]				*Ogmogaster pentalineata**	PO, Arc	[239, 242, 314, 246, 306, 171, 115, 66]
*Corynosoma strumosum**	Arc	[246, 306]							*Ogmogaster plicata*	Arc	[246, 306]
*Corynosoma validum**	Arc	[246, 306]							*Orthosplanchnus pygmaeus**	PO	[316, 246, 306]

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Odontoceti: Family *Delphinidae*											
***Cephalorhynchus commersonii* (Lacépède, 1804). Commerson's dolphin**											
-	-	-	*Monorygma grimaldii**	§	[17]	*Anisakis simplex* s.l.*	AO	[115, 36]	*Braunina cordiformis*	AO	[115, 36]
			*Phyllobothrium delphini**	§	[17]	*Halocercus brasiliensis*	AO	[115, 190]	*Pholeter gastrophilus*	AO	[36]
			Strobilocephalus triangularis	AO	[36]				*Synthesium delamurei*	AO	[35]
***Cephalorhynchus eutropia* (Gray, 1846). Chilean dolphin**											
Corynosoma cetaceum	PO	[287, 20]	-	-	-	*Anisakis* sp.	PO	[287]	*Braunina cordiformis*	PO	[105, 287]
						Pseudoterranova sp.	PO	[287]	*Nasitrema globicephalae*	PO	[43]
									Synthesium tursionis	PO	[287]
***Cephalorhynchus heavisidii* (Gray, 1828). Haviside's dolphin**											
-	-	-	*Monorygma grimaldii**	AO	[17]	*Anisakis pegreffii*	SA	[181]	-	-	-
			*Phyllobothrium delphini**	AO	[17]						
***Cephalorhynchus hectori* (Van Beneden, 1881). Hector's dolphin**											
Corynosoma sp.	PO	[188]	*Monorygma grimaldii**	PO	[17]	*Contracaecum* sp.	PO	[188]	*Campula* sp.	§	[128]
			*Phyllobothrium delphini**	PO	[188, 17]	*Phocascaris* sp.*	PO	[188]	*Braunina cordiformis*	PO	[188]
***Delphinus capensis* Gray, 1828. Long-beaked common dolphin**											
-	-	-	*Monorygma grimaldii**	§	[17]	*Anisakis simplex* s.l.*	AO, PO	[194, 147]	-	-	-
			*Phyllobothrium delphini**	§	[17]						
***Delphinus delphis* Linnaeus, 1758. Short-beaked common dolphin**											
*Bolbosoma vasculosum**	MS, BlS	[295, 76, 77, 306]	*Diphyllobothrium lanceolatum*	§	[218]	*Anisakis pegreffii*	AO, MS	[181]	*Amphimerus lancea**	AO	[166]
Corynosoma cetaceum	AO, IO, BlS	[134, 76, 77, 313, 254, 306, 34]	*Diphyllobothrium stemmacephalum*	BlS	[31, 77, 38]	*Anisakis simplex* s.l.*	AO, PO, BlS, SA	[76, 77, 73, 306, 71, 115, 1, 116, 38, 21, 34]	*Brachycladium atlanticum*	MS	[178]
*Corynosoma strumosum**	§	[218]	*Monorygma grimaldii**	AO, PO, BlS	[23, 76, 77, 306, 281, 62, 71, 115, 67, 229, 1, 116]	*Anisakis simplex* s.s.	AO	[181]	*Brachycladium delphini*	AO, MS, BlS	[31, 219, 76, 77, 127, 314, 306, 229]

Acanthocephala	Locality	Ref.	*Cestoda*	Locality	Ref.	*Nematoda*	Locality	Ref.	*Trematoda*	Locality	Ref.
			Phyllobothrium delphini[*]	AO, PO, BlS	[31, 119, 76, 77, 263, 303, 171, 306, 281, 62, 71, 115, 67, 229, 1, 116]	*Anisakis typica*	AO, PO, BlS	[31, 76, 77, 73, 306, 71]	*Brachycladium palliatum*	AO, BlS	[31, 219, 76, 77, 314, 306, 229, 116, 38]
			Scolex pleuronectis[*]	PO, MS	[222, 71]	*Crassicauda crassicauda*	AO	[306]	*Braunina cordiformis*	AO, BlS	[76, 77, 275, 289, 314, 306, 229, 34, 109]
			Strobilocephalus triangularis	PO	[67]	*Goezia* sp.[*]	§	[237]	*Campula oblonga*	AO	[5, 116]
			Tetrabothrius diplosoma[*]	AO	[306]	*Halocercus delphini*	AO, PO, MS, BlS	[31, 94, 310, 76, 77, 283, 306, 115, 1, 116, 278]	*Galactosomum erinaceum*[*]	AO, BlS	[31, 76, 77, 306, 229]
			Tetrabothrius forsteri	PO, MS, BlS	[31, 119, 76, 77, 306, 67, 222]	*Halocercus invaginatus*	§	[218, 13]	*Nasitrema delphini*	PO	[204, 314, 171, 12, 71, 62, 278]
						Halocercus kleinenbergi	BlS, SA	[76, 77, 306, 38]	*Nasitrema globicephalae*	PO	[62, 71, 67]
						Halocercus lagenorhynchi	AO, IO	[190, 286]	*Nasitrema lanceolatum*	PO	[62]
						Pseudoterranova decipiens[*]	§	[218]	*Nasitrema stenosoma*	PO	[71]
						Skrjabinalius cryptocephalus	PO, BlS	[76, 77, 278, 38]	*Oschmarinella rochebruni*	AO, PO, BlS	[31, 219, 76, 77, 314, 247, 306, 62, 71, 67, 229, 116, 34]
						Skrjabinalius guevarai	AO, MS	[227, 190]	*Pholeter gastrophilus*	AO, BlS	[306, 92, 213, 229, 116, 38, 34]
						Stenurus minor	§	[218]	*Synthesium delamurei*	MS	[222]
									Synthesium tursionis	PO	[280]

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Feresa attenuata* Gray, 1874. Pygmy killer whale**											
Bolbosoma vasculosum[*]	PO	[194]	*Monorygma grimaldii*[*]	PO	[194]	*Anisakis simplex* s.l.[*]	PO	[319]	*Nasitrema lanceolatum*	PO	[106]
			Phyllobothrium delphini[*]	§	[17]	*Anisakis typica*	PO	[319, 184]			
			Scolex pleuronectis[*]	PO	[106]	*Terranova* sp.[*]	PO	[194]			
			Tetrabothrius forsteri	PO	[106]	*Halocercus brasiliensis*	PO	[120]			
			Trigonocotyle sexitesticulae[*]	PO	[126, 194]	*Stenurus globicephalae*	PO	[106, 194]			
***Globicephala macrorhynchus* Gray, 1846. Short-finned pilot whale**											
Bolbosoma capitatum	AO	[194, 55]	*Anophryocephalus* sp.[*]	§	[226]	*Anisakis simplex* s.l.[*]	AO, SA	[138, 137, 115, 55]	*Brachycladium nipponicum*	PO	[308, 314, 306]
			Diphyllobothrium sp.	AO	[47]	*Anisakis simplex* s.s.	AO	[48]	*Campula gondo*	PO	[308, 314, 306]
			Monorygma grimaldii[*]	AO	[194, 55]	*Anisakis typica*	AO	[194, 181, 47, 48]	*Nasitrema attenuatum*	PO	[204]
			Phyllobothrium delphini[*]	AO	[17, 47]	*Crassicauda* sp.	AO	[194]	*Nasitrema globicephalae*	AO, PO	[204, 314, 171, 12, 196]
			Plicobothrium globicephalae	§	[315]	*Halocercus kleinenbergi*	AO	[47]	*Nasitrema gondo*	PO	[309, 314, 306]
			Trigonocotyle globicephalae	§	[312]	*Stenurus globicephalae*	AO	[94, 306, 196, 194, 55, 47]	*Nasitrema lanceolatum*	PO	[204, 314, 171]
			Trigonocotyle lintoni	PO	[306]	*Stenurus minor*	AO	[196]			
						Torynurus convolutus	AO, PO	[94]			

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Globicephala melas* (Traill, 1809). Long-finned pilot whale**											
Bolbosoma capitatum	AO, MS	[31, 76, 313, 59, 306, 115, 97, 27, 232, 28, 25]	*Diphyllobothrium polyrugosum*	AO	[27]	*Anisakis berlandi*	SA	[181, 180]	*Campula gondo*	PO	[76]
			Diphyllobothrium stemmacephalum	AO	[26]	*Anisakis pegreffii*	PO	[180]	*Nasitrema globicephalae*	PO	[63, 99]
			Monorygma grimaldii[*]	AO, MS	[76, 306, 229, 232]	*Anisakis simplex* s.l.[*]	AO	[88, 232, 182, 1, 116]	*Orthosplanchnus arcticus*[*]	AO	[59, 229, 232]
			Phyllobothrium delphini[*]	AO, MS	[119, 76, 263, 303, 306, 288, 115, 229, 232, 1]	*Anisakis simplex* s.s.	AO, SA	[115, 182, 181, 180]	*Pholeter gastrophilus*	AO, MS	[237, 229, 232]
			Plicobothrium globicephalae	AO	[241, 229, 232]	*Anisakis typica*	PO	[31, 76, 73, 306, 184]	*Synthesium delamurei*	AO, MS	[230, 229, 232, 28]
			Scolex pleuronectis[*]	AO	[59]	*Crassicauda carbonelli*[*]	MS	[231, 232]	*Synthesium subtile*	AO	[29, 232, 28, 35]
			Trigonocotyle globicephalae	AO, MS, SA	[23, 306, 115, 27, 28]	*Stenurus globicephalae*	AO, MS	[31, 76, 59, 306, 13, 115, 237, 232, 1, 116]			
			Trigonocotyle lintoni	AO, MS	[76, 59, 306, 229]	*Torynurus convolutus*	AO, PO	[31, 76, 306, 13]			
***Grampus griseus* (G. Cuvier, 1812). Risso's dolphin**											
-	-	-	*Monorygma grimaldii*[*]	PO	[69, 237, 229, 58]	*Anisakis pegreffii*	MS	[39]	*Brachycladium palliatum*	AO	[318, 229]
			Phyllobothrium delphini[*]	AO	[31, 76, 263, 303, 318, 306, 229, 1, 194, 58]	*Anisakis physeteris*	PO	[147]	*Nasitrema gondo*	PO	[197]
			Scolex pleuronectis[*]	MS	[96,100, 9, 17]	*Anisakis simplex* s.l.[*]	PO, SA	[115, 147]	*Synthesium delamurei*	MS	[96]
			Tetrabothrius forsteri	MS	[96]	*Crassicauda grampicola*	AO, PO, MS	[135, 76, 162, 306, 235, 225, 197, 279]	*Synthesium tursionis*	AO	[318]
			Trigonocotyle globicephalae	MS	[96]	*Pseudoterranova ceticola*	PO	[147]	*Pholeter gastrophilus*	AO, MS	[318, 229, 58, 98]
						Stenurus globicephalae	AO, MS	[13, 237, 98]			
						Stenurus minor	AO	[285, 306, 190]			

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Lagenodelphis hosei* Fraser, 1956. Fraser's dolphin**											
Bolbosoma sp.	PO	[284]	*Monorygma grimaldii**	AO, IO	[186, 294, 194, 55, 47]	*Anisakis pegreffii*	AO	[48]	*Campula* sp.	IO	[186]
			*Phyllobothrium delphini**	AO	[281, 294, 229, 194, 55, 47]	*Anisakis simplex* s.l.*	AO, IO	[186, 294]	*Nasitrema* sp.	PO	[278]
			Strobilocephalus triangularis	AO	[223, 294, 229]	*Anisakis typica*	AO	[48]			
			Tetrabothrius forsteri	AO	[194]	*Stenurus ovatus*	PO	[186, 278]			
***Lagenorhynchus acutus* (Gray, 1828). Atlantic white-sided dolphin**											
Bolbosoma sp.	AO	[37]	*Diphyllobothrium stemmacephalum*	AO	[208]	*Anisakis simplex* s.l.*	AO	[137, 115, 237, 116]	*Oschmarinella laevicaecum*	AO	[37]
			*Monorygma grimaldii**	AO	[23, 31, 76, 115, 229, 248, 116]	*Anisakis typica*	AO	[137]	*Pholeter gastrophilus*	AO	[37, 229, 248]
			*Phyllobothrium delphini**	AO	[23, 306, 248, 116]	*Pseudalius inflexus*	AO	[115, 190]			
			Strobilocephalus triangularis	AO	[23, 31, 76, 306, 229]	*Stenurus globicephalae*	AO	[306, 13, 37, 248]			
			Tetrabothrius forsteri	AO	[37, 229]	*Torynurus convolutus*	AO	[115]			
						Crassicauda grampicola	AO	[112]			
***Lagenorhynchus albirostris* (Gray, 1846). White-beaked dolphin**											
-	-	-	*Monorygma grimaldii**	§	[17]	*Anisakis simplex* s.l.*	AO	[31, 76, 73, 306, 116]	*Pholeter gastrophilus*	AO	[116]
			*Phyllobothrium delphini**	§	[17]	*Anisakis simplex* s.s.	AO	[182]			
			Plicobothrium globicephalae	AO	[170]	*Halocercus lagenorhynchi*	AO	[31, 94, 310, 76, 306]			
						Pseudoterranova sp.	§	[15]			
***Lagenorhynchus australis* (Peale, 1848). Peale's dolphin**											
-	-	-	*Trigonocotyle prudhoei**	AO	[177, 115]	-	-	-	-	-	-
***Lagenorhynchus cruciger* (Quoy and Gaimard, 1824). Hourglass dolphin**											
-	-	-	*Phyllobothrium delphini**	AO	[103]	*Anisakis simplex* s.l.*	AO	[76, 306, 103]	*Oschmarinella* sp.	AO	[103]
			*Trigonocotyle prudhoei**	Ant	[306]	*Anisakis typica*	AO	[76, 306]	*Synthesium subtile*	AO	[35]

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Lagenorhynchus obliquidens* Gill, 1865. Pacific white-sided dolphin**											
-	-	-	*Diphyllobothrium fuhrmanni*	PO	[26]	*Anisakis simplex* s.l.*	PO	[62, 71]	*Brachycladium* sp.	PO	[247]
			*Monorygma grimaldii**	PO	[67, 71]	*Crassicauda* sp.	PO	[67]	*Nasitrema globicephalae*	PO	[204, 171, 12, 62, 71, 67]
			*Phyllobothrium delphini**	PO	[136, 171, 71, 67]						
			*Scolex pleuronectis**	PO	[71]						
			Strobilocephalus triangularis	PO	[71]						
			Tetrabothrius sp.	PO	[71]						
***Lagenorhynchus obscurus* (Gray, 1828). Dusky dolphin**											
*Corynosoma australe**	AO	[72]	*Monorygma grimaldii**	§	[17]	*Anisakis simplex* s.l.*	AO	[134, 72, 21]	*Braunina cordiformis*	AO, PO	[297, 72]
			*Phyllobothrium delphini**	AO, PO, SA	[115, 297, 201, 160]	*Anisakis typica*	SA	[31, 73, 115]	*Nasitrema* sp.	PO	[297]
			*Scolex pleuronectis**	PO	[177, 297]				*Pholeter gastrophilus*	AO, PO	[297, 72]
			*Trigonocotyle prudhoei**	AO	[177, 115]				*Synthesium subtile*	AO	[35]
***Lissodelphis borealis* (Peale, 1848). Northern right-whale dolphin**											
-	-	-	*Monorygma grimaldii**	PO	[71]	*Anisakis berlandi*	PO	[181]	*Nasitrema globicephalae*	PO	[204, 314, 171, 12, 62, 71]
			*Phyllobothrium delphini**	§	[17]	*Anisakis simplex* s.l.*	PO	[62, 71]			
			*Scolex pleuronectis**	PO	[199]						
***Lissodelphis peronii* (Lacépède, 1804). Southern right-whale dolphin**											
-	-	-	*Strobilocephalus triangularis*	PO	[201]	-	-	-	*Delphinicola tenuis*	PO	[201]
			Tetrabothrius forsteri	PO	[201]						
***Orcaella brevirostris* (Owen in Gray, 1866). Irrawaddy dolphin**											
-	-	-	-	-	-	-	-	-	*Amphimerus lancea**	PO, IO	[31, 219, 76, 306]

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Orcinus orca* (Linnaeus, 1758). Killer whale**											
Bolbosoma nipponicum	PO	[262, 306]	*Diphyllobothrium fuhrmanni*	PO	[257]	*Anisakis simplex* s.s.	PO	[181]	*Oschmarinella albamarina*	AO	[114, 116]
Bolbosoma capitatum	AO, PO	[262, 306, 114, 116]	*Diphyllobothrium orcini*[*]	PO	[123]	*Anisakis simplex* s.l.[*]	AO, PO	[76, 73, 171, 306, 114, 116]	*Synthesium subtile*	AO, PO, Arc	[261, 314, 306, 292, 35]
			Diphyllobothrium polyrugosum	AO	[83, 306, 115, 229, 114, 116]						
			Monorygma grimaldii[*]	AO	[89, 229]						
			Phyllobothrium delphini[*]	AO	[115, 229]						
			Trigonocotyle spasskyi[*]	PO	[76, 306]						
***Peponocephala electra* (Gray, 1846). Melon-headed whale**											
Bolbosoma capitatum	AO	[55]	*Diphyllobothrium* sp.	AO	[194]	*Anisakis physeteris*	PO	[147]	*Nasitrema gondo*	PO	[195]
			Monorygma grimaldii[*]	AO	[55, 47]	*Anisakis simplex* s.l.[*]	PO	[147]			
			Phyllobothrium delphini[*]	AO	[47]	*Anisakis typica*	AO	[129, 184]			
			Strobilocephalus triangularis	AO	[47]	*Halocercus* sp.	PO	[278]			
						Stenurus globicephalae	AO, PO	[194, 190, 278, 47]			
***Pseudorca crassidens* (Owen, 1846). False killer whale**											
Bolbosoma capitatum	AO, IO	[31, 76, 313, 306, 115, 97, 11]	-	-	-	*Anisakis berlandi*	PO	[182]	*Nasitrema attenuatum*	PO	[204, 314, 171]
						Anisakis simplex s.l.[*]	AO	[76, 73, 318, 306, 115, 182, 11]	*Nasitrema globicephalae*	PO	[204, 314, 171, 12]
						Anisakis simplex s.s.	PO	[182]	*Nasitrema gondo*	PO	[198]
						Anisakis typica	AO, PO	[318, 209]	*Odhneriella elongata*	AO, PO	[229]
						Stenurus auditivus	AO	[318]	*Synthesium elongatum*	AO	[318]
						Stenurus globicephalae	AO	[190, 320]			

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Sotalia fluviatilis* (Gervais and Deville in Gervais, 1853). Tucuxi**											
-	-	-	*Monorygma grimaldii*[*]	§	[17]	*Anisakis simplex* s.l.[*]	AO	[21]	*Amphimerus lancea*[*]	AO, AR	[31, 219, 76, 289, 115]
			Phyllobothrium delphini[*]	§	[17]	*Anisakis typica*	AO	[253, 184, 192, 317]	*Braunina cordiformis*	AO	[253, 192]
						Halocercus brasiliensis	AO	[115, 253, 190, 192]	*Nasitrema attenuatum*	AO	[115]
***Sotalia guianensis* (Van Bénedén, 1864). Guiana dolphin**											
-	-	-	-	-	-	*Anisakis typica*	AO	[130, 163]	*Braunina cordiformis*	AO	[163, 176, 93]
						Halocercus brasiliensis	AO, AO	[94, 310, 76, 306,192, 176, 47, 163, 120, 93]	*Nasitrema attenuatum*	AO	[95]
									Synthesium tursionis	AO	[176]
***Sousa chinensis* (Osbeck, 1765). Indo-Pacific humpback dolphin**											
-	-	-	*Monorygma grimaldii*[*]	PO	[17]	*Halocercus pingi*	PO	[212]	-	-	-
			Phyllobothrium delphini[*]	PO	[17]						
***Stenella attenuata* (Gray, 1846). Pantropical spotted dolphin**											
Bolbosoma balaenae	PO	[68]	*Monorygma grimaldii*[*]	PO	[68, 67, 207]	*Anisakis simplex* s.l.[*]	PO	[65, 68]	*Brachycladium pacificum*	PO, SA	[68, 115, 67]
Bolbosoma vasculosum[*]	PO	[68]	*Phyllobothrium delphini*[*]	PO	[68, 67]	*Anisakis typica*	AO	[181, 48]	*Braunina cordiformis*	PO	[76, 314, 306]
			Scolex pleuronectis[*]	PO	[207]	*Halocercus delphini*	PO	[68]	*Nasitrema globicephalae*	PO	[67, 207]
			Strobilocephalus triangularis	PO	[68, 67]				*Nasitrema stenosoma*	PO	[204]
			Tetrabothrius forsteri	PO	[68, 67, 207]				*Oschmarinella rochebruni*	PO, SA	[68, 115, 67]
									Synthesium elongatum	SA	[115]
***Stenella clymene* (Gray, 1850). Clymene dolphin**											
Bolbosoma sp.	AO	[47]	*Monorygma grimaldii*[*]	AO	[17, 47]	*Anisakis* sp.	AO	[47]	-	-	-
			Phyllobothrium delphini[*]	AO	[17, 47]	*Anisakis typica*	AO	[129]			
			Scolex pleuronectis[*]	AO	[47]	*Halocercus brasiliensis*	AO	[47, 120]			
			Strobilocephalus triangularis	AO	[47]	*Skrjabinalius guevarai*	AO	[6]			
			Tetrabothrius forsteri	AO	[47]						
			Trigonocotyle sp.	AO	[47]						

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Stenella frontalis* (G. Cuvier, 1829). Atlantic spotted dolphin**											
Bolbosoma vasculosum[*]	AO	194	*Phyllobothrium delphini*[*]	AO	[194, 47]	*Anisakis typica*	AO	[194]	*Brachycladium palliatum*	AO	[318]
			Strobilocephalus triangularis	AO	[47]	*Halocercus delphini*	AO	[318]	*Braunina cordiformis*	AO	[318]
									Pholeter gastrophilus	AO	[318]
***Stenella coeruleoalba* (Meyen, 1833). Striped dolphin**											
Bolbosoma capitatum	AO	[251]	*Monorygma grimaldii*[*]	AO, PO, MS	[115, 67, 233, 229, 169, 1, 116, 58, 17, 47, 207]	*Anisakis pegreffii*	MS	[181, 180, 39]	*Brachycladium atlanticum*	AO, MS	[4, 99, 178]
Bolbosoma turbinella	AO	[251]	*Phyllobothrium delphini*[*]	AO, PO, MS, SA	[92, 318, 115, 229, 169, 1, 116, 58, 17, 47, 207]	*Anisakis simplex* s.l.[*]	AO, PO, MS	[138, 73, 318, 169, 1, 116]	*Brachycladium delphini*	AO, MS	[127, 92, 229]
Bolbosoma vasculosum[*]	MS	[233, 169, 8, 179]	*Scolex pleuronectis*[*]	AO, PO, MS	[8, 251, 9, 17, 207, 159]	*Anisakis simplex* s.s.	AO	[181, 39]	*Brachycladium pacificum*	PO	[67]
			Strobilocephalus triangularis	AO, PO, MS	[68, 69, 233, 169, 229, 8, 207, 179]	*Anisakis typica*	AO, MS, SA	[73, 115, 3, 184, 48]	*Brachycladium palliatum*	AO, PO, MS	[233, 158, 229, 169, 207]
			Tetrabothrius forsteri	AO, PO, MS, SA	[115, 233, 229, 169, 8, 207, 179]	*Halocercus brasiliensis*	AO	[251]	*Nasitrema globicephalae*	PO	[67, 207]
			Trigonocotyle globicephalae	MS	[179]	*Halocercus delphini*	AO, MS	[299, 237]	*Oschmarinella rochebruni*	AO, MS	[224, 229, 169, 99, 116, 178, 109]
						Halocercus lagenorhynchi	AO, PO, SA	[252, 115, 116, 207]	*Pholeter gastrophilus*	AO, MS	[92, 233, 234, 229, 169, 168, 58, 159]
						Skrjabinalius guevarai	AO, MS	[233, 227, 169]			
						Stenurus minor	MS	[16]			
						Stenurus ovatus	MS	[169]			

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
					***Stenella longirostris* (Gray, 1828). Spinner dolphin**						
Bolbosoma balaenae	PO	[68]	*Diphyllobothrium fuhrmanni*	PO	[76, 306]	*Anisakis simplex* s.l.*	PO	[68]	*Brachycladium nipponicum*	PO	[308, 76, 306]
Bolbosoma hamiltoni	PO	[7]	*Diphyllobothrium stemmacephalum*	PO	[26]	*Anisakis typica*	AO, PO	[7, 48, 129]	*Brachycladium pacificum*	PO	[76, 68, 67, 7, 207]
*Bolbosoma vasculosum**	PO	[68]	*Monorygma grimaldii**	AO, PO	[68, 67, 55, 47]	*Halocercus brasiliensis*	AO	[47]	*Oschmarinella laevicaecum*	PO	[308, 76, 314, 306]
			*Phyllobothrium delphini**	AO, PO	[68, 67, 47]	*Halocercus delphini*	PO	[68]	*Oschmarinella rochebruni*	PO	[68, 67, 7]
			*Scolex pleuronectis**	AO, PO	[7, 47]				*Synthesium tursionis*	PO	[7]
			Strobilocephalus triangularis	AO, PO	[68, 67, 7, 47]						
			Tetrabothrius forsteri	AO, PO	[68, 67, 47]						
			Trigonocotyle sp.	AO	[47]						
					***Steno bredanensis* (G. Cuvier in Lesson, 1828). Rough-toothed dolphin**						
Bolbosoma capitatum	AO, MS	[31, 76, 313, 306, 115]	*Phyllobothrium delphini**	AO	[55]	*Anisakis simplex* s.l.*	PO	[138, 73, 209, 147]	*Brachycladium palliatum*	AO	[107]
			Strobilocephalus triangularis	AO	[76, 306, 229]	*Anisakis simplex* s.s.	AO	[48]	*Nasitrema attenuatum*	AO	[95]
			Tetrabothrius forsteri	AO	[23, 31, 76, 306, 107]	*Anisakis typica*	AO	[107, 55, 48, 129]	*Nasitrema globicephalae*	AO	[95]
			*Trigonocotyle prudhoei**	AO	[177, 306, 115]				*Synthesium tursionis*	AO	[107]
									Braunina cordiformis	AO	[253]
									Pholeter gastrophilus	AO	[107]
					***Tursiops aduncus* (Ehrenberg, 1833). Indo-Pacific bottlenose dolphin**						
-	-	-	*Diphyllobothrium fuhrmanni*	RS	[142]	*Anisakis typica*	RS	[142]	*Brachycladium curilense*	RS	[142]
						Crassicauda sp.	IO	[151]	*Nasitrema attenuatum*	RS	[142]
						Halocercus lagenorhynchi	IO	[286]	*Pholeter gastrophilus*	RS	[142]
						Stenurus ovatus	IO	[286]			

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Tursiops truncatus* (Montagu, 1821). Common bottlenose dolphin**											
Bolbosoma sp.	§	[40]	*Diphyllobothrium fuhrmanni*	PO	[257]	*Anisakis pegreffii*	MS, SA	[182, 39]	*Brachycladium atlanticum*	AO	[222]
Corynosoma australe[*]	AO	[250]	*Diphyllobothrium stemmacephalum*	BlS	[81, 86, 38]	*Anisakis simplex* s.l.[*]	AO, PO, BlS	[76, 115, 40, 1, 116, 38, 201, 250]	*Brachycladium delphini*	AO	[318]
Corynosoma cetaceum	AO, IO, BlS	[134, 76, 77, 313, 254, 306, 250]	*Monorygma grimaldii*[*]	AO, PO, MS, BlS	[31, 76, 77, 89, 306, 229, 298, 1, 116, 58, 207]	*Anisakis simplex* s.s.	AO	[48, 39]	*Brachycladium palliatum*	AO	[304, 318, 229]
			Phyllobothrium delphini[*]	AO, PO, MS, BlS	[31, 76, 77, 89, 263, 303, 306, 229, 298, 1, 116, 58, 55]	*Anisakis typica*	AO, PS, MS	[318, 73, 298, 184, 55, 48]	*Braunina cordiformis*	AO, PO, BlS	[76, 275, 255, 136, 314, 318, 171, 306, 118, 298, 253, 38, 250]
			Scolex pleuronectis[*]	AO	[17, 222]	*Contracaecum multipapillatum*[*]	AO	[48]	*Campula oblonga*	AO	[318]
			Strobilocephalus triangularis	MS	[222]	*Crassicauda crassicauda*	MS, BlS	[31, 76, 77, 162, 306]	*Nasitrema attenuatum*	AO	[145, 115]
			Tetrabothrius forsteri	PO	[298, 222]	*Crassicauda grampicola*	BlS	[38]	*Nasitrema dalli*	SA	[115]
						Halocercus delphini	AO	[1, 190]	*Nasitrema delphini*	AO	[318]
						Halocercus lagenorhynchi	AO, PO, BlS	[135, 76, 77, 282, 304, 193, 318, 306, 115, 278]	*Nasitrema globicephalae*	PO	[298]
						Pharurus alatus	IO	[286]			
						Pseudoterranova decipiens[*]	AO	[250]	*Pholeter gastrophilus*	AO, PO, MS, BlS	[304, 193, 92, 115, 234, 229, 298, 38, 222, 250, 109]

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
						Skrjabinalius cryptocephalus	PO, BlS	[40, 190, 278]	*Synthesium tursionis*	AO, PO, MS, BlS	[31, 219, 76, 77, 306, 229, 298, 102,101, 99, 194, 280, 38, 174, 222, 250]
						Skrjabinalius guevarai	MS	[111, 237, 190]			
						Stenurus minor	AO, PO, BlS	[190]			
						Stenurus ovatus	AO, PO, MS, BlS, IO	[31, 94, 75, 76, 77, 306, 115, 298, 278, 38, 286]			
Family *Iniidae* *Inia geoffrensis* (Blainville, 1817). Amazon river dolphin											
-	-	-	-	-	-	*Anisakis insignis*[*]	AR	[31, 76, 152, 115, 163, 317]	*Hunterotrema caballeroi*[*]	AR	[187, 152, 304, 317]
									Hunterotrema macrosoma[*]	AR	[60]
									Pholeter gastrophilus	AR	[317]
Family *Kogiidae* *Kogia sima* (Owen, 1866). Dwarf sperm whale											
-	-	-	*Monorygma grimaldii*[*]	§	[17]	*Anisakis brevispiculata*	AO	[55, 48, 221]	-	-	-
			Phyllobothrium delphini[*]	AO, SA	[318, 115, 55, 47]	*Anisakis paggiae*	AO	[181, 55, 48, 221]			
						Anisakis physeteris	PO, SA	[115, 147]			
						Anisakis simplex s.l.[*]	AO, PO, SA	[115, 147, 48]			
						Anisakis typica	AO	[318, 48, 221]			
						Anisakis ziphidarum	AO	[221]			
						Pseudoterranova ceticola	AO, PO	[147, 55, 48]			

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Kogia breviceps* (Blainville, 1838). Pygmy sperm whale**											
Bolbosoma vasculosum[*]	§	[215, 15]	*Monorygma grimaldii*[*]	AO, IO	[23, 31, 115, 229, 1, 47]	*Anisakis brevispiculata*	AO, SA	[2, 181, 48]	-	-	-
			Phyllobothrium delphini[*]	AO, MS[∞], SA	[76, 263, 91, 303, 318, 306, 281, 115, 229, 1, 194, 55, 47]	*Anisakis paggiae*	AO, SA	[183, 48]			
			Scolex pleuronectis[*]	AO	[9]	*Anisakis physeteris*	AO, PO, SA	[115, 194, 147, 48]			
						Anisakis simplex s.l.[*]	AO, PO, SA	[296, 73, 115, 1, 147, 48]			
						Anisakis simplex s.s.	AO	[48]			
						Anisakis typica	AO, SA	[318, 115, 129]			
						Pseudoterranova ceticola	AO, PO	[194, 147, 2, 48]			
						Pseudoterranova kogiae[*]	SA	[76, 91, 306, 115]			
						Crassicauda magna[*]	AO, PO	[76, 162, 91, 306, 1]			
[∞]**Single record of the host species in the Mediterranean Sea**											
Family *Monodontidae*											
***Monodon monoceros* Linnaeus, 1758. Narwhal**											
-	-	-	-	-	-	*Anisakis simplex* s.l.[*]	AO	[31, 76, 73, 306]	-	-	-
						Pseudoterranova decipiens[*]	AO	[76, 306]			
						Halocercus monoceris	Arc	[302, 165]			
						Pharurus alatus	Arc	[31, 94, 76, 306, 13]			

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Delphinapterus leucas* (Pallas, 1776). Beluga**											
Bolbosoma sp.	AO	[191]	*Diphyllobothrium lanceolatum*	Arc	[82, 80, 306]	*Anisakis simplex* s.l.*	AO, Arc	[31, 164, 76, 73, 306, 115, 301, 191]	*Odhneriella arctica**	Arc	[82, 141, 80, 314, 306, 292, 301]
*Corynosoma cameroni**	AO, Arc	[295, 203, 306, 293]	*Diphyllobothrium phocarum**	Arc	[82, 306]	*Anisakis simplex* s.s.	AO	[182]	*Orthosplanchnus sudarikovi**	Arc	[290, 306, 292, 293]
*Corynosoma wegeneri**	Arc	[203, 84]	*Monorygma grimaldii**	§	[17]	*Crassicauda giliakiana*	PO	[76, 162, 80, 306]	*Oschmarinella albamarina*	Arc	[291, 314, 306, 292, 293]
*Corynosoma reductum**	AO	[295]	*Phyllobothrium delphini**	§	[17]	*Halocercus monoceris*	AO, Arc	[191, 148]	*Synthesium mironovi*	AO, PO	[219, 144, 76, 141, 285, 80, 314, 306]
*Corynosoma semerme**	PO, Arc	[203, 171, 293, 301]				*Halocercus taurica*	AO	[191, 148]	*Synthesium seymouri**	AO, PO, Arc	[31, 309, 76, 141, 80, 171, 306, 292, 293, 301, 191, 174]
*Corynosoma strumosum**	AO, PO, Arc	[164, 76, 203, 171, 306, 293, 301]				*Pharurus pallasii**	AO, PO, Arc	[76, 306, 13, 293, 301, 191]	*Synthesium subtile*	PO, Arc	[261, 306, 292]
						Porrocaecum sp.*	AO	[82, 256]			
						*Pseudoterranova decipiens**	Arc	[32, 80, 306]			
						*Stenurus arctomarinus**	AO, Arc	[306, 13, 293, 301, 191, 93]			
						Stenurus minor	AO	[80, 306]			
Family *Phocoenidae*											
***Phocoena dioptrica* Lahille, 1912. Spectacled porpoise**											
*Corynosoma australe**	AO	[33]	*Scolex pleuronectis**	AO	[33, 205]	*Anisakis simplex* s.l.*	AO	[217, 33]	-	-	-
			Tetrabothrius sp.	AO	[205]						

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Neophocaena phocaenoides* (G. Cuvier, 1829). Indo-Pacific finless porpoise**											
Corynosoma sp.	PO	[146]	*Diphyllobothrium fuhrmanni*	PO, IO	[306, 76, 133]	*Anisakis simplex* s.l.*	PO	[147]	*Campula folium**	PO	[210, 76, 314, 306, 133]
			*Monorygma grimaldii**	§	[17]	*Anisakis typica*	§	[133]	*Campula oblonga*	PO	[146, 133]
			*Phyllobothrium delphini**	§	[17]	*Halocercus pingi*	PO	[31, 94, 310, 76, 306, 146, 212, 133]	*Delphinicola tenuis*	PO	[146, 133]
						*Halocercus sunameri**	PO	[310, 306, 146, 133]	*Nasitrema spathulatum**	PO	[210, 309, 76, 314, 306, 146, 133]
						Halocercus taurica	PO	[146, 133]	*Nasitrema sunameri**	PO	[309, 314, 306, 146, 133]
						*Pharurus asiaeorientalis**	PO, YR	[146, 133]	*Odhneriella elongata*	PO	[210, 314]
						*Pharurus sunameri**	PO	[310, 306, 146, 133]	*Synthesium elongatum*	PO	[210, 76, 306, 133]
						Stenurus auditivus	PO	[94, 76, 306]	*Synthesium nipponicum*	PO	[146, 133]
						*Stenurus nanjingensis**	PO	[133]	*Synthesium tursionis*	IO	[121, 133]
***Phocoena sinus* Norris and McFarland, 1958. Vaquita**											
-	-	-	-	-	-	-	-		*Synthesium tursionis*	PO	[150]

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Phocoena phocoena* (Linnaeus, 1758). Harbor porpoise**											
Bolbosoma capitatum	AO	[116]	*Diphyllobothrium lanceolatum*	AO, BlS	[76, 77, 306, 229]	*Anisakis simplex* s.l.*	AO, PO, BlS, BaS	[31, 76, 77, 73, 306, 115, 53, 236, 170, 24, 41, 45, 125, 116, 1, 155, 153]	*Brachycladium nipponicum*	PO	[306]
*Corynosoma alaskensis**	PO	[117, 171, 306]	*Diphyllobothrium latum**	BlS	[76, 77, 306, 38]	*Anisakis simplex* s.s.	AO, PO	[182]	*Campula oblonga*	AO, PO, Arc, MS, BlS, BaS	[31, 219, 76, 77, 51, 314, 171, 306, 115, 229, 237, 53, 236, 170, 24, 41, 45, 249, 104, 116, 155, 109, 153]
*Corynosoma semerme**	BlS	[76, 77, 306]	*Diphyllobothrium phocarum**	PO	[240, 171]	*Anisakis typica*	AO, BlS	[31, 76, 77, 73, 306, 184]	*Opisthorchis tenuicollis**	AO, Arc	[76]
*Corynosoma strumosum**	BlS	[76, 77, 306]	*Diphyllobothrium stemmacephalum*	AO, BlS	[31, 76, 77, 312, 79, 306, 53, 229, 41, 45, 125, 116, 38]	*Phocascaris* sp.*	AO	[41]	*Pholeter gastrophilus*	AO, BlS, BaS	[31, 76, 77, 306, 92, 115, 229, 24, 125, 116, 38, 153]
			*Monorygma grimaldii**	AO	[17, 155]	*Pseudoterranova decipiens**	AO	[76, 32, 306]	*Synthesium mironovi*	PO	[69]
			*Phyllobothrium delphini**	AO	[17, 155]	*Porrocaecum* sp.*	AO	[256]	*Synthesium nipponicum*	PO	[51, 171, 69, 170]
			Tetrabothrius sp.	AO	[41]	*Halocercus invaginatus*	AO, PO, Arc, BlS BaS	[94, 76, 77, 171, 306, 13, 115, 24, 199, 249, 1, 116, 38, 154, 155, 153, 214]			
						Halocercus kirbyi	PO	[190]			
						Halocercus taurica	AO, PO, BlS	[76, 77, 306, 13, 116, 38, 214]			
						Pseudalius inflexus	AO, PO, BlS, BaS	[31, 94, 76, 77, 304, 306, 13, 115, 53, 236, 24, 45, 249, 116, 154, 153]			
						Stenurus minor	AO, PO, BlS, BaS	[31, 76, 77, 171, 306, 13, 115, 237, 53, 236, 24, 45, 249, 116, 38, 154, 155, 153, 214]			
						Torynurus convolutus	AO, PO, BlS BaS	[31, 76, 76, 171, 306, 13, 115, 236, 24, 45, 249, 116, 154, 153]			
						*Hysterothylacium aduncum**	AO	[125]			
						Crassicauda sp.	AO, PO	[69, 170, 116]			

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Phocoena spinipinnis* Burmeister, 1865. Burmeister's porpoise**											
Corynosoma australe[*]	AO, PO	[33, 287]	*Monorygma grimaldii*[*]	§	[17]	*Anisakis simplex* s.l.[*]	AO, PO	[21, 33, 287]	*Braunina cordiformis*	PO	[244]
Corynosoma cetaceum	AO, PO	[33, 57, 287]	*Phyllobothrium delphini*[*]	§	[17]	*Anisakis typica*	PO	[244]	*Nasitrema globicephalae*	PO	[244]
						Pseudalius inflexus	AO, PO	[50, 57, 244, 280]	*Pholeter gastrophilus*	AO, PO	[57, 244, 33]
						Pseudoterranova sp.	PO	[287]	*Synthesium tursionis*	PO	[287, 244, 280]
						Stenurus australis[*]	AO, PO	[190, 201, 280, 320]			
						Stenurus minor	AO, PO	[57, 280]			
***Phocoenoides dalli* (True, 1885). Dall's porpoise**											
-	-	-	*Monorygma grimaldii*[*]	§	[17]	*Anisakis simplex* s.l.[*]	PO	[138, 61, 171, 115, 132, 170]	*Campula oblonga*	PO	[309, 51, 314, 61, 193, 306, 71, 56, 132, 170]
			Phyllobothrium delphini[*]	PO	[17, 300]	*Crassicauda boopis*	PO	[279]	*Nasitrema dalli*	PO	[309, 314, 61, 306, 71, 132]
			Tetrabothrius forsteri	PO	[306, 311]	*Halocercus dalli*[*]	PO	[310, 306, 56, 132]	*Nasitrema delphini*	PO	[62]
						Halocercus kirbyi	PO	[76, 171, 306, 115]	*Nasitrema globicephalae*	PO	[62]
						Halocercus pingi	PO	[190]	*Synthesium nipponicum*	PO	[309, 314, 306]
						Placentonema sp.	§	[132]			
						Stenurus minor	PO	[136, 193, 171, 62, 71, 132]			
						Stenurus truei[*]	PO	[190]			
						Stenurus yamagutii[*]	PO	[190]			
						Torynurus dalli[*]	PO	[306, 13, 71, 132, 170]			

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Family *Physeteridae*											
***Physeter macrocephalus* Linnaeus, 1758. Sperm whale**											
Bolbosoma brevicolle	AO	[31, 76, 84, 306]	*Diphyllobothrium* sp.	Ant	[70]	*Anisakis pegreffii*	§	[182]	*Brachycladium curilense*	PO	[76, 84, 306, 314,]
Bolbosoma capitatum	AO, MS, Ant	[31, 70, 76, 84, 306]	*Diplogonoporus* sp.	§	[84]	*Anisakis physeteris*	AO, PO, Ant, MS, SA	[31, 33, 47, 70, 73, 76 115, 138, 147, 170, 171, 172, 181, 200, 201, 306]			
Bolbosoma tuberculata	AO, IO	[266, 273]	*Hexagonoporus calyptocephalus*[*]	PO	[86, 306]	*Anisakis simplex* s.l.[*]	AO, PO, SA	[73, 76, 115, 138, 170, 171, 172, 306]			
Bolbosoma turbinella	PO	[84, 306]	*Hexagonoporus giganticus*[*]	Ant	[264, 273, 306]	*Crassicauda anthonyi*	AO	[229]			
Corynosoma bullosum[*]	AO, Ant	[33, 70]	*Hexagonoporus physeteris*[*]	PO	[76, 84, 306]	*Placentonema gigantissimum*[*]	AO, PO	[70, 76, 84, 115, 245, 263, 170, 171, 306]			
Corynosoma curilense[*]	PO	[76, 84, 306]	*Monorygma grimaldii*[*]	PO, Ant	[70, 115, 269, 306]	*Stenurus ovatus*	AO	[229]			
Corynosoma strumosum[*]	PO	[84, 305]	*Multiductus physeteris*[*]	PO	[115]						
			Phyllobothrium delphini	AO, PO, Ant, MS, SA	[31, 46, 70, 76, 84, 115, 116, 170, 171, 190, 227, 243, 261, 268, 302, 305]						
			Priapocephalus grandis	AO, Ant	[52, 70, 84, 115, 177, 229, 243, 306]						
			Scolex pleuronectis[*]	PO	[9, 177, 306]						
			Tentacularia sp.[*]	§	[273]						
			Tetrabothrius affinis	AO, PO, Ant	[84, 115, 177, 306]						
			Tetrabothrius curilensis[*]	Ant	[70, 76, 277, 306]						
			Tetrabothrius wilsoni	Ant	[54, 70, 243]						
			Trigonocotyle sp.	PO	[76]						

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Family *Platanistidae*											
***Platanista gangetica* (Lebeck, 1801). South Asian river dolphin**											
-	-	-	-	-	-	*Contracaecum lobulatum*[*]	GR	[31, 65, 76, 216]	*Campula oblonga*	GR	[31, 65, 76]
Family *Pontoporiidae*											
***Pontoporia blainvillei* (Gervais and d'Orbigny, 1844). Franciscana**											
Bolbosoma turbinella	AO	[10]	*Monorygma grimaldii*[*]	AO	[17]	*Anisakis simplex* s.l.[*]	AO	[18, 21]	*Pholeter gastrophilus*	AO	[18]
Corynosoma australe[*]	AO	[10, 20]	*Phyllobothrium delphini*[*]	AO	[17]	*Anisakis typica*	AO	[10, 22, 139, 192]	*Synthesium pontoporiae*[*]	AO	[10, 18, 35, 173, 174, 175, 228]
Corynosoma cetaceum	AO	[18, 10, 19, 20, 139, 254]				*Procamallanus* sp.[*]	AO	[22]			
Family Ziphiidae											
***Berardius bairdii* Stejneger, 1883. Baird's beaked whale**											
-	-	-	*Phyllobothrium delphini*[*]	PO	[171, 245]	*Anisakis simplex* s.l.[*]	PO	[171, 172, 306]	*Oschmarinella sobolevi*	PO	[306]
						Crassicauda giliakiana	PO	[279, 306]			
						Halocercus hyperoodoni[*]	PO	[190]			
***Hyperoodon ampullatus* (Forster, 1770). Northern bottlenose whale**											
Bolbosoma balaenae	AO	[76, 306]	*Strobilocephalus triangularis*	AO	[23, 31, 76, 229, 306]	*Anisakis physeteris*	§	[73, 84]	*Oschmarinella sobolevi*	AO	[76, 260, 314]
Bolbosoma turbinella	AO	[31, 76, 78, 270, 306]				*Anisakis simplex* s.l.[*]	AO	[31, 73, 76]			
						Anisakis typica	§	[137]			
						Crassicauda giliakiana	§	[76]			
***Hyperoodon planifrons* Flower, 1882. Southern bottlenose whale**											
-	-	-	-	-	-	*Crassicauda giliakiana*	Ant	[306]	-	-	-

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Mesoplodon bidens (Sowerby, 1804). Sowerby's beaked whale											
Bolbosoma vasculosum[*]	AO, MS[∞]	[76, 295, 306]	*Phyllobothrium delphini*[*]	AO, MS[∞]	[76, 229, 263, 303, 306]	*Anisakis simplex* s.l.[*]	AO	[31, 73, 76, 115, 306]	-	-	-
			Strobilocephalus triangularis	AO	[76, 229]						
			Tetrabothrius forsteri	MS[∞]	[23, 31, 76, 229, 306]						
[∞]**Single record of the host species in the Mediterranean Sea**											
Mesoplodon bowdoini Andrews, 1908. Andrews' beaked whale											
-	-	-	-	-	-	*Anisakis nascettii*	PO	[185]	-	-	-
						Anisakis ziphidarum	PO	[185]			
Mesoplodon densirostris (Blainville, 1817). Blainville's beaked whale											
Bolbosoma vasculosum[*]	AO	[55, 318]	*Phyllobothrium delphini*[*]	AO	[318]	*Anisakis simplex* s.l.[*]	AO	[116, 318]			
			Tetrabothrius sp.	AO	[116]	*Anisakis ziphidarum*	SA	[181]			
						Crassicauda sp.	PO	[279]			
Mesoplodon europaeus (Gervais, 1855). Gervais' beaked whale											
-	-	-	*Monorygma grimaldii*[*]	§	[17]	*Anisakis simplex* s.s.	AO	[48]	*Brachycladium parvulum*[*]	AO	[108]
			Phyllobothrium delphini[*]	§	[17]	*Anisakis ziphidarum*	AO	[48, 181]	*Cetitrema meadi*[*]	AO	[64]
Mesoplodon grayi von Haast, 876. Gray's beaked whale											
-	-	-	*Phyllobothrium delphini*[*]	AO	[33]	*Anisakis nascettii*	PO	[185]	-	-	-
			Scolex pleuronectis[*]	AO	[33]	*Anisakis ziphidarum*	§	[181]			
Mesoplodon hectori (Gray, 1871). Hector's beaked whale											
Bolbosoma sp.	AO	[46]	*Tetrabothrius hobergi*[*]	AO	[205]	*Anisakis* sp.	AO	[46]	*Braunina cordiformis*	AO	[46]
Mesoplodon layardii (Gray, 1865). Layard's beaked whale											
-	-	-	-	-	-	*Anisakis nascettii*	PO	[185]	-	-	-
						Anisakis berlandi	SA	[182]			
						Anisakis ziphidarum	SA	[185]			
Mesoplodon mirus True, 1913. True's beaked whale											
-	-	-	*Monorygma grimaldii*[*]	§	[17]	*Anisakis nascettii*	SA	[185]	-	-	-
			Phyllobothrium delphini[*]	§	[17]	*Anisakis simplex* s.l.[*]	SA	[115]			
						Crassicauda anthonyi	AO	[49, 306]			
Mesoplodon stejnegeri True, 1885. Stejneger's beaked whale											
-	-	-	*Tetrabothrius forsteri*	Arc	[259]	*Crassicauda* sp.	PO	[279]	*Oschmarinella macrorchis*	PO	[87]

Table 2. (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
***Ziphius cavirostris* G. Cuvier, 1823. Cuvier's beaked whale**											
Bolbosoma vasculosum[*]	MS	[98]	*Monorygma grimaldii*[*]	AO	[55]	*Anisakis pegreffii*	MS	[39, 182]	*Oschmarinella albamarina*	PO	[207]
			Phyllobothrium delphini[*]	AO	[55, 194]	*Anisakis physeteris*	MS	[39, 162]			
			Scolex pleuronectis[*]	MS	[9]	*Anisakis simplex* s.l.[*]	PO	[147]			
			Tetrabothrius sp.	MS	[98]	*Anisakis typica*	MS	[229]			
						Anisakis ziphidarum	AO, MS, SA	[55, 181]			
						Crassicauda anthonyi	AO, PO, MS	[49, 55, 90, 194, 207, 306]			
						Crassicauda boopis	MS	[31, 162]			
						Crassicauda crassicauda	AO, MS	[76, 115, 162]			

No helminths reported from the following cetacean species:

Family Balaenidae, North Pacific right whale, *Eubalaena japonica* (Lacépède, 1818).

Family Balaenopteridae, Antarctic minke whale, *Balaenoptera bonaerensis* Burmeister, 1867.

Omura's whale, *Balaenoptera omurai* Wada, Oishi and Yamada, 2003.

Family Delphinidae, Australian snubfin dolphin, *Orcaella heinsohni* Beasley, Robertson and Arnold, 2005.

Atlantic humpback dolphin, *Sousa teuszii* (Kükenthal, 1892).

Indian Ocean humpback dolphin, *Sousa plumbea* (G. Cuvier, 1829).

Australian humpback dolphin, *Sousa sahulensis.*

Family Lipotidae, Baiji, *Lipotes vexillifer* (Miller, 1918).

Family Phocoenidae, Narrow-ridged finless porpoise, *Neophocaena asiaeorientalis* (Pilleri and Gihr, 1972).

Family Ziphiidae, Arnoux' beaked whale, *Berardius arnuxii* Duvernoy, 1851.

Longman's beaked whale, *Indopacetus pacificus* (Longman, 1926).

Hubbs' beaked whale, *Mesoplodon carlhubbsi* Moore, 1963.

Ginkgo-toothed beaked whale, *Mesoplodon ginkgodens* Nishiwaki and Kamiya, 1958.

Deraniyagala's beaked whale, *Mesoplodon hotaula* Deraniyagala, 1963.

Perrin's beaked whale, *Mesoplodon perrini* Dalebout, Mead, Baker, Baker and van Helden, 2002.

Pygmy beaked whale, *Mesoplodon peruvianus* Reyes, Mead and Van Waerebeek, 1991.

Spade-toothed whale, *Mesoplodon traversii* (Gray, 1874).

Shepherd's beaked whale, *Tasmacetus shepherdi* Oliver, 1937.

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Chapter 39

INFLUENZA VIRUSES: A THREAT TO MARINE MAMMALS POPULATIONS

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ABSTRACT

Marine mammals may have an important influence on the marine communities and their conservation has a great impact on balance in the ocean's ecosystems. Marine mammals mortality due to infectious disease and algal biotoxins are increasing worldwide. Morbilliviruses and influenza viruses are two major viral infections that caused several outbreaks with high morbidity and mortality among marine mammals. Influenza viruses are among few zoonotic pathogens known to have caused infections in marine mammals. Direct transmission of influenza A and B viruses from humans to seals and vice versa, and mass die-off of seals due to influenza virus infections in several occasions accentuates their importance for the conservation of marine mammals population.

Keywords: marine mammals, influenza viruses, zoonotic diseases

INTRODUCTION

Marine mammals are an important part of the marine communities and their conservation has a great impact on balance in the ocean's ecosystems. Marine mammals face a wide range of threats from human activities, hunting, climate change, pollution, diseases, and habitat degradation. Marine mammals mortality due to infectious disease and algal biotoxins are increasing worldwide.

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Various disease outbreaks and mass mortality events, some involving hundreds to thousands of animals, affected the marine mammal populations. Mass mortality events due to outbreaks of phocine distemper virus (PDV) infections occurred in European harbour seals (Phoca vitulina) in 1988 with more than 23000 and again in 2002 with more than 30000 dead cases (Härkönen et al., 2006; Kreutzer et al., 2008). Outbreaks of dolphin morbillivirus infections in bottlenose dolphins along the Atlantic coast accounted for 750 dead dolphins in 1987-1988 (Lipscomp et al., 1994; Taubenberger, et al., 1996) and again in 2013-2015 with approximately 2000 dead cases. In the Mediterranean Sea also dolphin morbilliviruses caused striped dolphins die-off in 1990-1992 with more than 1000 found carcasses (Aguilar & Raga, 1993), and again in 2006-2007 with approximately 100 striped dolphins and additional 60 long-finned pilot whales in the Spanish Mediterranean coast (Fernández et al., 2008). Morbilliviruses also killed Baikal seals in Lake Baikal in 1988 (Mamaev et al., 1996) and thousands of Caspian seals in the Caspian Sea in 2000 (Kennedy et al., 2000).

The importance of marine mammals as hosts or carriers of potential zoonotic pathogens such as influenza viruses is not well understood. The fact that influenza viruses are some of the few zoonotic pathogens known to have caused infections in marine mammals and evidence for direct transmission of influenza viruses from seals to humans and vice versa, underlines the importance of investigation and monitoring of influenza viruses in marine mammal populations (Fereidouni et al., 2014).

INFLUENZA OUTBREAKS IN SEAL AND WHALE POPULATIONS

Our knowledge of disease outbreaks in seal populations is restricted to how efficient are reporting systems in the countries that are close to marine mammals communities. Therefore the real rate of infection most probably be higher than we expect. Influenza viruses were partly isolated from marine mammals during outbreaks with clinical symptoms and mortality, and partly just during conventional monitoring studies from apparently healthy animals (Fereidouni et al., 2014). The first reported case of influenza virus isolation from marine mammals dates back to 1975/76. In this study, tissue samples were collected from 72 whales hunted in the South Pacific and influenza viruses were isolated from 13 lung and one liver specimens. Only detailed data on one H1N3 subtype that isolated probably from a striped whale (family Balaenopteridae) was reported (Lvov et al. 1978).

The first reported case of influenza virus induced mortality in marine mammals dates back to December 1979 (Figure 1). Approximately 500 mainly juvenile harbour seals (Phoca vitulina) carcasses were found along the north-eastern coast of the United States. The causal virus was identified as an influenza A virus of the H7N7 subtype (Webster et al. 1981a; Lang et al. 1981). This epizootic infection affected at least 20% of the local seal population and some animals developed severe acute haemorrhagic viral pneumonia (Geraci et al. 1982). The causative virus was antigenically similar to an avian H7N7 virus. However, as its biological properties was more like a mammalian strain, the virus was probably adapted to mammalian hosts during replication in seals (Webster et al. 1981a; Kida et al. 1982; Naeve & Webster, 1983; Callan et al. 1995).

The next reported influenza outbreak occurred in the same location in Massachusetts, from June 1982 to August 1983 (Figure 1). Approximately 60 harbour seals died due to the

infection with an H4N5 influenza A virus. The main pathological findings was pneumonia and the causative virus was isolated from the lungs and brains of dead seals (Hinshaw et al. 1984). This outbreak focused the scientific attention on the role of marine mammals in the ecology and epidemiology of influenza viruses in nature (Webster et al. 1992).

In 1984, two influenza A viruses (H13N2 and H13N9) were isolated simultaneously from the hilar lymph nodes and lungs of a sick long-finned pilot whale (Globicephala melas) near Portland, Maine, USA. In post-mortem examination, pathological findings included a large hilar lymph node and haemorrhagic lungs (Hinshaw et al. 1986). It was speculated that these H13 influenza viruses had probably been related with two mass stranding of long-finned pilot whales (97 whales in October, 23 whales in November 1984) along the New England coast (Hinshaw et al. 1986; Van Bressem et al. 1999; Waltzek et al. 2012).

In 1991, two influenza viruses of the H4N6 subtype were isolated from the lung tissue of two dead harbour seals. Pathological lesions consistent with influenza virus infection, such as acute interstitial and/or haemorrhagic pneumonia and subcutaneous emphysema were observed. There was no indication for an extensive outbreak in that period along the New England coast, and the viruses were found within the framework of an surveillance study (Callan et al. 1995).

Again in September 1991 to April 1992, an increase in the number of stranded seals along Cape Cod was reported. A severe epizootic of viral pneumonia in the seal population was not observed, but pathologic lesions in respiratory system, including acute interstitial pneumonia and subcutaneous emphysema were found in dead seals. For the first time influenza viruses of the H3N3 subtype were isolated from the harbour seals (Callan et al. 1995).

An influenza B virus was isolated in 1999 from a throat swab of a juvenile seal in a rehabilitation centre in the Netherlands. Phylogenetic analysis of the HA gene showed a close relation to viruses which had been circulating in humans in 1995 (Osterhaus et al. 2000). No influenza virus infection-specific clinical symptom was reported in that juvenile seal. A retrospective study of seal serum samples, collected before and after 1995 indicated the introduction of influenza B virus into the seal population in 1995. Less than 2% of sera collected after 1995 tested positive for antibodies to the influenza B virus, which indicated low incidence of infection (Fouchier et al. 2001).

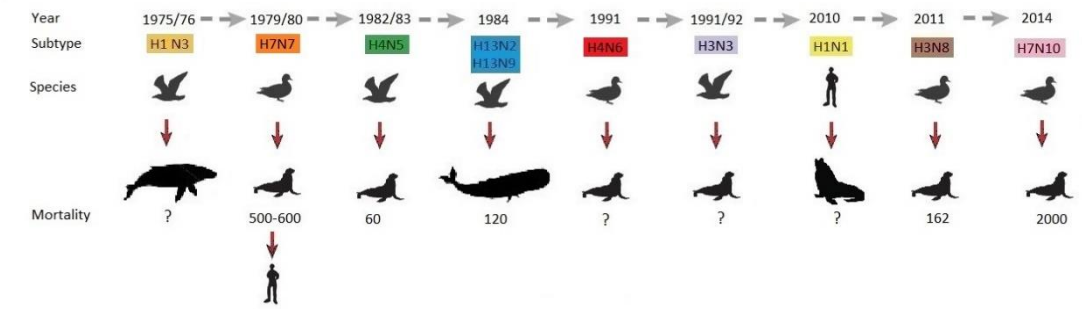

Figure 1. Influenza virus outbreaks/infections reported during 1975-2015. Arrows indicate known transmission routes between each species based on reported cases, phylogenetic analysis and experimental studies.

Following the human pandemic caused by a new H1N1 influenza virus (H1N1pdm09), nasals swabs were collected from 42 female free-ranging Northern elephant seals (Mirounga angustirostris) in a surveillance study along California's central coast in April 2010 (Goldstein et al. 2013). Two swab samples tested positive and the isolated H1N1 viruses showed an homology greater than 99% to influenza viruses isolated from humans in 2009. Serological analyses of serum samples collected between January 2010 and April 2011 indicated a recent introduction of the virus into the local seal population in April 2011. No mortality was seen in that seal population during the period of study (Goldstein et al. 2013).

In September - December 2011 higher mortality of harbour seals compared to previous years was reported from the New England coast. An avian influenza virus of the H3N8 subtype was isolated from several tissues of five out of 162 dead harbour seals. The main pathological findings were pneumonia and ulcerations of the skin and oral mucosa.

The first reported mass mortality of harbour seals in Europe due to influenza virus infections occurred in the Baltic Sea in 2014. From March to October, 425 carcasses were detected at the west coast of Sweden (Zohari et al., 2014), 152 carcasses on the small island of Anholt in Denmark (Krog et al., 2015), and approximately 1,400 carcasses in the north coastal waters of Germany (Bodewes et al., 2015). Avian influenza A virus (H10N7) was detected in the lungs of affected animals in all three occasions with close genetic relatedness. This subtype had not been reported in seals until 2014 and was associated with the highest mortality ever has been reported in seals due to influenza viruses. In addition, preliminary analysis of the haemagglutinin gene (HA) sequence of the causative influenza virus suggested the presence of molecular determinants that indicated mammalian adaptation (Bodewes et al., 2015).

Several serological studies have been carried out since 1978 to estimate the prevalence of influenza virus infections among marine mammals. Different methods such as haemagglutination inhibition (HI), indirect enzyme linked immunosorbent assay (ELISA) and competitive ELISA were used. Each method had its intrinsic sensitivity and specificity, positive predictive values (PPV) and negative predictive value (NPV); however, in most cases these methods were never validated for marine mammal serum samples (Fereidouni et al., 2014). Nevertheless, positive serum samples were found for harbour seals and sea lions (de Boer et al., 1990), harp and hooded seals (Stuen et al., 1994), ringed seal and beluga whales (Austin & Webster, 1993; Nielsen et al., 2001), Caspian seals, common minke whales and Dall's porpoise (Ohshi et al., 2002, 2006), Pacific walrus and bearded seals (Calle et al., 2002, 2008), Kuril harbour seals (Fujii et al., 2007), and fur seals (Blanc et al., 2009). In addition, influenza B antibodies were found in harbour and gray seals (Fouchier et al., 2001; Bodewes et al., 2013).

GENETIC BACKGROUND OF MARINE MAMMALS INFLUENZA VIRUSES

Several influenza viruses have been isolated from marine mammals since the 1970s and sequence data of one to eight genome segments of them were obtained using different sequencing methods. Phylogenetic, genetic and/or antigenic analyses using different methods has been indicated that the majority of marine mammal influenza viruses showed high genetic relatedness with those viruses reported from wild birds.

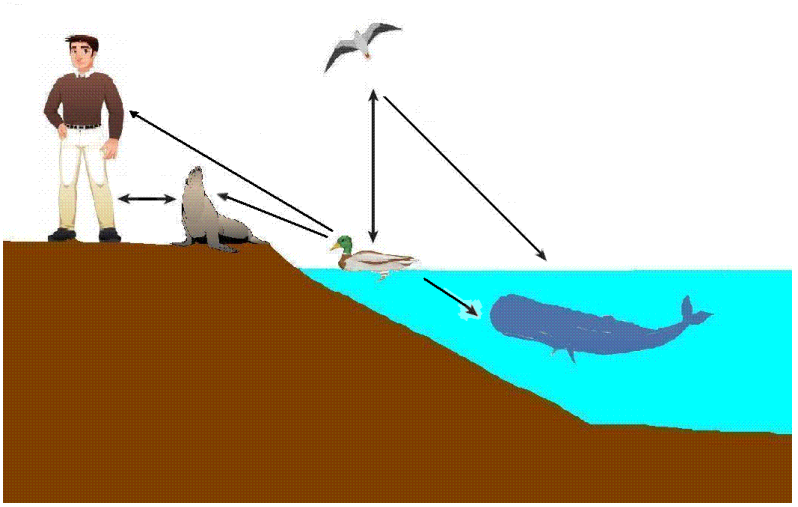

Figure 2. Inter-species transmission of influenza viruses between marine mammals and other species based on genetic relatedness of the isolated influenza viruses. Arrows indicate known transmission routes between each species based on reported cases, phylogenetic analysis and experimental studies (adapted from Fereidouni et al., 2014).

Probably the first phylogenetic analysis for marine mammals influenza viruses was performed in 1982 using the full sequenced haemagglutinin gene of H7N7 virus and its association with avian H7 viruses was proved (Kida et al. 1982; Naeve & Webster, 1983).

Another phylogenetic analysis was performed in 1990, to make a comparison between the nucleoprotein (NP) gene sequence of three influenza viruses, H1N3, H13N2 and H7N7 isolated from stripped whales, long-finned pilot whales and harbour seals, respectively. The results indicated again high genetic relatedness between marine mammal viruses and those reported from wild birds (Mandler et al. 1990).

No sequence of seal H4N5 virus is available in GenBank and only based on the HI assay of the virus and due to the high replication of the virus in avian hosts, it was concluded that the HA was antigenically and biologically similar to avian viruses (Hinshaw et al. 1984). In 1991, when two H4N6 influenza viruses were isolated from harbour seals, based on presence of H4 HA, it was concluded that they should originated from wild bird isolates (Callan et al. 1995).

In 1984, two whale influenza viruses, H13N2 and H13N9, were shown to be closely related to the H13 influenza viruses circulating among seagulls in the USA (Hinshaw et al. 1986). The seal H3N3 influenza isolate was first suspected to be related to seasonal human H3 influenza viruses, but later phylogenetic analysis demonstrated a close relationship to North American avian influenza viruses (Callan et al. 1995). The phylogenetic analyses of all eight segments of H3N8 seal influenza virus demonstrated also the closest relationship to waterfowl influenza viruses (Anthony et al. 2012).

At last, influenza viruses of H10N7 subtype, commonly found in migratory waterfowl, caused highest reported influenza-related mortality among harbour seals in 2014. Phylogenetic analyses showed that the HA of this virus is genetically closely related to Eurasian H10 viruses recently found in migratory ducks in Sweden, Denmark and Georgia (Zohari et al., 2014; Krog et al., 2015; Bodewes et al., 2015).

The 2010 influenza viruses isolated from free-ranging Northern elephant seals were the only influenza A viruses showed an homology to human and not avian viruses. Those viruses showed an homology greater than 99% to the pandemic influenza viruses (H1N1pdm09) isolated from humans in 2009 (Goldstein et al. 2013).

The evidence indicated that introduction of influenza viruses from wild birds and humans into marine mammal populations might have occurred on several occasions independently. High mortality of marine mammals, in several occasions, after transmission of avian influenza viruses to their population pose a great concern regarding the conservation of the population. Avian influenza viruses are known to be circulating at high prevalence in aquatic birds, and there is a continuous potential for transmission of viruses through direct or indirect contact of marine mammals with wild birds or their droppings at hauling-out sites or during feeding on the same food resources, although establishment of infection requires numerous factors (Fereidouni et al., 2014).

MARINE MAMMALS AND PUBLIC HEALTH

Circulation of avian influenza viruses in marine mammals may potentially have implications for public health, although the only reported evidence for transmission of influenza viruses from marine mammals to humans dates back to the 1979 outbreak of influenza in harbour seals with H7N7 subtype, which caused purulent keratoconjunctivitis in humans who handled the dead seals (Webster et al. 1981b). The virus raised concern as a zoonotic threat but it did not spread further. Affected people recovered without complications, and antibodies to the virus were not detectable in the serum samples of infected persons.

On the other hand, isolation of H1N1pdm09 influenza viruses from healthy seals in 2010 provided evidence for crossover transmission of influenza viruses from humans to marine mammals. The isolated viruses replicated in human epithelial respiratory cells not as efficient as human reference strains (Goldstein et al. 2013). This finding may indicating that these isolates adopted to elephant seal and lost potential to infect humans efficiently.

Genetic analyses of the isolated H3N8 viruses from harbour seals in 2011 indicated presence of mutations in the viral PB2 protein that increase transmissibility and virulence of influenza viruses in mammalian (Anthony et al. 2012). However, there is no evidence for transmission of those H3N8 viruses to other mammalian species, including humans.

Serological and virological data have shown that influenza B viruses could have persisted in the seal population for a long time without significant changes, a fact which may be considered as a risk for the re-introduction of the viruses to the human populations. However, at present no evidence is convincing enough to conclude that seals play a role as reservoir species for human influenza A or B viruses (Fereidouni et al., 2014).

CONCLUSION

In summary, interspecies transmission of influenza viruses is an important event in the evolution and ecology of these viruses. Influenza viruses are among the few zoonotic pathogens known to have caused infections in seals and whales. Infection of seals and other

marine mammals with influenza A and B viruses have been reported on several occasions. Many constraints made it difficult to determine the real incidence of influenza virus infections in marine mammals. Nevertheless, data from several outbreaks and also serological results show that this is not a rare event, and probably the real prevalence has been underestimated (Fereidouni et al., 2014). Close genetic relatedness of influenza viruses isolated from marine mammals and wild birds suggests that wild birds are the main source of influenza virus infections of marine mammals (Figure 2). However, our knowledge is yet insufficient to make any general conclusion about transmission routes of the influenza viruses between wild birds and marine mammals or the role of them in ecological persistence of influenza viruses in the nature; therefore more detailed and validated monitoring as well as experimental studies are necessary to clarify the ecological and zoonotic importance of influenza viruses circulating in marine mammals.

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Chapter 40

ECOLOGICAL INTERDEPENDENCE IN MARINE HABITATS: BIO-ECONOMIC MANAGEMENT OF A SPANISH MIXED FISHERY

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ABSTRACT

Ecological interactions amongst fish stocks can play a dominant role in how marine ecosystems are structured, and they can have a significant impact on the populations' growth dynamics. The analytical framework for proposing management guidelines has been developed based on bio-economic models that only account for a single species, both in terms of biological and well as economic aspects, and this diminishes the importance of interactions among the various marine community components. Bearing in mind the implications of the ecological dependencies that are created in multi-species fisheries, that bio-economic model can be extended. A better understanding of trophic relationships among fish stocks enables the development of management models for multi-species fisheries—that is, for most European fisheries. This chapter examines a fishery encompassing two of the Spanish fleet's target species that exhibit a significant ecological predatory interdependence: the blue whiting and the southern stock of European hake. Such interactions should be considered when establishing the Total Allowable Catch (TAC) because the catch level for one species will alter populations of the other, thereby affecting long-run sustainability of commercial fishing and of the marine environment. Within this fishery, both the catch and the spawning biomass for each species are trending downward. The trophic interaction between predator (hake) and prey (blue whiting) is included into the management problem of jointly determining fishing quotas for each species. The estimations indicate that increases in blue whiting biomass (prey) have generated a positive effect on hake biomass along that period 1988-2014, it is represented by a plus sign in the parameter for interdependence in the hake growth function. However, increases in hake biomass (predator) lead to a reduction in

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blue whiting biomass and this is represented by a minus sign in the parameter for interdependence in the blue whiting growth function. These results are consistent with a relationship of interdependence predator-prey. Incorporating this interaction into stock dynamics for both species and applying optimum control theory, we obtain the optimal spawning stock biomass values for both species and the corresponding catch values. The results show catch levels are lower than prevailing EU-determined levels for both species. In addition, as the social discount rate increases and, therefore society's preferences in the near future, the biomass of both species decreases, while catches or potential landings increase.

Keywords: mixed fisheries management, predator-prey model, European fisheries, Southern hake, blue whiting

INTRODUCTION

The effects of fishing are reflected not only in the target species but also in the entire ecosystem that inhabit. There are scientific evidences that it is occurring in the marine ecosystems and this imposes to find alternative approaches to the current fisheries management scheme, so it has been expressed the need that fisheries research and management to move towards an ecosystem-based approach [1]. The introduction of ecological considerations in fisheries management looks like this as a strategy to ensure the sustainability of fisheries and marine ecosystems. More and more scientists are recommending multi-species approaches to fishery management, due to the complex series of interactions between a fishery's different species [1, 2]. And in practice, fisheries are exploited as a multi-species resource [3]. Biological interactions amongst fish stocks can play a dominant role in how marine ecosystems are structured, and they can have a significant impact on the fish populations' dynamics [2, 4]. More specifically, predation and cannibalism are sources of mortality that can regulate a population's size and recruiting [2, 5-7]. Therefore, fishery management can largely benefit from better comprehension of the role of biological interactions—including trophic relationships—in the resource's dynamics [8-26].

Within the context of ecosystem management, the multispecies approach occupies an important place in fisheries management, since biological species interactions regulate the recruitment and the size of the populations of the ecosystem. The study of trophic relationships as sources of mortality may improve the understanding of the population dynamics and the management of marine resources. Fish stock assessment methods have evolved from monospecific models, studying the population of a single species whose dynamics is affected only by its own biomass and the effect of fishing on the same, towards multispecies models. The multispecies models take into account ecological or technological interactions to optimize yields form the ecosystem as a whole. The multispecies models are in many cases extensions of monospecific models, as it is the case of the excess production models, aggregate production models, multispecies per recruit models, virtual population analysis model, and food and trophic network models. Ecological interdependence occurs when there is a certain relationship between two exploited stocks [16, 17] or between two different population segments of the same stock. From there, we can go on to define two types of ecological interactions: competition and predation. Competition is defined according to the negative effects that one organism exerts over another by consuming or interfering with

its access to a resource that has a limited availability (supply) [18]. This interaction leads to a reduction in the survival, growth, and/or reproduction of competitors, particularly those that, hierarchically speaking, are subordinate [19, 20]. And predation is the consumption of one organism or species (prey) by another organism/species (predator) through carnivory, herbivory or omnivory [20]. On other hand, technological interaction occurs when two or more fishing gears are operating on the same population of fish.

Multispecies bioeconomic models arise from the extension of Gordon-Schaefer model for a single target stock, incorporating logistic equations predator-prey. Predator-prey models constitute a particular case within the multispecies models. They are based on the equations of Lotka-Volterra, describing the trophic interactions between the predator and the prey. The predator-prey basic model assumes exponential or Malthusian growth for the population of prey in absence of predators. This model incorporates the intrinsic growth rates of the predator and the prey and the carrying capacity of the ecosystem to each of the two species. The predator-prey model with capture poses a situation of commercial exploitation of the species and takes into account the discount rate of the fishery. This model aims to maximize the present value of the net benefit from the exploitation of both species: predator and prey.

This chapter deals with the management of a multispecies fishery (mixed fishery) with two target stocks that exhibit significant trophic interaction (ecological interdependence) as well as predation: the southern stock of European hake (*Merluccius merluccius*) and the blue whiting stock (*Micromesistius poutassou*). At this North Atlantic fishery, Southern hake is the predator and blue whiting the prey.

THE EUROPEAN HAKE AND BLUE WHITING MIXED FISHERY

The European hake and blue whiting are two target species for the Spanish longline and trawl fleet in Iberian Atlantic waters, although the latter is also used for horse mackerel, Atlantic mackerel, and octopus. Both species are primarily distributed across the continental shelf, where they spawn during the winter months.

European hake is widely distributed throughout the north-eastern Atlantic Ocean [27]. It is a demersal benthopelagic species that can reach depths that vary from 30 to 1000 meters, although they generally dwell at depths ranging between 70 to 370 meters. They form schools that stick close to the coastline in the summer and keep a greater distance during the winter. They reproduce at depths of 100 to 300 meters. Egg-laying season runs from January to May in the Bay of Biscay. Juveniles live in the muddy depths until about three years of age, at which point they move closer to the coast. Adults can typically be found on the continental slope. The European hake is a predator at the top of the northeast Atlantic demersal trophic pyramid. It primarily feeds on other species of fish like the anchovies (*Engraulis encrasicholus*), sardines (*Sardina pilchardus*), and blue whiting (*Micromesistius poutassou*). According to the organization that analyses the fish stocks in the Northeast Atlantic grounds (ICES), there are two stock units for this species: the southern stock, in zones VIIIc and IXa, and the northern stock, in the remaining NE Atlantic zones.

Table 1. Hake biomass and landings in in ICES zones VIIIc and IXa. 1988-2014

	Spanish landings (tons)	Total landings (tons)	SSB (tons)
1988	10600	16700	27035
1989	9200	13800	19905
1990	9800	13200	16287
1991	8900	12800	16462
1992	8840	13800	15527
1993	7840	11500	12775
1994	6800	9900	8908
1995	8600	12200	7104
1996	7000	9700	8552
1997	6100	8500	6557
1998	5000	7700	5812
1999	4000	7200	7545
2000	4700	7900	8823
2001	4400	7580	8973
2002	3900	6700	9379
2003	4700	6740	9075
2004	4900	6940	8962
2005	6200	8300	9286
2006	8700	10800	10816
2007	12800	14930	12786
2008	14600	16770	12695
2009	16900	19240	13830
2010	13000	15740	12940
2011	6500	17062	15229
2012	5800	14573	14756
2013	7200	13231	14036
2014	7300	12011	18836

Source: Authors compilation from International Council for Exploration of the Sea (ICES) [27].

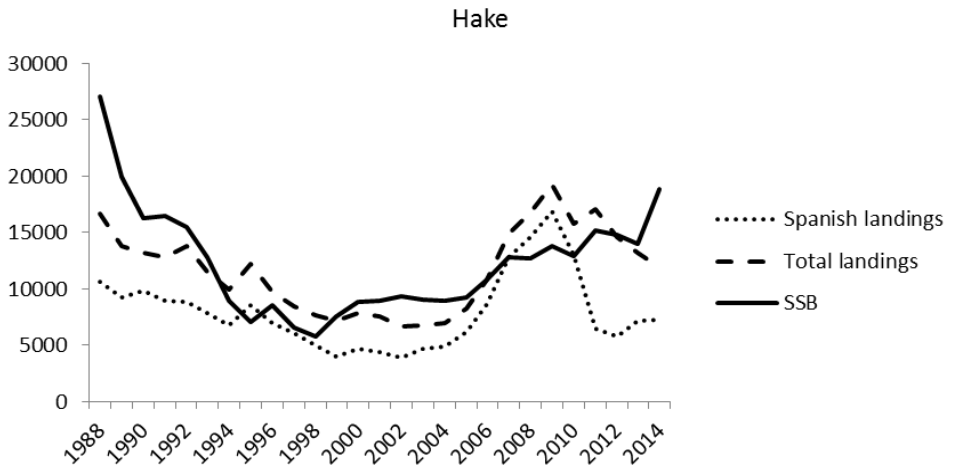
Blue whiting is a demersal species from the gadidae family, and it can be found throughout the northern Atlantic [28]. Its habitat is the ocean, and as a benthopelagic species, it lives mainly along the continental shelf and slope, distributed vertically at depths of between 150 and 1000 meters, though it is most commonly found at 300-400 meters below the surface. Blue whiting spawns between February and June and reaches first maturity at three years of age. Growth is rapid, and females are typically larger than males. The blue whiting's diet consists primarily of various types of crustaceans.

A number of studies have confirmed the ecological interdependence of the hake and blue whiting populations in Iberian waters analyzing the diet of the European hake in the Cantabrian Sea (zone VIIIc), and based on a seasonal analysis of the stomach contents of the fish sampled [29-31]. They highlight the importance of the blue whiting as the hake's primary prey, particularly amongst the adult population, while most juveniles consume cupleoids, at more shallow depths, since at such depths there are fewer blue whiting. Other studies confirm the existence of a significant relationship between the size of the hake and the size of the blue whiting using statistical regression analysis [9, 32]. Likewise, hake is far more dependent upon blue whiting in Iberian fishing grounds (zone VIIIc) than in the northern part of the Bay

of Biscay and the Celtic Sea [2, 9, 32]. Other authors studied the diet of the blue whiting, hake, horse mackerel and Atlantic mackerel in Portuguese waters (Zone IXa) and analyzed their stomach contents [8], and they concluded that in terms of percentage, occurrence, and weight, the blue whiting is the hake's most important prey.

Hake landings and biomass in ICES zones VIIIc and IXa are shown in Table 1 for period 1988-2014. These variables have changed over the past 25 years in zones VIIIc and IXa, as Figure 1 demonstrates. The landings, in general, exhibit a downward trend throughout most of this period, both in terms of the total catch for the industry overall as well as the Spanish fleet. However, beginning in 2007 there was significant recovery, though the numbers once again declined at the end of the period. Spawning stock biomass (SSB) also exhibited a downward trend up until 2006, at which point a notable recovery was made. Note that between 1994 and 1998, the landings surpassed the reproductive biomass; this scenario surfaced once again between 2007 and 2010.

As a result of the elevated hake landings, the species has been outside biosafety level limits since the 1990s [27]. This situation has created an incentive for implementing a recovery plan for the species aimed at recovering the stock and bringing it back within the established biosafety level limits, that is 35,000 tons of reproductive stock biomass (European Council-EC Regulation 2166/2005). According to that plan, in 2006 the European Commission (EC) applied an annual reduction of 10% to the total allowable catch (TAC) and fishing effort for the fishing fleets (measured as the sum of the engine power in kilowatt and the number of days fishing in the area). Over the last few years, the positive effects of this plan have started to become noticeable, to such an extent that the EC began increasing the TAC for this species in 2011.



Source: Authors compilation from Table 1.

Figure 1. Hake landings (tons) and estimated SSB (tons) in ICES zones VIIIc and IXa. 1988-2014.

As for the blue whiting, Table 2 shows the biomass and landings for this species in ICES zones VIIIc and IXa for period 1988-2014. The evolution of these variables is showed more clearly in Figure 2. Spanish landings in zones VIIIc and IXa have stayed relatively stable, at around twenty thousand tons since 2010, at which time a considerable decline was noted, due to the decreased TAC established for this species in 2010. The SSB demonstrates a sustained

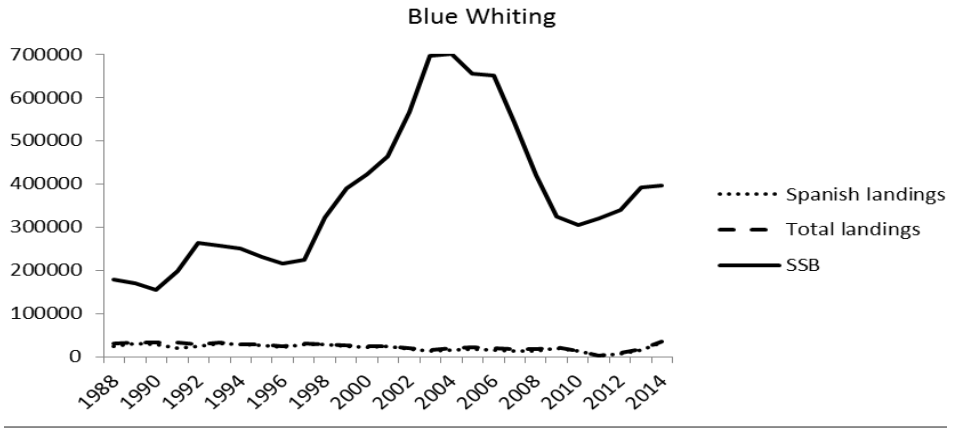
increase until the end of the 1990s, where a significant decrease is observed. Nevertheless, this variable shows signs of recovery in the last few years due to a drop in catches over the past decade, in combination with an increase in recruitment since 2009. It is estimated that in 2013, the SSB was already above the precautionary limits [28]. This has allowed European Council agreed a TAC higher in 2014 and 2015 than in previous years.

Table 2. Blue whiting biomass and landings in in ICES zones VIIIc and IXa. 1988-2014

	Spanish landings (tons)	Total landings (tons)	SSB (tons)
1988	24847	30826	177889
1989	30108	33665	170549
1990	29490	32354	154157
1991	19180	31993	197768
1992	23794	28722	264631
1993	31020	32256	257187
1994	28118	29468	249801
1995	25379	27664	231837
1996	21538	25099	216611
1997	27683	30122	224706
1998	27490	29390	322130
1999	23777	26402	390036
2000	22622	24654	422364
2001	23218	24964	463867
2002	17506	19165	565858
2003	13825	16476	697320
2004	15612	19549	700133
2005	17643	22833	655494
2006	15173	20496	649828
2007	13557	17454	541748
2008	14342	18562	420634
2009	20637	22680	324617
2010	12891	14373	304926
2011	2416	3019	321000
2012	6726	8681	339600
2013	15274	17330	391800
2014	32065	34215	396500

Source: Authors compilation from International Council for Exploration of the Sea (ICES) [28].

The Spanish fishing fleet has also exhibited a downward trend over the past 25 years, going from 279 ships in 1988 to 180 in 2013 [28, 33]. As far as economic data for the fishing grounds is concerned, Table 1 shows a standard ship's average revenue and costs between 2010 and 2014. The largest segment of the costs corresponds to personnel expenses (48% of the ship's average revenue), while the gross cash flow is positive and accounts for approximately 18% of the total revenue.



Source: Authors compilation from Table 2.

Figure 2. Blue whiting landings (tons) in ICES zones VIIIc and IXa and estimated SSB (10 tons). 1988–2014.

Table 3. Average technical characteristics and economic figures for Spanish fishery vessels

Variable	Average 2010–2014
Number of vessels	180
Crew	11
Length (m)	28.4
Power (kW)	466.29
Gross tonnage (GT)	224.2
Income (€)	514,414.17
Crew share (€)	248,101.95
Other vessel costs (€)	176,186.85
Gross cash flow / mixed incomes (€)	90,125.36
Landings of hake per vessel (tons) [a]	19.95
Landings of blue whiting per vessel (tons) [a]	83.02
Unit price for hake landings (€/kg) [a]	4.11
Unit price for blue whiting landings (€/kg) [a]	1.87
Unit cost for hake landings (€/kg) [a]	2.87
Unit cost for blue whiting landings (€/kg) [a]	1.06

[a] Figures estimated from the landings of the fleet in the port of Vigo.
Notes: Monetary values in €2014. kW = kilowatts; m = meters.
Source: Own compilation from [33, 38, 39].

Lastly, Table 3 also shows the technical characteristics and the main economic data for this Spanish fleet and, in particular, the unit costs and prices per landed tons for each species. Data are shown as an average for the period 2010-2014. Since we do not know the composition of the catches for the entire Spanish fleet in this fishery, the costs have been determined based on a representative sample. More specifically, the unit costs have been estimated using the weight that corresponds to each species (13.5% hake and 20.8% blue whiting) in the landings of vessels based in the Port of Vigo in 2010-2014 (these ships represent 37.8% of the total of the fishery's Spanish fleet), bearing in mind the average

tonnage of each species that these ships landed. Additionally, the unit prices were estimated based on prices obtained in the Vigo market for each species.

AN APPLIED MANAGEMENT MODEL

The ecological interdependence between hake and blue whiting in this fishery, which in this case is a predatory relationship, influences the marine ecosystem structure and trophic chain. Increasing or decreasing the TAC for one of the two species will impact this structure and its sustainability in the long run and, ultimately, the sustainability of the industry in this multi-species fishery. Therefore, it is important to take a simultaneous decision regarding catch levels for both stocks, based on their ecological relationship.

To that end, based on [34], we propose the following net growth functions for each species, taking into account the ecological relationships between hake and blue whiting:

$$\frac{dX}{dt} = r_X X\left[1 - \frac{X}{K_X}\right] - H_X + \alpha XY = F(X) - H_X \tag{1}$$

$$\frac{dY}{dt} = r_Y Y\left[1 - \frac{Y}{K_Y}\right] - H_Y + \beta XY = G(Y) - H_Y \tag{2}$$

where X stands for hake spawning biomass; Y is for blue whiting spawning biomass; r and K are biological and ecological parameters and denote the intrinsic growth rate and the average load capacity corresponding to each species, respectively; H is for catches (or landings); and α and β denote the co-efficient for ecological interaction between hake and blue whiting, respectively. According to the traditional notation in the bio-economic literature on fisheries, the function of net fishery benefits at moment t is determined by the sum of the net benefits generated by the catch for both species for that moment t (most commonly t is considered as a year). Formally, it is defined as the following expression [35]:

$$\pi\,(t) = (p_X - c_X)H_X + (p_Y - c_Y)H_Y \tag{3}$$

And where p and c stand for the catch unit price and cost of the corresponding species, both parameters are usually assumed constant along the period of study. The problem that the manager or regulator must solve is setting catch levels for each species that will yield greater potential benefits in the long term, given the biological relationships between both species and their net growth limits. Formally, this is expressed as follows [35, 36]:

$$max_{H_X, H_Y} \int_0^{\infty} [(p_X - c_X)H_X + (p_Y - c_Y)H_Y]\, e^{-\delta t} dt \tag{4}$$

$$s.t. \quad \frac{dX}{dt} = F(X) - H_X$$

$$\frac{dY}{dt} = G(Y) - H_Y$$

where δ stands for the social discount rate. This parameter collects the preferences of the society (or country) by the time and the conservation of the living aquatic resources: the higher this parameter, the greater preference for the present moment and greater exploitation of marine resources; and the lower the discount rate, more preference for the future and sustainability of marine biological resources. The yield of government bonds to long-term (ten years) is often used as the discount rate in bio-economic models.

In order to apply the theoretical model to the case study (hake and blue whiting mixed Spanish fishery), we need to know the value of the parameters of the net growth functions in addition to parameters shown in Table 3 (unit price and cost for catches of both species). Econometric regressions were calculated based on the SSB and catch data for the 1988-2014 period gathered by ICES [27, 28] and shown in Tables 1 and 2. The results of these regressions can be seen in Table 4. These estimations indicate that increases in blue whiting biomass (prey) have generated a positive effect on hake biomass along that period 1988-2014 (a plus sign in the parameter for α interdependence in the hake growth function). However, increases in hake biomass (predator) lead to a reduction in blue whiting biomass (a minus sign in the parameter β for interdependence in the blue whiting growth function). These results are consistent with a relationship of interdependence predator-prey; and they are, in general, in line with results from other studies that have also showed this relation analyzing the stomach contents of the fish sampled [2, 8, 9, 29-32].

By adding the value of the parameters shown in Tables 3 and 4 to the above management problem and applying the theory of optimal control [37], we can obtain the optimal SSB values for both species and the corresponding catch values. The results for different discount rate values are shown in Table 5.

As the social discount rate increases—and, therefore, society's preferences in the near future—the biomass of both species decreases, while catches (or potential landings) increase. In 2015, the TAC was set at 13,826 tons for hake and 1,260,000 tons for blue whiting by European Council. These fishing opportunities are slightly higher than they would be if catches for both species were estimated jointly by incorporating their trophic interaction (see Table 5).

Table 4. Statistical results for net growth function of hake and blue whiting

Coefficient	Hake (X)	Blue whiting (Y)
r	1.0983 *** (0.0000)	1.75482 *** (0.0000)
r/K	−3.42881e-0.5 ** (0.0077)	−3.15675e-0.7 *(0.0350)
α	1.9879e-0.7 * (0.0292)	—
β	—	−1.42684e-0.5 *(0.0279)
R^2	0.968498	0.957652

Notes: p-values are reported in parentheses. Coefficient estimates are statistically significant at ***1%, ** 5% or *10% level.

Table 5. Estimated tons of spawning stock biomass (SSB) and total allowable catch (TAC) for different discount rates

Discount rate	SSB		TAC	
	Hake	Blue whiting	Hake	Blue whiting
3%	40499	3016874	10915	670808
4%	38951	2834582	11213	856307
5%	37392	2636035	11344	1020481
6%	35954	2459279	11467	1139988

CONCLUSION

The rapid growth of the world population is generating a strong pressure on the marine resources as a source of food. Not only overfishing has impact on the target species, but also on the whole of the ecosystem where these species live. It is necessary to use multispecies approaches in fisheries management due to the complex set of interactions among species that occurs in the marine ecosystems. The ecological or biological interactions between species can play a key role in the structuring of the marine communities, and can significantly affect the dynamics of fish populations. Specifically, predation and cannibalism are sources of mortality that can regulate the recruitment and the size of the population. This chapter has developed a multispecies bio-economic model of predator-prey type applied to the mixed fishery for hake and blue whiting in European waters. Both species have a significant trophic interaction, as well as commercial importance for the Spanish fishing fleet which makes them suitable for applying a model of this type.

Hake and blue whiting stocks caught by the Spanish fleet in domestic fishing grounds exhibit significant prey-predator interaction, where hake is the predator and blue whiting the prey. By developing a multi-species bio-economic model for managing both populations in a mixed fishery, instead of contemplating two single-species fisheries, this biological interdependence can be considered as a biological technical externality. When this externality is positive, the optimal stock levels for predator and prey alike are greater than if they were independent populations. More specifically, increases in blue whiting biomass have a positive effect on hake biomass and vice-versa. Similarly, optimal populations of both species increase when the discount rate decreases, while catches would increase if this parameter were increased. The results obtained demonstrate that catches—where the existing trophic interaction is factored into the estimation thereof—are below the TAC levels established by the European Council.

In particular, Spanish fishing boats are currently responsible for 64% and 3% of the TAC established by the European Council for both species in ICES zones VIIIc and IXa. That is, the fleet would bring in approximately 7,000 and 30,000 tons of hake and blue whiting, respectively, if the catches for both stocks were determined in light of their ecological interaction (and while using the 5% discount rate typical of bio-economic studies). However, the TAC established for the Spanish fleet in 2015 was about 9,000 tons of hake and 38,000 tons of blue whiting, or amounts in excess of those that would be allowed had the TAC for both species had been estimated while factoring in their ecological interdependence. Although the 2015 TAC for both species is slightly greater than the fishing opportunities actually

obtained for these species, we remark that excess blue whiting catches could well lead to a reduction in the biomass of this species and could therefore—given the species' trophic relationship—also reduce the long-term biomass of hake, which is one of the Spanish fleet's primary target species.

In short, in mixed fisheries where there is an ecological interdependence between species, when the TAC for a given species is estimated on its own, it could have a greater impact on other target species that are not factored into in the management problem. Therefore, it is a good idea to determine the TAC jointly for the species involved and thus internalize the effect of their trophic interaction. This study has provided a very simple example of two stocks, but it is one that is quite relevant to the Spanish fleet, as hake is one of its primary target species. We must continue to gather information on trophic relationships in order to fine tune EU fishery policy recommendations and generate the least impact possible on marine ecosystems, thereby helping to ensure the conservation and sustainability of marine biodiversity as well as sustainability of the fishing activity.

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Publications in the Last Three Years:

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Chapter 41

MARINE SPONGE COMPOUNDS WITH ANTI-INFLAMMATORY ACTIVITY IN 2012–2016; AND THEIR MECHANISM OF ACTION

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ABSTRACT

This chapter presents an overview of the development and study of marine sponge's bioactive compounds for anti-inflammatory activity for last four years i.e., 2012-2016. Marine pharmacology during 2009–2011 remained a global enterprise contributing to the preclinical pharmacology of 262 marine compounds which are in pharmaceutical pipeline. There is no updated review for the studies done on marine sponge's compounds showing anti-inflammatory activity after 2011. This chapter is a sincere effort to present a systematic review of the preclinical pharmacology of marine sponge and its associated microbes/symbionts compounds for potential activity in inflammation.

Keywords: porifera, inflammation, inflammatory mediators, IL-1, IL-6, TNF-α, PGE2

1. INTRODUCTION

An inflammatory response is a natural defensive mechanism triggered by body tissues on being damaged by invading pathogens, physical stress, injury, persistent foreign bodies and by autoimmune reactions. Depending on the stimuli and the causative agents, it can be short or long lasting. Chronic inflammation involves the release of a number of mediators such as monocytes, macrophages, lymphocytes, plasma cells and fibroblasts that are not prominent in the acute response (Beg et al., 2011). The defence elements of body is found majorly in blood

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and during inflammation these cells and chemicals leave the blood and enter the tissue around the injured or infected site to protect and fight damage. Any type of infection caused by a bacterium, virus or fungus, commonly causes inflammation; while inflammation is the body's own response to it. It is part of many other common acute and chronic diseases. The therapeutic strategy for the treatment of patients with inflammation has been first, the relief of symptoms and the maintenance of function; and second the slowing or arrest of the tissue-damaging process. Nonsteroidal anti-inflammatory drugs (NSAIDs) relieves pain for significant periods, nonopioid analgesics are appropriate for the treatment of both acute and chronic inflammatory conditions. The glucocorticoids also have powerful anti-inflammatory effect but their toxicity limits their use mostly in cases of rheumatoid arthritis. The identified targets for inflammation are the pro-inflammatory mediators cyclo-oxygenase 1 and 2 (COX-1/-2), mitogen-activated protein kinases (MAPKs), janus protein tyrosine kinases (JAKs), nuclear transcription factor (NF-κB) and signal transducers and activators of transcription (STAT) which all directly or indirectly lead to the production of a vast number of pro-inflammatory cytokines and regulatory proteins such as IL-1/6, TNF-α, MIF, IFN-γ, MMPs (Kulkarni et al., 2006).

The role of natural products as therapy in inflammation has been recognized since ancient ages, and major progress has been done using combinatorial chemistry. Natural products with anti-inflammatory activity have been traditionally used for inflammatory conditions such as fevers, pain, migraine and arthritis (Yuan et al., 2006). Many such natural products and their derivatives with anti-inflammatory activity have been studied in detail like curcumin, parthenolide, resveratrol, cucurbitacins, 1, 8-cineole, salicylate, epigallocatechin-3-gallate, pseudopterosins, quercetin, lyprinol, bromelain, flavonoids, saponins and *Boswellia serrata* gum resin. Recently there is also lot of interest in multi-target drug concept to use the huge array of natural products with privileged scaffolds for developing safe anti-inflammatory multi-target drugs (Koeberle et al., 2014). In investigation of extracts from a marine organisms- sea urchin, *Echinus esculentus* for anti-inflammatory activity, UPLC-ToF-MS and NMR analysis concluded the purified compound with a phosphocholine group with an unknown side chain. A bryozoan, *Eucratea loricata*'s organic extract revealed anti-inflammatory activity in NFκB, TNF-α and IL-1β screening (Do Thuy, 2012). There are many anti-inflammatory natural compounds reported from marine sponge, varying widely in both chemical structure and biological activity. The anti-inflammatory compounds from marine sponge are dominated by terpenoid compounds, especially sesterterpenes (Keyzers and Davis-Coleman, 2005).

In this chapter we review the development of marine sponge's anti-inflammatory compounds for last four years i.e., 2012-2016. A systematic review of the preclinical pharmacology of marine sponge and its associated microbes/ symbionts compounds for potential activity in inflammation is discussed.

2. MARINE SPONGE'S ANTI-INFLAMMATORY ACTIVITY

Twenty four anti-inflammatory compounds were reported during the year 2008-11, which may be classified into diverse structural types: terpenes, non-terpenes, alkaloids and peptides. Marine sponges are the richest source of anti-inflammatory compounds among other marine

invertebrates such as ascidian, soft coral, cnidarians and gorgonian. The anti-inflammatory sponge natural product is dominated by isoprenoid derived metabolites, especially sesterterpenes followed by nitrogenous compounds- alkaloids, peptides etc. (Keyzers and Davis-Coleman, 2005). New treatment approaches for acute and chronic inflammation is investigating novel anti-inflammatory molecules which can target Toll-like receptor (TLR) signaling pathways. Fung et al. (2014) worked on crude marine sponge extracts and identified compound Girolline which targeted TLR5 signalling. It inhibited signaling through both MyD88-dependent and independent TLRs (i.e., TLR2, 3, 4, 5, and 7) and reduced cytokine (IL-6 and IL-8) production in human peripheral blood mononuclear cells and macrophages. In other study, *Geodia cydonium* extract induced a reduction in VEGF (vascular endothelial growth factor) and proinflammatory cytokines (CCL2, CXCL8, CXCL10, IFN-γ, and TNF-α) levels in human breast cancer cell line MCF-7 cells, indicating an anti-inflammatory effect (Costantini et al., 2015). *Spongia officinalis* was investigated for in vivo anti-inflammatory activity using the carrageenan-induced paw edema model in rats. The methanol extract (25, 50 and 100 mg/kg) produced a significant reduction of the edema and the semi-purified fraction F3 from methanol extract exhibited significant activity at the dose of 50 mg/kg, at the third hour after carrageenan injection, with 72.85% reduction in paw volume (Dellai et al., 2012). The methanolic extract of the Red sea marine sponge *Xestospongia testudinaria* prevented carrageenan-induced acute local inflammation in rats. Methanolic extract (100 mg/kg) of red sea sponge *Xestospongia testudinaria* decreased % increase in paw weight measured at 1, 2, 3 and 4 h after carrageenan injection. The extract decreased paw malondialdehyde (MDA) and nitric oxide (NO) and decreased the inflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin-1 β (IL-1β) and IL-6 (El-Shitany et al., 2015). Phorbaketal A, a tricyclic sesterterpenoid was isolated from the marine sponge *Phorbas* sp. It significantly inhibited the production of nitric oxide (NO), by showing suppression in the expression of inducible NO synthase at both the mRNA and protein levels in LPS- induced RAW 264.7 cells. Further, it also reduced production of inflammatory cytokines such as tumor necrosis factor-alpha, interleukin (IL)-1beta, IL-6, and monocyte chemotactic protein-1 (Seo et al., 2015). The ethanol extract of the demosponge *Homaxinella* cf. *balfourensis* showed activity by reducing the release of IL-1β and PGE2 only at the highest, but not cytotoxic concentration of 250μg/mL significantly. The extract of *Isodictya erinacea* inhibited the release of IL-1β and PGE2, although not affecting LTB4in a dose dependent manner. The extracts of the sponge *Isodictya toxophila* and *Mycale* (Oxymycale) *acerata*'s decreased the release of IL-1β, LTB4 and PGE2 release at the lower concentration of 50μg/mL, which was not cytotoxic (Moles et al., 2014). Solomonsterol A, a selective pregnane X receptor (PXR) agonist was isolated from the marine sponge *Theonella swinhoei*. It exerted anti-inflammatory activity along with attenuation of systemic inflammation and immune dysfunction in a mouse model of rheumatoid arthritis. It reduced the degree of joint damage by inhibiting the expression of pro-inflammatory mediators. (Mencarelli et al., 2014). In another study on the anti-inflammatory effect of compound 11-oxoaerothionin isolated from the Verongida sponge *Aplysina fistularis*, a concentration-dependent inhibition of iNOS (inducible nitric oxide synthase) protein, NO^{-}_{2} (nitrite), proinflammatory cytokines and PGE2 (prostaglandin E2) production was observed, when RAW264.7 cells and primary macrophages were preincubated with the compound after stimulation with lipopolysaccharide (Medeiros et al., 2012).

Aeroplysinin-1 is a secondary metabolite isolated from marine sponge *Aplysina* genus with a wide spectrum of bio-activities. It is a chiral, optically active molecule. The enantiomer (-)-aeroplysinin-1 was isolated from *Ianthella ardis* and (+)-aeroplysinin-1 was the first brominated derivative from *Aplysina aerophoba* isolated by Fattorusso & Minale (1972). The dextrorotatory enantiomer has been investigated extensively. It has potent antibiotic effects on gram-positive bacteria and several dinoflagellate microalgae. In preclinical studies, (+)-aeroplysinin-1 have shown promising anti-inflammatory, anti-angiogenic and anti-tumor effects. Due to its versatility, it is of pharmaceutical interest for treatment of different pathologies (Garcia-Vilas et al., 2015). Another marine sponge *Neopetrosia* (order Haploscleridae, family Petrosiidae) whole crude extract and its isolated compounds revealed antimicrobial, anti-fouling, anti-HIV, cytotoxic, anti-tumor, anti-oxidant, anti-protozoal, anti-inflammatory activities. Total extracts of *N. proxima* (100 mg/Kg) and *N. rosariensis* (100 mg/kg) significantly inhibited the paw edema of rats 60% and 72% resp. Dichloromethane and methanol fractions of *N. proxima* reduced myeloperoxidase activity and dichloromethane fraction of *N. rosariensis* significantly inhibited nitric oxide (66%), prostaglandin E2 (30.5%) and tumor necrosis factor alpha production (72%) (Franco et al., 2012; Qaralleh 2016). The crude extract of sponge *Reniera sarai*, also displayed anti-inflammatory activities (Dhinakaran et al., 2014). Red sea sponges *Scalarispongia aqabaensis* and *Callyspongia siphonella* are poorly investigated genera for their compounds and biomedicinal value. Sterols scalaristerol (5α, 8α- dihydroxycholest-6-en-3β-ol) from *Scalarispongia aqabaensis*, and callysterol (ergosta-5, 11-dien-3β-ol) from *Callyspongia siphonella* were isolated for its anti-inflammatory activity in rat-hind paw edema. The results indicated that callysterol has a strong anti-inflammatory activity, which was comparable to cortisone, while compound scalaristerol showed moderate anti-inflammatory activity. Callysterol affected the release of superoxide anion and thromboxane B2 in a concentration-dependent manner. It showed close to 50% inhibition at approximately 10 μM (Youssef et al., 2010).

3. Chemical Structures of Compounds Isolated and Their Mechanism of Action

A wide range of chemical compounds from various marine invertebrates have been investigated for their anti-inflammatory properties (Table 1). Most of them exhibit phospholipase A2 inhibitory activity. They also control nuclear factor-B activation and inflammatory gene expression (Alcaraz & Paya, 2006). Some sesquiterpenes were found to inhibit human neutrophil degranulation, superoxide generation, leukotriene B4 production (variabilin), TPA (12-o-tetradecanoylphorbol-13-acetate)-induced ear oedema (variabilin, bolinaquinone, topically) as well as carrageenan induced paw oedema (variabilin, petrosaspongiolide M and bolinaquinone, p.o.) in mice (Giannini et al., 2001; Haefner 2003).

Table 1. Compounds isolated from marine sponge and fungus and their mechanism of action

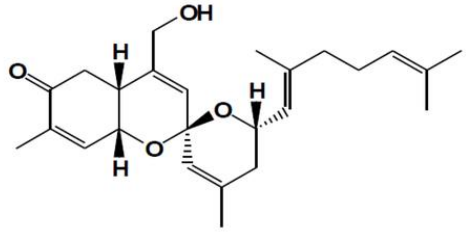
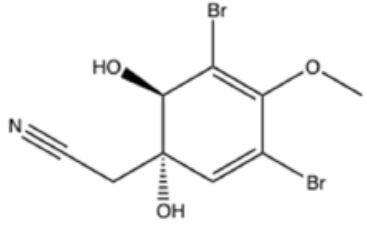
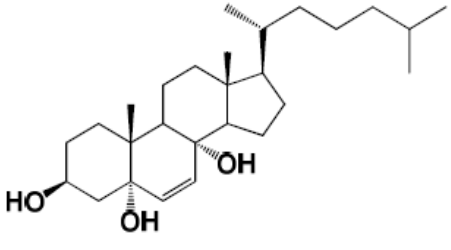
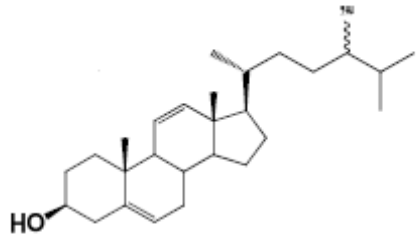
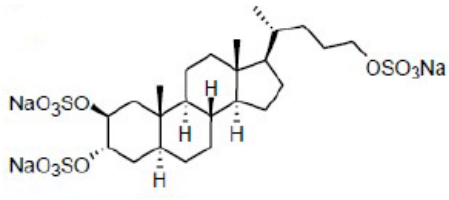
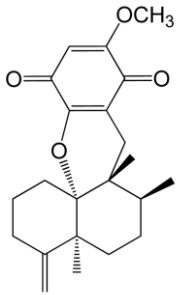
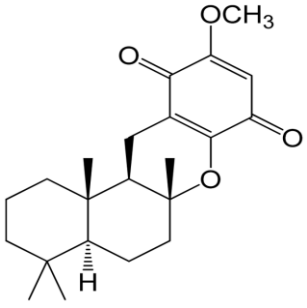
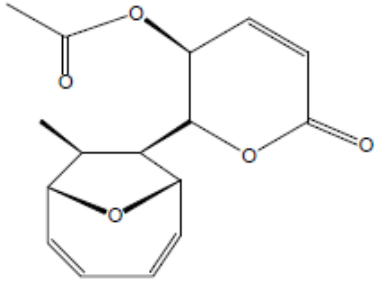
Sr. no.	Compound	Sponge	Molecular mechanism	Reference
1.	Phorbaketal A 	*Phorbas* sp.	Suppressed the expression of inducible NO synthase, reduced the LPS-induced production of inflammatory cytokines - tumor necrosis factor-alpha, interleukin (IL)-1beta, IL-6, and monocyte chemotactic protein-1.	Seo et al., 2015
2.	(+) Aeroplysinin- 1 	*Aplysina aerophoba*	Decreased the expression levels of mRNA and protein levels of MMP-2, MCP-1, TSP-1, COX-2, Il-1α and MMP-1.	Garcia-Vilas et al., 2015
3.	Scalaristerol 	*Scalarispongia aqabaensis*	No effect on release of superoxide anion and thromboxane B2	Youssef at al., 2010

Table 1. (Continued)

Sr. no.	Compound	Sponge	Molecular mechanism	Reference
4.	Callysterol 	*Callyspongia siphonella*	Inhibition of release of superoxide anion and thromboxane B2	Youssef at al., 2010
5.	Solomonsterol A 	*Theonella swinhoei*	Reduces the expression of TNFα, IFNγ and IL-17 and chemokines-MIP1α and RANTES.	Mencarelli et al., 2014
6.	Dactyloquinone B 	*Dactylospongia elegans*	Mixture of both compounds decreased production of TNF-α, IL-1β, and IL-6 production. decreased the production of NO production, and iNOS and COX-2 expression	Lee et al., 2015

Sr. no.	Compound	Sponge	Molecular mechanism	Reference
7.	Cyclospongiaquinone-1 	*Stelospongia conulata*		Kazlauskas et al., 1978
8.	 Mycoepoxydiene	Marine fungus *Diaporthe* sp.	inhibition of NF-κB and MAPK pathway activation	Chen et al., 2012

4. Anti-Inflammatory Activities of Bacteria Associated with Marine Sponges

Anti-inflammatory activity of secondary metabolites produced by *Theonella* sp. was studied using RAW 264.7 macrophages. Inhibition of nitric oxide (NO) production in lipopolysaccharide stimulated RAW 264.7 cells is a well-established assay to screen anti-inflammatory activity. The inhibition level of NO released by RAW 264.7 was estimated from nitrate standard curve. Two isolates TM 1.8 and TM 1.9 possessed anti-inflammatory activities of 112.06% and 109.7% respectively for every 1ug/L sample. The result was compared in inhibiting NO production compared to positive control N-Monomethyl-L-arginine Monoacetate (L-NMMA) which showed an inhibitory activity at 87.41% for every 1ug/L sample (Radzi et al., 2015). New tanzawaic acid derivative tanzawaic acid Q, and four known analogues, tanzawaic acids A, C, D, and K were investigated from a marine-derived fungus *Penicillium steckii* 108YD142. These compounds significantly inhibited nitric oxide production and the new tanzawaic acid Q inhibited the lipopolysaccharide (LPS)-induced inducible nitric oxide synthase and cyclooxygenase-2 proteins and mRNA expressions in RAW 264.7 macrophages. The result of this study demonstrated that the new tanzawaic acid derivative inhibits LPS-induced inflammation (Shin et al., 2016).

5. Synthetic Compounds from the Marine Sponge Compounds Lead

Several inflammatory diseases most notably rheumatoid arthritis, is related to P2X7R activation, which has earned great attention and interests in the application of new treatments through the inhibition of this receptor (Toulme et al., 2010). The functional role of the P2X7R in the inflammatory event is well known as an important mediator in the expression and release of important cytokines and inflammatory mediators. Because of its significant role, it is recently considered as an important anti-inflammatory and pain target, and it has received lot of attention from pharmaceutical companies for evaluating clinical trials based on it (Soares-Bezerra et al., 2013**)**.

Table 2. Synthetic compounds from the marine sponge compounds as a lead

	Target effects	Compounds	Reference
1.	P2X3R and P2X2/3R and.	AF-353 (Roche Pharmaceuticals)	Gever et al., 2006
		A-317491 (Abbott Laboratories)	
2.	P2X7 receptors	Cyanoguanidines A-438079, A-740003 and A804598	Nelson et al., 2006
3.	Anti-inflammatory action in human neutrophils	Carteramine A	Kobayashi et al., 2007

Several new synthetic compounds have been developed for both the P2X3R and P2X2/3R and P2X7 receptors. AF-353 is the most notable synthetic compound developed by Roche Pharmaceuticals (Gever et al., 2006) and A-317491 developed by Abbott Laboratories

for P2X3R (Gunosewoyo & Kassiou, 2010). Disubstituted tetrazoles, the cyanoguanidines A-438079, A-740003 and A804598 with action on hP2X7R (human)/mP2X7R (mouse) and rP2X7R (rat) are the candidates for a potential antagonist for P2X7R (Nelson et al., 2006). The compounds are listed in Table 2. TerraMarine Pharmaceuticals, New Zealand has already identified two promising anti-inflammatory compounds from marine organisms. The first one was patented in September 2005, and is now being improved for its effectiveness and usability. Further, they also plan to study the compound's action in the body and its pharmacokinetic properties. The study will help to determine the correct dosage of the compound and also its development into a usable drug. TerraMarine is also in process to file the provisional patent for the second compound. Owning a patent will achieve TerraMarine the rights to use the chemical structure of the compound as an anti-inflammatory drug or any other for 16 years (Proffitt 2006).

CONCLUSION

Research work summarized in this chapter highlights the ongoing search and development in the field of marine sponge's bioactive compounds and their extraordinary potential as a source of novel bioactive compounds and drugs. In the meantime, much work is in progress with reference to the clinical trials and bringing the drug in the market. However, this ultimate step is dependent on the extent to which the pharmaceutical industry shows its preparedness to grasp such opportunities. The recent policy adopted by most governments worldwide is encouraging and aims at funding basic research, clinical and the manufacturing industry. There is need of continuous effort in preclinical and clinical studies of the marine anti-inflammatory compounds to be tested so that more and more marine drugs are launched. The continuous search for marine sponge leads, and its transformation into a drug with such financial support will ensue good results.

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Chapter 42

TROPHIC ECOLOGY OF THE SHORTFIN MAKO *ISURUS OXYRINCHUS* (LAMNIFORMES: LAMNIDAE) IN THE EASTERN PACIFIC OCEAN

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ABSTRACT

Shortfin mako sharks *Isurus oxyrinchus* are important organisms in the pelagic ecosystem. In spite of their vulnerable status, they are captured by fishermen in the Eastern Pacific Ocean. The proper management and conservation of this population in the Pacific is critical and thus, more biological knowledge of this species is needed. In this work, the trophic habits and consumption of key prey by *I. oxyrinchus* were studied through stomach content analysis of samples collected off the coast of Ecuador, and a literature review of its feeding habits was conducted for the eastern Pacific. The index of relative importance of prey items, and the consumption of key prey were calculated for the three areas. The diet was composed of 78 prey taxa, and cephalopods were the main prey items in the north and central areas, with the jumbo squid *Dosidicus gigas* being the most important prey. For the southern area, *I. oxyrinchus* fed on fish with the bigeye cigarfish *Cubiceps pauciradiatus* being the most important prey species. The trophic niche of *I. oxyrinchus* was composed of squids and fish as the main prey items. The niche breadth for the three areas was reduced, and the trophic level calculated with stomach contents and stable isotopes of $\delta^{15}N$ showed values of 3.68 to 4.44 suggesting that this shark is a tertiary predator in the ecosystem. The jumbo squid *D. gigas* and fish of the family Scombridae were described as key species in the diet of *I. oxyrinchus*. The consumption estimate from 2000 to 2013 was 39 128 tons, with 55.8% of this consumption represented by *D. gigas*. These results demonstrate that *I. oxyrinchus* is an

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active and top predator in the trophic webs of the Eastern Pacific, with opportunistic feeding habits on the more available and abundant prey species.

Keywords: *Isurus oxyrinchus*, shortfin mako, trophic ecology, Eastern Pacific Ocean

1. INTRODUCTION

The shortfin mako shark *Isurus oxyrinchus* (Rafinesque, 1810) is a species of the family Lamnidae, which inhabits the waters of tropical and subtropical oceans (Compagno, 2001; Compagno et al. 2005; Nelson, 2006), with a distribution in the Atlantic, Indic and Pacific Oceans (Nelson, 2006). Sharks have a slow growth rate, late maturity, and long gestation period, biological characteristics that are sensitive to marine changes (Cortés, 2000; Frisk et al. 2001; Frisk et al. 2005).

In the early 1920s, the shark fisheries showed an increase in captures (Barker and Schluessel, 2005). As a result of this fishery activity, ocean shark populations have been declining (Dulvy et al. 2008; Ferreti et al. 2008; Mucientes et al. 2009; Dulvy et al. 2014). In spite of the fisheries regulation, the over-fishing continues as the main factor influencing the decline of sharks (Ferrati et al. 2010). According to the Food and Agriculture Organization of the United Nations (FAO, 2015), in the Eastern Pacific Ocean *I. oxyrinchus* is an important species in the total catches of sharks, and according to the International Union for the Conservation of the Nature (IUCN), it is considered a vulnerable species (Polidoro et al. 2012).

Sharks, such as *I. oxyrinchus*, are key top species that regulate the food webs in the ecosystems (Motta and Wilga, 2001; Navia et al. 2010; Bornatowski et al. 2014). *Isurus oxyrinchus* is an active predator of bony fishes and cephalopods in the Atlantic Ocean and in the south Pacific off the coast of Chile (Stillwell and Kohler, 1982; Vaske-Júnior and Rincón-Filho, 1998; Cortés, 2000; Maia et al. 2006; Mucientes-Sandoval and Saborido-Rey, 2008; Lopez et al. 2009; Wood et al. 2009). Its diet may include other sharks, dolphins and marine mammals (Cliff et al. 1990; Velasco-Tarelo, 2005; Mucientes-Sandoval and Saborido-Rey, 2008; Lopez et al. 2009; Lopez et al. 2012; Preti et al. 2012; Biton-Porsmoguer et al. 2015). As *I. oxyrinchus* is a top predator, its depletion from the oceans promotes changes in top-down control (Ferreti et al. 2008; Ferreti et al. 2010), and modifies the structure and flux of biomass and energy in the ecosystems (Ferreti et al. 2008; Heupel et al. 2014). Thus, it is necessary to identify their food sources and feeding habits at a local and regional scale, and perform a global analysis to identify trends in the trophic ecology of this shark.

Research on the diet and feeding behavior, as well as the predation strategy, of sharks has been conducted in the North and South Pacific Ocean (Yatsu, 1995; Mucientes-Sandoval and Saborido-Rey, 2008; Vetter et al. 2008; Lopez et al. 2009). In the central Pacific off the coast of Ecuador, greater knowledge of *I. oxyrinchus* and other sharks is needed. Trophic ecology studies are conducted to determinate the function and position of species in the food web, and are important to understanding the impacts of predators on the abundance and biomass of trophic levels (Motta and Wilga, 2001; Aguiar and Valentin, 2010; Hussey et al. 2014). Thus, the objective of this research was to investigate biological aspects and the trophic ecology of *I. oxyrinchus* caught in the Ecuadorian Pacific and compare these results with those from the

North and South Eastern Pacific Ocean, identifying key prey species, and inferring the biomass consumption of key prey species.

2. METHODS AND STATISTICAL ANALYSES APPLIED IN THE TROPHIC ECOLOGY OF *ISURUS OXYRINCHUS*

2.1. Stomach Content Analysis Applied to the Trophic Ecology of *Isurus oxyrinchus*

2.1.1. Prey Identification

The stomach contents of sharks have generally been collected from the by-catch of the artisanal or industrial fleets (Stillwell and Kohler, 1982; Vaske-Júnior and Rincón-Filho, 1998; Cortés, 2000; Maia et al. 2006; Mucientes-Sandoval and Saborido-Rey, 2008; Lopez et al. 2009; Wood et al. 2009; Galván-Magaña et al. 2013). Fishing methods used by fishermen are long-line and surface and middle water gillnet. The stomachs are extracted and their fullness is estimated as a percentage of stomach capacity. The stomach content is placed in a labeled bag, then keep on ice for transfer to the Laboratory, and frozen directly for later analysis. Prey are separated and identified to the lowest taxonomic level using different keys of identification and weighted with a precision of 0.1 g. For the identification of complete bony fishes, squids and cartilaginous fishes, the keys of Fischer et al. (1995a, b), and Jered and Roper (2010) are used. Fish remains are identified using the skeleton according to Clothier (1950), and the reference collection of fish skeletons of each institution. Hard structures such as otoliths and squid beaks are identified according to Wolff (1984), North et al. (1984), Clarke (1986), García-Godos (2001) and Xavier and Cherel (2009). Additional, the total length or fork length with precision in centimeters and the sex of each shark are recorded.

2.1.2. Cumulative Prey Curves

Cumulative prey curves have been constructed to determine if an adequate number of stomachs had been analyzed. These curves are constructed at all taxonomic levels and excluded those prey that occurred only once (Preti et al. 2012). A vegan package (Oksanen et al. 2010) of the statistical software R (R Development Core Team, 2010) is used for this analysis. When the curve reached the asymptote, the sample size is considered sufficient to describe the diet (Hurtubia, 1973). When the asymptote is not evident a straight line to the last 4 points is fitted and compared the slope of the line with a line of slope zero, with the asymptote indicated when the lines did not differ significantly (Bizzarro et al. 2007).

2.1.3. Diet Analysis

To evaluate the feeding rate, vacuity index has been used (Consoli et al. 2008), with the following equation $V = N_e/N_s*100$, where, N_e = number of empty stomachs and N_s = total number of stomachs analyzed.

Stomach contents have been analyzed by frequency of occurrence, and numeric and gravimetric methods to quantify the diet. Frequency of Occurrence (%FO) is calculated as the percentage of predators that fed on a certain prey. The number of individuals of a certain prey

relative to the total number of individual prey is represented by Number (%N), and the weight of a certain prey relative to the total weight of all prey is calculated as Weight (%W) (Cailliet, 1976). The percentage of the Index of Relative Importance (%IRI) is calculated to describe the contribution of each prey in the diet (Pinkas et al. 1971), as follows %IRI = ((IRI*100)/∑IRI). Graphics of the most important prey items by area have generally been plotted.

Comparisons between groups have been tested using contingency tables and by estimating G statistics (Crow, 1981). This statistic has a $\chi 2$ distribution.

$$G = 2 * \sum_{i.j} X_{ij} \ln\left(X_{ij} / \left(X_i X_j / N\right)\right)$$

Where Xij is the FO of prey of the i category ingested by the j predator category, Xi is the FO of prey of the i category ingested by all predators, Xj is the total FO of prey ingested by the j predator category, and N is the total number of prey items ingested by all predators.

2.1.4. Trophic Overlap

The index of Morisita-Horn has been used to calculate the trophic overlap between the food and feeding habits, as follow, $C\lambda = 2\sum(Pxi*Pyi)/(\sum Pxi^2 + \sum Pyi^2)$ where $C\lambda$ is the Morisita-Horn index between the *x* and *y* predators, *Pxi* is the proportion of prey *i* in the total prey consumed by predator *x*, *Pyi* is the proportion of prey *i* in the total prey consumed by predator *y*, *n* is the number of total prey (Smith and Zaret, 1982). Values of this index vary from 0 to 1, where values >0.6 indicate high overlap, 0.3 to 0.6 indicate a medium overlap, and 0.0 to 0.29 indicate low overlap (Langton, 1982).

2.1.5. Niche Breadth and Trophic Level

The %IRI has been used to obtain the niche breadth (*Bi*) by calculating the standardized Levin's index (Hurlbert, 1978; Krebs, 1999), as follows $Bi = ((1/\sum Pij^2))-1)/(n-1)$, where *Bi* is the Levin's index of predator *i*, *Pij* is the diet proportion of predator *i* that uses prey *j* and *n* is the number of prey categories. This index takes the values of 0 to 1; when $Bi < 0.6$ the niche breadth is narrow, and when $Bi > 0.6$ the niche breadth is wide (Krebs, 1999).

The trophic level has been calculated with the following equation: $TL_k = 1 + (\sum Pj*TLj)$, where TL_k is the trophic level, *Pj* is the proportion of the prey category in the predator diet, *TLj* is the trophic level of the prey categories (bony fishes *TLj* = 3.24, cephalopods *TLj* = 3.20, crustaceans *TLj* = 2.52, invertebrates *TL j* = 2.5, seabirds *TLj* = 3.87, chondrichthyan fishes *TLj*=3.65 and marine mammals *TLj* = 4.02) (Cortés, 1999).

2.1.6. Feeding Strategy

The feeding strategy of *I. oxyrinchus* has been described using the method of Amundsen et al. (1996). This is a two-dimensional method based on the specific abundance of prey (%SAP) and the frequency of occurrence (%FO). Results of this method describe the prey importance as rare or dominant, the feeding strategy as a generalist or specialist predator, and the inter- and intra-specific components of the niche breadth. The values are calculated with the following equation: $Pi = (\sum Si/\sum Sti)*100$, where *Pi* is the specific abundance of prey *i*, *Si* is the weight in grams of prey *i*, and *Sti* is the total weight of all stomach contents of stomachs

with prey *i*. It was not possible to apply this method to the other two areas because there were no data on individual feeding.

2.1.7. Consumption by Sharks of Key Prey Species

Estimates of consumption by sharks have been calculated using catches as a measure of abundance in the ocean because biomass estimates of sharks are not available. The analysis assumes that the proportion of each prey species does not vary over time. The consumption equation is represented as follows: $Qi = \sum Fj*(Q/B)*DCij$, where Q_i is the consumption of prey species, *Fj* represents fishery catches of sharks in tons, $(Q/B)j$ is the Consumption/ Biomass relationship of sharks, and ($DCij$ = %IRI) is the diet proportion of each prey species *i* in the diet of sharks *j*.

2.2. Stable Isotopic Analysis

2.2.1. Sampling and Isotopic Analysis

A small portion of muscle (dorsal portion of the head, or caudal portion) is cleaned with distilled water and lipids are extracted with chlorophorm and methanol (Bligh and Dyer 1959). Samples are freeze-dried and powdered, and 0.285-0.315 mg of each sample is packed into tin capsules. Isotopic analyses are performed at different laboratories (Laboratory of Stable Isotopes at the Estación Biológica de Doñana, Plant Science Department at University of California, Laboratorio de Isótopos estables UNAM, Laboratorio de Isotópos Estables CICIMAR-IPN). Samples are generally heated to 1020°C using a continuous flow isotope-ratio mass spectrometer (Thermo Electron) by means of a Flash HT Plus elemental analyser interfaced with a Delta V Advantage mass spectrometer. Stable isotope ratios are expressed in the standard δ-notation (‰) relative to Vienna Pee Dee Belemnite ($\delta^{13}C$) and atmospheric N_2 ($\delta^{15}N$). Based on laboratory standards, the measurement error is ± 0.1 and ± 0.2 for $\delta^{13}C$ and $\delta^{15}N$, respectively. The C:N ratio of tissues has to be always lower than 3‰. ANOVA tests have been used when comparing isotope values between groups.

2.2.2. Trophic Level Based on Isotope Values

The trophic level position of each species has been calculated according to Kim et al. (2011) and Carlisle et al. (2012), using the following equations:

$$TL = [(\delta^{15}N_{predator} - \delta^{15}N_{base})/TEF] + 3,$$

where TL is the trophic level of that consumer, $\delta^{15}N_{predator}$ represents the values of the consumer´s muscle tissue; $\delta^{15}N_{base}$ is the mean $\delta^{15}N$ of *Benthosema panamense* (9.9‰; for the Pacific off Ecuador; Calle, 2010, with a trophic level of 3; Dambacher et al., 2010), and *Pleuroncodes planipes* (12.3‰; for the north Pacific with a trophic level of 2.6; Madigan et al., 2012) as reference species that share the same habitat as predators, and TEF = 3.7 is the transfer energy factor of each predator (Sweeting et al. 2007).

2.3. Trophic Ecology of *Isurus oxyrinchus* in the Eastern Pacific

2.3.1. Feeding Habits of Isurus oxyrinchus

For description of the feeding habits of *I. oxyrinchus* off Ecuadorian waters (central Pacific), samples were collected in the fishing port of Santa Rosa, Santa Elena, Ecuador 02°13´S; 80°58´W, from July 2013 to April 2015, (Figure 1). For the north and south Pacific, reports of the food and feeding habits of *I. oxyrinchus* were reviewed. For the northern area of Eastern Pacific Ocean, the reports of Vetter et al. (2008) and Preti et al. (2012) were consulted, and for the southern area, the reports of Yatsu, (1995), Mucientes-Sandoval and Saborido-Rey, (2008), Lopez et al. (2009), Lopez et al. (2012), and Rosas-Luis et al. (2016) were consulted. To perform an adequate comparison of the prey items of *I. oxyrinchus*, the diet values reported off the coast of United States of America by Lopez et al. (2009), and off the coast of Chile by Preti et al. (2012) and the present study (Figure 1) were used to compare and estimate the feeding habits and consumption of sharks.

Figure 1. Eastern Pacific Ocean. Gray dark polygons represent the northern (Off the coast of the United States of America), central (off the coast of Ecuador), and southern (off the coast of Chile) areas where stomach content analyses were reported for *Isurus oxyrinchus*.

The consumption by *I. oxyrinchus* of the jumbo squid *D. gigas* and fishes of the family Scombridae (all scombrid fishes were unified into a single group; species included were *K. pelamis*, *T. albacares*, *Auxis* spp., *Scomber japonicus*, *Sarda chiliensis*, and unidentified scombrid fishes) were calculated for the period 2000 to 2013 based on the available fishery data of each area. The Q/B value was taken from Rosas-Luis et al. (2008).

2.3.2. Stable Isotopic Analysis of Isurus oxyrinchus

Stable isotopic analysis of $\delta^{15}N$ and $\delta^{13}C$ was applied to muscular samples collected in the fishing port of Santa Rosa, Santa Elena, Ecuador 02°13´S; 80°58´W, from July 2013 to April 2015. Reported data of Malpica-Cruz et al. (2013) and Maya-Meneses et al. (2016) for the North Pacific, and Clarian et al. (2016) for the south Pacific, were consulted and compared with those of the central area. Three size groups were used in the comparison, small sharks <112 cm total length, medium sharks 112-196 cm, and large sharks >196 cm.

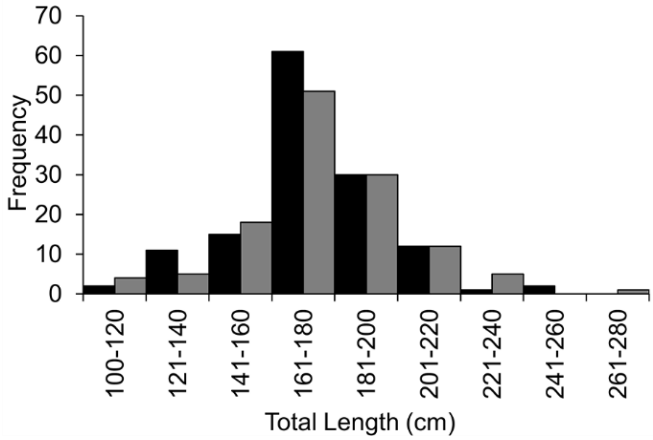

Figure 2. Size distribution of the shortfin mako *Isurus oxyrinchus* in centimeters (cm) caught off the coast of Ecuador. Black bars represent males and dark gray bars represent females.

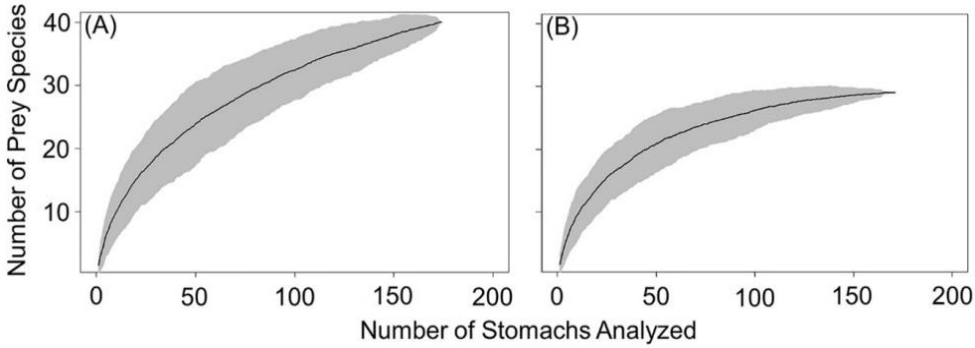

Figure 3. Cumulative curve of prey (black line) and standard deviation (gray) calculated for *Isurus oxyrinchus* caught off the coast of Ecuador. (A) represents the curve when all stomachs analyzed with all prey items were included, and (B) represents the curve calculated when uncommon prey were deleted (prey that occurred once in the stomachs).

3. FEEDING DESCRIPTION OF *ISURUS OXYRINCHUS* IN THE EASTERN PACIFIC OCEAN

3.1. Results of Stomach Content Analysis

3.1.1. Central Area "Ecuadorian Pacific"

General Description of *I. oxyrinchus* Caught in the Central Area

The feeding habits of 281 shortfin mako sharks were analyzed from the Ecuadorian Pacific Ocean: 134 were males, 129 females and 18 undetermined, with a sex proportion of 1.04M:1H ($\chi^2 = 0.095$; $p = 0.75$). The size of males ranged from 106 to 257 cm total length (Mean = 174.51 ± 23.60) and females from 105 to 267 cm total length (Mean = 176.16 ± 25.40) (Figure 2). The undetermined individuals ranged from 108 to 213 cm total length, and no size differences were observed among males and females ($t = -0.54$; $p = 0.58$). Figure 3 shows the cumulative prey curve. When all the prey items were included, the curve did not reach the asymptote ($R^2 = 0.95$, $p = 0.01$), but did when prey that occurred once were excluded ($R^2 = 0.85$, $p = 0.05$).

Prey Identification

Forty taxa were identified in the stomach contents of *I. oxyrinchus* caught in the Ecuadorian Pacific (Table 1). Cephalopoda was the main prey group (%IRI = 58.28%), with squids of the family Ommastrephidae being the most important in the diet (%IRI = 48.36). The second most important group in the diet was Osteichthyes (%IRI = 41.07%), with fishes of the family Scombridae being the most important (%IRI = 20.62) (Figure 4, Table 1). The jumbo squid *D. gigas* was the main prey species in the diet (%IRI = 48.28), followed by the skipjack *K. pelamis* (%IRI = 11.39), the scombrid *Auxis* spp. (%IRI = 7.47), and finally, the squid *Ancistrocheirus lesueurii* (%IRI = 7.26) (Figure 4 and Table 1).

3.1.2. Northern and Southern Pacific Areas

Prey Identification

Values of %IRI, %FO, %N from the north and south Pacific areas are shown in Table 1. For the northern area, 42 taxa have been reported in the diet of *I. oxyrinchus*. Cephalopods have been reported as the main prey items, with squid *D. gigas* of the family Ommastrephidae being the most important, followed by the group composed of Osteichthyes, with the fish *Cololabis saira* of the family Scomeresocidae and the chub mackerel *Scomber japonicus* of the family Scombridae being the most important. Other prey groups such as Chondrichthyes, mammals, and marine birds have also been reported (Figure 4, Table 1).

Twenty-two prey taxa have been reported in the diet of *I. oxyrinchus* in the southern area (Table 1). Osteichthyes have been reported as the main prey group, with the fish *Cubiceps puciradiatus* of the family Nomeidae being the most important. The second most important group was Cephalopoda, with the jumbo squid *D. gigas* of the family Ommastrephidae being the most important. Other groups of less importance such as Chondrichthyes, Mammalia and Crustacea have also been reported in the diet (Table 1, Figure 4).

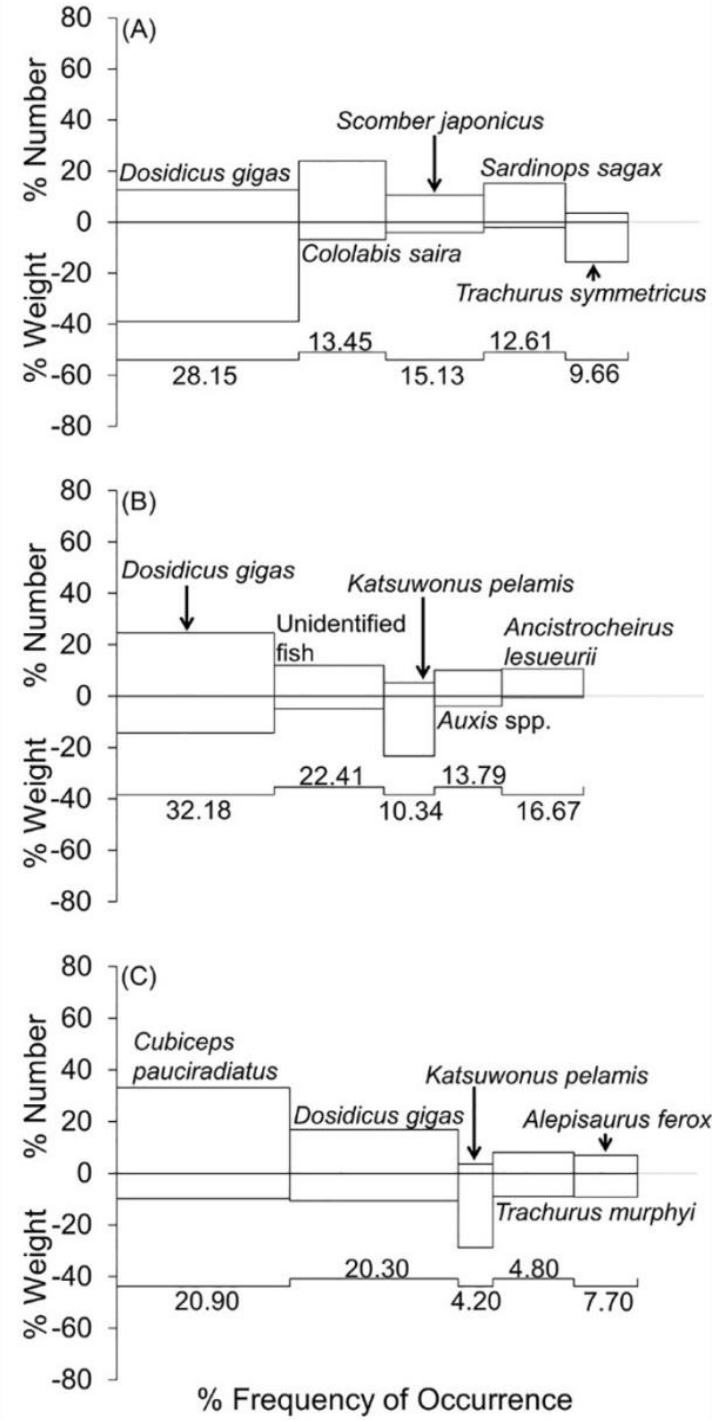

Figure 4. Diet composition of the main prey found in the stomach contents of *Isurus oxyrinchus* in the Eastern Pacific Ocean, North (A), Central (B) and South (C), expressed in the percentage of Number (%N) and Weight (%W) (vertical axis) and the Frequency of Occurrence (%FO) (horizontal axis).

Table 1. Index of Relative Importance (%IRI), Frequency of Occurrence (%FO), Number (%N) and Weight (%W) of prey in the stomach contents of shortfin mako *Isurus oxyrinchus* in the Eastern Pacific Ocean. Un = Unidentified. Northern and southern areas values were calculated based on Preti et al. (2012) and Lopez et al. (2009)

	Southern area				Central area				Northern area			
Prey species	%N	%P	%FO	%IRI	%N	%P	%FO	%IRI	%N	%P	%FO	%IRI
Crustacea												
Solenoceridae												
Haliporoides diomedae	0.60	1.00	0.70	0.05								
Cephalopoda												
Ancistrocheiridae												
Ancistrocheirus lesueurii					10.59	0.69	16.67	7.26				
Argonautidae												
Argonauta spp.									1.55	0.00	0.84	0.05
Bolitaenidae												
Japetella sp.									0.24	0.00	0.84	0.01
Enoploteuthidae												
Abraliopsis sp.									0.12	0.00	0.42	0.00
Gonatidae												
Gonatopsis borealis									0.48	0.00	1.26	0.02
Gonatus sp.					0.52	0.00	1.15	0.02	4.04	0.00	9.24	1.29
Histioteuthidae												
Histioteuthis dofleini					1.81	0.01	4.02	0.28	2.97	0.03	7.98	0.83
Histioteuthis heteropsis									0.48	0.00	1.68	0.03
Histioteuthis sp.	0.60	0.40	0.70	0.03	1.81	0.37	2.87	0.24	1.43	0.02	3.78	0.19
Loliginidae												
Loligo opalescens									1.31	0.00	3.36	0.15
Mastigoteuthidae												
Mastigoteuthis dentata					0.52	0.00	1.15	0.02				
Octopoteuthidae												
Octopoteuthis sicula					1.03	0.00	2.30	0.09				
Octopoteuthis sp.									1.55	0.00	3.78	0.20
Ommastrephidae												
Dosidicus gigas	16.90	10.70	20.30	26.19	24.55	14.3	32.18	48.28	12.6	38.97	28.15	50.21
Hyaloteuthis pelagica					0.78	0.05	0.57	0.02				

	Southern area				Central area				Northern area			
Prey species	%N	%P	%FO	%IRI	%N	%P	%FO	%IRI	%N	%P	%FO	%IRI
Ommastrephidae sp. 1					0.26	0.00	0.57	0.01				
Ommastrephidae sp. 2					0.26	0.00	0.57	0.01				
Sthenoteuthis oualaniensis					0.52	0.56	1.15	0.05				
Toradores filipovae	2.30	0.50	2.80	0.37								
Onychoteuthidae												
Onykia robusta									0.12	0.00	0.42	0.00
Onychoteuthis sp.					3.36	0.10	6.90	0.58	0.36	0.00	0.84	0.00
Opistoteuthidae												
Opistoteuthis sp.					0.26	0.02	0.57	0.01				
Thysanoteuthidae												
Thysanoteuthis rhombus					2.58	0.41	4.60	0.53				
Unidenfied Cephalopoda	5.80	2.70	7.00	2.78	2.84	0.83	6.32	0.89	2.62	0.50	5.88	0.56
Osteichthyes												
Alepisauridae												
Alepisaurus ferox	6.90	9.20	7.70	5.80								
Belonidae												
Belonidae Un					4.13	1.66	1.72	0.39				
Bramidae												
Brama australis	0.60	0.10	0.70	0.02								
Brama japonica									0.95	4.77	2.94	0.58
Carangidae												
Caranx sp.					0.26	0.13	0.57	0.01				
Decapterus macarellus					0.26	0.03	0.57	0.01				
Decapterus macrosoma					0.52	0.09	1.15	0.03				
Decapterus sp.					0.52	0.29	1.15	0.04				
Trachurus murphyi	8.10	9.00	9.80	7.83								
Trachurus symmetricus									3.45	15.64	9.66	6.38
Clupeidae												
Sardinops sagax									15.2	2.11	12.61	7.56
Coryphaenidae												
Coryphaena hippurus					2.07	7.40	4.60	1.68				
Echeneidae												
Remora remora					0.26	0.40	0.57	0.01				

Table 1. (Continued)

	Southern area				Central area				Northern area			
Prey species	%N	%P	%FO	%IRI	%N	%P	%FO	%IRI	%N	%P	%FO	%IRI
Engaulidae												
Engraulis mordax									1.31	0.00	2.10	0.10
Haemulidae												
Haemulidae Un					0.52	0.23	1.15	0.03				
Kyphosidae												
Medialuna californiensis									0.12	0.00	0.42	0.00
Macrouridae												
Coryphaenoides acrolepis									0.24	0.38	0.84	0.02
Coryphaenoides sp.									0.24	0.00	0.84	0.01
Merlucciidae												
Merluccius gayi					0.26	0.03	0.57	0.01				
Monacanthidae												
Aluterus monoceros					0.26	0.41	0.57	0.01				
Mugilidae												
Mugil cephalus					0.26	0.18	0.57	0.01	3.33	12.46	10.5	5.73
Nomeidae												
Cubiceps pauciradiatus	33.10	9.80	20.90	41.91								
Cubiceps paradoxus					0.78	0.09	0.57	0.02				
Cubiceps sp.	1.70	1.10	2.10	0.27								
Ophidiidae												
Chilara taylori									0.12	0.00	0.42	0.00
Paralepididae												
Magnisudis atlantica									2.26	0.23	1.68	0.14
Paralepididae Un									0.59	0.14	1.26	0.03
Paralichthyidae												
Citharichthys sordidus									0.12	0.00	0.42	0.00
Priacanthidae												
Pristigenys serrula					0.52	0.87	1.15	0.06				
Regalecidae												
Ragalecus glesne					0.26	1.33	0.57	0.04				
Scomberesocidae												

	Southern area				Central area				Northern area			
Prey species	%N	%P	%FO	%IRI	%N	%P	%FO	%IRI	%N	%P	%FO	%IRI
Cololabis saira									23.9	6.89	13.45	14.32
Scombridae												
Auxis spp.	0.60	0.90	0.70	0.05	10.08	3.97	13.79	7.47	0.24	0.98	0.42	0.02
Katsuwonus pelamis	3.50	28.8	4.20	6.34	5.17	23.4	10.34	11.39	0.36	0.99	1.26	0.06
Sarda chiliensis									0.48	0.97	1.26	0.06
Scomber japonicus	6.40	2.60	7.70	3.24	0.52	0.26	1.15	0.03	10.6	4.09	15.13	7.68
Scombridae Un	0.60	1.00	0.70	0.05	0.78	1.97	1.72	0.18	0.12	0.08	0.42	0,.03
Thunnus albacares					1.55	12.3	2.87	1.54				
Scorpaenidae												
Sebastes jordani									0.71	0.79	0.42	0.02
Sebastes goodei									0.12	0.11	0.42	0.00
Sebastes spp.									0.24	0.04	0.84	0.01
Tetraodontidae												
Lagocephalus lagocephalus					5.94	4.06	9.20	3.55				
Xiphiidae												
Xiphias gladius	1.20	1.50	1.40	0.18								
Unidenfied Osteichthyes	6.40	5.30	7.00	3.83	11.89	4.96	22.41	14.57	4.88	3.31	12.61	3.57
Chondrichthyes												
Carcharhinidae												
Prionace glauca									0.24	3.48	0.84	0.11
Lamnidae												
Isurus oxyrinchus	1.20	2.10	1.40	0.22								
Lamna nasus	1.20	7.40	1.40	0.56								
Mobulidae												
Mobula japanicus					0.26	13.8	0.57	0.31				
Pseudocarchariidae												
Pseudocarcharhias kamoharai	0.60	2.20	0.70	0.09								
Unidentified Chondrichthyes	0.60	0.80	0.70	0.05								
Delfinidae												
Delphinus delphis									0.12	2.88	0.42	0.04
Delfinidae Un					0.52	2.25	1.15	0.12				
Tursiops truncatus	0.60	3.50	0.70	0.13								
Unidentified mammals					0.78	2.45	1.72	0.21				

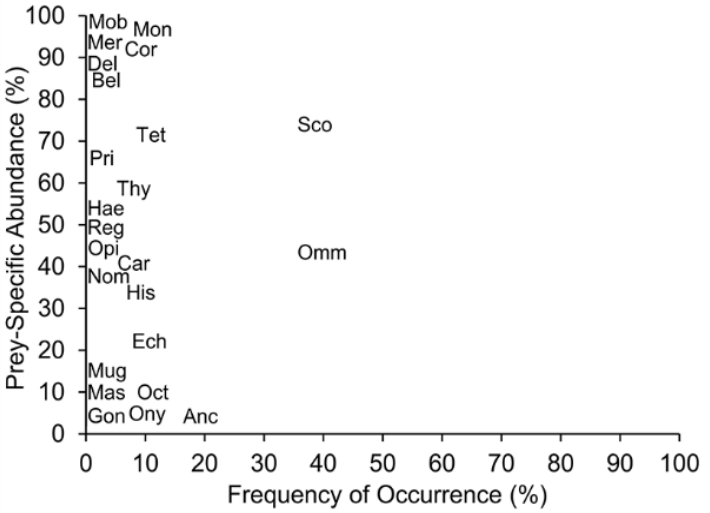

Figure 5. Diagram of the feeding strategy of the shortfin mako *Isurus oxyrinchus* in the Pacific Ecuadorian, expressed in the Specific Prey Abundance and the Frequency of Occurrence of family prey. Bel: Belonidae, Car: Carangidae, Cor: Coryphaenidae, Ech: Echeneidae, Hae: Haemulidae, Mer: Merluciidae, Mon: Monacanthidae, Mug: Mugilidae, Nom: Nomeidae, Pri: Priacanthidae, Reg: Ragalecidae, Sco: Scombridae, Tet: Tetraodontidae. Anc: Ancistrocheiridae, Gon: Gonatidae, His: Histioteuthidae, Mas: Mastigoteuthidae, Oct: Octopoteuthidae, Omm: Ommastrephidae, Ony: Onychoteuthidae, Opi: Opistoteuthidae, Thy: Thysanoteuthidae. Mob: Mobulidae. Del: Delfinidae.

3.1.3. Trophic Overlap

Prey items, found in the stomach contents of sharks from the Ecuadorian Pacific and those reported for the northern and southern Pacific, overlap with five specific prey (*Katsuwonus pelamis*, *Auxis* sp., *S. japonicus*, *D. gigas*, *Histioteuthis* sp.). Based on these prey items, a high trophic overlap was found between the northern and central areas $C\lambda$ = 0.87, while between the central and southern areas, and the northern and southern areas, trophic overlap was moderate ($C\lambda$ = 0.52, and $C\lambda$ = 0.48 respectively).

3.1.4. Niche Breadth and Trophic Level

The niche breadth for *I. oxyrinchus* in the north was *Bi* = 0.06 with 42 prey taxa. In the central area, it was *Bi* = 0.07 with 40 prey taxa, and for the south it was *Bi* = 0.14 with 21 prey taxa. The trophic level calculated for *I. oxyrinchus* in the northern area was 4.22, and for the central and southern areas, the trophic level was 4.23.

3.1.5. Feeding Strategy

The Amundsen graphical analysis, considering 24 prey families, indicated that most of the families found in the stomach contents, such as Echeneidae, Mugilidae, Gonatidae, Mastigoteuthidae, Octopoteuthidae and Onychoteuthidae, were consumed as rare items. The families Ommastrephidae and Scombridae showed the greatest abundance and frequency of occurrence in the Ecuadorian Pacific Ocean.

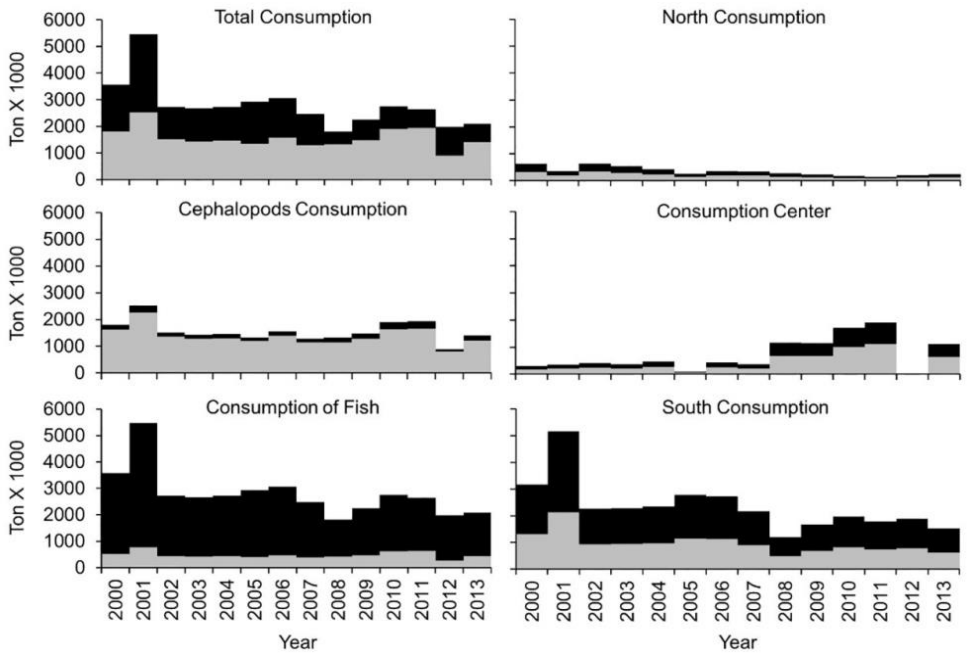

Figure 6. Total consumption of *Isurus oxyrinchus* in the Eastern Pacific Ocean: black area represents the consumption of fish, and gray area represents the consumption of cephalopods. Cephalopod Consumption in the Eastern Pacific Ocean: black area represents the total consumption, and gray area is the consumption of *Dosidicus gigas*. Consumption of fish in the Eastern Pacific Ocean: black area represents the total consumption, and gray area represents the consumption of Scombrid fish. Consumption of fish and cephalopods in the Eastern Pacific, North (United States of America), Central (Ecuador) and South (Chile): black area represents fish, and gray area represents cephalopods.

3.1.6. Consumption of Fish and Squid by Isurus oxyrinchus

Figure 6 shows the consumption by *I. oxyrinchus* of specific prey. The total consumption of fish in the three areas during the period analyzed was 39 128 tons. For squid, consumption was calculated to be 21 839 tons. The consumption of scombrid fishes was 6 657 total tons, and this value represented 17.01% of the total fish consumption. The total consumption of the jumbo squid *D. gigas* was 19 299 representing 88.37% of the total consumption of squids.

3.2. Results of Stable Isotope Analysis

$\delta^{15}N$ values of *I. oxyrinchus* of the central area (own unpublished data) were higher for sharks of medium size than larger size (one-way ANOVA, $F_{19} = 4.67$, $P < 0.05$)(Figure 7). Results of the comparison between N values of all size groups in the three areas showed no difference (one-way ANOVA, $F_6 = 2.46$, $P > 0.05$).

Trophic position of *I. oxyrinchus* based on $\delta^{15}N$ isotopes showed the highest values for medium sharks, and the lowest values for small sharks in the central and north areas (Table 2). The highest value of the trophic position was calculated for the south area, but no size class was reported. The trophic position values varied around 1 unit when the prey at the base of the food web changed (Table 2).

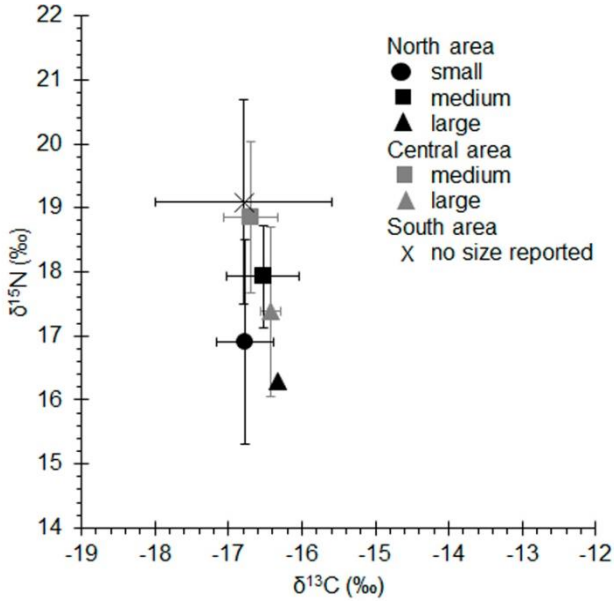

Figure 7. $\delta^{15}N$ and $\delta^{13}C$ values (mean ± s.d.) for *Isurus oxyrinchus* of the eastern Pacific ocean.

Table 2. Trophic position (TP) of *Isurus oxyrinchus* by size interval and studied areas. TP-1 was calculated by using the pelagic red crab *Pleuroncodes planipes* as base of the trophic web, and TP-2 was calculated by using the myctophid *Benthosema panamense*. * no size reported

Pacific area	Size	Number of samples	TP-1	TP-2
North	small	15	3.84	4.89
	medium	4	4.12	5.17
	large	6	3.68	4.73
Central	medium	41	4.37	5.42
	large	1	3.97	5.02
South	*	50	4.44	5.49

Values of $\delta^{13}C$ between medium and large sharks for the central area (own unpublished data) did not vary (one-way ANOVA, $F_{19} = 1.78$, $P > 0.05$). Results of the comparison of $\delta^{13}C$ values between the three areas did not vary (one-way ANOVA, $F_6 = 0.54$, $P > 0.05$) (Figure 7).

4. DISCUSSION

The high metabolic rates of Lamnid sharks (Newton et al. 2015) make the identification of food items difficult. However, this can be mitigated if sharks are feeding when they are caught. The stomachs analyzed in each study showed that the sharks fed actively before they were caught, and it was confirmed with the low vacuity percentage of stomachs for the three areas of the Eastern Pacific Ocean. Moreover, the feeding behavior of *Isurus oxyrinchus* is characterized by the consumption of complete or large pieces of prey (Wood et al. 2009),

further improving the identification of stomach contents. In this case, prey species were well-preserved and in earlier digestion stages, improving the identification of ingested species. The number of stomachs was adequate to describe the diet in the three areas: the United States of America, the South Pacific off the coast of Chile, and the Central Pacific Ocean off the coast of Ecuador.

4.1. Feeding Habits in the Eastern Pacific Ocean

The diet of *I. oxyrinchus* from different regions was composed of a similar number of prey items. Forty prey items were identified in the stomachs of sharks from the central area and 42 were reported for sharks off the coast of the United States (Preti et al. 2012). These may indicates the availability and abundance of similar prey in these areas, such as squids and fish (Clarke, 1996; Ebert and Stehmann, 2013; Galván-Magaña et al. 2013).

The feeding habits of *I. oxyrinchus* showed that these sharks are predators of fishes and squids, in agreement with the results previously reported for this species in the Pacific Ocean (Stillwell and Kohler, 1982; Velasco-Tarelo, 2005; Mucientes-Sandoval and Saborido-Rey, 2008; Lopez et al. 2009, 2012; Preti et al. 2012; Galván-Magaña et al. 2013). High consumption of bony fishes by *I. oxyrinchus* in the Ecuadorian Pacific and similar feeding behavior in the southern area suggests that these prey are abundant in the ecosystems and available to sharks (Stillwell and Kohler 1982; Lopez et al. 2009, 2012; Gorni et al. 2012, 2013). Nevertheless, as indicated by our results, the FO of squid was similar to fish, suggesting that these cephalopods are also important in their diets (Preti et al. 2012). The importance of fish and squid appears to vary between sampled areas and among seasons. There are reports showing fishes as the most important food source, indicating a feeding strategy based on ichthyophagous habits (Stillwell and Kohler, 1982; Velasco-Tarelo, 2005; Mucientes-Sandoval and Saborido-Rey, 2008, Lopez et al. 2009, 2012; Preti et al. 2012). In other areas, teutophagous habits are suggested (Galván-Magaña et al. 2013).

There is evidence of a trophic overlap between the food sources used by *I. oxyrinchus* since the squid *D. gigas* and the fishes *K. pelamis* and *Auxis* spp. were the most important prey in the Ecuadorian Pacific and in the northern area off the US coast (Velasco-Tarelo, 2005; Mucientes-Sandoval and Saborido-Rey, 2008; Vetter et al. 2008; Lopez et al. 2009; Preti et al. 2012). Squids are consumed by other sharks such as *Alopias pelagicus*, *Alopias superciliosus*, and *Prionace glauca* in Ecuadorian waters, and three species have been reported in their diet: *D. gigas* and *Sthenoteuthis oualaniensis* of the family Ommastrephidae, and *Ancistrocheirus lesueurii* of the family Ancistrocheiridae (Galván-Magaña et al. 2013; Rosas-Luis et al. 2016). The use of *D. gigas* as prey by *I. oxyrinchus* may be a result of the sharks´ vertical migration to deep waters during the day, which can be up to 800 m deep (Bress, 1993; Abascal et al. 2001; Loefer et al. 2005; Field et al. 2008). These deep waters are where *D. gigas* is found (Nigmatullin et al. 2001; Bazzino et al. 2010). On the other hand, the use of scombrid fishes such as *K. pelamis* and *Auxis* spp. may be a result of their high abundance and distribution in the Pacific (Allen and Robertson, 1998). Contrary to the results for the northern and central areas where cephalopods are the most important prey, in the southern area the bigeye cigarfish *Cubiceps pauciradiatus* was reported as the main prey source (Lopez et al. 2009). However, as presented in this work, the second most important prey was *D. gigas*. The inclusion of cephalopods in the diet of *I. oxyrinchus* in the southern

area varies year to year, as they were reported as the main prey item in 2008 (Mucientes-Sandoval and Saborido Rey, 2008), and were not important in 1995 (Yatsu, 1995). The important prey species in the diet suggests that *I. oxyrinchus* is an opportunistic feeder, and that its diet may change as a consequence of movements and abundance of prey.

4.2. Variation in Diet in the Three Areas of the Eastern Pacific Ocean

Crustaceans, marine birds, rays, sharks, and marine mammals are uncommon prey species in the diet of *I. oxyrinchus*. Crustaceans and birds have been reported as prey sources of this shark for the southern and northern areas (Vetter et al. 2008; Lopez et al. 2009; Preti et al. 2012), but they were not found in the stomachs analyzed for the central area. It is known that crustaceans, such as the pelagic red crab *Pleuroncodes planipes* in the north Pacific and *Pleuroncodes monodon* in the southern hemisphere, are abundant (Aurioles-Gamboa 1992; Roa et al. 1995; Robinson et al. 2004) and preyed upon by large predators such as *D. gigas* (Rosas-Luis et al. 2008). Thus, these prey are indirectly consumed by *I. oxyrinchus* via squid predation. Birds as food sources are preyed upon in the southern hemisphere by sharks such as *Galeocerdo cuvier* (Cliff and Dudley, 1991; Heithaus, 2005), but for *I. oxyrinchus* these prey sources may be ingested when they are dead and floating on the surface waters.

The presence of uncommon prey species in the diet of *I. oxyrinichus* in each area suggests that the primary distribution of these prey species overlaps with that of *I. oxyrinchus*. A large manta ray *M. japonica* was found in the stomach contents of a shark, and some large sharks had consumed small marine mammals. However, as our results and those previously reported by Lopez et al. (2009) and Preti et al. (2012) showed, these prey were consumed primary by large sharks. The consumption of these prey types by *Carcharodon carcharias*, *Carcharhinus leucas*, *Galeocerdo cuvier*, *Hexanchus sixgill* and *Notorynchus cepedianus* suggests that these species are active predators of marine mammals and birds (Lowe et al. 1996; Heithaus, 2002; Moteiro et al. 2006; Bornatowski et al. 2012). Considering that marine mammals and large fish were consumed in low proportions by *I. oxyrinchus*, we cannot conclude that this shark is an active predator of this kind of prey in the eastern Pacific Ocean. Finally, the consumption of other sharks by *I. oxyrinchus* was found in the three studied areas in agreement with similar reports in the Atlantic and Indian Oceans (Cliff et al. 1990; Maia et al. 2006).

4.3. Niche Breadth and Trophic Level

Measures of trophic position and niche breadth are essential in the interpretation of trophic ecology (Hussey et al. 2014). These results demonstrated that *I. oxyrinchus* in the Eastern Pacific Ocean has a narrow niche breadth, as a result of a selectivity for prey of the families Scombridae and Ommastrephidae. Its estimated trophic level (4.23) reflects its high level of predation activity in the ecosystem. This is a value similar to other Lamnidae species such as *Carcharodon carcharhias* and *Lamna nasus* with high trophic levels of 4.5 and 4.2, respectively (Cortés, 1999), that indicates, *I. oxyrinchus* is a top predator and a tertiary consumer in the ecosystem (Cortés, 1999; Estrada et al. 2003).

4.4. Feeding Strategy

The feeding strategy of *I. oxyrinchus* has been conditioned by prey availability, as this shark is a highly migratory species that exploits different areas and consumes a wide range of prey (Lopez et al. 2009). In the north (Velasco-Tarelo, 2005; Preti et al. 2012) and central Pacific, *I. oxyrinchus* fed mainly on *D. gigas*, *Katsuwonus pelamis*, *Auxis* spp., *Thunnus albacares* and *Scomber japonicus*, coinciding with the high abundance and distribution of these species in the Pacific Ocean (Punsly and Deriso, 1991; Markaida and Gilly, 2016). Nevertheless, feeding habits differed in the southern area off the coast of Chile where this consumer showed a heterogeneous and generalized diet (Lopez et al. 2009, 2012). This suggests that the niche breadth may vary according to geographic area, and that this shark preys opportunistically on abundant and available prey species.

4.5. Consumption of Fish and Squids by *Isurus oxyrinchus*

Isurus oxyrinchus is a top predator in the eastern Pacific Ocean, with an active vertical and horizontal migration (Fowler, 2014) that determines its impact on the food web. Captures of this shark for the analyzed areas are the only indicators permitting calculations of the trophic consumption of prey (Rosas-Luis et al. 2016). Considering this indicator, the consumption of fish is higher than squid, but as our results showed, these values vary between northern, central and southern areas. We focused our study on the most important prey to demonstrate how the decline of this shark may affect the abundance of other marine organisms (Chang and Liu 2009; Ferreti et al. 2010). If the actual tendency in the decline of *I. oxyrinchus* continues, the population of *D. gigas* and other prey will be affected positively. On the contrary, if the prey populations increase, the population of sharks may be affected positively. It is known that the estimates of biomass between prey and predator are overestimates (Jennings and Collingridge, 2015), but these can be used to create models and measurements for the management and conservation of endangered species.

The high consumption rates of the jumbo squid *D. gigas* by *I. oxyrinchus* suggests that this squid is an important component in the food web (Nigmatullin et al. 2001; Rosas-Luis et al. 2008; Alegre et al. 2014), and confirms its abundance and distribution patterns in the Eastern Pacific Ocean (Markaida and Gilly, 2016). On the other hand, the importance of scombrid fishes in the diet increases latitudinally, confirming their wide distribution in this region (Allen and Robertson, 1998), and its availability as a food source for *I. oxyrinchus*. So that, the trophic relationships between prey and predator are fragile, and small disturbances may affect both components in the ecosystem, resulting in disturbances throughout the trophic webs.

4.6. Stable Isotopic Analysis of *Isurus oxyrinchus*

Values of $\delta^{15}N$ and $\delta^{13}C$ of *I. oxyrinchus* in the central, north, and south eastern Pacific were interpreted in addition of the results of the analysis of stomach contents. Isotopes resulted in no difference between sharks sampled in the three areas. This result suggests that *I. oxyrinchus* has similar feeding habits in the whole area, being cephalopods and fish the

main prey groups (Stillwell and Kohler, 1982; Velasco-Tarelo, 2005; Mucientes-Sandoval and Saborido-Rey, 2008; Lopez et al. 2009, 2012; Preti et al. 2012; Galván-Magaña et al. 2013). Malpica-Cruz et al. (2013) and Maya-Meneses et al. (2016) mentioned that small and medium size *I. oxyrinchus*, caught off the coast of Mexico, had no variation in the $\delta^{15}N$ values, contrasting with the result of the central area where medium and large *I. oxyrinchus* had significant variation. The difference in $\delta^{15}N$ values for sharks in the central area results of the ontogenetic shift to a broader diet (Preti et al. 2012), and a more diverse feeding preferences of medium size sharks (Malpica-Cruz et al. 2013). The trophic position of *I. oxyrinchus*, based in $\delta^{15}N$ values of *P. planipes* as the base of the food web, coincided with the trophic position calculated with the results of stomach content analysis and that reported by Cortés, (1999), but it was different when the myctophid *B. panamense* was used as the base of the food web. It suggest that the selection of the organism as base of the food web is determinant for the trophic position in the ecosystem, and that more research is needed in order to define similar organisms as key species for isotopic analysis in each studied area. Despite this, the trophic position of *I. oxyrinchus* vary according to size, habitat and availability of food, and caution must be consider when make conclusions of the trophic position of this species.

Regarding the $\delta^{13}C$ values, no differences were found in comparison between size and areas, which confirms a same use of habitat of this species in the eastern Pacific. *Isurus oxyrinchus* are known to migrate between inshore and offshore environments (Stillwell and Kohler, 1982), this patters have been documented when sex segregation is present (Maya-Meneses et al. 2016). Unfortunately, our results and those reported for the south and north areas are not adequate to perform an analysis by sex, and nothing can be concluded. Thus, isotopic concentrations of muscular tissue of females and males are needed in order to prove the sexual segregation in and offshore of this species.

CONCLUSION

In conclusion, our results demonstrate that the short fin mako shark *I. oxyrinchus* feeds mainly on bony fishes and squids, but its trophic habits include other prey such as rays, sharks, marine mammals, marine birds and crustaceans. As described, *I. oxyrinchus* feeds on abundant and available prey sources, demonstrating its role as an opportunistic feeder in the studied areas. Variation in the prey items identified in the stomach contents is a result of the latitudinal distribution of sharks and the distribution areas of the potential prey, but as these results show, the jumbo squid *D. gigas* and fish of the family Scombridae are the most important food sources across the region. The squid *D. gigas* may be considered a key species in the development of *I. oxyrinchus* populations as this squid has shown demographic explosions and traverses both hemispheres having a positive impact on the populations of its predators.

Additional trophic ecology studies in the Pacific Ocean are necessary to identify the variation in the use of food sources by *I. oxyrinchus*. There are few studies on this shark in the north and south Pacific, and this is the first report with a full description of the feeding habits of this shark in the central Pacific. These results and those reported for the same species in the Atlantic Ocean increase our understanding of the importance of this species in the ecosystem.

We suggest integrating this information into ecological models for the better management of the shark population.

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Chapter 43

BIOMARKER RESPONSE IN BLACK SEA SCORPION FISH *SCORPAENA PORCUS* TO ANTHROPOGENIC IMPACT

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ABSTRACT

Long-term and large-scale monitoring studies indicate the changes of anthropogenic impact on the water ecosystems, which can be chronically stressed by multiple environmental factors. Fish are very sensitive to anthropogenic pollution, and some of them may be tested as biomonitors for the evaluation of the ecological status and risk assessment of marine environments. Fish biochemical parameters could be directly related to the area the fish were collected. In polluted areas the exposure of fish to xenobiotics results to interaction between these compounds and biological systems which may give elevation to biochemical and physiological damage or/and adaptive mechanisms via the induction of defense immune and antioxidant systems. Biochemical and physiological parameters are used as biomarkers for contaminants and could be applied for the evaluation of environmental stress and its after-effects in fish. Biomarkers exposure to environmental stressors vary widely depending on the type of anthropogenic activity involved. Sewage and chemical pollution from industrial, agricultural, maritime transport and domestic effluents are the main sources of pollution in Black Sea ecosystem. Coastal waters are the main recipients of discharges and combine effects of the various kinds of contaminants result dramatic ecological consequences such as eutrophication, biodiversity loss, elimination of some species, worsening of their health and decline the population size. Biochemical biomarkers variables seem to be useful indicators of the health status of fish and their habitats in monitoring studies. Scorpion fish (*Scorpaena porcus*) is among the most common fish species in Black Sea coastal waters, and it was selected as biomonitor species. The biomarker response in scorpion fish from several Sevastopol bays characterizing different level of pollution was studied

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related to anthropogenic impact. Selected biomarkers of exposure and effect, including parameters of oxidative stress and oxidative damage, were good tools for the evaluation of fish health and ecological status of their habitats.

Keywords: Black Sea, bays, pollution, biomarkers, oxidative stress, monitoring

1. INTRODUCTION

Marine environment and especially the coastal waters play an important role in human activity, because more than 2 000 000 000 people live in the coastal part of the sea and ocean and use their resources directly (The World Health Report, 2002). However, despite marine coastal ecosystems are extremely productive areas, they are impacted by great anthropogenic pressure, namely from pollutants, containing in domestic, agricultural, maritime transport and industrial effluents, entering marine water. Aquatic organisms, and especially fish are very important for commercial purposes, including fishery and aquaculture. Additionally, they play the key role in ecosystem, because they belong to the vertebrates, which are at the top of the food chain. Fish health characteristics, and their responses to unfavorable living conditions, have been extensively used as indicators of habitat quality in assessment tools developed for scientific and management purposes.

Fishes' response to environmental changes is complex, depending on the intricate relations between environmental conditions and the individual ability to adapt, which depends on their life history, age, physiological status, taxonomic position (Martinez-Alvarez et al., 2005; Fonseca et al., 2011a). Usually the investigators report the elevated levels of the man-made pollution and its deleterious effects on marine organisms in all levels of their biological organization. Long-term and large-scale monitoring studies indicate the changes of anthropogenic impact on the water ecosystems, which can be chronically stressed by multiple environmental factors. Environmental pollutants transfer to fish organism and accumulate in it, caused early biological effects, especially in cell and molecular levels. Accumulation of these alterations provokes damage of organ structure, tissues and systems disfunction, biochemical changes, including ROS generation (Gillis, 2012; Gillis et al., 2014; Grinevicius et al., 2009; Kaptaner et al., 2014).

For analysis of negative factors effects on fish many different criteria are used. Fish behavior, morphological characteristics, physiological and biochemical responses to specific and multiple stressors have been extensively used to determine individual health and population status, and to assess habitat quality (Fonseca et al., 2011b; Serafim et al., 2012). A multi-biomarker approach was applied in a dynamic environment with a significant chemical contamination. Moreover, biochemical responses in natural populations of different fish species depend on their ecological and biological features, which can result from different physiological processes in them (Solé et al. 2009; Rudneva, 2012).

The present multi-biomarker approach identified of anthropogenic pollution, including industry, agriculture, fishery, aquaculture, tourism, petroleum and gas production, coastal domestic infrastructure, maritime transport impact on marine environment in Black Sea. The specificity of Sevastopol bays including their geographical position, geomorphological structure, hydrological fluctuations and different level of pollution allow to understand the

main trends of anthropogenic impact on fish health and to establish the biomarkers response on stress living conditions.

In the present work the level of several biomarkers was assessed in highly distributed in Black Sea fish species *Scorpaena porcus*. For assessment of multiple biomarker responses in fish we determined the biomarkers of exposure: (1) antioxidants level in the form of enzymes activity, that reflects the response on the toxicity of reactive oxygen species (ROS): superoxide dismutase (SOD), catalase (CAT), peroxidase (PER), glutathione reductase (GR); (2) biotransformation enzyme, phase II glutathione S-transferase (GST), which metabolizes and conjugates xenobiotics; effect biomarkers: (1) lipid peroxidation (LPO) in the form of TBARS and chemiluminescence parameters; (2) level of oxidized proteins, measured as carbonyl groups concentration; (3) aminotransferase activity, which attributed with liver function. These biochemical responses were determined in scorpion fish *Scorpaena porcus*, sampled in four Sevastopol bays. It is resident species and it is also abundant in numerous other bays and coastal waters in Black Sea coast line.

The main objective of the present study was to evaluate the potential of fish biochemical response as indicators of habitat quality in marine bays, considering both natural and anthropogenic stressors.

2. MATERIALS AND METHODS

2.1. Materials

Scorpion fish *Scorpaena porcus* is highly distributed benthic species in Black Sea (Figure 1). Because this fish is among the most common fish species in Black Sea coastal waters, it was selected as biomonitor organism. It is demersal form, inhabiting benthic biotops, its abundance is at the depth of 1-40 m. However, sometimes it is found at the depth of 1-10 m. Scorpion fish is a settled form, however, it migrates at the bottom of the sea and its activity is high at night. Spawning time begins at the end of May and finish is at the middle of September. Female spawns the eggs in twin sacks, which dissolve in marine water, and the embryos in the eggs develop in pelagic zone. Scorpion fish is a batch spawner, individual female spawns many portions and the eggs hatch every 1-2 day. Individual spawning portion contains 1100-27200 eggs (Svetovidov, 1964).

Figure 1. Scorpion fish *Scorpaena porcus*.

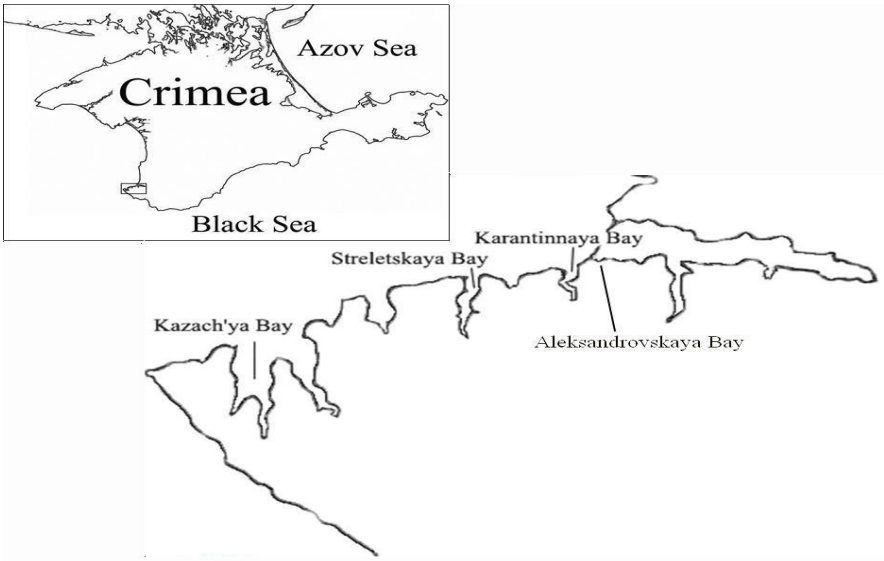

Figure 2. Sampling sites of *Scorpaena porcus* in Sevastopol bays, Black Sea.

Fish were caught in spring period of 2014 in four Sevastopol bays Streletskaya Bay (n = 17), Aleksandrovskaya Bay (n = 18), Karantynnaya Bay (n = 20) and Kazach'ya Bay (n =10), characterizing different level of anthropogenic impact and pollution (Figure 2). The animals were immediately placed in the aerated tank, transfer in the laboratory and anesthesy. For biochemical determinations liver samples were washed several times by cold 0.85% solution of NaCl, homogenized and centrifuged at 8000 g during 15 min at cool conditions. In obtained extracts biomarkers were determined.

2.2. Biochemical Assays

Antioxidant Enzyme Activities

Antioxidant enzyme activities in the scorpion fish liver in this study were determined according to methods described previously (Rudneva, 2012), with a few minor modifications. *Superoxide dismutase* (SOD) was assayed on the basis of inhibition of the reduction of nitroblue tetrasolium (NBT) with NADH mediated by phenazine methosulfate (PMS) under basic conditions (Nishikimi et al., 1972).All measurements were performed in 0.017 M sodium pyrophosphate buffer pH 8.3 at + 25°C. The reaction mixtures contained 5 μM NBT, 78 μM NADH, 3.1 μM PMS, and a 0.1 ml sample; the final volume was 1.5 ml. The reaction was carried out in the cuvette of spectrophotometer at 560 nm. *Catalase* (CAT) was measured by the method involving the reaction of hydroperoxide reduction. *Peroxidase* (PER) activity was detected by spectrophotometric method using benzidine reagent (Litvin, 1981). The reaction mixture contained 1 ml acetate buffer pH 5,4, 0.4 ml 0.09% benzidine, 0.2 ml 0.03% H_2O_2, and 0.2 ml sample. The reaction followed in a spectrophotometer for 1 min at 20°C and at 600 nm. *Glutathione reductase* (GR) activity was assayed spectrophotometrically using a method modified after Goldberg and Sparner (1987). The reaction mixture contained 0.1 ml mM NADPH, 0.5 ml 7.5 mM oxidized glutathione, 0.2 ml mM EDTA, and 2 ml 0.05 M

phosphate buffer pH 8.0. After incubation for 10 min, the extinction of the mixture was determined at 340 nm. *Glutathione-S-transferase* (GST) activity was determined by the method of Habig et al. (1974) by following the increase in absorbance at 340 nm due to the formation of the conjugate 1-chloro-2,4-dinitrobenzene (CDNB) using as substrate at the presence of reduced glutathione (GSH). The reaction mixture was prepared by mixing 1.5 ml sodium phosphate buffer 0.1 M pH 6.5, 0.2 ml GSH 9.2 mM, 0.02 ml CDNB 0.1 M and 0.1 ml of the sample. The absorbance was measured at 340 nm and at the temperature + 25°C spectrophotometrically using Specol-211 (Germany). The increase in absorbance was recorded for a total 3 min. The reaction solution without the hepatic homogenates was used as blank.

Aminotransferases Activity

Aminotransferases (ALT and AST) activity was also determined spectrophotometrically with 2,4-dinitrophenylhydrazine using the standard kit (Filicit - Diagnosis, Ukraine). Specifically, 0.2 ml substrate-buffer solution was added to 0.04 ml of liver extract or serum and incubated at room temperature for 1 h. The reaction was stopped by 0.2 ml 1,4-dinitrophenylhydrazine and the solution was incubated for 20 min. Then 2 ml 0.4 N NaOH was added to the mixture and the OD of the sample was measured at 500-530 nm. The ratio AST/ALT (de Rytis coefficient) was calculated also.

Cholinesterase (ChE) Activity

Cholinesterase (ChE) was measured on the basis on hydrolysis of acetylcholinchloride to acetic acid and choline. The alterations of pH changes the colour of the mixture which was determined spectrophotometrically used the standard kit (Felicit - Diagnosis, Ukraine).

Oxidized Proteins (OP) Concentration

The analysis of the concentration of was processed according the method of Dubinina et al. (1995). Optical density of carbonyl groups reacted with 2,4-dinitrophenil hydrazones was assayed at the following wave length 346, 370, 430, 530 nm used the spectrophotometer Specol-211 (Carl Zeiss, Iena, Germany).

TBARS Level

In obtained extracts thiobarbituric acid (TBA)-reactive substances concentration was determined spectrophotometrically, according the standard procedure (Stalnaya & Garishvily, 1977), the optical density was measured at 535 nm, as we described previously (Rudneva et al., 2012).

Chemiluminescence Analysis

Parameters of spontaneous hemiluminescence (SChL) and inducible by H_2O_2 and $FeSO_4$ chemiluminescence (IChL) were measured used Luminometer 2010 (LKB, Sweden) (Vladimirov, 2001). The value of hemiluminescence was estimated in arbitrary units per protein concentration.

Total Soluble Protein Concentration

Total soluble protein concentration was quantified spectrophotometrically used the kits of the Filicit-Diagnosis (Ukraine). The enzyme activities were calculated per mg protein in the liver extracts.

2.3. Statistical Analysis

Biochemical measurements were detected in duplicate for each sample. Simple, descriptive statistics were performed using an ANOVA to determine means (+/- SEM) (Halafyan, 2008). *P* value of 0.05 was used for determination of statistical significance in all cases. The graphs were made using the computer program EXELL. Statistical correlations between studied biochemical parameters in tested animals and tissues were calculated by the least-squares method using the computer program CURVEFIT (Version 2.10-L).

3. RESULTS

3.1. Ecological Characteristics of Tested Sites

Tested Sevastopol bays have significant anthropogenic influence from multiple sources (industrial, agricultural, shipping activities, domestic, recreation), which was evident from sediment chemical characterization. The bays are differed each from other by hydrological characteristics, hydrochemical parameters and the level of anthropogenic impact and pollution.

Figure 3. Streletskaya Bay.

For the purpose of the study of pollution effects on *Scorpaena porcus* we collected fish from four Sevastopol bays, which were ranked from most (Streletskaya Bay) to medium (Aleksandrovskaya Bay and Karantinnaya Bay) and least impacted (Kazach'ya Bay).

Streletskaya Bay (Figure 3) is the most susceptible to human impacts, because of the intense navigation and recreation that occurs there. Fuel docks and shipyards are located on its coasts; their drainage, along with the discharges of two municipal treatment enterprises and two storm water sewer systems, considerably pollute the marine environment (Ovsyanyi et al., 2001; Sergeeva, et al., 2010). According to several authors (Kiryukhina and Mironov, 2004), water and sediments in Streletskaya Bay contain considerably greater amounts of oil, heavy metals, chlorinated hydrocarbons, nutrients and suspended matter than the other three bays. The level of petroleum hydrocarbons at the bottom sediments was estimated as 705 mg/100 g wet weight, which was in 10-fold higher than in Karantynnaya Bay (Eremeev et al., 2008). The mean concentration of chlorinated hydrocarbons and petroleum hydrocarbons in the bottom sediments was estimated as 1,33 и 715,5 mg/100 g wet weight (Osadchaya, 2013). To this connection the level of petroleum hydrocarbons elevated during of 2003-2009 in 1,2-1,5-fold as compared to 1990s (Osadchaya, 2013). Concentration of 6 indicative congeners of PCBs ($\sum PCB_6$) and $\sum$ DDT in bottom sediments in Streletskaya Bay was estimated as 121 and 51 ng/g respectively (Malahova & Malahova, 2009), while mercury level was higher in 5-10-fold than the legal levels and ranged from 460 to 1063 ng/g dry weight (Kostova & Plotitcina, 2011). However, in the recent decade a change of the "quality" of the anthropogenic load was recorded in Streletskaya Bay, due to intensive building of tourist hotel complexes and shipyards on its coast.

Aleksandrovskaya Bay is involved in the great Sevastopol Bay and it is located before the pier, which separates Sevastopol Bay from the open part of the sea (Figure 4). The sediments of Aleksandrovskaya Bay were contaminated to a greater extent and they classify as the third level of oil contamination (Rubtzova, et al., 2013). Level of $\sum PCB_6$ и $\sum$ DDT in the bottom sediments in this bay was estimated as 97,4 ng/g dry weight and 14,8 ng/g dry weight respectively (Shcurova & Malahova, 2012). At the period of 1985 to 2009 concentration of petroleum hydrocarbons (PAH) in bottom sediments tended to increase in Sevastopol Bay, including Aleksandrovskaya Bay also, which is located at the entrance of Sevastopol Bay (Osadchaya, 2013).

Figure 4. Aleksandrovskaya Bay (1) and Karantinnaya Bay (2).

Karantinnaya Bay is located near Sevastopol Bay. They are separate each from other by the pier (Figure 4). In this bay the level of chlorinated hydrocarbons and petroleum hydrocarbons in the bottom sediments is estimated as 165,0 и 65,0 mg/100 g wet weight respectively (Eremeev et al., 2008). However, water in this bay contains NO_3^- - 17-116µg/l and $N_{org.}$ – 552 µg/l, which was greater than in the other Sevastopol bays (Kuftarkova et al., 2008). That was due to the different locations of the areas and the discharges of domestic wastewater in Karantinnaya Bay.

Figure 5. Kazach'ya Bay.

Kazach'ya Bay is located at 15 km from the center of the city Sevastopol between the Kamyshovaya Bay and the cape Phiolent (Figure 5). Kazach'ya Bay is recognized as reference site in the systems of Sevastopol bays, because the level of toxicants in its sediments is less then in other sampling sites. For instance, the level of mercury was estimated as 33.7 ng/g dry weight and PCB content was 152.0 ng/g wet weight (Malahova et al., 2003). Concentration of chlorinated hydrocarbons and petroleum hydrocarbons was estimated as 157.0 mg/100 g and 36.0/mg/100 g (Eremeev et al., 2008) respectively, which is the evidence of its low polluted status as compared with the other tested sites (Rubtzova et al., 2013). According the data of the chemical analysis, concentration of NO_2^-in the water was estimated as 1,0 µ/L, NO_3^- 6 µ/L, NH_4^- 11-14 µ/L, and $N_{org.}$ 447 µ/L, that is the evidence of the least anthropogenic press on the ecosystem of Kazach'ya Bay as compared to the other tested sites (Kuftarkova et al., 2008).

Despite of the additional non-persistent source of pollution, which is dolphinarium, located in the bay, according the chemical characteristics of the water and sediments, we suggest that Kazach'ya Bay can be used as reference area in comparative studies of biomarkers in scorpion fish for the evaluation of pollution effects caused anthropogenic activity. Moreover, the processes of the self-cleaning in this site are very intensive, which provides to save natural environment in general (Moiseenko, 2012).

Therefore, based on the analysis of the published data, we can conclude that among the tested Sevastopol bays Streletskaya Bay is the most negatively affected environmentally, Karantinnaya Bay and Aleksandrovskaya Bay are less contaminated, and Kazach'ya Bay is

least pressed. To the other hand, according the data of Kiryukhina and Mironov (2004), sediments in Aleksandrovskaya Bay were contaminated to a greater extent than those in Karantinnaya Bay, and the surface layers of the water, in contrast, were not polluted. That was due to the different locations of the areas and the discharges of domestic wastewater in Karantinnaya Bay.

3.2. Biomarkers Response

Antioxidant Enzyme Activity

High SOD activity ($p < 0.05$) was indicated in the liver of fish from Streletskaya Bay and Aleksandrovskaya Bay in comparison to the values of the individuals from the other sampling sites (Figure 6). No significant differences were found in SOD activity in fishes from Karantinnaya Bay and Kazach'ya Bay. Enzymatic level was the similar in the animals, caught in Streletskaya Bay and Aleksandrovskaya Bay. The greatest CAT activity ($p < 0.05$) was measured in the fish from Streletskaya Bay. The lowest activity ($p < 0.05$) was observed in the animals from Karantinnaya Bay, while no significant differences were shown between the CAT level in scorpion fish inhabiting Aleksandrovskaya Bay and Kazach'ya Bay. PER activity was more, than in 10-fold greater ($p < 0.01$) in the liver of fish from Kazach'ya Bay, the lowest value was shown in fish caught in Aleksandrovskaya Bay, no significant differences were indicated in the animals from Karantinnaya Bay and Streletskaya Bay. High GR activity was shown in the fish from Streletskaya Bay and Karantinnaya Bay, the lowest value was indicated in the animals from Kazach'ya Bay, while GR activity in the fish from Aleksandrovskaya Bay was greater in 2-fold ($p < 0.05$). The highest GST activity was shown in the individuals from Streletskaya Bay ($p < 0.05$), enzyme activity was the similar in the fish from Kazach'ya Bay and Karantinnaya Bay, while the value of the individuals from Aleksandrovskaya Bay was higher ($p < 0.05$). No relationships were observed between antioxidant enzyme activities in tested fish.

Cholinesterase Activity

ChE activity was significant higher ($p < 0.05$) in the liver of fish from Kazach'ya Bay as compared to the values of the animals from the other tested bays (Figure 7). No significant differences were observed in ChE level in the fish from Streletskaya Bay, Karantinnaya Bay, and Aleksandrovskaya Bay.

Aminotransferase Activity

Aminotransferase activity is present in Figure 8. The lowest ALT activity was indicated in the fish from Karantinnaya Bay ($p < 0.05$), while no differences were observed between enzyme activities in the liver of the fish from the other tested bays. High AST activity was shown in the scorpion fish caught in Kazach'ya Bay and Aleksandrovskaya Bay ($p < 0.05$), the lowest value was measured in the fish from Streletskaya Bay, hepatic AST activity in the individuals from Karantinnaya Bay was intermediate as compared with the values of the fish from the other tested sites.

TBARS Concentration

The greatest TBARS level was indicated in the liver of fish, collected in Streletskaya Bay ($p < 0.01$), while no significant differences were observed between the values of the individuals from the other examined bays (Figure 9).

Chemiluminescence Level

The values of spontaneous chemiluminescence (SChL) level was significantly greater ($p < 0.05$) in the hepatic extracts of the fish, caught in Streletskaya Bay and Aleksandrovskaya Bay, as compared with the values of the individuals from Kazach'ya Bay and Karantinnaya Bay (Figure 10). The response of inducible chemiluminescence (IChL) varied widely in hepatic extracts of fish in tested bays: it was the highest in the fish from Streletskaya Bay and progressively decreased in the individuals as following: Kazach'ya Bay → Karantinnaya Bay → Aleksandrovskaya Bay.

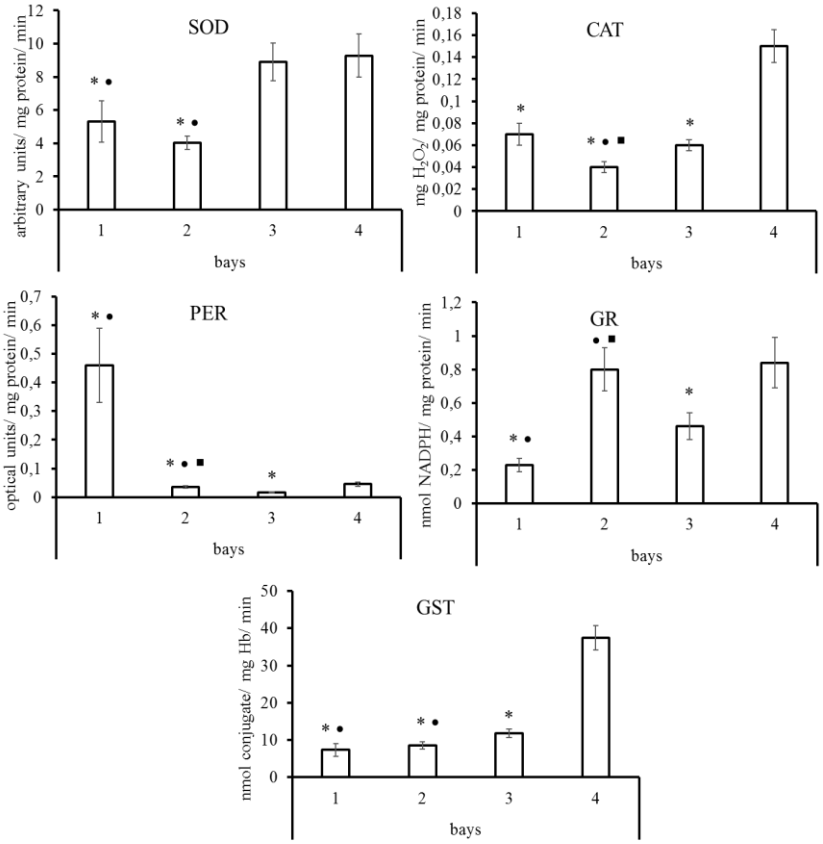

Figure 6. Antioxidant enzyme activities in the liver of scorpion fish, mean + SEM (1 – Kazach'ya Bay, 2 – Karantinnaya Bay, 3 – Aleksandrovskaya Bay, 4 – Streletskaya Bay; * – significant differences between fish from Streletskaya Bay and the values in fish from another bays; ● – with Aleksandrovskaya Bay, ▪ –with Kazach'ya Bay).

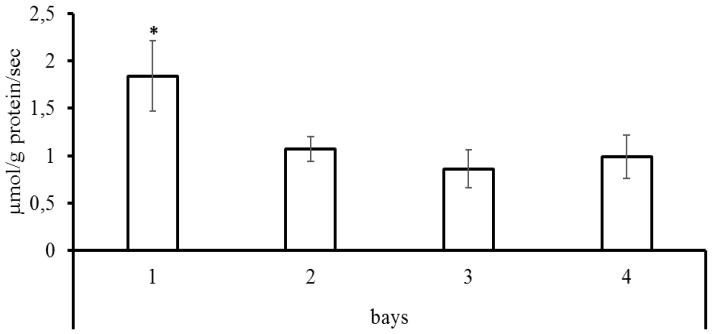

Figure 7. Cholinesterase activity in the liver of scorpion fish, mean ± SEM, 1-4 - bays, see Figure 6 (* - significant ($p < 0.05$) difference with the values of the fish captured from tested bays).

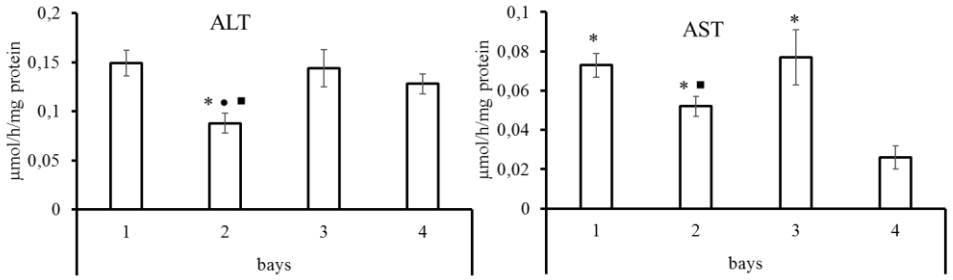

Figure 8. Aminotransferase activity in the liver of scorpion fish, mean + SEM, 1 – 4 bays, see Figure 6. * – significant differences between fish from Streletskaya Bay with another bays; ● – Aleksandrovskaya Bay, ▪ – Kazach'ya Bay).

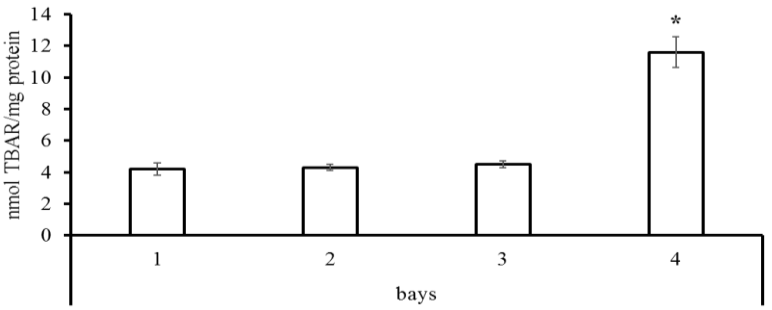

Figure 9. Concentration of TBA-reactive substances in the liver of scorpion fish, mean + SEM. 1-4 bays, see Figure 6. * - significant ($p < 0.05$) difference with values of the fish captured from tested bays).

Oxidized Proteins (OP) Concentration

Concentration of oxidized proteins (OP) in the liver of scorpion fish from checked bays is present in the Figure 11. Level of oxidized proteins, measured in all tested wave length (OD_{346}, OD_{370}, OD_{430}, and OD_{530}) was the greatest in the fish from Streletskaya Bay, high values were shown in the fish from Karantinnaya Bay and Aleksandrovskaya Bay, and the lowest level was found in the individuals caught in Kazach'ya Bay.

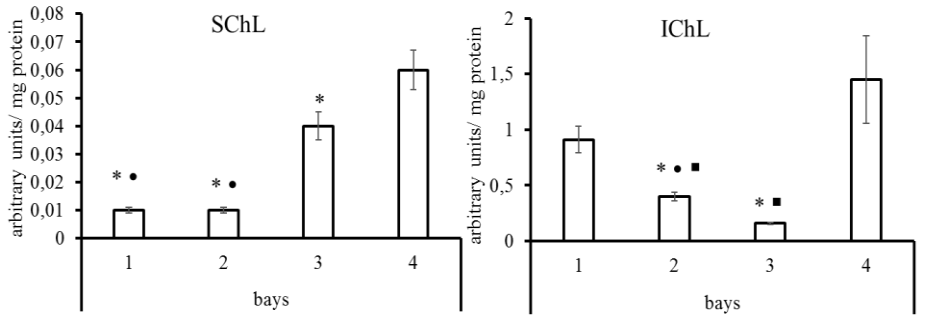

Figure 10. Chemiluminescence level in the liver of scorpion fish, mean ± SEM. SChL – spontaneous, IChL – inducible ChL. 1 – 4 – bays, see Figure 6. * – significant differences between fish from Streletskaya Bay with the values in fish from another bays; ● – with Aleksandrovskaya Bay, ▪ – with Kazach'ya Bay.

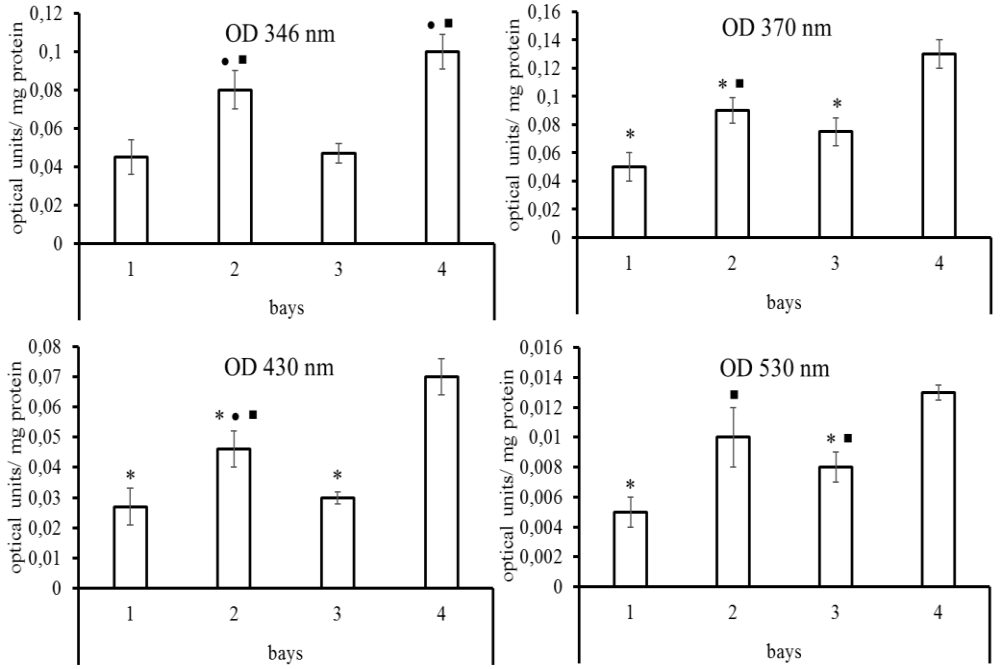

Figure 11. Concentration of oxidized proteins in the liver of scorpion fish, mean + SEM. 1-4 bays, see Figure 6. * – significant differences between fish from Streletskaya Bay and the values in fish from another bays; ● – with Aleksandrovskaya Bay, ▪ – with Kazach'ya Bay.

Therefore, we could conclude the different response of the hepatic biomarkers in scorpion fish, caught in examined Sevastopol bays.

4. DISCUSSION

The ecological characterization of examined bays in the coastal part of Sevastopol city showed the presence of diverse contaminants in the sediments and in the water, which are the result of human activities. The main anthropogenic sources of these pollutants are attributed

with industrial and agricultural discharges, domestic effluents, shipping and recreation activities. High concentration of xenobiotics in the bottom sediments and in the water in the biotops of scorpion fish causes oxidative stress in fish resulted their transfer via water and food in the organism.

In polluted areas the exposure of aquatic organisms to xenobiotics results to interaction between these compounds and biological systems which may give elevation to biochemical and physiological damage or/and adaptive mechanisms via the induction of defense immune and antioxidant systems (Goksoyr et al., 1996). Therefore, biochemical and physiological characteristics are used as biomarkers for contaminants and could be applied for evaluation of environmental stress and its after-effects. However, biomarkers exposure to environmental stressors vary widely depending on the type of anthropogenic activity involved (Adams, 2005). Biomarkers response depends on tissues and organs specificity and functions. In this context, liver is the main organ of pollutants accumulation and biotransformation, generating ROS production and its metabolites (Sole et al., 2009; Grinevicius et al., 2009; He et al., 2012; Morachis-Valdez et al., 2015).

For the evaluation of oxidative stress in fish caught in different bays, we used various parameters, and among them we studied the biomarkers of exposure - the activities of antioxidant enzymes namely SOD, CAT, PER, GR and GST, which was also recognized as phase II biotransformation enzyme (Livingstone, 2001; Van der Oost et al., 2003). Enzymatic antioxidant response in the liver of scorpion fish was characterized by a higher variability of SOD and GR activities among tested bays, while CAT, PER and GST values were more constant. In this case our results agree with the data of other researchers, who also reported the similar trends of antioxidant enzymatic response in the liver of *Dicentrarchus labrax*, caught in the estuaries, impacted by human activity (Fonseca et al., 2011a).

Antioxidant enzyme activity in the liver of fish from the most contaminated Streletskaya Bay was significantly higher as compared with the values of the animals, caught in other tested sites, with the exception of PER. Furthermore, antioxidant enzyme activity had positive correlation (with the exception of PER) with the level of anthropogenic impact in the examined bays, suggesting that pollution level increases the ROS production in fish liver and the antioxidant enzymatic system responds to an increase of environmental stress. At the other hand, increase of antioxidant enzyme activities in the fish from the most polluted bay demonstrated their ability to resist against oxidative stress and to adapt to the unfavorable living conditions. The similar responses of enzyme antioxidant system were documented in the tissues of fish affected different levels of pollutants in the laboratory and field conditions: heavy metals (Cao et al., 2010, 2011), petroleum and oil compounds resulting oil spills (Martinez-Gomez et al., 2009), organic chemicals (Al-Mamoori et al., 2014), complex urban effluents (Hansson et al., 2006), etc. We could note the specificity response of antioxidant enzymes in fish liver to different toxicants and their mixture, containing in the water and sediments as well as synergic and antagonistic effects at the case of interactions between xenobiotics and water. However, extremely high level of hepatic PER activity in scorpion fish from free contaminated Kazach'ya Bay could be attributed with the presence of short term local pollution source, which induced enzyme activity in the hepatocytes of the animals in the least impacted site.

GST activity was significantly higher (approximately in 4.5-5-fold) in the liver of fish from the most impacted Streletskaya Bay, while no significant differences were observed among the values of the individuals from less polluted and reference sites. We also noted high

positive correlation between CAT and GST activity (r = 0.87), while no relationships were observed between the other examined antioxidant enzyme values. GST plays an important role in the detoxification of lipid peroxides and demonstrates the functions such as glutathione peroxidase activity towards reactive oxygen species in the cells under oxidative stress. Induction of GST activity in some aquatic organisms such as mussels has been also found in high polluted marine environments (Hansson et al., 2006), after the wreckage and oil spills of the tankers (Martinez-Gomez et al., 2009). Increase of GST activity was documented in some fish species and invertebrates collected in environments impacted by complex discharges of contaminants and accidents (Hamed et al., 2004). Because enzyme plays an important role in biotransformation of xenobiotics, it may be used as a valid bioindicator for evaluation of pollution level. GST clearly responds on the pollution level and its response is linked with the antioxidant enzyme activities and induction of biotransformation processes in the hepatocytes. At the other hand, different factors both biotic, abiotic and anthropogenic may be determined GST activity and in our previous study we described the fluctuations of GST level in several fish species, inhabiting different biotops in the coastal waters of Black Sea (Rudneva et al., 2010). The researchers reported also, that the spatial patterns in GST activity were complex, given that high enzyme activity was observed in the liver of teleost species in highly polluted sites and in the least polluted site. The investigators suggest a strong GST inhibition by organic compounds in the liver of two species *S. senegalensis* and *P. microps* from the site, where the highest PAH concentrations were measured (Fonseca et al., 2011b).

Oxidative damage in scorpion fish liver measured as effect biomarkers, namely TBARS, oxidized proteins and chemiliuminescence level both spontaneous and inducible, showed the variability for between tested bays. Our findings indicated significant increasing (more than in 2-fold) the level of TBARS in the liver of scorpion fish caught in the most polluted Streletskaya Bay, than the corresponding values of the animals from the less polluted sites, which were comparable. High positive correlation was observed between CAT activity and TBARS level (r = 0.89) and GST activity and TBARS (r = 0.98). Therefore, we suggest, that TBARS level is a good biomarker for the evaluation of fish response to pollution. The researchers also reported the increase of LPO values in the aquatic animals exposed to pollutants (Javed et al., 2015; Jiang et al., 2011).

Similar trends were shown in chemiluminescence levels, which were attributed with the increasing of lipid peroxidation in the liver of fish, inhabiting polluted environment (Vladimirov, 2001). Both IChl and SChL values were significantly greater in the liver of the animals from the most impacted Streletskaya Bay. We found high positive correlation between ChL values in fish hepatocytes and TBARS concentration (r = 0.51) and between ChL level and CAT activity (r = 0.67). However, we measured high IChL level in the fish from the reference site Kazach'ya Bay, which could be explained for the complex influence of biotic and abiotic factors on the ROS production. Different food spectra, hydrochemical and hydrological fluctuations may be the reasons of high level of inducible chemilumioniscence in the liver of scorpion fish from Kazach'ya Bay, while the spontaneous (SChL) was lower as compared with the values of the fish from less impacted sites Aleksandrovskaya Bay and especially high polluted Streletskaya Bay. Therefore, we could conclude that ChL level in hepatocytes is also good biomarker in the procedure of the evaluation of fish health in examined sites.

Concentration of oxidized proteins (OP) in tissues is an indicator of the chronic pollution impact on fish organism resulted oxidative stress also. Oxidative stress caused unfavorable living conditions is an imbalance toward the pro-oxidant side of the pro-oxidant/antioxidant homeostasis. The content of protein carbonyl groups are good biomarker of oxidative stress because they have some advantages in comparison with the measurement of other oxidative products: they are the relative early formation and the relative stability of carbonylated (oxidized) proteins (Dalle-Donne et al., 2003; Luschak, 2007). Carbonyl (CO) groups (aldehydes and ketones) are produced on protein side chains when they are oxidized. These moieties are chemically stable, therefore protein carbonyl content is the most general indicator of protein oxidation. The accumulation and increase of carbonyls in proteins has been observed in several human diseases (Dalle-Donne et al., 2003; Luschak, 2007). Oxidative damage of proteins may result negative consequences because among proteins there are enzymes, hormones, mediators and other biomolecules, which play an important role in the metabolism and homeostasis of the organism.

In our studies we showed significant increase of oxidized proteins concentration in the liver of fish from the most contaminated Streletskaya Bay. The total level of oxidized proteins increased progressively in the hepatocytes of the individuals from Kazach'ya Bay → Karantinnaya Bay → Aleksandrovskaya Bay → Streletskaya Bay (0.127 → 0.226 → 0.16 → 0.313 correspondingly). We found high direct correlation between TBARS level and oxidized proteins concentration ($r = 0.63$). However, high correlations between GR, GT and OP were shown also ($r = 0.76$ and $r = 0.61$ respectively), which could reflect the interactions between the antioxidant enzyme activities and OP production. Growth of LPO parameters and oxidized proteins level in tissues of the animals in contaminated habitats are postulated as the non-specific response of the organism to the stress effects, and it was documented at the case of environment pollution by various chemical agents and their complex (Martinez-Porchas et al., 2011; Liu et al., 2006; Kaptaner et al., 2014).

Oppositely, we found that hepatic ChE activity progressively decreased in fish as following Kazach'ya Bay → Karantinnaya Bay → Aleksandrovskaya Bay → Streletskaya Bay. Inhibition of enzyme activity may reflect the pollution of biotope of organophosphate chemicals, metals, detergents which were recognized in domestic sewage and maritime effluents entering in Streletskaya Bay. Our results agree with the data of several researchers, who documented the inhibition of ChE activity in tissues of fish in polluted locations (Van der Oost et al., 2003; Napierska et al., 2009). ChE inhibition by organophosphorus (OP) compounds follows different behaviors depending on pesticide chemical structure. Ions can alter ChE activity inhibiting or activating so that it was proposed that enzymes could be used as biomarkers of heavy metals pollution. Another concern about application fish ChEs as biomarker of pesticides is that in eutrophed sites some species of cyanobacteria produce anticholinesterasic metabolites such as anatoxin-a which can be considered natural OP compounds and which toxicity can be approximately in 1000-fold higher than that of the insecticide paraoxon (Assis et al., 2011). Cholinesterases may reflect the disturbance of neural functions of the organism in stress conditions. Monitoring of ChE inhibition has been measured in the tissues of marine fish seems to be a valid indicator for chemical pollution of the marine environment as well (Kirbi et al., 2000). In our previous study we described the differences of ChE activity in the blood of scorpion fish caught in different polluted areas, and we also showed significantly decrease of the enzymatic activity in the blood serum of the individuals in the most impacted site (Rudneva et al., 2012).

AST is used as clinic diagnostic tool and it is attributed with cell necrosis of the liver and skeletal or cardiac muscle, starvation and lacking of vitamin E. ALT is an acute hepatic damage good marker (Coppo et al., 2001-2002). Hepatic ALT activity in scorpion fish varied less, than AST. The lowest ALT activity was indicated in the fish in Karantinnaya Bay without further site variability. AST activity varied independently and the lowest value was shown in the animals in the most polluted Streletskaya Bay. Both ALT and AST activities were lower in fish caught in Karantinnaya Bay. Therefore, at present study we reported significantly decrease of AST activity in the liver of fish in Streletskaya Bay, which can be attributed with the great intoxication, caused accumulation in the organ high concentration of toxicants. Enzyme activity could be inhibited by the complex mixture of environmental contaminants in Streletskaya Bay. In our previous study we also reported that aminotransferase activities (both ALT and AST) were lower in blood serum of fish in polluted sites (Streletskaya Bay and Karantinnaya Bay) than those in the samples, collected in free polluted areas (Rudneva et al., 2012). We propose that the reason of this phenomena was attributed with the damaged of hepatocytes in the liver of fish from highly impacted sites. To the other hand, aminotransferases are the organ-specific indicators for toxic effects and determination of transaminases in the liver has proved in diagnosis of liver damage (Van der Oost et al., 2003). Thus, the response of hepatic transaminases is not uniform and could be used as indicator of fish physiological status and health.

Therefore, we can conclude, that tested biochemical parameters in scorpion fish are good biomarkers for the evaluation of oxidative stress, caused anthropogenic pollution. However, they respond clearly at the case of the strong differences in pollution level between examined sites. Oppositely, the response was not uniform in fish from both less polluted bays and, probably, it depends on various xenobiotic combinations in them. Additionally, in these sites we could have so-called “mask effect” of pollutants which was associated with the complex effects of pollutants mixture on fish enzymatic activities. Some of toxicants induce oxidative stress and increase the level of antioxidant enzyme activities, while the others (heavy metals) bind with protein amino acids, SH-groups, carbonyls and other reactive groups, damage enzyme structure and inhibit its activity. We observed this effect in fish blood from highly polluted area, where lack or so-called “normal” enzyme activities were the result of summary effects of combinations of pollutants and their interactions (Rudneva et al., 2012). These interactions could modulate organism response and lead not only adaptive effect, but toxic one, characterizing the inhibition of defense systems, including immune and antioxidant components. Thus, in some cases in tissues of fish inhabiting high polluted water bodies the researchers observed the decrease of the activity of antioxidants and other key enzymes, which characterized clearly toxic effect caused high level of heavy metals and chlororganic compounds accumulation.

CONCLUSION

Biomarkers variables in the liver of scorpion fish from different sites of Sevastopol coastal waters, reported in this study, seem to be useful indicators of the health status of fish and water quality in monitoring studies. The best biomarkers are the following: ChE activity and GST activity, TBARS, oxidized proteins and chemiluminescence level, which are highly

sensitive to anthropogenic pollution and reflect the response of the organism on stressful environment. Antioxidant enzyme and aminotransferase activities response was not uniform and depends on examined sites and the combinations of pollutants in them. The obtained results can be applied for development monitoring management and for perspectives of conservation ecology and fish status in impacted aquatic ecosystems. The analysis of the hepatic biomarkers is important for the evaluation of fish health in human impacted water bodies and their abilities to protect against chemical pollution and keep their life in the pollute environments. As the response of biomarkers are not uniform and only measuring a battery of bioindicators can provide insight into the fish health and ecological status of their habitats.

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Chapter 44

AQUATIC INVASIVE SPECIES: ADDITIONAL STEPS COULD HELP MEASURE FEDERAL PROGRESS IN ACHIEVING STRATEGIC GOALS*

United States Government Accountability Office

WHY GAO DID THIS STUDY

Aquatic invasive species—harmful, nonnative plants, animals, and microorganisms living in aquatic habitats—damage ecosystems or threaten commercial, agricultural, and recreational activities. The Nonindigenous Aquatic Nuisance Prevention and Control Act of 1990 created the Task Force and required it to develop an aquatic nuisance (which GAO refers to as invasive) species program. The Water Resources Reform and Development Act of 2014 includes a provision that GAO assess federal costs of, and spending on, aquatic invasive species.

This chapter examines (1) how much Task Force member agencies expended addressing aquatic invasive species for fiscal years 2012-2014; (2) activities conducted by Task Force member agencies and challenges in addressing aquatic invasive species; and (3) the extent to which the Task Force has measured progress in achieving the goals of its 2013-2017 strategic plan. GAO sent a questionnaire to member agencies to obtain expenditures for fiscal years 2012-2014; interviewed member agency officials; and analyzed laws and strategic planning documents.

WHAT GAO RECOMMENDS

GAO recommends that the Task Force develop a mechanism to measure progress toward its strategic goals and help meet certain statutory requirements. Most member agencies

* This is an edited, reformatted and augmented version of a United States Government Accountability Office, Publication No. GAO-16-49, dated November 2015.

generally concurred or had no comments, but NOAA disagreed. GAO believes its recommendation is valid as discussed further in this chapter.

WHAT GAO FOUND

The 13 federal member agencies of the Aquatic Nuisance Species Task Force (Task Force) estimated expending an average of about $260 million annually for fiscal years 2012 through 2014 to address aquatic invasive species. However, several member agencies identified in their questionnaire responses challenges in developing their estimates. For example, some member agencies reported that their activities to address aquatic invasive species were often integrated into larger projects, making it difficult to isolate the portion of expenditures specific to aquatic invasive species out of total expenditures for the projects. As a result, expenditure information reported by GAO generally reflects member agencies' best estimates of total expenditures, rather than actual expenditures.

Task Force member agencies conducted a wide range of activities and identified several challenges in addressing aquatic invasive species. Member agencies reported conducting activities across several activity categories, including taking actions to prevent introductions, control the spread of existing invaders, and research ecological impacts of aquatic invasive species. For instance, most conducted prevention activities—such as constructing a series of electric barriers to prevent the entry of Asian Carp from the Mississippi River Basin into the Great Lakes—recognizing that prevention activities may be the most cost-effective method of addressing aquatic invasive species. Additionally, officials from several member agencies expressed concern that their activities, though numerous, may not be adequate relative to the growing magnitude and impacts of aquatic invasive species amid decreasing or constrained agency resources.

The Task Force—which is co-chaired by the U.S. Fish and Wildlife Service and National Oceanic and Atmospheric Administration (NOAA)—developed a 2013- 2017 strategic plan to guide its member agencies but has not taken key steps to measure progress in achieving the goals laid out in its strategic plan. As called for in its strategic plan, the Task Force in 2012 planned to develop an operational plan to track and measure aquatic invasive species activities and progress. However, the Task Force did not develop an operational plan because of constrained funding and limited resources, according to Task Force representatives. The Task Force also did not meet several of the 1990 Act's requirements including describing its members' roles and activities and reporting annually to Congress on the program's progress. The representatives agreed that a mechanism to track activities and measure progress is important and said they plan to discuss the possibility of doing so at their November 2015 meeting. Task Force representatives, however, had not established a time frame or specifics for their approach. Developing and regularly using a tracking mechanism could help the Task Force measure progress in achieving its strategic goals, as well as help the Task Force meet the 1990 Act's requirements to describe its members' roles and specific activities and to report annually to Congress on the program's progress.

ABBREVIATIONS

Corps	U.S. Army Corps of Engineers
EPA	Environmental Protection Agency
FWS	U.S. Fish and Wildlife Service
NOAA	National Oceanic and Atmospheric Administration
Task Force	Aquatic Nuisance Species Task Force
The 1990 Act	The Nonindigenous Aquatic Nuisance Prevention and Control Act of 1990
USGS	U.S. Geological Survey

November 30, 2015

Congressional Committees

Invasive species[1]—harmful, nonnative plants, animals, and microorganisms—are pervasive throughout the United States and cause major economic losses to segments of the economy and significant environmental damage each year to crops, rangelands, waterways, fisheries, and ecosystems.[2] Invasive species, called a national crisis by the Department of the Interior,[3] number in the thousands and are expected to increase, with about 250 new species having invaded the United States since 2011 and more than 750 invasive species expanding their range since that time.[4] As we have found, the impact of invasive species in the United States is widespread, and their consequences for the economy and the environment are profound, although this can be difficult to measure.[5] A widely cited academic study from 2005—the most recent comprehensive study of its kind—estimated that the environmental impacts and economic costs associated with invasive species amount to almost $120 billion per year.[6]

Addressing aquatic invasive species is a complex, interdisciplinary issue with the potential to affect many sectors and levels of government operations. Multiple federal agencies, often in coordination with state and local governments, industry, international parties, and nongovernmental agencies, work to prevent, manage, eradicate, and raise awareness about invasive species. The National Invasive Species Council, which was established by an Executive Order in 1999 to, among other things, coordinate federal agencies' activities concerning invasive species,[7] reported that estimated expenditures for invasive species activities by more than 20 federal agencies were over $2 billion dollars in fiscal year 2014.[8] This estimate encompasses expenditures for all invasive species, however, and does not separate out expenditures specific to aquatic invasive species—species found in marine, freshwater, estuarine, and riparian areas, such as fish, mollusks, snakes, plants, and pathogens or parasites of aquatic animals and plants. Aquatic invasive species, which are one type of invasive species, harm native ecosystems or commercial, agricultural, or recreational activities dependent on these ecosystems, such as by threatening commercially or recreationally important fish species, according to the National Invasive Species Council. Officials from National Oceanic and Atmospheric Administration (NOAA) and the Department of the Interior have likened aquatic invasive species to an oil spill that will

continue to spread unless promptly and completely contained—once they have arrived and become established, aquatic invasive species are difficult to eradicate.[9]

The Nonindigenous Aquatic Nuisance Prevention and Control Act of 1990, as amended (the 1990 Act), was enacted to, among other things, prevent the unintentional introduction and dispersal of nonindigenous (which we refer to as nonnative) species into waters of the United States through the management of ballast water (water in a ship's holding tank used for stability and safety that may be taken on in one location and discharged in another) and other requirements, and to understand and minimize economic and ecological impacts of nonnative aquatic nuisance species that become established in the United States.[10] The 1990 Act notes that, if preventive management measures are not taken nationwide to prevent and control unintentionally introduced nonnative aquatic species in a timely manner, further introductions and infestations of destructive species may occur. The 1990 Act created the Aquatic Nuisance Species Task Force (Task Force), which coordinates governmental efforts dealing with aquatic invasive species in the United States through regional panels, special committees, and work groups. The Task Force consists of 13 federal member agencies along with state, regional, and nongovernmental organizations.[11] Each of the Task Force's federal member agencies has a different set of responsibilities related to aquatic invasive species.[12]

The 1990 Act requires, among other things, the Task Force to develop and implement an aquatic invasive species program for waters of the United States. Specifically, the 1990 Act requires the Task Force to develop a program that identifies the goals, priorities, and approaches for aquatic invasive species prevention, monitoring, control, education, and research to be conducted or funded by the federal government. The act requires the Task Force to (1) describe the specific prevention, monitoring, control, education, and research activities to be conducted by each Task Force member; (2) describe the role of each Task Force member in implementing the elements of the program; and (3) include recommendations for funding to implement elements of the program.[13] The act also requires that the Task Force report to Congress annually on the progress of its program.[14] In 1994, The Task Force developed a program overview that established the core elements of its aquatic invasive species program and served to guide the work of the Task Force. The Task Force subsequently developed a series of strategic plans starting in the early 2000s to further guide its membership—the federal, state, regional, and nongovernmental organizations that conduct aquatic invasive species activities—in implementing the aquatic invasive species program. In 2012, the Task Force developed its most recent strategic plan, covering 2013 through 2017, which identified eight goals for the program.

The Water Resources Reform and Development Act of 2014 includes a provision in section 1039(a)(2) that GAO conduct an assessment of the federal costs of, and spending on, aquatic invasive species.[15] We briefed your offices on our preliminary results on June 3, 2015. This report transmits our final results related to this review. This report examines (1) how much Task Force member agencies expended addressing aquatic invasive species for fiscal years 2012 through 2014; (2) activities conducted by Task Force member agencies and challenges in addressing aquatic invasive species; and (3) the extent to which the Task Force has measured progress in achieving the goals of its 2013-2017 strategic plan.

For all three objectives, we reviewed aquatic invasive species-related laws, regulations, and academic studies. To determine how much Task Force member agencies expended addressing aquatic invasive species and to obtain information on activities conducted, we conducted interviews with, and obtained documentation from the Task Force and its 13

federal member departments and agencies (member agencies) regarding any expenditure information they maintain related to aquatic invasive species. We also interviewed staff from the National Invasive Species Council to learn about their efforts to collect information on federal expenditures for invasive species activities. We then developed and disseminated a questionnaire to the 13 Task Force member agencies to obtain each member agencies' estimated annual expenditures to address aquatic invasive species for fiscal years 2012 through 2014 (the most recent years for which reliable data were available) and examples of these activities.[16] The expenditures reflect the agencies' best estimates of how much they spent on aquatic invasive species activities during these years. Based on our assessment of the estimated annual expenditures reported by Task Force member agencies, we found the estimates for fiscal years 2012 through 2014 were sufficiently reliable for purposes of this report—to provide general estimates of total annual expenditures by these agencies on activities related to aquatic invasive species.

To further describe activities conducted by Task Force member agencies and any challenges in addressing aquatic invasive species, we built on the information gathered through our questionnaire and conducted a series of interviews with officials from the 13 member agencies; the federal ex-officio member of the Task Force, the Smithsonian Environmental Research Center; and each of the Task Force's six regional panels. Through these interviews, we collected information and documentation on the agencies' aquatic invasive species activities and any challenges they face in addressing aquatic invasive species. Many of the activities reported by agencies were ongoing or span multiple fiscal years, and thus, the information we collected often highlights, but is not limited to, fiscal years 2012 through 2014. We also conducted site visits in Southern Florida, Northern California, and Western Washington to observe activities and interview local federal officials at the sites. We selected these locations based on the number and variety of aquatic invasive species present and federal agencies involved, as well as the types of activities conducted in those locations.

To determine the extent to which the Task Force has measured progress in achieving the goals of its 2013-2017 strategic plan, we conducted interviews with and obtained documentation from Task Force representatives, officials from the 13 Task Force member agencies, and officials representing the six regional panels. We reviewed the Task Force's 2013-2017 strategic plan and other documentation related to its strategic plan. We then compared this information to program requirements identified in the 1990 Act, our previous reports on leading practices provided by the Government Performance and Results Modernization Act of 2010, and our executive guide on strategic planning,[17] as appropriate. Appendix I presents a more detailed description of our objectives, scope, and methodology.

We conducted this performance audit from November 2014 to November 2015 in accordance with generally accepted government auditing standards. Those standards require that we plan and perform the audit to obtain sufficient, appropriate evidence to provide a reasonable basis for our findings and conclusions based on our audit objectives. We believe that the evidence obtained provides a reasonable basis for our findings and conclusions based on our audit objectives.

BACKGROUND

Aquatic invasive species can be found in all U.S. states and territories. They can enter and travel in aquatic habitats by several common pathways, including through the discharge of ships' ballast water; hull fouling, such as barnacle growth, on commercial vessels and recreational boats; and accidental or intentional release of organisms into aquatic habitats through aquaculture, bait, aquaria (fish tanks), or the pet trade. Once established in a particular location, an aquatic invasive species can spread to other locations and ecosystems. Scientists and officials from several federal agencies said that the presence and impacts of aquatic invasive species are, and are likely to continue, growing, such as from the warming of ocean waters and the opening of shipping channels through the Arctic, allowing new species to potentially thrive in habitats previously too cold or inaccessible.

See Appendix II for more information on species examples, their known locations, and invasion pathways.

The Task Force, created by the 1990 Act, is co-chaired by the U.S. Fish and Wildlife Service (FWS) and NOAA. FWS provides funding for the administration of the Task Force, including conducting annual meetings, publishing *Federal Register* notices, and supporting an Executive Secretary and other FWS staff that work as regional coordinators.[18] To implement its aquatic invasive species program, the Task Force relies on its 13 member agencies—each of which has a different set of responsibilities related to aquatic invasive species, based on their overall mission and areas of programmatic responsibility (see table 1). These member agencies conduct aquatic invasive species activities and commit resources to achieve the goals of the aquatic invasive species program.[19] According to the Task Force's 1994 program overview, implementation of the program is a cooperative effort that will build on and fill gaps in existing activities and programs, and individual agencies will implement the program in line with their specific authorities, priorities, expertise, and funding. In addition, the Task Force is advised by six regional panels— consisting of representatives of state, tribal, and nongovernmental organizations, commercial interests, and neighboring countries—that help identify regional priorities and coordinate regional activities.[20] Some funding is provided to each regional panel as well as to state governments and other entities to support implementation of species- or region-specific aquatic invasive species management plans and other activities.[21] Together, these federal, state, and nonfederal agencies and organizations work to prevent and control aquatic invasive species and implement the 1990 Act.

Activities to address aquatic invasive species can be categorized using the seven general activity categories developed by the National Invasive Species Council. These categories reflect common activities agencies conduct along the continuum of an invasion of a species, from preventing the arrival or spread of an invading species to controlling or eradicating that species from the ecosystem. Table 2 describes each activity category.

Table 1. Aquatic Nuisance Species Task Force Member Agencies' key roles and responsibilities

Department or agency	Component agency	Key roles and responsibilities for aquatic invasive species
Department of Agriculture	Animal and Plant Health Inspection Service	Manage the Federal Noxious Weed Lista and inspect imports for prohibited aquatic plants. Control and manage aquatic animals and animal pathogens with significant impact on commercial aquaculture.
	U.S. Forest Service	Control and prevent aquatic invasive species on national forest land and participate in prevention efforts through educational outreach.
Department of Commerce	National Oceanic and Atmospheric Administration	Co-chair the Task Force. Responsible for prevention, monitoring, control, education, and research to prevent introductions and spread of aquatic invasive species in aquatic habitats such as National Marine Sanctuaries.
Department of Defense	U.S. Army Corps of Engineers	Control and manage the spread of aquatic invasive species on or around the facilities, ports, harbors, and other navigable waterways it manages. Support research programs on aquatic invasive species.
Department of Homeland Security	U.S. Coast Guard	Regulate biofouling (the accumulation of organisms on a vessel's exterior surfaces) and ballast water to help ensure aquatic invasive species are not discharged into waters of the United States.
Department of the Interior	Bureau of Land Management	Prevent and control the impacts of aquatic invasive plants and animals on fish and wildlife habitat throughout the public lands it manages.
	Bureau of Reclamation	Manage programs to control aquatic invasive species in water systems it manages, including reservoirs, rivers, and distribution canals.
	National Park Service	Manage and control aquatic invasive species established in national parks and prevent the introduction of new species in national parks.
	U.S. Fish and Wildlife Service	Co-chair the Task Force. Manage multiple programs addressing prevention, management, and control of aquatic invasive species. Enforce the Lacey Act,b including its prohibitions on the importation and interstate transport of injurious wildlife (which includes certain aquatic invasive species).
	U.S. Geological Survey	Conduct research and provide scientific information on aquatic invasive species to support the efforts of federal agencies and other partners.
Department of State		Fund the Great Lakes Fishery Commission and other agencies' efforts to address aquatic invasive species and coordinate with international parties.
Department of Transportation	Maritime Administration	Research ballast water and hull fouling issues and participate in ship hull cleaning efforts for ship disposal.
Environmental Protection Agency		Regulate discharges incidental to the normal operation of certain vessels, such as ballast water, under Sec. 402 of the Clean Water Act. Chair the Great Lakes Interagency Task Force overseeing the Great Lakes Restoration Initiative, which includes efforts to address aquatic invasive species.

Sources: Draft, Aquatic Nuisance Species Task Force 2014 Report to Congress (information); GAO (analysis). | GAO-16-49

Note: This list of agency roles and responsibilities for aquatic invasive species is not comprehensive; it is intended to illustrate key roles and responsibilities of each agency as they relate to aquatic invasive species.

[a]The Plant Protection Act authorizes the Secretary of Agriculture, who has delegated this authority to the Animal and Plant Health Inspection Service to publish, by regulation, a list of noxious weeds and to prohibit or restrict their importation, exportation, or movement in interstate commerce if necessary to prevent the introduction into the United States or the dissemination of a noxious weed within the United States (7 U.S.C. § 7712). Noxious weeds are any plant or plant product that can directly or indirectly injure or cause damage to crops, livestock, poultry, or other interests of agriculture, irrigation, navigation, the natural resources of the United States, the public health, or the environment.

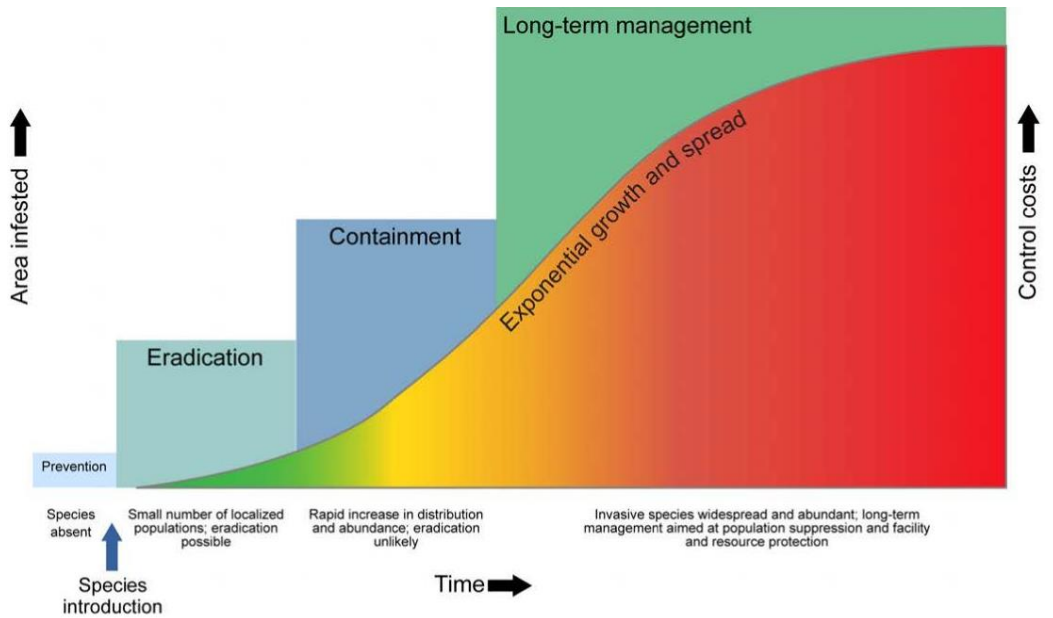
[b]Among other things, the Lacey Act, as amended, prohibits the importation into the United States and interstate transport of certain animals, including aquatic invasive species listed as injurious wildlife.

Table 2. Categories of activities to address invasive species

Activity category	Definition
Prevention	Actions taken to prevent the entry, establishment, dispersal, and dissemination of invasive species.
Early detection and rapid response	Actions taken to detect incipient invasions and assess the current and potential impact of invasions; to eradicate, contain, or control a potentially invasive nonnative species introduced into an ecosystem while the infestation of that ecosystem is still localized, and to eradicate or contain invasive species populations while they are still localized.
Control and management	Actions taken to lessen and manage the impact of invasive species within their established ranges and limit their spread.
Restoration	Actions taken to assist the recovery and reestablishment of plant and animal communities that have been overwhelmed by invasive species.
Research	Actions taken to identify, evaluate, control, and understand invasive species and their interactions with the biotic (i.e., living things that shape an ecosystem) and abiotic (i.e., not derived from living organisms) elements of the environment.
Education and public awareness	Actions taken to maintain and increase public awareness of invasive species and related programs and to promote public activities that reduce the spread and impact of invasive species.
Leadership and international cooperation	Actions taken to provide leadership, oversight, and coordination to maintain and enhance the capabilities to prevent, control, manage, and understand invasive species and invasion pathways with relevant state, local and international partners, and provide for public input and participation.

Sources: The National Invasive Species Council Invasive Species Interagency Crosscut Budget Summary (2014); GAO questionnaire of 13 Task Force member agencies. | GAO-16-49

Note: These activity categories apply to all invasive species, including both aquatic and terrestrial species.



Sources: National Invasive Species Council; U.S. Department of Agriculture; National Park Service, U.S. Fish and Wildlife Service; Rodgers, L., South Florida Water Management District; Department of Primary Industries, State of Victoria; Australia; and GAO. | GAO-16-49.

Figure 1. Invasive species invasion curve.

Preventing the introduction of aquatic invasive species into ecosystems is generally the most effective means of avoiding their establishment and spread, according to numerous academic reports, as well as the Task Force and several of its member agencies.[22] According to a 2006 study, the difficulties and expense of reversing biological invasions means investment in prevention is likely to be the most successful and cost-effective response to biological invasions.[23] Further, eradication (the elimination of an invading species from the ecosystem) and control (limiting an invasive species to a specific ecosystem) becomes increasingly difficult and costly as a species becomes established and spreads, as shown in figure 1.[24]

TASK FORCE MEMBER AGENCIES ESTIMATED EXPENDING AN AVERAGE OF ABOUT $260 MILLION ANNUALLY TO ADDRESS AQUATIC INVASIVE SPECIES IN FISCAL YEARS 2012 THROUGH 2014

Task Force member agencies estimated expending an average of about $260 million annually for fiscal years 2012 through 2014 to address aquatic invasive species. Several of the member agencies identified challenges and limitations associated with the expenditure information they provided in response to our questionnaire. As a result, the information reported by Task Force member agencies on annual expenditures through our questionnaire generally reflects the agencies' best estimates, rather than actual expenditures. Table 3 provides the estimated annual expenditures for each Task Force member agency during fiscal years 2012 through 2014.

Based on information reported through our questionnaire, estimated expenditures by Task Force member agencies for fiscal year 2014 ranged from a high of about $149 million by the U.S. Army Corps of Engineers (Corps) to a low of $70,000 by the Bureau of Land Management. Specifically, the Corps reported that the majority of its estimated annual expenditures were for controlling and managing existing aquatic invasive species at multiple projects it manages, and mostly came from the respective project's operations and maintenance funding. The Bureau of Land Management's estimates for fiscal year 2014 comprised the annual cost to develop and place aquatic invasive species awareness advertisements in print materials focusing on outdoor activities such as hunting, fishing, and boating. Also, for fiscal year 2014, the Bureau of Land Management reported that it did not have funding to provide to its state offices to coordinate or carry out aquatic invasive species activities in their local areas, as it did in fiscal years 2012 and 2013. Estimates for Task Force member agencies generally reflected a variety of activities undertaken or funded by the respective agency spanning multiple species and regions within their areas of programmatic responsibility. In contrast, estimates for some agencies reflected efforts specific to a particular region or activity. For example, the Environmental Protection Agency (EPA) reported that its estimates mostly reflected expenditures of funding transferred to other agencies to carry out activities in support of the Great Lakes Restoration Initiative—a program launched in 2010 to protect and restore the Great Lakes ecosystem. One of the Initiative's main focus areas includes prioritizing efforts to prevent the introduction of new invasive species into the Great Lakes.[25]

Table 3. Estimated annual expenditures to address aquatic invasive species, by Task Force Member Agency, Fiscal Years 2012 through 2014

Dollars in thousands			
	Fiscal year 2012 estimated expenditures	**Fiscal year 2013 estimated expenditures**	**Fiscal year 2014 estimated expenditures**
Department of Agriculture			
Animal and Plant Health Inspection Service	$3,618	$3,454	$3,467
U.S. Forest Service	a	a	a
Department of Commerce			
National Oceanic and Atmospheric Administration	3,855	3,454	3,528
Department of Defense			
U.S. Army Corps of Engineers	134,886	133,139	148,571
Department of Homeland Security			
U.S. Coast Guard	2,809	3,995	3,362
Department of the Interior			
Bureau of Land Management	298	205	70
Bureau of Reclamation	4,728	5,352	6,335
National Park Service	3,967	4,078	5,437
U.S. Fish and Wildlife Service	13,049	13,096	15,049
U.S. Geological Survey	10,229	10,034	11,325
Department of State[b]	18,575	18,106	19,341
Department of Transportation			
Maritime Administration	5,137	4,965	4,473
Environmental Protection Agency[c]	56,709	44,946	54,599
Total	**$257,860**	**$244,824**	**$275,557**

Sources: GAO questionnaires of 13 Task Force member agencies. I GAO-16-49

Note: The data in this table were provided by Task Force member agencies and reflect their best estimates of annual expenditures to address aquatic invasive species for fiscal years 2012 through 2014.

[a]The U.S. Forest Service reported that it was unable to develop estimated annual expenditures for its aquatic invasive species activities. The agency cited several reasons, including that its program management and financial accounting systems do not separately track aquatic invasive species expenditures.

[b]The Department of State estimates reflect amounts mostly provided to the Great Lakes Fishery Commission, largely for Sea Lamprey control and research efforts. The Great Lakes Fishery Commission provided financial resources to other Task Force member agencies and therefore to minimize double-counting, we have to the extent possible, excluded this funding from the estimates reported by the other Task Force member agencies.

[c]The Environmental Protection Agency's estimates reflect amounts for the Great Lakes Restoration Initiative (a program launched in 2010 to protect and restore the Great Lakes ecosystem), most of which was provided to other Task Force member agencies. To minimize double-counting, we have, to the extent possible, excluded this funding from the estimates reported by the other Task Force member agencies.

In responding to our questionnaire, several of the Task Force member agencies identified challenges and limitations in collecting information on how much they estimated expending to address aquatic invasive species. These included the following:

- **Expenditures on aquatic invasive species activities are not specifically tracked.** Seven of the 13 Task Force member agencies reported that their budget structures and financial accounting systems were not designed to specifically track expenditures on aquatic invasive species activities. For instance, the U.S. Forest Service reported that many aquatic invasive species related activities are conducted throughout the agency, but the agency's program management and financial accounting systems do not separately track aquatic invasive species expenditures. Specifically, U.S. Forest Service officials said they could not identify the portion of funding expended directly for aquatic invasive species because these activities were often integrated into larger projects—such as inspecting and cleaning equipment used in fighting wildfires. For

example, the agency has developed specific protocols to inspect, assess, and decontaminate equipment, such as the inside of a fire pump, to help make sure it is clear of any invasive algae or mussels that may be unintentionally transferred to a new watershed when moving water between areas to fight fires. U.S. Forest Service officials further explained that this is one step of many in cleaning and preparing the equipment for its next use, and its management and financial accounting systems are not set up to capture or break out activities to this level of detail. Similarly, the Bureau of Reclamation reported that expenditures for aquatic invasive species activities at its water projects—such as clearing water control structures to maintain water delivery through pipes and canals—are funded mostly through the operations and maintenance budget for each project and are not tracked as expenditures specific to aquatic invasive species.

- **Decisions on expenditures for aquatic invasive species are made at the local or regional level.** Four of the 13 member agencies reported that decisions on expenditures for aquatic invasive species activities are delegated to a regional or local level and are not tracked at the national level. For example, the National Park Service reported that once funding is provided to a national park, headquarters management does not generally direct how the funding is expended at that park. Instead, park management generally determines how the funding will be used to accomplish park objectives, including whether and how to prioritize funding for aquatic invasive species activities. Similarly, the Bureau of Land Management reported that numerous decisions and activities take place at its local or state office level that are not tracked by headquarters, including expenditures on aquatic invasive species, and, therefore, annual expenditures on aquatic invasive species across the agency are unknown. The U.S. Forest Service also reported in its questionnaire that many of its aquatic invasive species activities are conducted through cooperative partnership agreements at the local and regional level and expenditures for these activities are not reported at the national level.

TASK FORCE MEMBER AGENCIES CONDUCTED A WIDE RANGE OF ACTIVITIES AND IDENTIFIED SEVERAL CHALLENGES IN ADDRESSING AQUATIC INVASIVE SPECIES

Through our questionnaire and interviews with officials from the Task Force and its member agencies, we found that member agencies conducted a wide range of activities and faced several challenges in addressing aquatic invasive species. Most member agencies reported conducting activities across the seven general activity categories developed by the National Invasive Species Council, including taking actions to prevent introductions of new aquatic invasive species and control the spread of existing ones (see app. III). Task Force member agencies also identified several challenges in addressing aquatic invasive species. Some of these challenges are overarching, and others relate to how member agencies plan or conduct aquatic invasive species activities specific to the activity categories.

Regarding overarching challenges, several Task Force member agencies—including officials from the Departments of the Interior and Agriculture, the Corps, and NOAA—

expressed concern that their activities, though numerous, may not be adequate relative to the growing magnitude and impacts of aquatic invasive species amid decreasing or constrained agency resources. Task Force representatives further said that many of the member agencies have faced competing priorities in carrying out aquatic invasive species-related activities, with some member agencies having limited flexibility to conduct work in multiple areas. According to officials from the U.S. Geological Survey (USGS), for example, much of the agency's aquatic invasive species activities have been focused on identifying methods to treat and control Asian Carp in accordance with the Great Lakes Restoration Initiative and other funding for this work. USGS officials said that though their work on Asian Carp has been critical, it has sometimes meant that they have not been able to prioritize other needs, such as identifying marine invaders from nonballast water sources or new marine and arctic threats given the warming of ocean waters.

The following are examples of activities Task Force member agencies conducted to address aquatic invasive species along with challenges they identified related to specific activity categories, based on the responses we received to our questionnaire and interviews with officials from the Task Force and its member agencies. These examples include activities from each of the seven activity categories—(1) prevention, (2) early detection and rapid response, (3) control and management, (4) restoration, (5) research, (6) education and public awareness, and (7) leadership and international cooperation. These examples do not represent all activities conducted or challenges identified by member agencies, but rather they illustrate the nature and type of activities and challenges discussed.

Prevention

Eleven of the 13 Task Force member agencies reported conducting a range of prevention activities, often related to managing specific pathways to help prevent the introduction of aquatic invasive species into new aquatic habitats. Task Force member agencies repeatedly highlighted the importance of conducting prevention-oriented activities as a cost-effective means of addressing aquatic invasive species. Officials from some member agencies also said that they would like to conduct more prevention-oriented activities, but that they have faced challenges in doing so, in part because of policy or funding decisions within their respective agencies.[26] For example, Corps officials said they believed that it would be most cost-effective to treat certain aquatic invasive plants upstream from project boundaries before the species spreads downstream and potentially threatens project infrastructure; however, it is generally the agency's policy to treat areas within rather than outside project boundaries.[27] Some Task Force member agencies also told us that prevention activities cannot be conducted at the expense of activities aimed at controlling aquatic invasive species already established, and that a more balanced approach between prevention and control activities may be warranted.

Examples of prevention activities include the following:

- **Regulations.** The U.S. Coast Guard and EPA regulate the management of ballast water—a primary pathway for the introduction of new aquatic invasive species into and within the United States— and other vessel discharges into waters of the United States. In 2012, the Coast Guard updated its ballast water regulations to include a

standard for the allowable concentrations of living organisms allowed in a vessel's ballast water discharged in waters of the United States. In 2013, EPA issued a general permit that contains numeric technology-based limitations on acceptable concentrations of living organisms in ballast water discharge.[28]

- **Inspections.** FWS's Office of Law Enforcement inspects certain wildlife shipments to help ensure that prohibited species, including certain aquatic invasive species, do not enter the country. FWS's has about 120 inspectors at 49 ports of entry nationwide that review import documentation and conduct visual inspections of some shipments to help prevent species listed as injurious wildlife under the Lacey Act from being illegally brought into the country or across state lines.[29]
- **Physical barriers.** The Corps operates a series of electric barriers in the Chicago Area Waterway System located approximately 25 miles from Lake Michigan to prevent the entry of Asian Carp and other aquatic invasive species from the Mississippi River Basin into the Great Lakes. These barriers send out pulses to form an electric field in the water that discourages fish from crossing.

Prevention Efforts to Control the Spread of Quagga and Zebra Mussels

Several Task Force member agencies are involved in activities to prevent the spread of invasive Quagga and Zebra Mussels throughout the western United States. For example, U.S. Fish and Wildlife Service (FWS) and the National Park Service support implementation of the Quagga-Zebra Mussel Action Plan, which was developed by several state and federal agencies, as well as nongovernmental organizations in the western United States. This plan serves as a road map for identifying and prioritizing specific actions needed to prevent the further spread of Quagga and Zebra Mussels, respond to new infestations, and manage existing ones. FWS has installed signs at National Wildlife Refuges to alert boaters about the risk of these species and has funded training in 18 states on inspecting boats and other watercraft to identify and remove the mussels. The National Park Service expended approximately $2 million in fiscal year 2014 on mussel prevention and control and monitoring at nine western parks. In addition, the Bureau of Reclamation has conducted a series of public education and outreach efforts, including the dissemination of informational pamphlets at boat shows, designed to educate the public on practices they can follow to help prevent the spread of Quagga and Zebra Mussels.

Source: Bureau of Reclamation. | GAO-16-49
Quagga Mussels covering infrastructure.

Early Detection and Rapid Response

Ten of the 13 Task Force member agencies reported conducting early detection and rapid response activities—activities to detect the presence of aquatic invasive species in an area and remove any newly detected species while they are localized and before they become established and spread to new areas. Aside from preventing introductions, the most cost-effective way to address an invasive species is to detect and respond to invasions early, according to documents from the U.S. Forest Service and NOAA. However, coordinated rapid response efforts have been challenging to implement due, in part, to constraints in existing funding, according to officials from some agencies. Consequently, 11 Task Force member agencies are part of a federal work group, co-led by the Department of the Interior and the National Invasive Species Council, that in January 2015 started developing a framework for a national early detection and rapid response program and a plan for an emergency rapid response fund.[30] The work group reported in July 2015 that it plans to issue a report of recommendations to implement an early detection and rapid response framework, including mechanisms for funding, to the White House and the Council on Climate Preparedness and Resilience in the fall of 2015.

Early Detection Technique Using Environmental DNA

Detection methods such as the use of environmental DNA have become widespread among Task Force member agencies, such as the U.S. Geological Survey (USGS), the U.S. Army Corps of Engineers (Corps), the National Park Service, the Bureau of Reclamation, and the U.S Fish and Wildlife Service (FWS). Environmental DNA—genetic material shed into the environment by organisms that can be detected in samples of air, water, or soil—is a relatively new tool being used to detect invasive species, particularly in areas where the species is not abundant or is difficult to detect. For example, because they are well camouflaged in the environment, visual detection of Burmese Pythons in South Florida is difficult, with detection rates of less than 1%. Use of environmental DNA methods, however, can increase python detection rates to more than 90%, according to USGS officials. Since spring 2015, USGS researchers have been working with FWS to test water from the Loxahatchee National Wildlife Refuge in Florida to determine whether Burmese Pythons may have spread to the refuge. Although environmental DNA helps confirm the presence of an aquatic invasive species in an area, it neither confirms whether the species has become established in the area, nor does it provide information on the number or current location of any species detected.

Examples of early detection and rapid response activities include the following:

- **National early detection database.** The USGS maintains the Nonindigenous Aquatic Species Database, a publicly accessible database, to track information on the locations of aquatic invasive animals throughout the United States. Federal agencies, as well as state and local agencies and the public, can report aquatic invasive species sightings and when verified, the sightings are added to the database and updated daily by the USGS.[31]
- **Rapid response strike teams.** The FWS has five regional strike teams in place to help eradicate any new invasions as soon as possible after they are detected in the nation's 563 wildlife refuges. These strike teams survey a small portion of the acreage within national wildlife refuges when new invasions are suspected,

according to FWS officials, to determine the presence of any invasions and then take actions to eradicate or contain confirmed invasions before populations spread.

Control and Management

Eleven of the 13 Task Force member agencies reported conducting activities designed to lessen and mitigate the impact or spread of aquatic invasive species on the facilities or areas they manage. Such activities may be designed to eradicate an invading species, but where eradication is not deemed feasible, such activities are designed to manage the invader by controlling the impact of the species and its spread. Activities aimed at controlling or managing the impact and spread of invasions represent a substantial portion of overall aquatic invasive species-related activities conducted, in terms of both effort and funding, according to Task Force representatives and officials from several member agencies. Some of these officials stressed the importance of sustaining efforts to control and manage aquatic invasive species to avoid reintroductions or spread of the species. For example, Corps officials said that, after eliminating infestations of Melaleuca, an invasive wetland tree, over a prescribed 10- year treatment period, periodic treatments would still be necessary to ensure new populations do not become established. Officials from several member agencies including the Corps noted, however, that limited or inconsistent funding has, at times, made it challenging to consistently manage areas as prescribed—potentially leading to the reemergence of aquatic invasive species.

Examples of control and management activities include the following:

- **Biological controls.** To control and manage the spread of Alligatorweed, a leafy aquatic invasive plant found in the southeastern United States and California, officials from the Corps told us they are using a beetle that feeds and reproduces only on Alligatorweed. According to officials from the Corps and the U.S. Department of Agriculture, the beetle has been successful in controlling the weed, and the need for additional treatments, such as herbicide applications, has been nearly eliminated in Florida.
- **Chemical controls.** The Department of State, through the Great Lakes Fishery Commission, along with the Corps, FWS, USGS and other federal and state partners, are primarily using chemicals called lampricides to kill Sea Lamprey, an invasive fish, in their larval stage before they can attach and prey upon native fish. According to Department of State officials, as of 2015, chemical controls have led to a 90 percent reduction in the Sea Lamprey population over its historical high level.
- **Physical and mechanical controls.** The Bureau of Reclamation uses physical and mechanical control methods to remove Water Hyacinth, an aquatic invasive plant, from one of its California facilities. Bureau of Reclamation officials said that, if left untouched, Water Hyacinth clogs canals, pumps, and fish screens, which can kill the fish they are working to protect. Bureau of Reclamation officials told us that, between 2013 and 2015, they removed between 10,000 and 20,000 truckloads of Water Hyacinth from the area surrounding the facility—with a dump truck filled with

Water Hyacinth leaving the facility every 5 minutes during the height of its growing season.

Multipronged Method to Control and Manage Melaleuca

Melaleuca, an Australian tree that has destroyed many southern Florida wetlands, can be managed through a combination of biological, chemical, and physical and mechanical controls. For instance, through the introduction of weevils, a type of beetle that serves as a biological control, Melaleuca can be controlled. Researchers from the U.S. Department of Agriculture said, however, that the ability of Melaleuca trees to grow in various water depths has prevented the weevils— which require ground to burrow in—from successfully reproducing and eating the Melaleuca in swampy areas. According to National Park Service officials, Melaleuca can also be controlled if it is consistently treated over a 10-year period using the method in which the trees are first cut or hacked down with a machete or mechanical device and then sprayed with herbicides designed to kill them on the first, second, fourth, seventh, and tenth years of treatment. If this process is not followed as prescribed, however, the trees may regrow and spread. The National Park Service Exotic Plant Management Team and Everglades National Park have contributed to control of Melaleuca in South Florida, as shown in the photo below.

Source: National Park Service. | GAO-16-49
Partially treated Melaleuca forest.

Restoration

Ten of the 13 Task Force member agencies reported conducting a variety of activities to restore aquatic habitats adversely affected by aquatic invasive species. Officials from a few Task Force member agencies said that it may be possible to begin restoring habitats or ecosystems while control and management activities are under way, but in some cases aquatic invasive species may need to first be controlled or contained. According to a few member agencies, this creates a challenge in that restoration activities must wait until control activities are finished, meaning that restoration may be delayed.

Examples of restoration activities include the following:

- **Habitat restoration.** NOAA reported providing funding and technical expertise for community-based habitat restoration projects, such as providing about $925,000 in 2012 for the Lower Black River Habitat Restoration Project in Ohio. The goal of this project is to restore fish and wildlife habitat in the lower Black River through actions such as the removal of aquatic invasive plants by chemical and manual techniques followed by the planting of native shrubs.

- **Native fish restoration.** The National Park Service reported removing nonnative fish from waters in a number of parks to restore native species and enhance natural aquatic biodiversity. Officials told us that they have been expending about $1 million per year since 2013 at Yellowstone National Park on lake trout removal efforts in Yellowstone Lake. These efforts include contracting with commercial fishing crews to remove invasive lake trout that have caused a significant decline in populations of the native Yellowstone Cutthroat Trout.

Research

All 13 Task Force member agencies reported conducting or sponsoring research designed to support activities to help prevent, detect, or control the impacts or spread of aquatic invasive species, as well as determine their impacts on aquatic habitats. Research is critical to identify effective techniques for prevention, detection, control, and management of aquatic invasive species and to help clarify and quantify the effects aquatic invasive species have on native species and habitats, as well as economic costs and impacts to human health, according to Task Force documents. Officials from several member agencies and Task Force representatives noted that significant gaps in knowledge in certain areas related to aquatic invasive species is a challenge and, therefore, would like to see additional research, such as a comprehensive study to identify and assess the environmental impacts and economic costs associated with invasive species in the United States. Such information is critical to understanding the magnitude of the impacts from aquatic invasive species and for obtaining funding to address problems they are causing, according to these officials. In addition, limits in scientific knowledge about newly introduced species and the levels at which they may become established or harmful, especially in ballast water, affect member agencies' ability to manage the ballast water pathway, according to officials from NOAA and the Smithsonian Environmental Research Center. Officials from the U.S. Coast Guard said that it is difficult to set regulations or establish allowable concentrations of organisms that can be safely released in ballast water when the threshold for establishment of a new potentially invasive species may not be well understood.

Federal Research on Hydrilla

Federal research on Hydrilla, a submerged invasive plant that has clogged navigation channels and other water systems across the United States, involves efforts by several Task Force member agencies. For example, the U.S. Army Corps of Engineers (Corps) conducted research on the biology of Hydrilla during 2015 to provide a better understanding of the invasion ecology of this species in northern rivers and glacial lakes. The Corps has also researched chemical treatments and application strategies to control or alter the reproduction of Hydrilla. Chemical treatments developed through research have been successful in controlling some strains of Hydrilla, according to Corps officials. Aquatic herbicides developed through research have also been successful in controlling Hydrilla, but some strains have become resistant. In addition, the Animal and Plant Health Inspection Service, in collaboration with the Corps, is researching biological controls for Hydrilla, such as releasing insects that will eat the plant.

Examples of research activities include the following:

- **Species research.** The Corps is researching various types of invasive aquatic vegetation and options for managing such species through its Aquatic Plant Control Research Program, which is authorized by statute.[32] In 2014, Corps' researchers completed field studies in Montana that used selective management strategies to control Eurasian Watermilfoil, a plant that is invasive throughout most states, including Alaska.
- **Impacts research.** Officials from USGS and NOAA have conducted research aimed at improving scientific knowledge about how aquatic invasive species may be adversely affecting ecosystems. In 2015, USGS continued research to identify whether newly established nonnative species may warrant being considered "high priority invaders," such as the Burmese Python in the Everglades. Since 2009, NOAA has conducted research to determine how certain aquatic invasive species have affected endangered salmon feeding behavior and habitat in the Pacific Northwest as part of its effort to understand the impacts that aquatic invasive species have on these native species and the ecosystems upon which they depend.
- **Pathways research.** The Maritime Administration sponsors the operation of three research facilities—in California, Maryland, and Wisconsin—that are testing the capability of treatment systems for ballast water to determine whether those systems may be approved by the U.S. Coast Guard pursuant to its ballast water regulations.

Education and Public Awareness

Eleven of the 13 Task Force member agencies reported engaging in education and public awareness activities to increase awareness about aquatic invasive species and their impacts and help minimize or prevent further introductions. According to Task Force documents, the lack of public awareness about the impacts and threats posed by some invasive species and how they are introduced is a substantial challenge for Task Force member agencies in addressing aquatic invasive species.

Lionfish Education and Public Awareness

Several Task Force member agencies are involved in raising awareness about Lionfish, a highly invasive fish that has spread throughout coastal waters of the southeast and the Caribbean. To help raise awareness, the National Oceanic and Atmospheric Administration, along with nonprofit partners, has sponsored numerous Lionfish derbies since 2010, including 10 public tournaments in 2014 in which divers could hunt the edible fish with spears. The National Park Service produced a Lionfish Response Plan in 2012 that aims to help inform the public about the Lionfish invasion and prevent and mitigate impacts to parks. Biscayne National Park, in Florida, conducts an education program in which Lionfish removed from the park are sent to classrooms for safe dissection by students. National Park Service officials told us that concentrated education efforts like this have been effective in educating the public about Lionfish. In addition, the Department of State provided funding to work with partners in the Gulf of Mexico and the Caribbean to launch a web portal that provides managers and the public with access to the latest information on Lionfish and impacts in the Atlantic Ocean.

Examples of education and public awareness activities include the following:

- **National awareness campaigns.** The Task Force, Bureau of Land Management, FWS, U.S. Forest Service, and the U.S. Coast Guard are among the federal agencies that collaborate on the "Stop Aquatic Hitchhikers!" campaign. Since 2002, this multimedia campaign has used television, billboards, and social and print media to encourage users of outdoor recreational areas to help stop the transport and spread of aquatic invasive species by, for example, making sure they clean, drain, and dry their boats and boat trailers before transporting them to different aquatic areas.
- **Local awareness events.** The National Park Service, along with state agencies and nongovernmental organizations, hosted the inaugural 5K "Race Against Invasives" run through Everglades National Park in February 2015 to raise awareness about invasive species, especially those in Florida.

Leadership and International Cooperation

Ten of the 13 Task Force member agencies have been involved in activities to provide leadership to the aquatic invasive species community—which includes federal and nonfederal as well as international agencies working on aquatic invasive species issues—and to enhance cooperation and collaboration, such as by participating and serving as members in a range of international, national, regional, state, and local task forces, councils, and other entities. Given the often complex and widespread nature of aquatic invasive species, working across jurisdictional boundaries is the most effective approach to combating aquatic invasive species, according to Task Force officials and documents. Moreover, working with other federal and nonfederal agencies and organizations helps the Task Force to identify areas where legislation may be needed to fill gaps in statutory authority, suggest priority policy issues, and define roles and responsibilities for managing aquatic invasive species, according to Task Force documents. Officials from the regional panels told us, however, that one challenge in such work is that constrained agency funding has meant that they have not been able to consistently attend Task Force, regional panel, or other cooperative meetings.[33]

Examples of leadership and international cooperation activities include the following:

- **Aquatic Nuisance Species Task Force activities.** The Task Force conducts semiannual meetings that provide an open and public forum for members to exchange information and coordinate their aquatic invasive species activities. For example, the Task Force's May 2015 meeting included presentations on a wide range of topics, from the adoption of species-specific national management plans to recommendations from its regional panels on issues of local significance.
- **International cooperation.** Officials from the Corps and the U.S. Department of Agriculture have collaborated with scientists in China, South Korea, and Switzerland to identify and develop insect biological control agents to target invasive aquatic plants such as Hydrilla and Eurasian Watermilfoil. For example, in fiscal year 2014, Corps officials reported expending about $450,000 on developing such control agents, which included collecting 350 plant samples from more than 90 field sites to

help match invasive plants located in the United States with their countries of origin to improve the success of identifying insects to control these species.

THE TASK FORCE HAS NOT TAKEN KEY STEPS TO MEASURE PROGRESS IN ACHIEVING ITS STRATEGIC GOALS

The Task Force has not taken key steps to measure progress in achieving the goals laid out in its 2013-2017 strategic plan.[34] In 2012, the Task Force developed its 2013-2017 strategic plan, which serves to guide Task Force member agencies in conducting aquatic invasive species-related activities to implement the aquatic invasive species program. The strategic plan identifies eight goals for the program—which generally align with the seven activity categories developed by the National Invasive Species Council—as well as a number of targeted action items for Task Force member agencies to achieve these goals (see table 4).[35]

The action items identified in the strategic plan were intended to be completed over the 5-year period of the plan, but the strategic plan also stated that accomplishing the items would be dependent upon the budgets of individual agencies. The strategic plan did not identify or describe roles or activities to be conducted by specific member agencies or measures to track progress in achieving its eight strategic goals. Rather, the strategic plan called for the Task Force to develop an operational plan to specify how Task Force member agencies would put the strategic plan into operation.[36] According to the strategic plan, the function of the operational plan was to ensure the strategic goals were measurable and accountable. Specifically, the operational plan was intended to contain the following elements: (1) a description of short-term efforts to support and implement the strategic plan and its goals; (2) the roles of Task Force member agencies; (3) when available, the time frames, lead agencies or groups, and funding; and (4) regular updates with its actions reported annually to measure progress toward accomplishing the goals of the strategic plan. The elements envisioned for the operational plan are also largely required by the 1990 Act.[37]

Before the strategic plan went into effect, however, the Task Force decided not to develop an operational plan as envisioned in the strategic plan. Instead, the Task Force decided to develop a reporting matrix in the form of a spreadsheet to collect information on member agencies' aquatic invasive species-related activities, according to the Task Force's autumn 2012 meeting minutes. This reporting matrix was designed to collect information on the aquatic invasive species activities that member agencies had planned to conduct related to the goals of the strategic plan. This reporting matrix was also designed to collect funding information associated with each of these activities, which could serve as a starting point for the Task Force to identify funding gaps and priorities and develop recommendations for funding to implement elements of its aquatic invasive species program as required by the 1990 Act. The reporting matrix was disseminated to Task Force member agencies in August 2012, but fewer than half (6 of 13) of the Task Force member agencies provided information to the Task Force.[38] According to Task Force representatives, the Task Force did not disseminate or collect additional information using the reporting matrix after 2012.

Table 4. Aquatic Nuisance Species Task Force's 2013-2017 strategic goals and examples of action items

Goal	Examples of action items
Coordination -Maximize the organizational effectiveness of the Aquatic Nuisance Species Task Force.	• Coordinate the development and implementation of species and pathways management plans.
	• Increase communication among members, regional panels, and committees of the Task Force to prioritize issues and activities.
Prevention -Develop strategies to identify and prevent the establishment of new aquatic invasive species and slow the spread of existing ones in the waters of the United States.	• Recommend amendments to the injurious wildlife provisions of the Lacey Acta to allow a proactive approach for preventing the establishment of new invasive species through the trade of live organisms.
	• Develop and maintain a priority list for aquatic invasive species pathways.
Early detection and rapid response -Identify and respond to aquatic invasive species within a timely manner following introduction to prevent their establishment and/or spread.	• Increase public and industry involvement in reporting sightings of aquatic invasive species through the U.S. Geological Survey's online reporting form for its publicly available database on locations of aquatic invasive species.
	• Develop a rapid response technical support network that can provide resources and technical support in response to newly detected species.
Control and management -Control established aquatic invasive species when feasible and when the benefits of managing the established species outweigh the costs of removing them with respect to harm to the environment, the economy, and public health.	• Increase the number of training workshops for natural resource managers and the total number of personnel and volunteers trained in control measures for aquatic invasive species. • Identify gaps in control efforts and tools.
Restoration -Protect and rehabilitate native species and ecosystems by conducting habitat restoration efforts on multiple scales.	• Ensure that federal land and water management guidance manuals consider aquatic invasive species issues during the planning and development of habitat restoration projects. • Compile, highlight, and share lessons learned for both restoration successes and failures within the United States.
Education and outreach -Increase awareness concerning the threats of aquatic invasive species, emphasizing the impacts, importance of prevention and containment, and recommendations for appropriate domestic and international actions.	• Utilize the internet and social media as well as traditional media sources to disseminate information and promote awareness of aquatic invasive species. • Cooperate with media outlets to reach a broad range of the public with aquatic invasive species messages.
Research -Facilitate research to address environmental, economic, and human health risks and impacts associated with aquatic invasive species.	• Develop and maintain a list of aquatic invasive species research priorities and communicate this list to the scientific community. • Improve data collection at ports of entry so numbers and identification of species entering the United States through commerce in living organisms are available and accessible.
Funding -Coordinate federal agency budgets to support the Aquatic Nuisance Species Task Force's priorities and establish a clear process that links state and regional needs in their areas of responsibility.	• Compose an annual report focused on aquatic invasive species and use it as an opportunity to reach decisionmakers and other leaders on the need for policies and funding for aquatic invasive species efforts. • Prioritize actions based on anticipated efficacy, threat level, and costs/benefits to natural resources.

Source: Aquatic Nuisance Species Task Force Strategic Plan (2013-2017). | GAO-16-49.

Note: The action items included in this table are not comprehensive, but rather they serve as examples of the action items associated with each strategic goal and are intended to illustrate the types of actions that Task Force member agencies may take to implement the strategic plan.

[a]Among other things, the Lacey Act, as amended, prohibits the importation into the United States and interstate transport of certain animals, including aquatic invasive species listed as injurious wildlife.

According to Task Force representatives, the Task Force decided not to develop an operational plan or use the reporting matrix after 2012 because of constrained funding and limited resources. In particular, they said they were limited in their efforts because of the constrained funding environment that emerged from sequestration in 2013 and 2014.[39] According to Task Force representatives, the retirement in 2013 and the continued vacancy of its Executive Secretary has resulted in the Task Force being without dedicated staff to support updates to the reporting matrix. Task Force representatives further explained that, given the limited staff devoted directly to the Task Force, they rely on staff from member agencies to contribute to the administration of the program, but member agencies have had competing priorities and have not had the resources to contribute to developing an operational plan in the way that was originally envisioned when the strategic plan was developed. In addition, Task Force representatives said that, since 2014, the Task Force along with member agency staff, has been focused on drafting a report to Congress, an annual requirement under the 1990 Act.[40] Since its inception, the Task Force has provided one report to Congress, in 2004.

Task Force representatives said they expect to finalize and issue their draft report by the end of 2015. In reviewing a draft of the report, we found that the draft provided an overview and examples of aquatic invasive species activities conducted by the Task Force, member agencies, regional panels, and states since the Task Force's 2004 report, as well as some information on the role of Task Force member agencies in aquatic invasive species management. After they finalize the 2015 report, Task Force representatives have not indicated that they would begin submitting reports annually to meet this reporting requirement in the future.

Task Force representatives also said they have no plans to develop an operational plan, as called for in the strategic plan, but acknowledged the importance of developing a means to regularly track various member agencies' aquatic invasive species activities and measure progress toward meeting the strategic goals. Specifically, in response to our inquiry into the status of an operational plan, Task Force representatives told us in May 2015 that they planned to discuss the possibility of reviving or modifying the reporting matrix they had used in 2012. Task Force representatives subsequently told us that, during a June 2015 meeting, member agencies agreed that a tracking mechanism was important. However, they also told us that they did not determine what such a mechanism would look like, how it would be implemented and by whom, or how to address concerns expressed by some member agencies that the mechanism not burden agency staff already working at capacity in light of constrained funding. Task Force representatives said they plan to further discuss the idea of reviving or modifying the reporting matrix at their next semiannual Task Force meeting in November 2015. But, representatives could not tell us when they planned to make a decision on the approach they would take or provide specifics on what information they would collect or how they would measure progress in achieving their strategic goals.

By developing and regularly using a tracking mechanism—that would include the elements envisioned for an operational plan and required by the 1990 Act—the Task Force could better position itself to (1) measure progress in achieving its strategic goals and (2) comply with certain requirements in the 1990 Act for the aquatic invasive species program. Addressing aquatic invasive species is a complex, interdisciplinary issue with the potential to affect many sectors and levels of government operations. Strategic planning is a way to respond to this governmentwide problem on a governmentwide scale. Our past work on crosscutting issues has found that governmentwide strategic planning can integrate activities

that span a wide array of federal, state, and local entities, as well as provide a comprehensive framework for making resource decisions and holding agencies accountable for achieving strategic goals.[41] With its strategic plan, the Task Force has a framework in place to guide and integrate the numerous and varied aquatic invasive species activities spanning many member agencies. In addition to measuring progress in achieving the Task Force's strategic goals, developing and regularly using a tracking mechanism could also help the Task Force meet the 1990 Act's requirements to describe its members' roles and specific activities and to report annually to Congress on the program's progress.

CONCLUSION

Aquatic invasive species, a serious and growing problem affecting all states and U.S. territories, have been likened to a never-ending oil spill, given that they are notoriously difficult to eradicate once they become established. Though hard to calculate, the economic and ecological harm caused by aquatic invasive species is vast. Capturing how much federal agencies have expended—and will likely need to expend—to effectively address aquatic invasive species is also challenging. Consequently, it is not possible to identify how much may be needed to fully address aquatic invasive species, both in terms of current invasions or measures to prevent future invasions. Capturing how much progress federal agencies have made in combatting aquatic invasive species is similarly challenging.

The Task Force and its member agencies have taken significant steps— including conducting a wide array of activities and developing a strategic plan to guide their efforts—to address the threats and impacts of aquatic invasive species. However, the Task Force has not met several of the 1990 Act's requirements, including reporting annually to Congress on the program's progress, or developed a mechanism to ensure its strategic goals are measurable and accountable, such as through an operational plan, as called for in its strategic plan, because of constrained funding and limited resources. Task Force member agencies agreed that a mechanism to track activities and measure progress was important, but the Task Force has not decided what the mechanism would look like, how it would be implemented and by whom, or how to address concerns that it not burden agency staff already working at capacity. Developing and regularly using a tracking mechanism could help the Task Force measure progress in achieving its strategic goals, as well as help the Task Force meet the 1990 Act's requirements to describe its members' roles and specific activities and to report annually to Congress on the program's progress. Moreover, such a mechanism could provide a starting point for identifying funding gaps and priorities, better positioning the Task Force to meet the 1990 Act's requirement to include recommendations for funding to implement elements of its aquatic invasive species program.

RECOMMENDATION FOR EXECUTIVE ACTION

As the Aquatic Nuisance Species Task Force considers how to measure progress toward accomplishing its strategic goals, we recommend that the Task Force develop and regularly

use a tracking mechanism, to include elements envisioned for an operational plan and to largely meet requirements in the 1990 Act, including:

- specifying the roles of member agencies related to its strategic plan,
- tracking activities to be conducted by collecting information on those activities and associated funding,
- measuring progress member agencies have made in achieving its strategic goals, and
- reporting to Congress annually on the progress of its program.

AGENCY COMMENTS AND OUR EVALUATION

We provided the Secretaries of Agriculture, Commerce, Defense, Homeland Security, Interior, State, and Transportation and the Administrator of the EPA a draft of this report for their review and comment. Only the Department of the Interior and the Department of Commerce's NOAA provided written comments. Interior generally agreed with the report's findings and recommendation, and NOAA disagreed, as further discussed below. The Department of Defense's U.S. Army Corps of Engineers, the Department of State, and EPA indicated that they had no comments on our report through e-mail communications provided through departmental audit liaisons on October 19, October 21, and October 23, 2015, respectively. We also received e-mails provided through audit liaisons from the following departments that stated that the departments agreed with the report's findings and recommendation and had no other comments: The Department of Agriculture's Animal and Plant Health Inspection Service and U.S. Forest Service (dated October 29, and October 30, 2015, respectively); the Department of Transportation (dated October 26, 2015); and the Department of Homeland Security (dated October 15, 2015).

In its written comments, the Department of the Interior stated that it generally agreed with the findings of our report and concurred with our recommendation. Interior stated that it appreciated our review of the challenges faced by the Task Force in addressing and managing risks posed by the introduction and proliferation of aquatic invasive species. Interior stated that the Task Force, of which its FWS is a co-chair, is currently evaluating the reporting matrix to improve its utility as a tracking mechanism. Additionally, Interior stated that, at its November 2015 meeting, the Task Force agreed to track accomplishments using a modified activity tracking tool while its members continue to evaluate how best to track their activities going forward. Interior also stated that the Task Force's report to Congress is undergoing final agency review, and it is expected to be delivered to Congress in the coming months, which, together with its tracking efforts, will help provide the Task Force with a mechanism to both measure and communicate progress toward its strategic goals, as called for in our report. We agree that using a modified activity tracking tool and completing the report to Congress will be positive first steps in the Task Force's measuring progress toward accomplishing its strategic goals and meeting requirements in the 1990 Act, in accordance with our recommendation. Interior also provided technical comments, which we incorporated, as appropriate.

In its written comments, NOAA disagreed with several aspects of our findings, conclusions, and recommendation. In addition, NOAA stated that our report did not

sufficiently address certain aspects of the mandate to conduct the review contained in section 1039(a)(2) of the Water Resources Reform and Development Act of 2014. First, NOAA stated that the report did not mention future costs to mitigate the impacts of aquatic invasive species and that, although it may be difficult to give specific numbers, some information could be speculated upon. In the opening paragraph of our report, we state that the impacts of invasive species in the United States are widespread and expected to increase, with profound consequences for the economy and the environment. We cite a 2005 academic study—the most recent comprehensive study of its kind— that estimates the environmental impacts and economic costs associated with invasive species at almost $120 billion per year. Additionally, through our questionnaire, we requested that federal member agencies provide planned activities and estimated expenditures for future years. However, as we describe in the scope and methodology appendix (app. I) of our report, we decided not to report future estimated expenditures given the limited information provided by some member agencies. We believe that reporting partial information could be misleading and could underestimate likely future expenditures.

Second, NOAA stated that our analysis could have gone into more detail about current federal spending on prevention activities. We limited our reporting of expenditures for fiscal years 2012 through 2014 to estimates of *total* annual expenditures for each Task Force member agency because many member agencies reported that they could not provide estimates of their expenditures by activity category, including prevention. Third, NOAA stated that we did not address whether federal spending is adequate for the maintenance and protection of services provided by federal facilities. As we note in our report, capturing how much federal agencies have expended—and will likely need to expend—to effectively address aquatic invasive species is challenging. Given the limited information available from the Task Force member agencies on current and planned expenditures related to aquatic invasive species, we determined we would not be able to reliably conduct an analysis of the adequacy of federal spending. Lastly, NOAA stated that we chose to focus on the Aquatic Nuisance Species Task Force and its strategic plan rather than documenting other legislative and programmatic efforts that target the prevention, control, and management of aquatic invasive species. The scope of our review includes all federal member agencies of the Task Force, and in discussing activities and challenges those member agencies face in addressing aquatic invasive species, our report highlights many of the legislative and programmatic efforts those agencies are undertaking, such as efforts by the U.S. Coast Guard and EPA to regulate and manage ballast water through updated regulations.

NOAA also stated that our report did not mention federal mandates intended to address aquatic invasive species other than the Nonindigenous Aquatic Nuisance Prevention and Control Act of 1990, the National Invasive Species Act of 1996, and Executive Order 13112. NOAA stated that at its exit conference with us on July 14, 2015, it noted that many federal agencies receive additional directions or mandates to address or respond to aquatic invasive species and their impacts and that each agency must balance these mandates. We agree that federal agencies may have multiple responsibilities in addressing aquatic invasive species—we outline many of these responsibilities in table 1 of the background of our report where we describe the key roles and responsibilities of Task Force member agencies under various federal laws. Also, in describing examples of the activities and challenges member agencies face in addressing aquatic invasive species in the second objective of our report, we identify and describe many of the requirements and mandates member agencies must follow. For

example, we describe efforts of the FWS' Office of Law Enforcement to enforce the Lacey Act, which prohibits the importation and interstate transport of wildlife listed as injurious, among other things. NOAA also stated that balancing and responding to various requirements ultimately affects the agencies' ability to adequately respond to this national issue. We agree with this statement, and in our discussion of challenges faced by member agencies in addressing aquatic invasive species, we report that many of the member agencies have faced competing priorities in carrying out aquatic invasive species-related activities, with some member agencies having limited flexibility to conduct work in multiple areas.

In addition, NOAA stated that the reported presence of a species in USGS' Nonindigenous Aquatic Species database (one of two key sources we used to prepare species' location information for the map) does not mean that the species is established in a particular state's waters as the map portrays. In our draft report, in a note to the figure, we included a statement to clarify that species distributions in the map represent the reported presence of a species in at least one, but not necessarily all, bodies of water in the state, and do not necessarily indicate establishment of the species in any part of the state. To further clarify this point so as not to potentially mislead readers, in response to NOAA's comment, we have updated the figure title and note and also added a statement to this effect in the body of the report. Second, NOAA stated that Caulerpa, one aquatic invasive species we highlighted in the figure, had been eradicated. Upon receipt of this information from NOAA and in light of obtaining additional supporting data, we removed Caulerpa from the figure. Third, NOAA stated that providing points of pathways of invasion was confusing or inaccurate in some cases. We agree that the manner in which we linked our description of the pathways of invasion to the map in the draft report could be misinterpreted; consequently, in response to NOAA's comment, we disassociated the description of pathways from the map. We believe that providing a description of various pathways aquatic invasive species may use to enter and spread into new areas is important context for our report.

Furthermore, concerning our recommendation that the Task Force develop and regularly use a tracking mechanism, to include elements envisioned for an operational plan and to largely meet requirements in the 1990 Act, NOAA stated that it does not believe the recommendation can address problems faced by the Task Force. NOAA stated that, with respect to measuring progress, the Task Force agreed to use an activity matrix to compile information, but the matrix has not been updated since 2012 for several reasons, including because of uncertainties in funding, shifting priorities, and the loss of the Task Force Executive Secretary position, which has not been filled since the former Executive Secretary retired in 2013. NOAA further stated that the report does not address the underlying causes that have hindered Task Force efforts to track progress, including the limited budget under which the Task Force operates, which has been reduced significantly in recent years. Our recommendation was not intended to comprehensively address the problems faced by the Task Force, but rather was more narrowly focused. Specifically, the intent of our recommendation is to help the Task Force regularly track progress toward achieving its strategic goals in a manner that ensures it also largely meets requirements in the 1990 Act, such as reporting to Congress annually on the progress of its program. In our report, we discuss the constrained funding environment and limited resources the Task Force and its member agencies reported working under, including having limited staff devoted directly to the Task Force and facing the constrained funding environment that emerged from sequestration in 2013 and 2014. We believe that by implementing our recommendation—that

is, by developing and regularly using a tracking mechanism to include the roles of member agencies, activities conducted and associated funding, and progress made in achieving strategic goals—the Task Force would be in a better position to identify and communicate its progress, as well as funding or resource needs to address problems faced by the Task Force. As we note in our report, capturing how much federal agencies have expended—and will likely need to expend—to effectively address aquatic invasive species is challenging. But by developing and regularly using a tracking mechanism, we believe the Task Force would be better-positioned to assess funding gaps and priorities and begin to identify solutions to address the challenges member agencies face in addressing aquatic invasive species.

Finally, NOAA identified examples where it stated information portrayed in our report could have evolved into recommendations. For example, NOAA commented that a recommendation that calls for a more balanced approach in conducting prevention activities would be beneficial. In our report, we state that member agencies repeatedly highlighted the importance of conducting prevention-oriented activities as a cost-effective means of addressing aquatic invasive species. We also note that officials from some member agencies said they would like to conduct more prevention-oriented activities, but that prevention activities cannot be conducted at the expense of activities aimed at controlling aquatic invasive species already established, and that a more balanced approach between prevention and control activities may be warranted. We include this and the other examples NOAA references in our report to provide context on an issue, provide examples of activities being undertaken by member agencies, or describe challenges faced by member agencies in addressing aquatic invasive species—consistent with the objectives and scope of work conducted for this review. Consistent with government auditing standards, we are to have sufficient, appropriate evidence to provide a reasonable basis for findings and conclusions before we can develop recommendations. Based on our work, we did not have sufficient evidence to provide a reasonable basis for making recommendations on the examples NOAA identified. We encourage NOAA to continue to work with Task Force member agencies and others to pursue areas they identify as needing additional work, such as identifying ways to take a more balanced approach across prevention and control activities. We believe that by implementing our recommendation, NOAA, as one of the co-chairs of the Task Force, would be in a better position to identify funding gaps and priorities, and determine recommendations for funding based on emerging needs.

NOAA also provided technical comments, which we incorporated, as appropriate.

Anne-Marie Fennell
Director,
Natural Resources and Environment

APPENDIX I: OBJECTIVES, SCOPE, AND METHODOLOGY

This report examines (1) how much the Aquatic Nuisance Species Task Force (Task Force) member agencies expended addressing aquatic invasive species from fiscal year 2012 through 2014; (2) activities conducted by Task Force member agencies and challenges in

addressing aquatic invasive species; and (3) the extent to which the Task Force has measured progress in achieving the goals of its 2013-2017 strategic plan.

For all three objectives, we reviewed aquatic invasive species-related laws, including the Nonindigenous Aquatic Nuisance Prevention and Control Act of 1990, as amended (the 1990 Act),[1] regulations, and academic studies. We conducted interviews with, and obtained documentation from, the co-chairs of the Task Force and other Task Force representatives; officials from the 13 Task Force federal member departments and agencies (member agencies);[2] and representatives from each of the Task Force's six regional panels to learn about their roles and responsibilities, aquatic invasive species-related activities, and any expenditure information they maintain related to those activities. In addition, we interviewed staff from the National Invasive Species Council to learn about their efforts to collect information on federal expenditures for invasive species activities.

To determine how much Task Force member agencies expended addressing aquatic invasive species for fiscal years 2012 through 2014 and obtain information on activities conducted, we developed and disseminated a questionnaire to the 13 Task Force member agencies, requesting information on their estimated expenditures and activities conducted to address aquatic invasive species. Specifically, the questionnaire requested member agencies to provide estimates of their expenditures for the activities they conducted in each of the following seven aquatic invasive species activity categories: (1) prevention, (2) early detection and rapid response, (3) control and management, (4) research, (5) restoration, (6) education and public awareness, (7) and leadership and international cooperation.[3] These were the same activity categories used by the National Invasive Species Council to collect and report information for its annual invasive species interagency "crosscut" budget summary. The council's annual budget summary includes estimates of federal agency expenditures and planned funding on activities to address all types of invasive species, but it does not include a breakdown of expenditure by type, including expenditures specific to aquatic invasive species. Therefore, the council's annual budget summary provided a framework for us to follow in developing our questionnaire, but we could not use information from the budget summary to obtain or report information on federal expenditures specific to aquatic invasive species. Several Task Force member agency officials recommended that we follow the council's framework for our questionnaire since many of the member agencies provide information to the council, and they suggested that following a similar framework would facilitate their ability to respond to our request. In developing our questionnaire, we worked with staff from the National Invasive Species Council and conducted pretests with three member agencies to obtain their comments, which were incorporated as appropriate.

In our questionnaire, we requested that each member agency provide (1) its estimated expenditures for fiscal years 2012 through 2014 (the most recent years for which member agencies reported reliable data were available), (2) examples of aquatic invasive species activities conducted during this time period, and (3) its planned activities and estimated expenditures for future years, which we defined as fiscal years 2015 and 2016. We also included questions about how the Task Force member agencies prepared their estimates, their sources of information, any challenges or limitations in preparing the estimates, and whether the estimates were reviewed by their budget or financial offices.

We received completed responses from all 13 of the Task Force member agencies. The member agencies provided information on their activities conducted to address aquatic invasive species, but member agencies varied in the level of detail they provided about their

estimated expenditures. Twelve of the 13 member agencies included at least some information on their estimated expenditures for fiscal years 2012 through 2014, but the U.S. Forest Service reported that it was unable to provide estimates. For the other 12 agencies, they varied in their ability to provide consistent and complete information on their estimated expenditures at the level of detail we requested in our questionnaire. With respect to the expenditure information for fiscal years 2012 to 2014, some agencies were able to provide estimates of their expenditures by activity category, but many reported that they could not provide estimates at this level of detail. For example, the Environmental Protection Agency reported its expenditures supported activities for five of the seven activity categories, but because it could not provide separate estimates for each of these categories it reported all of its expenditures under the prevention category. Similarly, the National Park Service reported conducting activities in all seven activity categories in fiscal years 2012 and 2013, but provided estimates for two activity categories (research and restoration) and reported that it was unable to determine how much of its estimated expenditures went toward the other five activity categories in these years. Based on inconsistencies and incomplete responses across the 13 member agencies, we decided to limit our reporting for fiscal years 2012 through 2014 to estimates of total annual expenditures for each Task Force member agency.

With respect to future expenditures for fiscal years 2015 to 2016, a few member agencies indicated they did not have estimates of expenditures for future years, though others had partial estimates. To avoid reporting potentially misleading information that could underestimate likely future expenditures compared to amounts reported for fiscal years 2012 through 2014, we decided not to report the future expenditure estimates provided to us. Similarly, 9 of the 13 member agencies reported that they were not able to provide estimates for how much they expended addressing specific aquatic invasive species, citing reasons such as expenditures being tracked at a project level rather than by a specific species. Therefore, we do not include species-specific expenditure information in our report.

After receiving completed questionnaires, we followed up with Task Force member agency officials to obtain clarification or additional information, as needed. We did not independently verify the accuracy of the estimated expenditures reported by the member agencies, which likely include some over- and some under-estimates. For example, in its response, the U.S. Fish and Wildlife Service (FWS) described various activities that were implemented through projects supported with grant funding from the Wildlife Sport Fish Restoration Program.[4] But, FWS did not include expenditure estimates for these project activities because it could not reliably estimate how much of the grant funding should be attributed to the aquatic invasive species component of the grant-funded projects. We asked each of the Task Force member agencies for their assessment of whether their estimated expenditures for fiscal years 2012 to 2014 were an underestimate, overestimate, or about right. Ten of the member agencies responded that their estimates were "about right," and two indicated they were underestimates (one member agency did not provide estimates). Accordingly, the expenditures reflect the agencies' best estimates of how much they expended on aquatic invasive species activities during these years. Based on our assessment of these responses, along with the responses provided through the questionnaire, we determined that the expenditure estimates for fiscal years 2012 through 2014 were sufficiently reliable for purposes of this report—to provide general estimates of total annual expenditures by Task Force member agencies on activities to address aquatic invasive species.

To describe the activities conducted by Task Force member agencies and any challenges in addressing aquatic invasive species, we built on the information gathered through our questionnaire and conducted a series of interviews with officials from the 13 member agencies, the federal ex-officio member of the Task Force (the Smithsonian Environmental Research Center), and each of the Task Force's six regional panels. Through these interviews, we collected information and documentation on aquatic invasive species activities conducted and any challenges agencies identified in addressing aquatic invasive species. Many of the activities and challenges relate to ongoing activities that span multiple fiscal years and thus the information we collected often highlights, but is not limited to, fiscal years 2012 through 2014. We also conducted site visits in Southern Florida, Northern California, and Western Washington to interview local federal officials and observe activities at the sites, such as inspections of shipments of live fish to search for aquatic invasive species and research being conducted at research facilities. We selected these locations based on the number and variety of aquatic invasive species and federal agencies, as well as the types of activities conducted in those locations. Information we obtained from our interviews and site visits on activities conducted and challenges identified are not generalizable, but we believe the examples we obtained provide important insights into the wide array of aquatic invasive species activities being undertaken across the 13 Task Force member agencies and the challenges agencies face in conducting those activities.

To determine the extent to which the Task Force has measured progress in achieving the goals of its 2013-2017 strategic plan, we conducted interviews with and obtained documentation from Task Force representatives, officials from the 13 Task Force member agencies, and officials representing the six regional panels. We reviewed the Task Force's 2013-2017 strategic plan, its 2012 reporting matrix, and other documentation related to the Task Force's efforts to collect information related to its strategic plan. We then analyzed and compared this information to program requirements identified in the 1990 Act,[5] our previous reports on leading practices provided by the GPRA Modernization Act of 2010,[6] and our executive guide on strategic planning,[7] as appropriate.

We conducted this performance audit from November 2014 to November 2015 in accordance with generally accepted government auditing standards. Those standards require that we plan and perform the audit to obtain sufficient, appropriate evidence to provide a reasonable basis for our findings and conclusions based on our audit objectives. We believe that the evidence obtained provides a reasonable basis for our findings and conclusions based on our audit objectives.

APPENDIX II: EXAMPLES OF AQUATIC INVASIVE SPECIES AND THEIR REPORTED PRESENCE BY STATE, AND COMMON PATHWAYS

Table 5 provides descriptions of the aquatic invasive species used as examples, and table 6 provides descriptions of common pathways of invasion.

Table 5. Description of examples of aquatic invasive species

Species	States affected	Species description
Common Water Hyacinth	Alabama; Arizona; Arkansas; California; Colorado; Connecticut; Delaware; Florida; Georgia; Hawaii; Illinois; Kentucky; Louisiana; Massachusetts; Mississippi; Missouri; New Jersey; New York; North Carolina; Oregon; South Carolina; Tennessee; Texas; Virginia; Washington	A free-floating flowering plant that forms dense colonies that block sunlight and crowd out native species, and can clog water intake structures, canals, and irrigation systems, therefore damaging ecosystems, raising operation costs, and impacting navigation of waterways. Common Water Hyacinth has become established in the Southeast, Northeast, and along the Pacific Coast of the United States after being introduced from South America through the ornamental aquarium trade.
Chinese Mitten Crab	California; Connecticut; Delaware; Louisiana; Maryland; Michigan; New Jersey; New York; Ohio; Oregon; Washington	Crustaceans that threaten fisheries and aquatic ecosystems, cause clogging at water intake structures, and have the potential to transport disease to humans. Chinese Mitten Crabs are native to the Pacific Coasts of China and Korea. They were introduced to California, the Great Lakes, and the Mid-Atlantic Coast through ballast water discharge and the intentional or accidental release of these crabs purchased as food. Chinese Mitten Crabs are listed as an injurious species under the Lacey Act, making it illegal to import or transport them between states.
Sea Lamprey	Illinois; Indiana; Michigan; Minnesota; New York; Ohio; Pennsylvania; Wisconsin	Fish that prey on native fish by attaching to the outside of a native host fish and draining its nutrients, often killing the host fish. Sea Lamprey can devastate native fish populations and harm fishing industries. They are native to the Atlantic Ocean, but invaded the Great Lakes by passing through canals.
Asian Carp	Every state but Alaska, Montana, and Rhode Island	Collectively refers to four species: Grass Carp, Bighead Carp, Black Carp, and Silver Carp. These fish harm ecosystems, threaten native species, and are also a danger to human health when they jump out of the water, which can injure or distract recreational boaters. Found naturally in Russia and China, each species of Asian Carp was intentionally introduced in the United States to improve water systems. Specifically, Grass Carp were intended for vegetation control, Bighead and Silver Carp for water quality improvement, and Black Carp for aquaculture-related work on parasite control. However, these fish escaped their introduction areas and, as a group, Asian Carp have invaded lakes in nearly all U. S. states, including the invasion of Grass Carp into the Great Lakes. Bighead Carp, Black Carp, and Silver Carp are listed as injurious species under the Lacey Act, making it illegal to import or transport them between states.
Zebra and Quagga Mussels	Alabama; Arizona; Arkansas; California; Colorado; Connecticut; Delaware; Georgia; Illinois; Indiana; Iowa; Kansas; Kentucky; Louisiana; Maryland; Massachusetts; Michigan; Minnesota; Mississippi; Missouri; Nebraska; Nevada; New Jersey; New York; North Dakota; Ohio; Oklahoma; Pennsylvania; South Dakota; Tennessee; Texas; Utah; Vermont; Virginia; West Virginia; Wisconsin	Distinct species of mussels that cause similar types of damage to native ecosystems by destroying native fish habitat and food webs. Both species of mussels colonize on water supply pipes, which can impact hydroelectric and nuclear power plants, public water supply plants, and industrial facilities. These mussels also affect navigation and boating by increasing the weight on vessels and sinking navigation buoys. They arrived in the United States from the Black, Caspian, and Azov Seas through ballast water discharge and have spread across much of the continental United States from recreational boats and fishing gear. Zebra Mussels (but not Quagga Mussels) are listed as an injurious species under the Lacey Act, making it illegal to import or transport them between states.

Table 5. (Continued)

Species	States affected	Species description
Northern Snakehead	Arkansas; California; Delaware; Florida; Illinois; Maryland; Massachusetts; New Jersey; New York; North Carolina; Pennsylvania; Virginia	A fish that competes with native species for food and habitat and can eat small birds and mammals in addition to other fish and aquatic reptiles. It originates from China, Russia, and Korea and has invaded numerous areas of the north and Mid-Atlantic Coast of the United States as well as Florida, Illinois, Arkansas, and California. The U.S. Fish and Wildlife Service estimates that snakeheads arrived in U.S. waters as an import for the live food market and became invasive by intentional release from the aquarium trade. Northern Snakehead is listed as an injurious species under the Lacey Act, making it illegal to import or transport them between states.
Nutria	Alabama; Arizona; Arkansas; California; Delaware; Florida; Georgia; Idaho; Iowa; Kansas; Kentucky; Louisiana; Maryland; Minnesota; Mississippi; Missouri; Nebraska; Nevada; New Mexico; New York; North Carolina; Ohio; Oklahoma; Oregon; Pennsylvania; Tennessee; Texas; Virginia; Washington; West Virginia	An aquatic invasive mammal, resembling a beaver, which has destroyed habitat and threatened endangered species in many states. Nutria overgraze wetland habitats, destroying ecosystems. It is native to southern South America and was originally imported into Louisiana for fur farming, but escaped to areas including Louisiana, Maryland, Oregon, Texas, and Washington. In Maryland, populations of Nutria have largely been eliminated, according to Animal and Plant Health Inspection Service officials.
Snowflake Coral	Hawaii	A soft, nonreef forming coral that grows over hard surfaces, but also grows quickly over native corals and reef surfaces, which kills native reefs, especially the native black coral that supports a vibrant economy and ecosystem in Hawaii. Snowflake Coral also feeds on zooplankton that supports reef ecosystems. It is native to the Caribbean and was introduced through hull fouling to Hawaii.
Water Lettuce	Arizona; California; Colorado; Connecticut; Delaware; Florida; Georgia; Hawaii; Kansas; Louisiana; Maryland; Mississippi; Missouri; New Jersey; New York; North Carolina; Ohio; South Carolina; Texas	A floating aquatic invasive plant that grows into dense mats that can clog waterways and water intake structures. Scientists disagree as to whether the plant is native to the continental United States or originated from South America or Africa, but agree that it was likely transported by ballast water across the United States and is invasive in Hawaii.
New Zealand Mudsnail	Arizona; California; Colorado; Idaho; Illinois; Minnesota; Montana; New York; Nevada; Ohio; Oregon; Pennsylvania; Utah; Washington; Wisconsin; Wyoming	A small freshwater snail that can cause declines in native snail populations by out competing them and can alter stream ecosystems through their rapid spread. The New Zealand Mudsnail originates from New Zealand and adjacent small islands and has become established in the western United States and the Great Lakes. It is believed that the snail was introduced to the Great Lakes and western states through ballast water discharge and hull fouling.
Alligatorweed	Alabama; Arkansas; California; Florida; Georgia; Illinois; Kentucky; Louisiana; Mississippi; North Carolina; Oklahoma; South Carolina; Tennessee; Texas; Virginia	A leafy aquatic plant that forms dense mats that crowd out native species and impedes recreational activities such as boating, swimming, and fishing. Alligatorweed originates from South America and was introduced through ballast water discharge in the southeastern United States and California.
Hydrilla	Alabama; Arizona; Arkansas; California; Connecticut; Delaware; Florida; Georgia; Indiana; Iowa; Kentucky;	A submersed aquatic plant that can grow in thick mats and has the ability to regrow from small pieces of the plant. It can clog canals, irrigation, and structure vents. Hydrilla is listed as a Federal Noxious Weed, which restricts its importation,

Species	States affected	Species description
	Louisiana; Maine; Maryland; Massachusetts; Mississippi; New Jersey; New York; North Carolina; Pennsylvania; South Carolina; Tennessee; Texas; Virginia; Washington	exportation, and interstate movement. According to U.S. Army Corps of Engineer officials, bacteria can grow on the surface of Hydrilla, such as Aetokthonos hydrillicola, and kill some waterfowl and Bald Eagles when they ingest the bacterium. Hydrilla is found in the Southeast, Northeast, Washington, California, Arizona, Indiana, and Iowa. It was introduced from Asia through the aquarium trade.
Lionfish	Alabama; Florida; Georgia; Louisiana; Mississippi; New Jersey; New York; North Carolina; Rhode Island; South Carolina; Texas; Virginia	A saltwater fish that preys on native fish communities and damages fragile coral ecosystems. Lionfish have several spines that contain potent venom that can administer a painful sting to humans and potential predators. As a result, it has few natural predators outside its native habitat. Lionfish are native in the western Pacific Ocean, Indian Ocean, and Red Sea; they are invasive in the western Atlantic Ocean, Caribbean Sea, and Gulf of Mexico. The arrival of Lionfish in the Atlantic Ocean is attributed to the aquarium trade and intentional release of pet Lionfish.
Melaleuca	Florida; Hawaii; Louisiana	An invasive tree that crowds out native species and causes damage to ecosystems, especially delicate wetlands. Melaleuca has adapted to grow at varying levels of water and each tree can hold millions of seeds, which allow this invasive tree to spread rapidly. It is originally from Australia and was brought to the United States as an ornamental plant used for erosion control. It is found in Hawaii, Florida, and Louisiana and is listed as a Federal Noxious Weed, which restricts its importation, exportation, or interstate movement.
Burmese Python	Florida	A constrictor snake that preys on and competes with native species. It is native to Southeast Asia and is invasive in Southern Florida. It was introduced by pet owners through escape or intentional release. The Burmese Python is listed as injurious wildlife under the Lacey Act, making it illegal to import or transport the snake between states.

Sources: USGS; NOAA; USFWS; EPA; USDA; Hawaii Invasive Species Council; Nonindigenous Aquatic Species Database; PLANTS Database; Global Invasive Species Database; GAO. | GAO-16-49.

Table 6. Examples of common pathways

Common pathways	Description of common pathways
Trade	The transport of items through commerce or other exchanges—by boat, airplane, the mail, or other transportation methods—increases the probability of introducing aquatic invasive species. Aquatic invasive species can be intentionally sent through trade or mail routes such as with Chinese Mitten Crabs for human consumption, or they can hitchhike by various transportation modes or on other species.
Ballast water	Water in a ship's holding tank that is used for stability and safety. When ballast water is acquired in one body of water or location and released in another, the potential for invasive species to be released and impact coastal communities is immense. Ballast water discharge is a common pathway through which aquatic invasive species are introduced into U.S. waters. Ballast water can introduce aquatic invasive species at any port in fresh or saltwater around the United States. For example, the introductions of Quagga and Zebra Mussels have been attributed to ballast water discharges.
Hull fouling	The accumulation of organisms on a vessel's exterior surfaces can occur on commercial, recreational, or military vessels. Also called biofouling, hull fouling can introduce aquatic invasive species into any water body where boats or other water vessels are used and species become detached from surfaces. Snowflake Coral, for example, was introduced to Hawaii through this pathway.

Table 6. (Continued)

Common pathways	Description of common pathways
Outdoor gear and bait	Fishing and hiking gear, as well as live bait, from outdoor activities has been known to spread aquatic invasive species to new waters. Fishing equipment, diving gear, and other recreational items that are transported among water bodies increase the risk of introduction. Discarding unused live bait after fishing can also introduce species that disrupt their new ecosystems and eliminate competing native species. For example, New Zealand Mudsnails have been unintentionally transported on fishing equipment across water bodies throughout the United States.
Boats and boat trailers	Recreational boats and boat trailers are a common pathway of introduction for aquatic invasive species, such as Hydrilla. Small pieces of this plant can survive on trailers and boat motors and regrow in new locations. Boat trailers are a common pathway for species to move from one water body to another and numerous federal and state agencies are invested in preventing species' spread through this pathway through the "Stop Aquatic Hitchhikers!" campaign and cleaning stations at boat launches across the United States. Boats and boat trailers, for example, are estimated to have spread Quagga and Zebra Mussels from the Great Lakes to other water bodies in the United States.
Aquarium release	Escape or intentional release of unwanted pets and ornamental or aquarium plants, as well as escape or intentional release of species from aquaculture farms, can be a source of invasive species in all parts of the country. Lionfish and Burmese Pythons, for example, were introduced to U.S. waterways after being released from aquaria.
Intentional introductions	Illegal stocking and the live food industry can introduce aquatic invasive species. Although it may be prohibited by law, people release fish into new waters for sport fishing, which can have severe impacts on ecosystems. The import of live, exotic plants and animals and the release of those species in new locations to create local live food sources is another pathway of intentional introduction. The Northern Snakehead, for example, is thought to have been introduced in Maryland from the live food industry after it became a popular food source.

Sources: USGS; NOAA; USFWS; EPA; USDA; Hawaii Invasive Species Council; Nonindigenous Aquatic Species Database; PLANTS Database; Global Invasive Species Database; GAO. | GAO-16-49.

APPENDIX III: AQUATIC INVASIVE SPECIES ACTIVITIES CONDUCTED BY TASK FORCE MEMBER AGENCIES

Through our questionnaire to the 13 federal member agencies of the Aquatic Nuisance Species Task Force (Task Force), we requested that member agencies identify the types of aquatic invasive species activities they conducted during fiscal years 2012 through 2014, including how those activities fell within the seven general activity categories developed by the National Invasive Species Council. The Task Force member agency responses are summarized in Table 7.

Table 7. Aquatic invasive species activities conducted by task force member agencies, by activity category, Fiscal Years 2012 through 2014

Agency	Prevention[a]	Early detection and rapid response[b]	Control and management[c]	Restoration[d]	Research[e]	Education and public awareness[f]	Leadership and international cooperation[g]
Department of Agriculture							
Animal and Plant Health Inspection Service	√	√	√	√	√	√	√
U.S. Forest Service	√	√	√	√	√	√	√
Department of Commerce							
National Oceanic and Atmospheric Administration	√	√	√	√	√	√	√
Department of Defense							
U.S. Army Corps of Engineers	√	√	√	√	√	√	√
Department of Homeland Security							
U.S. Coast Guard	√		√		√	√	√
Department of the Interior							
Bureau of Land Management			√	√	√	√	
Bureau of Reclamation	√	√	√	√	√	√	√
National Park Service	√	√	√	√	√	√	√
U.S. Fish and Wildlife Service	√	√	√	√	√	√	√
U.S. Geological Survey		√			√		√
Department of State	√	√	√	√	√	√	√
Department of Transportation							
Maritime Administration	√				√		
Environmental Protection Agency	√	√	√	√	√	√	
Total number of agencies conducting activity	11	10	11	10	13	11	10

Sources: GAO questionnaires of 13 Task Force member agencies. I GAO-16-49

Note: These seven activity categories were developed by the National Invasive Species Council Activities and reflect common activities agencies conduct along the continuum of an invasion of a species. These activity categories apply to all invasive species, including both aquatic and terrestrial species.

[a]*Prevention* includes actions taken to prevent the entry, establishment, dispersal, and dissemination of aquatic invasive species.

[b]*Early Detection and Rapid Response* includes actions taken to detect the presence of an aquatic invasive species and assess current and potential impact of the introduction. Rapid Response includes activities taken to eradicate, contain, or control a potentially invasive species introduced into an ecosystem before it spreads.

[c]*Control and Management* includes actions taken to lessen and manage the impact of aquatic invasive species within their established ranges and limit their spread.

[d]*Restoration* includes actions taken to assist the recovery and reestablishment of aquatic plant and animal communities that have been overwhelmed by aquatic invasive species.

[e]*Research* includes actions taken to identify, evaluate, control, and understand aquatic invasive species and their interactions with the biotic and abiotic elements of the environment.

[f]*Education and Public Awareness* includes actions taken to maintain and increase public awareness of aquatic invasive species and programs and to promote public actions that reduce the spread and impact of aquatic invasive species.

[g]*Leadership and International Cooperation* includes actions taken to provide leadership, oversight and coordination to maintain and enhance the capabilities to prevent, control, manage, and understand aquatic invasive species and invasion pathways with relevant state, local, and international partners, and provide for public input and participation.

END NOTES

[1] For the purposes of this report, we define an invasive species as a nonnative species—to include all taxa of animals, plants, and microorganisms—the introduction of which does or is likely to cause economic or environmental harm or harm to human health.

[2] GAO, *Invasive Species: Clearer Focus and Greater Commitment Needed to Effectively Manage the Problem*, GAO-03-1 (Washington, D.C.: Oct. 22, 2002); *Invasive Species: Cooperation and Coordination Are Important for Effective Management of Invasive Weeds*, GAO-05-185 (Washington, D.C.: Feb. 25, 2005); CRS, Invasive Non-Native Species: Background and Issues for Congress (Washington, D.C.: Nov. 25, 2002); and the Department of the Interior's *National Invasive Species Council Five-Year Review of Executive Order 13112 on Invasive Species* (Washington, D.C.: 2005).

[3] The Department of the Interior's 2014 Invasive Species Action Plan states that invasive species pose one of the greatest threats to the ecological, economic, and cultural integrity of U.S landscapes. The plan also states that the number and impacts of aquatic invasive species are expected to escalate in the coming decade due to, among other things, the global movement of people and materials from increased tourism and trade that will further disperse species around the world.

[4] EDDMapS. August 2015. The University of Georgia, Center for Invasive Species and Ecosystem Health. Available online at www.eddmaps.org/tools/query.

[5] GAO-05-185; GAO-03-1; and GAO, *Invasive Species: Federal and Selected State Funding to Address Harmful, Nonnative Species*, GAO/RCED-00-219 (Washington, D.C.: Aug. 24, 2000).

[6] D. Pimentel, R. Zuniga, and D. Morrison, "Update on the environmental and economic costs associated with alien-invasive species in the United States," *Ecological Economics* 52 (2005). This study is the most recent comprehensive assessment of the costs associated with invasive species on a national scale available, according to officials from several federal agencies that work on invasive species issues.

[7] 64 Fed. Reg. 6183 (Feb. 8, 1999). National Invasive Species Council, Invasive Species Interagency Crosscut Budget (May 27, 2015). In 1999, Executive Order 13112 established the National Invasive Species Council, to, among other things, provide national leadership regarding invasive species and coordination of federal agency activities concerning invasive species relying to the extent feasible and appropriate on existing organizations. As part of this effort, the National Invasive Species Council has been identifying funding sources and spending by federal agencies on invasive species activities through an annual "crosscut" budget summary.

[8] Expenditures are the actual spending of money; an outlay. GAO, *A Glossary of Terms Used in the Federal Budget Process*, GAO-05-734SP (Washington, D.C.: September 2005).

[9] As we have previously found, a fundamental concept to invasiveness is that invasive species have been introduced into an environment in which they did not evolve, and they usually have no natural predators to limit their spread. GAO-03-1.

[10] 16 U.S.C. § 4701(b).

[11] 16 U.S.C. § 4721(b).The Nonindigenous Aquatic Nuisance Prevention and Control Act of 1990, as amended, designated the following as Task Force members: the Director of the U.S. Fish and Wildlife Service, the Undersecretary of Commerce for Oceans and Atmosphere, the Administrator of the Environmental Protection Agency, the Commandant of the U.S. Coast Guard, the Assistant Secretary of the Army (Civil Works), the Secretary of Agriculture, and the head of any other federal agency the Task Force chairpersons deem appropriate. As of July 2015, the Task Force was co-chaired by representatives from the U.S. Fish and Wildlife Service and the National Oceanic and Atmospheric Administration, and membership consists of the following 13 federal agencies and departments: (1) Animal and Plant Health Inspection Service, (2) U.S. Forest Service, (3) National Oceanic and Atmospheric Administration, (4) U.S. Army Corps of Engineers, (5) U.S. Coast Guard, (6) Bureau of Land Management, (7) Bureau of Reclamation, (8) National Park Service, (9) U.S. Fish and Wildlife Service, (10) U.S. Geological Survey, (11) Department of State, (12) Department of Transportation's Maritime Administration, and (13) Environmental Protection Agency. In addition, the act, as amended, authorized the chairpersons to invite representatives from state agencies and other governmental entities to participate as ex-officio members of the Task Force. As of 2015, the Task Force included 13 state, regional, and nongovernment entities as ex-officio members, along with six regional panels.

[12] The Task Force uses the terms "aquatic invasive species" and "aquatic nuisance species" interchangeably. The 1990 Act uses the term "aquatic nuisance species." For purposes of this review, we use the term "aquatic invasive species." According to the definition we used in our questionnaire, aquatic species include all animals and plants as well as pathogens or parasites of aquatic animals and plants totally dependent on aquatic

ecosystems for at least a portion of their life cycle. Bacteria, viruses, parasites and other pathogens of humans are excluded.

[13] 16 U.S.C. § 4722(b).

[14] 16 U.S.C. § 4722(k)(2).

[15] Pub. L. No. 113-121, § 1039(a)(2), 128 Stat. 1193, 1237 (2014).

[16] We also requested that Task Force member agencies provide us with details about their aquatic invasive species related expenditures, such as expenditures by categories of activities and specific aquatic invasive species of concern. The agencies varied in the level of detail they were able to provide to us. Because of the incompleteness and inconsistency of the data reported across the Task Force member agencies, we did not include this information in our report.

[17] GAO/GGD-96-118.

[18] According to Task Force representatives and FWS officials, in fiscal year 2014, about $260,000 in funding was provided through the FWS' Branch of Aquatic Invasive Species program budget to support administration of the Task Force.

[19] Several agencies have included addressing invasive species (or aquatic invasive species in particular) as part of their strategic planning efforts that guide agency activities and resource expenditures. Specifically, the Department of the Interior, with five member agencies on the Task Force, includes addressing invasive species as a stated goal as part of its departmentwide goals contained in its 2014-2018 strategic plan. Similarly, in its 2015-2020 strategic plan, the U.S. Forest Service includes addressing invasive species as part of its goal to sustain the nation's forests and grasslands. In addition, in September 2015, FWS's Fish and Aquatic Conservation Program released its 2016-2020 strategic plan in which managing aquatic invasive species is identified as one of seven goals. This plan described specific challenges related to aquatic invasive species that program staff plan to conduct over the 5-year period to accomplish the plan's goals.

[20] Each of the six regional panels meets at least once a year to share information and coordinate activities, among other things, and each panel is responsible for reporting back to the Task Force on its progress in implementing aquatic invasive species activities and to provide recommendations. The 1990 Act directed the Task Force to establish two of the six regional panels and to encourage development of additional regional panels.

[21] According to Task Force representatives and FWS officials, in fiscal year 2014 FWS provided over $3 million in funding, including approximately $1 million to support implementation of 40 state and interstate aquatic nuisance species plans with Task Force approved management plans; $240,000 to six regional panels; $1 million for the Quagga-Zebra Mussel Action Plan; $300,000 for the National Asian Carp Management and Control Plan; and $700,000 for expenditures on other national species management and control plans.

[22] David M. Lodge, et al., "Biological Invasions: Recommendations for U.S. Policy and Management" in *Ecological Society of America*, 16(6) (Washington, D.C., 2006); Brian Leung et al., "An ounce of prevention or a pound of cure: bioeconomic risk analysis of invasive species" in *The Royal Society*, DOI 10.1098/rspb.2002.2179; and David Finnoff et al., "Take a risk: Preferring prevention over control of biological invaders," *Ecological Economics* 62 (2007) 216-222; U.S. Environmental Protection Agency, Office of Wetlands, Oceans, and Watersheds, "Overview of EPA Authorities for Natural Resource Managers Developing Aquatic Invasive Species Rapid Response and Management Plans" (Washington, D.C.: December 2005).

[23] David M. Lodge, et al., "Biological Invasions: Recommendations for U.S. Policy and Management" in *Ecological Society of America*, 16(6) (Washington, D.C., 2006).

[24] See also G.M. Ruiz and J. T. Carlton, *Invasive Species: Vectors and Management Strategies*, chapter 5, 2nd ed. (2003); and Daniel Simberloff, "How Much Information on Population Biology Is Needed to Manage Introduced Species?," *Conservation Biology* 17, no. 1 (February 2003). "The most effective way to deal with invasive species, short of keeping them out, is to discover them early and attempt to eradicate or at least contain them before they spread."

[25] Great Lakes Interagency Task Force, *Great Lakes Restoration Initiative Action Plan Fiscal Years 2010-2014*, 09-P-0231 (Washington, D.C.: Feb. 21, 2010.) The plan includes a "zero tolerance policy" for invasive species. We recently reported on information available about the Initiative's activities and results, including those addressing invasive species, see GAO, *Great Lakes Restoration Initiative: Improved Data Collection and Reporting Would Enhance Oversight*, GAO-15-526 (Washington, D.C.: July 21, 2015).

[26] The preference to do more prevention-oriented activities, according to officials from several member agencies, includes activities under both the *prevention* and *early detection and rapid response* activity categories.

[27] To treat aquatic invasive species outside project boundaries in the past, the Corps partnered with state and local agencies. Specifically, the River and Harbor Act of 1958, as amended, authorizes a comprehensive U.S. Army

Corps of Engineers program to provide for the prevention, control, and progressive eradication of noxious aquatic plant growths and aquatic invasive species from navigable and other waters of the United States. The law requires a cost share from local interests for the program's projects (known as the Aquatic Plant Control program). Before 1996, the Corps utilized this authority to fund aquatic invasive plant control projects with local and state governments. According to Corps officials, Corps management stopped requesting funding for this control program in 1996 based on a determination that the local and state governments receiving the benefits of this work should be responsible for paying all of the costs. From fiscal year 1998 through fiscal year 2012, the Corps conducted limited activities under this Aquatic Plant Control program when Congress would appropriate or direct funds for it, according to Corps officials.

[28] 78 Fed. Reg. 21938 (Apr. 12, 2013). In October 2015, the United States Court of Appeals for the Second Circuit ruled that EPA acted arbitrarily and capriciously with respect to five aspects of the permit and remanded these aspects to EPA to adequately address them; however, the permit remains in force while EPA does so.

[29] A species can be added to the list of injurious wildlife by statutory amendment or by FWS rulemaking. The most recent listings were in March 2015, when FWS added four reptiles to the list. See 80 Fed. Reg. 12702 (Mar. 10, 2015). As of April 2015, there were about 240 mammals, birds, reptiles, fish, crustaceans, and mollusks—of which about 150 are considered aquatic invasive species—that were listed as injurious wildlife. This includes species of walking catfish (100), snakeheads (28), zebra mussels (6), carp (4), mitten crab (3) and snakes (9). In addition, FWS officials indicated that all salmonids (approximately 170 species) are listed due to their pathogen risk, which renders them injurious. In October 2015, FWS finalized a change to the process by which it adds new species to its list of injurious wildlife to make it more efficient and allow the agency to better prevent the introduction of species that are injurious. 80 Fed. Reg. 66554 (Oct. 29, 2015).

[30] This work was initiated in response to the October 2014 Priority Agenda Enhancing the Climate Resilience of America's Natural Resources, issued by the White House Council on Climate Preparedness and Resilience.

[31] USGS officials told us that, before 2012, the Nonindigenous Aquatic Species Database also included data on aquatic invasive plants, but that they stopped including plant data in the database because of funding constraints at the time. In June 2015, USGS officials told us that an increase in funding to their Invasive Species Program in fiscal year 2015 has allowed them to resume their efforts to collect and report data on aquatic invasive plants as part of this database, and that adding plant information back to the database was a high funding priority for the agency partly due to requests from other federal agencies and partners to do so.

[32] 33 U.S.C. § 610(a)(1).

[33] Some regional panel representatives told us that it has been difficult for them to fully conduct activities, including attending semiannual Task Force meetings, due to decreased funding provided to the panels. Beginning in fiscal year 2012, funding for the regional panels decreased from $300,000 to $240,000 annually, which represents a decrease from $50,000 to $40,000 per panel. Similarly, Task Force representatives said that funding to support implementation of state and interstate management plans has remained level at about $1 million annually, but that the number of approved plans eligible for assistance has increased each year, resulting in less funding being awarded per state plan.

[34] For the Task Force's 2013-2017 strategic plan, see http://anstaskforce.gov/Documents/ ANSTF%20Strategic% 20Plan%202013-2017.pdf.

[35] We have previously found that, though a legal requirement specific to federal agencies and departments, the Government Performance and Results Modernization Act of 2010 serves to guide executive federal agency planning and provides leading practices for strategic planning at agencies and entities that work closely with or are a part of the federal government, including task forces. (See GAO, *Environmental Justice: EPA Needs to Take Additional Actions to Help Ensure Effective Implementation*, GAO-12-77 (Washington, D.C.: Oct. 6, 2011.) The identification of goals in the Task Force's Strategic Plan is consistent with such leading practices.

[36] We have previously found that the Government Performance and Results Modernization Act of 2010—which calls for a performance plan to implement an agency's strategic plan—provides leading practices for strategic planning for task forces. The operational plan described in the Task Force's Strategic Plan is consistent with such leading practices.

[37] The 1990 Act requires the Task Force to submit a report to Congress annually detailing the progress of its program, as well as to develop a program that (1) describes the specific prevention, monitoring, control, education and research activities to be conducted by each Task Force member; (2) describes the role of each Task Force member in implementing the elements of the program; and (3) includes recommendations for funding to implement elements of the program.

[38] All six regional panels also provided information to the Task Force for the reporting matrix in 2012.

[39] As a result of sequestration in 2013 and 2014, federal agencies reduced or delayed some services and disrupted agency operations. For information on 2013 sequestration, see GAO, *2013 Sequestration: Agencies Reduced Some Services and Investments, While Taking Certain Actions to Mitigate Effects*, GAO-14-244 (Washington, D.C.: Mar. 6, 2014).

[40] The 1990 Act requires the Task Force, beginning in 1992, to submit a report to Congress annually detailing the progress of its program.16 U.S.C. § 4722(k)(2).

[41] GAO, *Climate Change: Adaptation: Strategic Federal Planning Could Help Government Officials Make More Informed Decisions*, GAO-10-113 (Washington, D.C.: Oct. 7, 2009).

End Notes for Appendix I

[1] 16 U.S.C. §§ 4701-4751.

[2] We defined our scope to include the 13 federal department and agencies that are the federal members of the Aquatic Nuisance Species Task Force, as established by the 1990 Act. Other federal agencies may also conduct aquatic invasive species-related activities.

[3] We also included an eighth category labeled "other" for member agencies to report any activities they determined did not fit within any of the seven categories.

[4] 50 C.F.R. pt. 80-86.

[5] 16 U.S.C. § 4722(b).

[6] GPRA Modernization Act of 2010, Pub. L. No. 111-352, 124 Stat. 3866 (2011).

[7] GAO/GGD-96-118.

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Chapter 45

AQUATIC NUISANCE SPECIES TASK FORCE STRATEGIC PLAN (2013-2017)*

Aquatic Nuisance Species Task Force

EXECUTIVE SUMMARY

Aquatic nuisance species (ANS) are nonindigenous species that threaten the diversity or abundance of native species, the ecological stability of infested waters, and/or any commercial, agricultural, aquacultural, or recreational activities dependent on such waters. ANS include nonindigenous species that may occur within inland, estuarine, or marine waters and that presently or potentially threaten ecological processes or natural resources. The term ANS is often used interchangeably with aquatic invasive species, the preferred term of Federal and State managers. An aquatic invasive species is defined as a species not native to the ecosystem under consideration whereby introduction of this species does or is likely to cause economic or environmental harm or threaten human health.

ANS are one of the largest threats to the ecosystems and economies of the United States. Approximately 49% of the species on the threatened or endangered species lists are at risk primarily because of predation or competition with exotic species. In fact, impacts of invasive species are second only to habitat destruction as a cause of global biodiversity loss. ANS such as snakehead fish, sea lamprey, hydrilla, and the New Zealand mudsnail may prey upon, displace, alter habitat, or otherwise harm native species. Other ANS may reduce production of fisheries, decrease water availability to residential and commercial users, block transport routes, choke irrigation canals, foul industrial and public water supply pipelines, degrade water quality, accelerate filling of lakes and reservoirs, and decrease property values. The damages to human enterprises caused by ANS results in enormous economic costs. The United States invests more than $120 billion per year in damage and control costs to combat invasive species. As the world trade network continues to grow, the number and frequency of introduced species are expected to increase. Additionally, climate change may also allow

* This is an edited, reformatted and augmented version of a document approved by the ANS Task Force, May 3, 2012.

increased introductions. This plan presents the strategic priorities designed to address ANS in the United States under current and future conditions.

In 1990, Congress passed the Nonindigenous Aquatic Nuisance Prevention and Control Act to establish a broad national program to prevent introduction and control the spread of introduced ANS; this legislation was reauthorized and furthered with the National Invasive Species Act in 1996. The Aquatic Nuisance Species Task Force (ANSTF) is an intergovernmental organization dedicated to preventing and controlling aquatic invasive species and implementing these Acts. The ANSTF, co-chaired by the U.S. Fish and Wildlife Service and National Oceanic and Atmospheric Administration, consists of 13 Federal agency representatives and 13 *ex-officio* representatives. These members work in conjunction with Regional Panels and issue-specific committees to coordinate efforts amongst agencies as well as efforts of the private sector and other North American interests.

The ANSTF serves to develop and implement a program for waters of the United States to prevent introduction and dispersal of ANS, monitor, control, and study such species, and disseminate related information. In 1994, the *Aquatic Nuisance Species Program* document was drafted to guide the work of the ANSTF, establishing the core elements of the Task Force. A broader focus was established with the 2002-2007 and 2007-2012 Strategic Plans, placing more emphasis on prevention strategies. This *Aquatic Nuisance Species Task Force Strategic Plan 2013- 2017* (Strategic Plan) carries through many of the goals and objectives established in previous plans by remaining focused on prevention, monitoring, and control of ANS as well as increasing public understanding of the problems and impacts associated with invasive species. The Strategic Plan also calls attention to other areas of management including habitat restoration and research. The Strategic Plan establishes eight goals, each with objectives and action items to be completed in the next 5 years.

Coordination: The ANSTF was created to facilitate cooperation and coordinate efforts between Federal, State, tribes, and local agencies, the private sector, and other North American interests. The objectives for the coordination goal include strengthening cooperation at both national and regional levels within the ANSTF and the Regional Panels and encouraging the development and implementation of ANS plans and regulations.

Prevention: Prevention is the first-line of defense against ANS. This goal calls for developing strategies to identify and reduce the risk of ANS introduced by increasing development and use of risk assessments, Hazard Analysis and Critical Control Point programs (HACCP), and pathway assessment and interdiction options.

Early Detection and Rapid Response: Early Detection and Rapid Response programs are designed to monitor habitats to discover new species soon after introduction, report sightings of previously unknown species in an area, and work quickly to keep the species from becoming established and spreading. Objectives for the ANSTF include improving detection and monitoring programs and facilitating development and implementation of rapid response contingency plans.

Control and Management: Control and management tools are needed to assess, remove, and contain ANS populations as well as to guide management decisions. The ANSTF will implement this goal by evaluating and providing support to management plans, increasing training opportunities, and encouraging the development of management techniques.

Restoration: Habitat restoration is an essential to guard against future invasions and to minimize harm from invasive species. This goal focuses on restoring impacted ecosystems

and consideration of potential ANS during planning and implementation of restoration activities.

Education/Outreach: The lack of awareness concerning ANS impacts is one of the largest management obstacles. Few people understand the threat some nonindigenous species pose and how their actions might introduce them. Objectives by the ANSTF for education and outreach include reaching out to the general public, providing technical guidance to targeted audiences, and raising awareness among legislators and decision makers.

Research: Research supports all facets of the Strategic Plan and is necessary to increase the effectiveness of prevention, detection, response, and control and management of invasive species. To help ensure that research addresses critical needs, this goal focuses on coordination among government agencies, academia, and other participating entities.

Funding: Securing dedicated long-term and emergency funding is necessary to achieve the goals laid out in the Strategic Plan. The actions outlined by the ANSTF focus on coordinating Federal agency budgets to support ANSTF priorities, develop partnerships, and seek opportunities to leverage funds within Federal and State agencies, local governments, tribal entities, industry, as well as other entities including non-governmental organizations.

The Strategic Plan should not be considered a comprehensive list of all ANS strategic actions; it does contain a targeted set of priority strategic goals, objectives, and associated action items that are intended to be completed in the next 5 years**.** The accomplishment of specific objectives and action items will be dependent upon budgets of individual agencies and the Regional Panels; and in some cases, legal or regulatory changes as well as enforcement of these changes. An Operational Plan will be composed to depict short-term efforts to achieve the actions in the Strategic Plan to ensure the goals and objectives of the Strategic Plan are measurable and accountable. The Operational Plan will be completed by the ANSTF members working together and separately with support of the Regional Panels and committees. The actions in the Operational Plan will be updated regularly and reported on to measure the progress towards meeting the goals of the Strategic Plan.

Management of ANS is challenging; however, considerable success is being achieved in the prevention, detection, eradication, control, and outreach efforts of ANS along with increased emphasis for the restoration of ecosystems that have been impacted by ANS. Additional research and information exchange, new detection and eradication techniques, and innovative control methodologies are increasing our capacity to address invasive species problems. The *Aquatic Nuisance Species Task Force Strategic Plan 2013–2017* takes a deliberate, cooperative approach and builds on existing programs. The Task Force will strive to maximize efforts over the next 5 years to prevent and control invasive species with the purpose of protecting our environment, economy and human health.

INTRODUCTION

Aquatic nuisance species (ANS) are nonindigenous species that threaten the diversity or abundance of native species, the ecological stability of infested waters, and/or any commercial, agricultural, aquacultural, or recreational activities dependent on such waters. ANS include nonindigenous species that may occur within inland, estuarine, or marine waters and that presently or potentially threaten ecological processes or natural resources. In addition

to the severe and permanent damage to the habitats they invade, ANS may also adversely impact society by hindering economic development, preventing recreational and commercial activities, decreasing the aesthetic value of nature, and serving as vectors[1] of human disease. The table below provides a list of the three classes of adverse impacts caused by ANS.

The term ANS is often used interchangeably with aquatic invasive species (AIS), the preferred term of Federal and State managers. An aquatic invasive species is defined as a species not native to the ecosystem under consideration whereby introduction of this species does or is likely to cause economic or environmental harm or threaten human health.

Selected Examples of Aquatic Nuisance Species Impacts		
Environmental Effects	Economic Impacts	Public Health
Habitat Alterations	Industrial Water Supplies	Disease Epidemics
Water Quality	Municipal Water Supplies	Public Safety
Predation	Power Plants	Physical Injury
Competition	Commercial Fisheries	Bacterial Risks
Hybridization	Recreation	Harmful Algal Blooms
Parasitism	Navigation and Shipping	Parasites
Introduction of Pathogens	Aquaculture	Flooding

Environmental Harm

In the United States, approximately 49% of the species on the threatened or endangered species lists are at risk primarily because of predation or competition with exotic species[2]. In fact, impacts of invasive species are second only to habitat destruction as a cause of global biodiversity loss[3]. ANS impact the habitats they invade by reducing the abundance of native species and altering ecosystem processes. They can impact native species through predation, competition for food and space, hybridization, as well as the introduction of pathogens and parasites. Normal functioning of the ecosystem, including hydrology, nutrient cycling, or productivity, may also be altered by ANS. Aquatic weeds provide an excellent example of the severe impact an exotic organism may have on the environment. Non-native aquatic plants including Eurasian water-milfoil (*Myriophyllum spicatum*), hydrilla (*Hydrilla verticillata*), and water hyacinth (*Eichhornia crassipes*) can negatively impact the diversity of native aquatic plants and invertebrates, the efficiency for large predator fish to obtain prey, water quality, and aquatic recreational activities including swimming, fishing and boating. Invasive fish species also have the ability to alter aquatic ecosystems. For example, the common carp (*Cypinus carpio*) is capable of reducing native vegetation and increasing turbidity. These types of habitat alterations are responsible for the extinction of several native fish species[4].

Economic Harm

ANS are seen as a threat not only to native biodiversity and ecosystem functioning, but also to economic development. They can reduce production of fisheries, decrease water availability to residential and commercial users, block transport routes, choke irrigation canals, foul industrial and public water supply pipelines, degrade water quality, accelerate filling of lakes and reservoirs, and decrease property values. The damages to human enterprises caused by ANS result in enormous economic costs.

Over the past 200 years, more than 50,000 non-native plant and animal species have become established in the United States. Approximately one in seven has become invasive[5],

with damage and control costs estimated at more than $120 billion per year[6] - a cost higher than the total of all other natural disasters combined[7]. Zebra and quagga mussels (*Dreissena polymorpha, D. rostriformis bugensis*) alone cause one billion dollars per year in damages.[8] Another 100 million is spent annually in the United States to control nonnative aquatic weeds.[9] In two California lagoons, more than $5 million was spent in the first 3 years of an ongoing eradication program for the seaweed *Caulerpa taxifolia*.[10] As a final example, the Great Lakes States invested over $26.7 million toward prevention and control of aquatic invasive species in just 2 years, of which almost $900,000 was committed to Asian carp[11] control efforts. [12] These numbers are likely underestimated as they do not consider ecosystem health or the aesthetic value of nature, which can influence tourism and recreational revenue. Estimating the economic impact associated with ANS is further confounded as monetary values are difficult to estimate for the extinction of species or loss of native biodiversity and ecosystem services.

Harm to Human Health

Throughout history, epidemic diseases such as malaria, yellow fever, typhus, and bubonic plague have spread using organisms as vectors and reservoirs. Further, there has been conjecture that the ballast water of ships may transport waterborne pathogens and diseases[13] as well as causative agents of harmful algal blooms (HABs)[14, 15], however, additional study is needed to link the transport of microorganism to outbreaks of human disease. The effect of ANS on public health extends beyond the immediate effects of disease and parasites; human injury may also result from ANS. For instance, hazards may occur from collisions between boaters and jumping silver carp (*Hypophthalmichthys molitrix) or* from the sharp-edged mussel shells found in recreational areas. Additional risk to human is perceivable as chemicals used to control invasive species can pollute soil and water. Other ANS, such as invasive mussels, may increase human and wildlife exposure to organic pollutants such as polychlorinated biphenyls (PCBs), as these toxicants accumulate in their tissues and are passed up the food chain.

ANS - What Can Be Done?

Prevention is the most cost effective and environmentally protective tool to control ANS. Preventative measures include decontaminating boats and gear that could transport ANS and restricting the importation or release of potentially harmful species. However, even the best prevention efforts may not stop all invasions. When a new species is introduced, the best strategy is early detection and rapid response. This includes monitoring habitats to discover new species soon after introduction, reporting sightings of previously unknown species in an area, and working quickly to keep introduced species from becoming established and spreading. Once established, invasive species can be difficult to control and nearly impossible to eradicate. Control methods include mechanical, chemical, and biological approaches. The methods used will vary dependent upon the species and location; however, control efforts are typically costly, labor intensive, and indirectly affect native species. Furthermore, control efforts often create disturbance, which may render the habitat vulnerable to subsequent

invasions. Accordingly, habitat restoration is necessary following eradication or control efforts to minimize the chance an area will be reinvaded.

Education and outreach are critical tools to prevent and manage the impacts of ANS. The public must understand the problems and impacts associated with ANS so they can be active partners in solving the problem. More importantly, people need to know what they can do to help prevent the introduction and spread of ANS. Federal, State, and local programs and legislation have implemented regulations to prevent the introduction and reduce the spread of invasive species. These regulations include mandatory boat inspections at public boat ramps, live species prohibitions and restrictions, and ballast water regulations at shipping ports. To successfully address ANS issues collaboration, cooperation, and coordination are necessary among and between Federal and State agencies, local governments, tribal entities, industry, as well as other entities including non-governmental organizations.

Future Challenges

Global trade and intercontinental travel have been cited as major causes of biological invasion. For example, it has been estimated that 10,000 marine species are transported around the world in ballast water every day[16]. As the world trade network continues to grow, new markets and trade routes continually open. This growth will increase the number of new species that are introduced and the frequency with which such introductions are repeated. Managing this increased rate of aquatic bioinvasion will require the United States and other counties to strengthen approaches for preventing introductions while maintaining trade.

Additional challenges to ANS management result from changes in the Earth's climate that will likely continue, or even accelerate, over the next century. Very little is known of the impacts from ANS in relation to climate change, yet models suggest that the economic, energy, social, and environmental impacts may be profound. Fast growth, rapid reproduction, and the ability to survive in a wide range of environmental conditions are among some of the life history traits shared by ANS that may allow them to capitalize on the biotic and abiotic changes generated by global climate change. Furthermore, species that have long been “in motion,” but failed to establish and reproduce in hostile conditions, may soon be able to invade these once "off limit" thermal regimes. Other species will migrate to maintain the temperature conditions needed for reproduction, growth, and feeding. There is a growing concern that these shifting species will begin to function as invasive species, disrupting the structure and function of their new communities. Many communities have already experienced the impacts of warming coastal waters and have shown subsequent alterations in species proportions as well as changes to community structure and dynamics. Predictions of how species will respond to climate change will help guide conservation decisions and management of natural resources. Future ANS managers will need to develop tools that include both invasion biology and climate change impacts.

STRUCTURE OF THE AQUATIC NUISANCE SPECIES TASK FORCE

The Aquatic Nuisance Species Task Force (ANSTF) was established by Congress with the passage of the Nonindigenous Aquatic Nuisance Prevention and Control Act (NANPCA) in 1990 and reauthorized with the passage of the National Invasive Species Act (NISA) in

1996 (collectively, the Act). The ANSTF is an interagency committee established by Section 1201 of the Act and serves to develop and implement a program for waters of the United States[17] that:

- Prevents the introduction and dispersal of ANS;
- Monitors, controls and studies such species;
- Conducts research on methods to monitor, manage, control and/or eradicate such species;
- Coordinates ANS programs and activities of ANSTF members and affected State agencies; and
- Educates and informs the general public and program stakeholders about the prevention, management, and control of these species

Federal and Ex-Officio Members

The ANSTF's charter is authorized by the Federal Advisory Committee Act (FACA) of 1972. The charter provides the ANSTF with its core structure and ensures an open and public forum for its activities. To meet the challenges of developing and implementing a coordinated and complementary Federal program for ANS activities, the ANSTF members include 13 Federal agency representatives and 13 representatives from ex-officio member organizations. These members work in conjunction with Regional Panels and issue-specific committees to coordinate efforts amongst agencies as well as efforts of the private sector and other North American interests.

The Act designated the Director of the Fish and Wildlife Service and the Undersecretary of Commerce for Oceans and Atmosphere as the ANSTF Co-chairpersons. It specified six Federal agencies[18] that would constitute the ANSTF, but also gave the co-chairs legal authority to include other Federal agencies as members of the ANSTF, as appropriate. Members of ANSTF are responsible for committing resources to achieve the goals of the Strategic Plan and reporting annually on their progress. At the time of Plan adoption (May 3, 2012), the following were ANSTF member departments and agencies:

- United States Fish and Wildlife Service (USFWS)—co-chair
- National Oceanic and Atmospheric Administration (NOAA)—co-chair
- Army Corps of Engineers (ACOE)
- Bureau of Land Management (BLM)
- Bureau of Reclamation (BOR)
- Department of State (DOS)
- Environmental Protection Agency (EPA)
- United States Forest Service (USFS)
- Department of Transportation (DOT), Maritime Administration (MARAD)
- National Park Service (NPS)
- United States Coast Guard (USCG)

- United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS)
- United States Geological Survey (USGS)

The Act also named four organizations[19] to be included in the base ANSTF membership, yet authorized the co-chairs to invite representatives of specific regional organizations, State agencies, and other governmental entities to participate as *ex-officio* members of the ANSTF. At the time of Plan adoption (May 3, 2012), the following were ex-*officio* members of the ANSTF:

- Great Lakes Commission
- Lake Champlain Basin Program
- Chesapeake Bay Program
- San Francisco Estuary Project
- American Public Power Association
- American Water Works Association
- Association of Fish and Wildlife Agencies
- Gulf States Marine Fisheries Commission
- Mississippi Interstate Cooperative Resources Association
- Native American Fish and Wildlife Society[20]
- National Association of State Aquaculture Coordinators
- Smithsonian Environmental Research Center
- *Fisheries and Oceans Canada (invited observer)*

ANSTF Regional Panels

The ANSTF focuses its work on ANS issues of national concern that require or could benefit from collaborative solutions. It strives to create opportunities and synergies among members and participants to work collaboratively by sharing resources, expertise, and ideas across agency and organizational lines. While the ANSTF has a national focus, it recognizes the tremendous importance of actions taken at the regional and local level to achieve national ANS solutions. Section 1203 of NANPCA created the Great Lakes Regional Panel to identify priorities, to coordinate ANS program activities, and to advise public and private interests on control efforts in their region. The 1996 amendment required the ANSTF to encourage the development of additional regional panels to provide an intergovernmental mechanism for the development of a coordinated Federal program to prevent and control nonindigenous ANS as authorized by the Act. The Regional Panels are responsible for implementing actions that assist in achieving the Strategic Plan's goals and reporting annually on their progress[21]. At the time of Plan adoption (May 3, 2012), the ANSTF had established six Regional Panels:

- Great Lakes Regional Panel
- Western Regional Panel
- Gulf and South Atlantic Regional Panel

- Northeast Regional Panel
- Mississippi River Basin Regional Panel
- Mid-Atlantic Regional Panel

Regional Panel membership is composed of Federal, State, intergovernmental, tribal, industry, and environmental non-governmental organization representatives. In addition, some panels may include international representation (e.g. Canadian Federal and Provincial representatives) as observers.

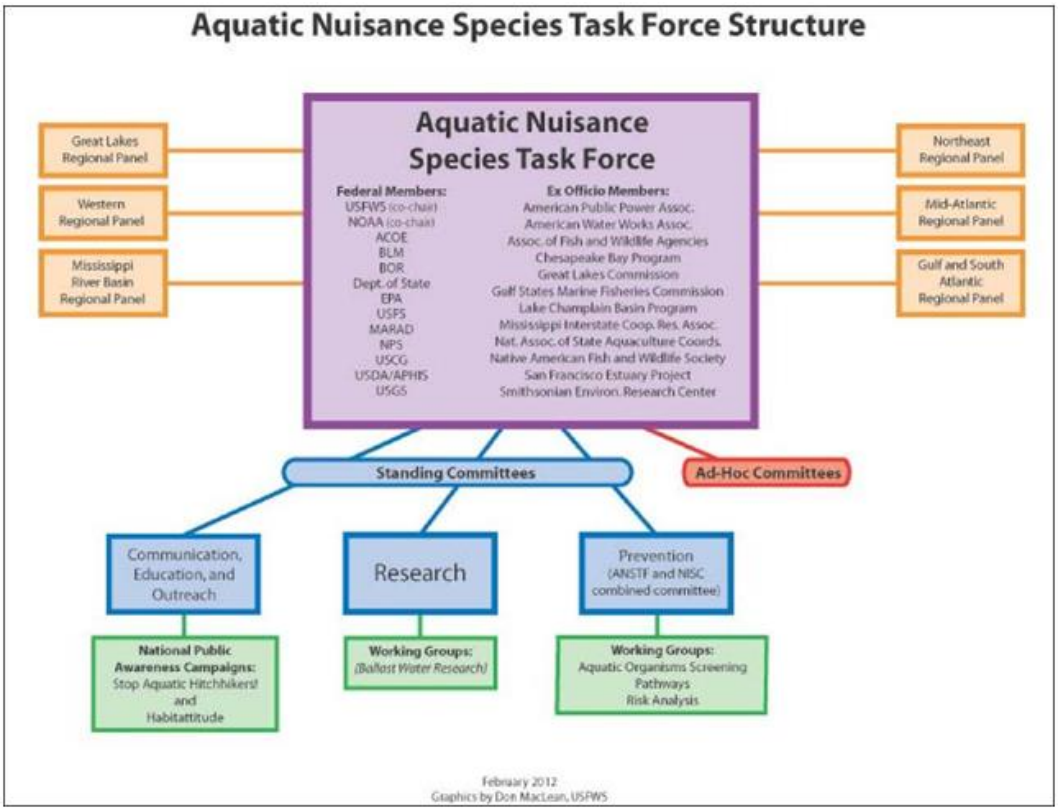
The Regional Panels make a concerted effort to involve a broad spectrum of stakeholders in order to provide balanced advice to the ANSTF on regional priorities and issues of local significance.

ANSTF Committees

To obtain the necessary technical coordination of various ANS efforts, the ANSTF has established several issue- and/or species-specific standing and ad hoc committees to carry out the Act. Committee membership includes all affected entities, such as Federal and State agencies, tribes, non-governmental organizations, industry groups, and academia. Committees are made up of member agency representatives and subject matter experts. Committee activities include the development of public awareness/action campaigns, species-specific control and management plans, standardized scientific protocols, research priorities, theoretical frameworks to screen organisms prior to their entry to the United States., and providing technical advice to the ANSTF. Committees, both standing and ad hoc, are responsible for reporting to the ANSTF on goal attainment under this Plan. At the time of Plan adoption (May 3, 2012), the ANSTF had established three standing committees that oversee related working groups: the Communication, Education and Outreach Committee, Research Committee, and Prevention Committee (the latter is a joint committee with the National Invasive Species Council (NISC)). In addition, ad hoc committees are formed as needed to focus on a specific discipline or issue that warrants the attention of the ANSTF.

ANSTF Strategic Plan

Section 1202 of the Act authorized the ANSTF to develop and implement a program for waters of the United States to prevent introduction and dispersal of ANS, to monitor, control, and study such species, and to disseminate related information. The *Aquatic Nuisance Species Program* document guided the work of the ANSTF from 1994 to 2002. The document tracked the requirements outlined in the NANPCA (1990). It established the core elements of the ANS program (prevention, detection and monitoring, control) and support elements (research, education, and technical assistance), provided for prioritization of activities, and charted a course for implementation of the Act.



The ANSTF Strategic Plans for 2002–2007 and 2007- 2012 maintained the key elements of the ANS Program, but provided a broader focus for activities consistent with provisions of NISA (1996). These plans provided more emphasis on prevention strategies, particularly for intentional introductions.

The ANSTF Strategic Plan for 2013 – 2017 (hereafter, the Strategic Plan) carries through many of the goals and objectives established in previous plans by remaining focused on prevention, monitoring, and control of ANS as well as increasing public understanding of the problems and impacts associated with invasive species. The Strategic Plan also calls attention to other areas of ANS management, including habitat restoration and research. The Strategic Plan establishes eight goals:

1. Coordination - Maximize the organizational effectiveness of the Aquatic Nuisance Species Task Force
2. Prevention - Develop strategies to identify and prevent the establishment of new and slow the spread of existing ANS in the waters of the United States
3. Early Detection and Rapid Response - Identify and respond to aquatic nuisance species within a timely manner following introduction in order to prevent their establishment and/or spread
4. Control and Management - Control established aquatic nuisance species when feasible and when the benefits of managing the established species outweigh the costs of removing them with respect to harm to the environment, the economy, and public health

5. Restoration – Protect and rehabilitate native species and ecosystems by conducting habitat restoration efforts on multiple scales
6. Education / Outreach - Increase awareness concerning the threats of aquatic nuisance species, emphasizing the impacts, importance of prevention and containment, and recommendations for appropriate domestic and international actions
7. Research - Facilitate research to address environmental, economic, and human health risks and impacts associated with aquatic nuisance species
8. Funding - Coordinate Federal agency budgets to support Aquatic Nuisance Species Task Force priorities and establish a clear process that links State and regional needs in their areas of responsibility

The strategic goals serve as a blueprint and coordination tool for the ANSTF. The order in which the goals are presented in the Plan represent the logical arrangement determined by the ANSTF; accordingly, the hierarchy of the goals do not represent individual importance or priority level. Under each goal, objectives describe what is to be accomplished over the next 5 years. Action items listed under the objectives describe how ANSTF expects to accomplish the goals and objectives. The accomplishment of specific objectives and action items will be dependent upon budgets of individual agencies and the Regional Panels; and in some cases, legal or regulatory changes as well as enforcement of these changes. The Strategic Plan should not be considered a comprehensive list of all ANS strategic actions; it does contain a targeted set of priority strategic goals, objectives, and associated action items that are intended to be completed in the next 5 years.

ANSTF Operational Plan

In addition to its Strategic Plan, the ANSTF will compose a separate Operational Plan. Its function is to ensure the goals and objectives of the Strategic Plan are measurable and accountable. In contrast to the action items in the Strategic Plan that outline long-term or continual actions, the actions listed in the Operational Plan will depict short- term efforts used to support and implement the Strategic Plan. The Operational Plan will be completed by the ANSTF members working together and separately with support of the Regional Panels and committees. To the greatest extent possible, implementation of the actions within the Operational Plan will build on and fill gaps in existing activities and programs rather than supplanting them. Responsibilities will be assigned to specific agencies, Regional Panels, or committees. Implementation will be assumed by those specified in line with their specific mandates, priorities, expertise, and funding. The Operational Plan will include, when available, the time frame, lead and supporting agencies or groups, and allocated funding. Further, the Operational Plan will be regularly amended to reflect changes in circumstances, plans, or priorities. The actions listed in the Operational Plan will be reported annually to measure the progress towards accomplishing the over-arching goals and objectives identified within the Strategic Plan.

COORDINATION WITH OTHER FEDERAL AND STATE INVASIVE SPECIES MANAGEMENT PLANS

The ANSTF recognizes that many Federal and State agencies, interagency groups, and local entities contribute to the management of invasive species. The largest and most comprehensive invasive species-focused committees and working groups are the National Invasive Species Council (NISC), Federal Interagency Committee for the Management of Noxious and Exotic Weeds (FICMNEW), and the Federal Interagency Committee on Invasive Terrestrial Animals and Pathogens (ITAP). As described below, these working groups facilitate communication and collaboration at all levels of the Federal government and with State, local and private partners by focusing on particular taxa and pathways.

The National Invasive Species Council (NISC) was established by Executive Order 13112 (Order). NISC is co-chaired by the Secretaries of Agriculture, Commerce, and the Interior and includes 10 member departments and their constituent agencies as well as a small staff assigned specifically to the Council. The Order directs the Secretary of the Interior to establish an Invasive Species Advisory Committee (ISAC) composed of non-federal experts and stakeholders to provide advice and recommendations to NISC on invasive species-related issues. NISC provides national leadership and oversight on both terrestrial and aquatic invasive species and ensures that Federal programs and activities to prevent and control invasive species are coordinated, effective, and efficient. NISC has specific responsibilities including promoting action at State, tribal, local, and ecosystem levels; identifying recommendations for international cooperation; facilitating a coordinated network to document, evaluate, monitor invasive species' effects; developing a web-based information network on invasive species; and developing guidance on invasive species for Federal agencies to use in implementing the National Environmental Policy Act. NISC is also responsible for preparing a National Invasive Species Management Plan which directs Federal efforts to prevent, control and minimize invasive species and their impacts.

The Federal Interagency Committee for the Management of Noxious and Exotic Weeds (FICMNEW) was established through a Memorandum of Understanding signed by agency leadership in August 1994. FICMNEW represents an unprecedented formal partnership between 16 Federal agencies with direct invasive plant management and regulatory responsibilities spanning across the United States and territories. FICMNEW members interact on important national and regional invasive plant issues and share information with various public and private organizations participating with the Federal sector to address invasive plant issues. FICMNEW's charter directs the committee to coordinate, through the respective Secretaries, Assistant Secretaries, and Agency heads, information regarding the identification and extent of invasive plants in the United States and to coordinate Federal agency management of these species. FICMNEW accomplishes these portions of its charter by developing and sharing scientific and technical information, fostering collaborative efforts among Federal agencies, providing recommendations for national and regional level management of invasive plants, and sponsoring technical and educational conferences and workshops concerning invasive plants.

ITAP is the Federal Interagency Committee on Invasive Terrestrial Animals and Pathogens. ITAP's mission is to support and facilitate more efficient networking and sharing of technical information for program planning and coordination among Federal agencies and

departments involved with invasive species research and management. ITAP focuses on several major taxonomic groups of invasive species for which improved technical coordination is essential to facilitate effective Federal responses.

Invasive species management plans prepared by these groups and others provide an opportunity to identify priorities and establish cooperative, well-coordinated approaches to invasive species management. Invasive species issues are significant in their breadth and scope; as this area involves all taxa of life and threatens natural ecosystems around the globe. A variety of pathways are capable of transporting species into new environments including ballast water and hulls of ships, materials associated with the trade of nursery stock, importation of fruits and vegetables, and the international movement of people. Furthermore, multiple efforts are necessary to encompass the various components of invasive species management, including prevention, monitoring, removal, restoration, research, and education. The great extent needed to manage invasive species and their impacts infers that one agency, task force, or work group cannot tackle it alone. Work can be done with greater effectiveness and efficiency if it is focused on specific taxonomic, ecosystem, or regional priorities. To ensure that redundancy and overlap does not occur while addressing invasive species-related issues, the ANSTF communicates with Federal and State agencies and interagency groups to create a big picture framework for existing management plans. Working with other agencies and organizations allows the ANSTF to identify areas where legislation is needed to fill gaps in statutory authority, suggest priority policy issues, and define roles and responsibilities for managing invasive species.

States also play a critical role in preventing and controlling the spread of invasive species and have numerous programs relating to the wide variety of invasive species found within their borders. ANSTF encourages State and interstate planning entities to develop management plans describing detection and monitoring efforts of ANS, prevention efforts to stop their introduction and spread, and control efforts to reduce their impacts. In addition, these plans serve to coordinate efforts between State agencies, local governments, tribal entities, industry, as well as other entities including nongovernmental organizations. Consequently, they are a valuable and effective tool for identifying and addressing ANS problems and concerns in a climate of many jurisdictions and other interested entities. Once a State or interstate ANS management plan is approved, the ANSTF monitors the activities of the planning entity to ensure the plans are implemented. This monitoring process allows the ANSTF to evaluate the capacity and capability at State and local levels to coordinate, detect, and respond to invasive species. This information allows the ANSTF to better identify strategies for monitoring, containment, outreach, and other ANS activities. It also allows for identification of priority activities and species, obstacles to fully implementing the ANS State management plans, and cooperative partnerships that exist among entities managing ANS. This information is critical for recognizing the amount and type of data and management methods available, which allows for an assessment of gaps, redundancies, and opportunities for collaboration among agencies that are not being realized. It is clear that actions and goals performed at the Federal level will not will not succeed unless they are undertaken in cooperation with stakeholders; for that reason, coordination and joint action with State partners is critical for addressing invasive species problems within the United States.

CONCLUSION

Management of ANS is challenging; however, considerable success is being achieved in the prevention, detection, eradication, control, and outreach efforts of ANS along with increased emphasis on the restoration of ecosystems that have been impacted by ANS. Additional research and information exchange, new detection and eradication techniques, innovative control methodologies, and collaborative models are increasing our capacity to manage ANS. Since the establishment of the ANSTF, awareness of the problems caused by ANS has dramatically improved, as evidenced by increased activity at Federal, State, and local levels. Despite the significant increase in activity and awareness, much remains to be done to prevent and mitigate the impacts of ANS. The intent of the ANSTF Strategic and Operational Plans is to create a strategic approach to minimize harm to the environment, economy, or human health that results from ANS. This involves taking advantage of what has been learned and creating next steps that are well planned and coordinated.

Goal 1: Coordination - Maximize the Organizational Effectiveness of the Aquatic Nuisance Species Task Force

The scope and complexity of ANS management summons the strengths of different government agencies and private organizations in different ways. A primary objective of the ANSTF is to facilitate cooperation and coordinate Federal government efforts relating to ANS in the United States with those of the private sector and other North American interests by utilizing Regional Panels and issue-specific committees and including bi-national bodies where applicable. The Regional Panels established by the ANSTF are a critical and effective mechanism for achieving the goals and objectives established by the ANSTF Strategic Plan. The memberships within each of the panels include representatives of States, Indian tribes, non-governmental organizations, commercial interests, and neighboring countries. The roles of each panel include, but are not limited to, identifying regional ANS priorities, coordinating ANS program activities in the region, making recommendations to the ANSTF, and providing advice to public and private interests concerning methods of ANS management and control.

To further increase coordination, the ANSTF encourages State and interstate planning entities to develop management plans describing detection and monitoring efforts of ANS, prevention efforts to avert their establishment and spread, and control efforts to reduce their impacts. In addition, management and control plans have been developed or are under development by the ANSTF and other partners for several species. Under Section 1204 of the Act, management plan approval from the ANSTF is required for both State and species plans to obtain funding. Regardless of financial incentives, plans are a valuable and effective tool for identifying and addressing ANS problems and concerns in a climate of many jurisdictions and interested entities.

Fulfilling Goal 1 requires ongoing cooperation, communication, and dialogue as well as an understanding of the views and roles of all agencies and organizations involved. The actions suggested below will allow the ANSTF to lower institutional barriers to efficiency and effectiveness, beginning with enhanced Federal agency collaboration. The actions also include a thorough analysis of the roles, responsibilities, and supporting legislation involving

ANS so gaps in authority can be identified. Further, strong monitoring and evaluation of the ANSTF Strategic and Operational Plans are encouraged to provide measures of success toward reaching goals and providing information for future revisions of the plans.

Objective 1.1: Strengthen the Coordination Capacity of the ANSTF

a) Increase communication among the members, Regional Panels, and committees of the ANSTF to prioritize issues and activities
b) Continue to build capacity within and among the Regional Panels in order to increase regional and international coordination, identify needs and emerging issues, and communicate recommendations to ANSTF members
c) Provide technical guidance and resource assistance to States though a coordinated effort by the Regional Panels
d) Strengthen the working relationship with NISC, working cooperatively to implement ANS activities identified in the ANSTF Strategic Plan and NISC National Management Plan
e) Identify, increase communication, and encourage participation with other interagency organizations dedicated to invasive and nuisance species
f) Identify, increase communication, and encourage participation with industry, tribes, and other stakeholders affected by ANSTF and Regional Panel activities
g) Annually report on the ANSTF Operational Plan; when warranted, utilize this information to update and amend the ANSTF Operational Plan

Objective 1.2: Evaluate the Ability of Statutory Authorities, Regulations, and Programs Necessary to Implement ANSTF Goals and Objectives

a) Identify gaps in statutory authorities, regulations, and programs necessary to meet ANSTF goals and objectives
b) Recommend revisions to statutory authorities, regulations, and programs when needed to meet ANSTF goals and objectives

Objective 1.3: Facilitate the Development and Continued Effectiveness of State and Interstate ANS Management Plans

a) Encourage plan development and provide technical drafting assistance to States without an ANS management plan
b) Encourage State and interstate ANS management plan review and revision every 5 years to reflect completed goals and objectives, new goals and objectives, new species and pathways, and management priorities
c) Seek opportunities to leverage funds to fully support implementation of ANSTF-approved State and interstate ANS management plans
d) Report on the number of State and interstate ANS management plans in place and under development
e) Review guidelines for the preparation of State and interstate ANS management plans
f) Annually summarize and report on State and interstate ANS management plan accomplishments and expenditures; utilize this information to compose a national assessment of ANS activity

Objective 1.4: Coordinate the Development and Implementation of ANSTF- Approved Species Control and Management Plans and Pathway Management Plans

a) Encourage the development of pathway management plans
b) Coordinate discussions on species and pathways for which plans may be needed; determine a lead entity for development of such plans
c) Distribute ANSTF-approved species management and control plans to State, tribal agencies, and relevant stakeholders to encourage State and tribal-level action
d) Monitor the development, evaluate the effectiveness of implementation, and report on the progress of ANS plan implementation
e) Report annually on the progress of existing ANSTF-approved species control and management plans
f) Support the implementation and long-term efforts of ANSTF approved plans

Objective 1.5: Cooperate with Nations That Have Neighboring Waters and Shared Pathways with the United States to Prevent, Detect, and Control ANS

a) Broaden involvement with international ANS activities and organizations
b) Continue and expand cooperation between ANSTF, Regional Panels, and foreign entities concerning the planning and implementation of prevention, monitoring, research, education, and control programs related to ANS that infest waters of the United States and neighboring nations

Goal 2: Prevention - Develop Strategies to Identify and Prevent the Establishment of New and Slow the Spread of Existing ANS in the Waters of the United States

Prevention is the first line of defense against ANS. Once a species becomes established, control efforts require significant and sustained resources. Since eradication may not be feasible, prevention is the most cost-effective means to avert the risk of harmful introductions. Investment in prevention avoids many of the long-term economic, environmental, and social costs associated with ANS. New species can arrive through many different ways, but most species that are considered to be invasive are a direct result of human activity. The movement of ANS may utilize pathways including, but not limited to, ballast water and hulls of ships, canals and waterways, aquaculture, the aquarium and pet trade, the bait industry, recreational activities, biological research, and habitat restoration projects.

Long-term success in prevention will reduce the rate of introductions, the rate of establishment, and the damage from additional ANS. One example of this success can be found in the Great Lakes. Beginning in 1970, one invader on average was recorded every 8 months in this region[22]; in 2006 more stringent measures were taken to regulate ballast water discharges, and since that time no new invasive species attributed to ballast water release and transoceanic shipping in general have been reported in the Great Lakes[23]. Additional successes in prevention will require Federal agency support and cooperation with State agencies and private organizations. Implementation of preventative measures may require broadening legislative mandates, strengthening the capacity of some departments, and refining or consolidating legislative and regulatory tools. The actions suggested below

identify the most efficient way to reduce ANS risks by supporting authorities and programs that address intentional and unintentional introductions from all pathways. The joint ANSTF and NISC Prevention Committee via the Pathways Work Team has completed a report on major invasive species pathways (*Training and Implementation Guide for Pathway Definition, Risk Analysis and Risk Prioritization*) as well as a *Pathways Ranking Guide*. These documents will be used to guide ANSTF efforts in pathway management.

Objective 2.1: Take Steps to Interdict Specific Pathways by Developing and Implementing Guidance and Appropriate Measures

a) Develop and implement risk mitigation measures to prevent introductions through priority pathways
b) Evaluate risk mitigation measures to prevent introductions to ensure they are effective and environmentally sound
c) Encourage State and Federal agencies to incorporate invasive species management into emergency response and contingency plans (e.g., wildfire management and spill response plans)
d) Recommend amendments to the injurious wildlife provisions of the Lacey Act (18 U.S.C. § 42) to allow a proactive approach for preventing the establishment of new invasive species through the trade of live organisms
e) Recommend amendments to legislation to ensure prevention of the establishment of nonnative aquatic plants though trade is addressed
f) Encourage improved implementation and enforcement of the Lacey Act provisions on injurious wildlife and other regulations relevant to the transport, propagation, sale, collection, possession, importation, purchase, cultivation, distribution, and introduction of ANS

Objective 2.2: Facilitate Use of Science-Based Risk Assessment and Screening Procedures to Assess and Prioritize Pathways for the Introduction of ANS or Potential Species of Concern

a) Identify authorities and regulations to carry out screening of specific pathways or species; recommend improvements based on identified gaps
b) Support continued application of pathway ranking tools and related management guidance
c) Develop and maintain a priority list for ANS pathways

Objective 2.3: Expand Training and Use of the Hazard Analysis and Critical Control Point (HACCP) Program into Work Conducted by Natural Resource Managers

Goal 3: Early Detection and Rapid Response - Identify and Respond to Aquatic Nuisance Species within a Timely Manner Following Introduction in Order to Prevent Their Establishment and/or Spread

Despite the best preventive efforts, new ANS are certain to be introduced into waters of the United States. When a new species is introduced, the best strategy is early detection and

rapid response (EDRR). This includes monitoring habitats to discover new species soon after introduction, reporting sightings of previously unknown species in an area, and working quickly to keep the species from becoming established and spreading. EDRR increases the likelihood that localized ANS populations will be found, contained, and eradicated before they become widely established. EDRR can slow range expansion of ANS, and avoid the need for costly long-term control efforts. Several States including Minnesota, Wisconsin, Indiana, and Virginia have successfully eradicated infestations preventing the establishment and spread of ANS. Moreover, novel approaches to enhance early detection, including genetics-based methods, are being actively developed. These methods have strong potential to improve the ability to identify likely invaders and susceptible habitats.

Providing fast access to information on taxonomy, control methods, and subject matter experts can allow new and existing ANS to be readily recognized and managed. EDRR must be supported with emergency funding and guided by contingency plans coordinated by Federal, State, and local agencies as well as other entities, including tribes and non-governmental organizations. Goal 3 also supports several other needs: it evaluates prevention and control programs, provides information on invasion patterns and future management needs, and emphasizes the value of taxonomic expertise as an essential component of EDRR efforts. The actions suggested below identify environmentally sound methods that can prevent further spread and minimize harm to public interests. In addition to increasing ANS monitoring efforts, actions include the development of rapid response capabilities as well as research and education programs specifically related to the early detection of and rapid response to ANS.

Objective 3.1: Facilitate Surveys and Monitoring to Detect ANS

a) Assess existing early detection monitoring programs; identify gaps and recommend improvements for a more integrated approach
b) Develop model protocols for universal or common practices for early detection monitoring
c) Work with agencies and organizations to incorporate invasive species monitoring into existing survey work
d) Identify high-priority areas for targeted monitoring efforts; develop new early detection and surveillance programs as needed, including monitoring the impact of climate change on the distribution of ANS
e) Further develop, refine, and support genetic and emerging tools based on novel approaches to improve early detection of ANS

Objective 3.2: Make Taxonomic and Ecological Information and Expertise Readily Available

a) Update the ANSTF Expert Database on a regular basis
b) Identify gaps in available expertise
c) Support establishment of new taxonomic expertise to identify native and exotic species

Objective 3.3: Increase Public and Industry Involvement in Early Detection and Rapid Response Programs

a) Increase public volunteer training opportunities utilizing existing programs and infrastructure
b) Promote use of the ANS National Hotline and USGS Online Reporting Form to report sightings of non-native species
c) Develop additional tools to encourage reporting of suspicious sightings

Objective 3.4: Facilitate Development of Rapid Response Contingency Plans

a) Review and make accessible existing model rapid response plans for aquatic invasions
b) Support development of additional model rapid response plans for aquatic invasions based on both taxonomic groups and jurisdictional authority where invasion occurred
c) Identify authorities and regulations to carry out emergency response actions
d) Support implementation of the National Incident Management System (NIMS) to prevent, protect against, recover from, and mitigate the effects of ANS incidents as mandated by Homeland Security Presidential Directive (HSPD)-5[24]

Objective 3.5: Build Capacity to Respond Rapidly to Invasions

a) Synthesize lessons learned from previous rapid response attempts to new ANS invasions and make it readily available
b) Make species-specific control and management information readily available
c) Develop a rapid response technical support network that that can provide resources and technical support in response to newly detected species
d) Increase training opportunities of the NIMS protocol
e) Increase number of mock-NIMS based rapid response exercises to identify additional steps needed for rapid response preparedness
f) Explore opportunities and identify obstacles for establishing an emergency rapid response fund

Goal 4: Control and Management - Control Established Aquatic Nuisance Species When Feasible and When the Benefits of Managing the Established Species Outweigh the Costs of Removing Them with Respect to Harm to the Environment, the Economy, and Public Health

Once ANS are established, under most conditions complete eradication is usually not feasible. A more realistic approach for established populations is using control measures to slow the rate of range expansion and lessen the impacts to public interests. Management objectives may include eradication within an area, suppressing a population, limiting spread, and reducing impacts. Control measures may include mechanical, chemical, biological, and integrated pest management strategies. Adequate funding, public awareness, and management expertise are critical to success, particularly because ANS can span geographic and jurisdictional boundaries and do not recognize political boundaries or agency jurisdictions.

Therefore, Federal and State agencies, Indian tribes, and private organizations should coordinate an ecosystem-level approach to managing ANS.

Multiple control and management tools are needed to assess, remove, and contain ANS populations as well as to guide management decisions. The actions suggested below seek to identify, improve, and execute these tools. Further, the Strategic Plan actions require inter-jurisdictional communication and regionally coordinated action through the continued development and implementation of control and management plans.

Objective 4.1: Support and Evaluate ANSTF-Approved Control and Management Plans

a) Identify gaps in control efforts and tools
b) When warranted, develop or broaden existing control methods and programs to achieve the target level of control

Objective 4.2: Increase Invasive Species Training for Natural Resource Managers and Leverage Participation

a) Increase the number of training workshops and total number of personnel and volunteers trained in control measures for ANS
b) Review and, as needed, develop ANS training materials used by natural resource managers

Objective 4.3: Evaluate the Benefits and Risks Associated with the Commercial Harvest of ANS as a Means of Control or Eradication

a) Develop guidelines to assist States and tribes in determining when commercial harvest may be beneficial for control of ANS
b) Develop guidelines to assist States and tribes in developing policies for new or existing commercial harvest programs for ANS
c) Encourage long-term monitoring and evaluation of commercial harvest activities

Objective 4.4: Encourage an Integrated Pest Management (IPM)[25] Approach to Manage Existing ANS Populations

Goal 5: Restoration – Protect and Rehabilitate Native Species and Ecosystems by Conducting Habitat Restoration Efforts on Multiple Scales

Habitat restoration is an essential part of the control and management efforts used to guard against future invasions or to minimize harm to native ecological communities and other public interests. Restoration of the natural habitat should be addressed whenever the control or eradication of ANS is planned since habitat rehabilitation is often necessary to avoid the replacement of one invasive species with another, control flooding, or avoid other problems associated with the absence of biological organisms. Restoration activities may also include planting or stocking organisms or improving predator-prey relationships to attain food webs more similar to pre-invasion conditions. ANS can be transported by materials, equipment, vehicles, or personnel used to conduct restoration activities; accordingly all habitat restorations, even those not focused on ANS control, should call attention to actions

that prevent establishment of invaders not yet present within the project site. Restoration efforts should make use of plant and animal species that are native to the particular habitat. One of the benefits of using native species includes their ability to thrive under the local conditions while being less likely to invade new habitats. Consequently, native species reduce maintenance costs and produce healthy natural communities, thus providing a practical and ecologically valuable option for restoration projects.

The actions suggested below focus on ANS concerns during habitat restoration efforts by targeting consideration of potential ANS during planning and implementation of restoration activities and encouraging post-restoration monitoring to ensure that any ANS introduced as a result of restoration are responded to in a rapid and efficient manner.

Objective 5.1: Restore Impacted Ecosystems

a) Identify and support agencies or programs that can assist in restoring areas impacted by ANS
b) Provide technical assistance on the species and methods to use in restoring native species, including means to enhance resilience against re-invasion, climate change, and other drivers of change
c) Compile, highlight, and share lessons learned for both restoration successes and failures within the United States

Objective 5.2: Address and Provide Technical Assistance for Invasive Species Management before, during, and after Habitat Restoration Projects

a) Ensure that Federal land and water management field and guidance manuals consider ANS issues during the planning and development of habitat restoration projects
b) Review and make accessible existing restoration project standards to mitigate impacts of ANS during restoration activities. Develop new guidelines when warranted
c) Encourage application of adaptive management principles and assessment of treatment regimes to improve and sustain restoration efforts over time
d) Encourage the development of Hazard Analysis and Critical Control Point (HACCP) plans for all federally funded or authorized restoration projects
e) Support the development and expansion of markets that supply native plants and certified weed-free materials; encourage use of these materials by agencies and other organizations Encourage post-restoration monitoring for ANS by agencies and other organizations conducting habitat restoration or landscaping projects
f) Encourage restoration of areas following ANS eradication or control efforts

Goal 6: Education and Outreach - Increase Awareness Concerning the Threats of Aquatic Nuisance Species, Emphasizing the Impacts, Importance of Prevention and Containment, and Recommendations for Appropriate Domestic and International Actions

The lack of awareness concerning ANS impacts is one of the largest management obstacles. Few people understand the threat some nonindigenous species pose and how their

actions might introduce them. Many ANS have been introduced through the actions of uninformed people; for example, disposing of bait, launching a boat, or stocking a private pond can each lead to the introduction of ANS if precautions are not taken. Further, the importation of organisms through trade has allowed species to spread by the receipt of unwanted organisms that hitchhike with the intentionally imported ones. Many policy makers, natural resource administrators, and private interest groups have facilitated the intentional introductions of species for certain economic or recreational purposes without understanding the effects these species may have on native species. These intentional and unintentional methods of introduction can be eliminated or curtailed by educating people about their potential to transfer ANS into new habitats.

Robust public awareness and action programs will help the public understand the impacts associated with invasive species so they can be partners in solving the problems. More importantly, people need to know what they can do to help prevent the introduction and spread of ANS in waters of the United States. ANSTF agency and *ex-officio* members, Regional Panels, States, tribes, and other entities have conducted workshops, created exhibits, pamphlets, information sheets, wallet identification cards, videos, websites, traveling displays, and other public education materials for distribution across the country. In recent years, many States have focused efforts on educating non-English speaking communities about ANS issues in general, and in respect to their culture. The actions suggested below focus on continuing to develop, review, and disseminate information to the general public as well as targeted user groups and businesses that may be potential vectors of ANS. Support and collaboration are necessary at many levels among and between Federal and State agencies, local governments, tribal entities, public and non-public sectors to successfully address ANS. As such, the actions below also focus on education of ANS threats and solutions for legislators and other decision makers.

Objective 6.1: Increase Understanding Among the General Public of the Problems and Impacts Associated with ANS and Actions That Can Be Taken to Prevent and Control ANS in Waters of the United States

a) Promote and expand new and existing national campaigns (e.g., Stop Aquatic Hitchhikers and Habitattitude™) with a proven track record of raising awareness and fostering behavior change among target audiences
b) Utilize the internet and social media as well as traditional media sources to disseminate information and promote awareness of ANS
c) Develop educational activities and products aimed at the general public regarding specific actions that can be taken to prevent, detect, and control ANS
d) Cooperate with media outlets to reach a broad range of the public with ANS messages
e) Participate in public affairs activities (e.g., conferences, shows, tournaments) to reach a broad range of the public with ANS messages
f) Raise the level of understanding and expertise on ANS worldwide by encouraging technical information exchange with other countries
g) Participate in international conferences and workshops

Objective 6.2: Disseminate ANS Outreach and Technical Guidance Materials to Target Audiences

a) Identify and prioritize targeted user groups and businesses that may be potential vectors of ANS.
b) Maintain and promote the ANSTF Recreational Guidelines
c) Leverage opportunities with relevant user groups and businesses to ensure awareness of the threats of ANS and reduce the risk of spread via emerging pathways

Objective 6.3: Promote the Use of Guidance Documents, Best Management Practices (Bmps)[26], and Other Outreach Materials Related to ANS

a) Encourage ANSTF member agencies, Regional Panels, and other stakeholders to submit guidance documents, BMPs, and other outreach materials to the ANSTF for review and endorsement
b) Identify gaps in outreach materials. Develop or update these materials when needed
c) Utilize the ANSTF website as a clearinghouse for guidance documents, BMPs, and other outreach materials endorsed by ANSTF

Objective 6.4: Promote Awareness of the ANSTF and Its Activities and Provide Educational Briefings on ANS Threats and Solutions and to Legislators and Other Decision Makers

a) Provide timely advice to the appropriate agencies concerning ANS that have been detected in waters of the United States, as well as waters of neighboring nations
b) Provide educational briefings and materials to Federal, State, tribal legislators and their staff members
c) Provide educational briefings and materials to Federal and State agency decision makers to build support for and incorporation of ANS programs into agency activities
d) Participate in and assist NISC with the national expansion of National Invasive Species Awareness Week

Goal 7: Research - Facilitate Research to Address Environmental, Economic, and Human Health Risks and Impacts Associated with Aquatic Nuisance Species

Information and research is needed to quantify and clarify the effects that ANS are having on native species and habitat as well as to socio-economics and human health. Although much research has been conducted for some aquatic invasive species, there are many species for which little is known. Increased knowledge of the biology, potential impacts, associated control methods, and interaction with climate change and other major drivers of change will allow for the most effective management of ANS. Research supports all facets of this Strategic Plan and is necessary to increase the effectiveness of prevention, detection, response, and control and management of invasive species. To help ensure that research addresses critical needs, the actions suggested below focus on coordination among Federal, State, and tribal governments; academia; and other participating entities. Economic

research is also highlighted in this section. There is a lack of knowledge on a worldwide scale of the economic impacts of ANS. In many cases, it is the economic impacts that will be the driving force in effecting change in personal and business actions, management, and policy.

Many of the actions below encourage the continued development of risk analysis[27] tools to characterize the likelihood and severity of potential ANS to the environment, the economy, and human health and the means and methods to manage identified risks[28] . Science-based risk analysis is needed to evaluate invasive species before they reach the jurisdiction of the United States and to prioritize appropriate responses once they do. Risk analysis requires a methodology that integrates environmental, economic, social, and human health considerations. The principal role of ANSTF will be to provide guidance to these institutions on research, monitoring, and risk analysis needs and to provide feedback to researchers on the effectiveness of the management tools they develop.

Objective 7.1: Develop and Maintain a List of ANS Research Priorities; Communicate This List to the Scientific Community

Objective 7.2: Develop and Maintain Guidance Documents, Protocols, and Best Management Practices (BMPs) Related to ANS

a) Maintain and promote the Federal Aquatic Nuisance Species Research Risk Analysis Protocol[29]
b) Evaluate effectiveness and identify gaps in guidance documents and BMPs. Develop or update these documents when needed

Objective 7.3: Track the Progress of Research Activities Funded or Prioritized by the ANSTF

a) Utilize the research page on the ANSTF website as a clearinghouse for research activities funded or prioritized by the ANSTF
b) Share information on Federal invasive species grant opportunities and programs by linking this information from agency web pages to the ANSTF website

Objective 7.4: Support Development of Socio-Economic Research and Methods to Quantify the Economic Impact of ANS

Objective 7.5: Support Research on Interdiction Methods for Specific Pathways of ANS[30]

a) Facilitate the development of technologies and practices used for ballast water treatment and vessel hull fouling
b) Support development and implementation of fully effective ANS barriers between the Mississippi River and Great Lakes Basin and other infested natural waterbodies

Objective 7.6: Support Efforts to Identify Gaps and Expand Research Relevant to Control and Eradication Measures to Address ANS That Have Become Established in Waters of the United States

Objective 7.7: Encourage Research to Develop Species Invasion-Risk Forecast Tools

a) Develop and implement risk analyses and forecast models to evaluate invasiveness of high priority species, taking into account climate change and other drivers of change
b) Improve data collection at ports of entry so numbers and identification of species entering the United States through commerce in living organisms are available and accessible
c) Support development of a Risk Analysis Clearinghouse based on the outcome of species invasion-risk forecast analyses

Objective 7.8: Support Existing Databases and Global Database Networks So National and Worldwide Decision-Support Information for Invasive Species Management Is Accessible, Transparent, and Accurate

Goal 8: Funding - Coordinate Federal Agency Budgets to Support the Aquatic Nuisance Species Task Force's Priorities and Establish a Clear Process That Links State and Regional Needs in Their Areas of Responsibility

The ANSTF operates within a limited budget to conduct semiannual meetings and provides a fraction of the support needed to achieve goals identified by the Regional Panels and ANSTF-approved management plans. It is the cornerstone of the ANSTF to provide resources that will allow the States, Regional Panels, and tribes to implement programs that reflect the goals within the Strategic Plan. The actions suggested below focus on obtaining dedicated, long-term funding for the ANSTF by developing partnerships, and seeking opportunities to leverage funds within State and Federal agencies, Indian tribes as well as public and private interests. The actions also encourage Federal agencies to continually review ANS priorities to find opportunities where agency authorities align with priority needs to create funding opportunities that can be met or communicated to the Office of Management and Budget.

Objective 8.1: Secure Dedicated, Long-Term Funding for the ANSTF Strategic Plan Actions

a) Compose an annual report focused on ANS and use it as an opportunity to reach decision makers and other leaders on the need for proper policies and funding for ANS efforts
b) Encourage Federal agencies to take ANSTF-approved Regional Panel recommendations into consideration as budgets are developed and to provide feedback
c) Encourage Federal agencies to continually review regional priorities for opportunities where agency authorities align with priority needs in order to create funding opportunities that can either be met or communicated to the Office of Management and Budget

d) Coordinate with NISC to ensure ANS priorities are recorded in the Interagency Invasive Species Performance Budget and communicated to the Office of Management and Budget
e) Seek opportunities to leverage funds for ANS activities from Federal agencies and additional partners.

Objective 8.2: Optimize Use of Current Funding for ANS Activities by Engaging Potential Resources and Programs Within Federal Agencies and Additional Partners

Objective 8.3: Develop a List of ANS Funding Priorities

a) Prioritize actions based on anticipated efficacy, threat level, and costs / benefits to natural resources
b) Annually assess the funding needs of the Regional Panels as well as species-specific and State ANS management plans

APPENDIX 1: LIST OF ACRONYMS

ACOE	Army Corps of Engineers
AIS	Aquatic Invasive Species
ANS	Aquatic Nuisance Species
ANSTF	Aquatic Nuisance Species Task Force
APHIS	Animal and Plant Health Inspection Service
BLM	Bureau of Land Management
BMP	Best Management Practice
BOR	Bureau of Reclamation
DOS	Department of State
DOT	Department of Transportation
EDRR	Early Detection and Rapid Response
EPA	Environmental Protection Agency
FACA	Federal Advisory Committee Act
FICMNEW	Federal Interagency Committee for the Management of Nuisance and Exotic Weeds
HACCP	Hazard Analysis and Critical Control Point
HSDP	Homeland Security Presidential Directive
ICS	Incident Command System
IPM	Integrated Pest Management
ITAP	Federal Interagency Committee on Invasive Terrestrial Animals and Pathogens
MARAD	Maritime Administration
NANPCA	Nonindigenous Aquatic Nuisance Prevention and Control Act
NIMS	National Incident Management System
NISA	National Invasive Species Act
NISC	National Invasive Species Council
NOAA	National Oceanic and Atmospheric Administration

NPS	National Park Service
USCG	United States Coast Guard
USDA	United States Department of Agriculture
USFS	United States Forest Service
USFWS	United States Fish and Wildlife Service
USGS	United States Geological Survey

END NOTES

[1] The term "vector" is continues to vary among agencies and organizations and is commonly confused with "pathway". The ANSTF defines a vector as the physical means or agent causing a species to translocate or spread (e.g. ship, car, waders).Pathway is defined as an activity or process through which a species may be transferred to a new location (e.g. shipping, animal trade, recreational activities).

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[11] For the purposes of this document the term “Asian carps” refers to four species: black carp (Mylopharyngodon piceus), bighead carp (Hypophthalmichthys nobilis), grass carp (Ctenopharyngodon idella), and silver carp (H. molitrix).

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[17] The term "waters of the United States" is defined by the Clean Water Act 40 CFR 230.3(s)

[18] Federal agencies specified within the Act include the U.S. Fish and Wildlife Service, National Oceanic and Atmospheric Administration, Environmental Protection Agency, United States Coast Guard, Army Corps of Engineers, United States Department of Agriculture.

[19] Organizations specified within the Act include the Great Lakes Commission, Lake Champlain Basin Program, Chesapeake Bay Program, and San Francisco Bay-Delta Estuary Program.

[20] Two members co-represent the Native American Fish and Wildlife Society

[21] Section 1202(k) (2) of NANPCA, requires the ANSTF to submit, on an annual basis, a report to Congress focusing on progress in carrying out the provisions of the Act. Under the Act, the Regional Panels are required to submit an annual report to the ANSTF describing activities in their regions related to ANS prevention, research and control activities. Additionally, contracts for funding the panels require an annual report.

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[24] Homeland Security Presidential Directive 5 (HSPD-5) required all Federal agencies and departments to adopt the National Incident Management System (NIMS) to coordinate emergency preparedness and incident management and response among the public and private sectors.

[25] Integrated pest management (IPM) is a broad based ecological approach that utilizes a range of practices to maximize control of a species. In IPM, one attempts to prevent introduction, to observe patterns of spread, and control as necessary by the most economical means, and with the least possible hazard to people, property, and the environment.

[26] The phrase "Best Management Practice" (BMP) was originally used in the U.S. Clean Water Act and associated Federal regulations to refer to procedures used for industrial wastewater control and stormwater management. Although the term was defined by the Environmental Protection Agency as a regulatory tool used to implement Federal wastewater permit regulations, it is now commonly used in the language of environmental management. The Aquatic Nuisance Species Task Force uses the term BMP in the Strategic and Operational Plans to describe any method or technique found to be the most effective and practical means in achieving an objective while making optimum use of resources.

[27] The term "Risk analysis" in this document includes both risk assessment and risk management. Risk assessment measures the likelihood of an event occurring and the severity of negative impacts from such an event. Risk management is the process of identifying, evaluating, selecting, and implementing actions to reduce risk and includes more subjective elements including risk prioritization, risk tolerance, and associated decisions that weigh the benefits and costs of risk minimization options.

[28] Additional information on ANS risk analysis methodology can be found within 1) National Research Council. 1983. Risk Assessment in the Federal Government: Managing the Process. National Academy Press, Washington DC. 2) National Research Council. 1993. Issues in Risk Assessment. National Academy Press, Washington DC. 3) Stern, P.C. and H.V. Fineberg (eds). 1996. Understanding Risk: Informing Decisions in a Democratic Society. National Academy Press, Washington DC. 4) National Research Council. 2009. Science and Decisions: Advancing Risk Assessment. National Academy Press, Washington DC.

[29] The ANSTF developed a research protocol as is required by the Nonindigenous Aquatic Nuisance Prevention and Control Act of 1990 (NANPCA, 101, 104 STAT. 4671, 16 U.S.C. 4701-4741), as amended by the National Invasive Species Act, 1996. Section 1202(f) (2) of NANPCA directs the ANSTF to establish a protocol "to ensure that research activities carried out under [NANPCA] do not result in the introduction of aquatic nuisance species to waters of the United States."

[30] The action items listed for Objective 7.5 are included in this document as they are mandated by the Nonindigenous Aquatic Nuisance Prevention and Control Act (NANPCA). Additional actions taken to support this objective will be included in the ANSTF Operational Plan.

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Chapter 46

A NEW LATE TRIASSIC (WAREPAN: MIDDLE TO LATE NORIAN) ORTHOCONIC NAUTILOID FROM NEW ZEALAND AND NEW CALEDONIA

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ABSTRACT

Stipamonotis herangiae n. gen. et sp. is described from the Murihiku Terrain, mainly the Kawhia Regional Syncline, New Zealand, and the Téremba Terrain of New Caledonia. It is a small, cylindrical, unornamented michelinoceratine, a rare accompaniment to the bivalve monotis ubiquitous in these rocks, ranging through all zones of the Warepan Stage (Middle to Late Norian) at the very end of the time-range of the Order Orthocerida.

The topmost, previously unnamed zone of the Warepan Stage is here named the Cotterallae Zone, characterised by the presence of the bivalve *Makoiamya cotterallae* Grant-Mackie, 2013 and the absence of species of monotis. Its fauna includes the last members of the new species.

Keywords: orthocerida, *Stipamonotis herangiae* new genus and species, Late Triassic, Norian, Warepan Stage, New Zealand, New Caledonia

INTRODUCTION

During fieldwork on the Kiritehere coast, southwest Auckland (Figure 1), I collected examples of a small orthoconic nautiloid not previously recorded in the fauna. I regarded it then (Grant-Mackie 1975) as related to the Timor Permian genus *Bitauniоceras* Shimizu & Obata, 1936 (v. also Campbell, in Spencer et al. 2009). The same taxon has since been found also in the South Island (Nelson) and New Caledonia (Figure 2) (Appendix 1). The purpose of

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this chapter is to document its distribution in the Late Triassic (Warepan Stage of the local chronostratigraphic scheme - Figure 3) and describe the species.

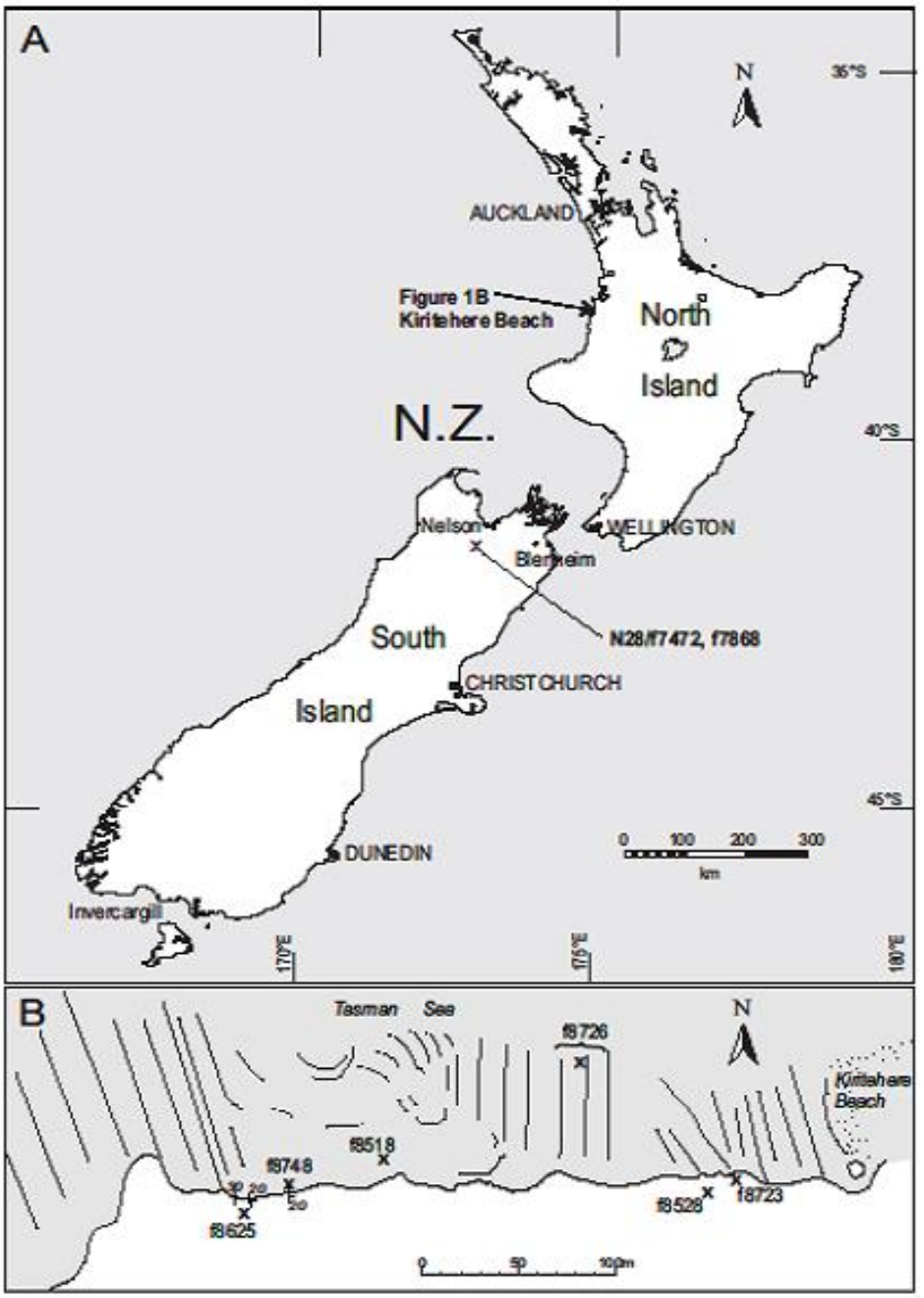

Figure 1. Locality maps. A. outline map of New Zealand showing collection sites of *Stipamonotis* in the Nelson area and the location of Map 1B. B. map of the coast SW of the mouth of the Kiritehere River (after Grant-Mackie 1981).

Figure 2. Outline map of New Caledonia showing *Stipamonotis* collections sites.

International			NZ - New Caledonia			Ma
	Stage	Zone	Stage	Substage	Zone	
Jur						201.3
Late Triassic	Rhaetian	Crickmayi	Otapirian			208.5
	Norian L	Amoenum	Warepan	Marokopan	Cotterallae	
		Cordilleranum			Calvata	
					Richmondiana	
					Acutecostata	213.3
	Norian M	Columbianus		Kiriteherean	Murihikuensis	
					Rauparaha	217.0

Figure 3. Divisions of the Late Triassic Warepan Stage (after Grant-Mackie 2015, with the Warepan-Otapirian boundary age adjusted according to Raine et al. 2015, and the zonal scheme altered as herein).

Orthoconic nautiloids are important elements of Paleozoic molluscan faunas. They appeared in the Early Ordovician, reached their acme in the Devonian, and four genera survived the Permo-Triassic extinction event (Bizzarini & Gnoli 1991, Gradinaru & Sobolev 2007), before finally succumbing at about the Norian-Rhaetian boundary c. 208.5 million years ago (Raine et al. 2015), except for an unconfirmed record of an Early Cretaceous genus, *Zhuravlevia* Doguzhaeva, 1994 from the Caucasus (Fam. Geisonoceratidae, otherwise Middle Ordovician - Early Triassic) (shown also in Ponder & Lindberg 2008, Figure 8.7, p. 178).

Doguzhaeva (1994) also recorded the orthoconic genus *Trematoceras*, without documentation, as occurring in the New Zealand Triassic, but a literature search has not provided any evidence for this claim, unless the record by Trechmann (1918) of *Orthoceras* sp. from the Southland Late Triassic was regarded as a *Trematoceras* by Doguzhaeva. Begg (1981) reported the presence of four species of *Michelinoceras* and one of *Orthoceras* from the Malakovian, Etalian and Oretian (Anisian-Early Norian) of the Wairaki Hills, Southland. None of these fits the generic characters outlined below for the Kiritehere material, the *Orthoceras* having a marginal siphuncle and the michelinocerids possessing episeptal or endosiphuncular deposits. Collections examined in GNS Science, Lower Hutt, included some 20 Triassic orthocone specimens (Etalian – Otapirian; Anisian-Rhaetian), some of which are of the coleoid *Aulacoceras.* Apart from determining that these are probably not congeneric with the Kiritehere specimens described herein, because of lacking any of their characteristic features, they are not further considered here.

SYSTEMATICS

Orthocerids have been subject to many classification schemes (v, e.g., Zakharov 1996) and that followed here is from Sweet (in Teichert et al. 1964) who also emphasised that any scheme of suprageneric classification of this group must so far be suspect and highly provisional because of the rarity of complete specimens and the inadequate state of current knowledge concerning their relationships.

The classification advocated by Zakharov (1996) has two subclasses of cephalopod, Nautilomorphi (or Nautiloda) and Coleoidea, with Superorder Pseudorthoceratoidea in the former, with a large embryonic shell, and Michelinoceratoidea in the latter, with a small embryonic shell. Both have a central or subcentral siphuncle; the Pseudorthoceratoidea possess various types of intracameral deposits and range Ordovician-Triassic whereas the Michelinoceratoidea are Paleozoic only.

This classification is radically different from that of the Treatise (Sweet 1964), and means that previously-identified '*Orthoceras'* sp. and *'Michelinoceras'* sp. are divided between two subclasses, calling for a more careful assessment of material before generic assignment. Furthermore, cephalopod classification probably continues in a fluid state and further changes are likely to be proposed. The more traditional/conservative scheme used in the Treatise (Sweet 1964) is for this reason followed here.

Class Cephalopoda Cuvier, 1798
Subclass Nautiloidea Agassiz, 1847
Order Orthocerida Kuhn, 1940
Superfamily Orthocerataceae M'Coy, 1844
Family Orthoceratidae M'Coy, 1844
Subfamily Michelinoceratinae Flower, 1945

Michelinoceras Foerste, 1932, (type *Orthoceras michelini* Barrande, 1966) is the most abundant and widespread of the Triassic orthoceratids and includes long slender nearly cylindrical orthocones with circular cross section, long camerae, central or nearly central

siphuncles free of organic deposits, straight septal necks, thin cylindrical connecting rings, and well developed hyposeptal and episeptal cameral deposits (Sweet 1964).

Genus *Stipamonotis* Grant-Mackie, n. gen.

Type

S*tipamonotis herangiae* Grant-Mackie, n. gen. et sp., Late Warepan Stage (Late Norian), Late Triassic, New Zealand, New Caledonia.

Derivation of Name

Latin 'stipare', to accompany, emphasising the association of these orthocones with the abundant co-occurring pterioid bivalves, collectively monotis (see Grant-Mackie 2015).

Diagnosis

Unornamented longiconic michelinoceratine with straight transverse sutures, long chambers, centrally located orthochoanitic siphuncle with no endosiphuncular deposits, and episeptal and mural cameral deposits taking campanulate form.

Comments

In terms of Zakharov's (1996) scheme, the new genus would belong to the Superorder Pseudorthoceratoidea Barskov, 1963, Order Pseudorthocerida Barskov, 1963, and probably Family Trematoceratidae Zakharov, 1996. Unfortunately, specimens to hand do not include early ontogenetic structures, so that differences between Pseudoceratoidea and Michelinoceratoidea are not preserved, but the age strongly suggests location in the former superfamily if that classification were to be preferred.

The type species of *Stipamonotis* is known only from conch fragments of up to 11 chambers with no indication of curvature, and the juvenile portion of the phragmocone is unknown. The other common Triassic michelinoceratine, *Trematoceras* Eichwald, 1851 (type *Orthoceras elegans* Münster, 1841), and the only other genus known in the Late Triassic, possesses mural and episeptal deposits within the camerae but the latter produce stellate or petaloid impressions on internal molds of septal faces, and none was seen in specimens of the new material. In addition, some species show significant taper of the phragmocone and others possess annular ridges. Of the other genera included in this subfamily none possesses the combination of characters seen in the new genus, and most have a middle Paleozoic (Ordovician-Devonian) range. The other Triassic orthoceroid genera, *Paratrematoceras* Schastlivtseva, 1981, *Pseudotemperoceras* Schastlivtseva, 1986 and *Romanorthoceras* Gradinaru & Sobolev, 2007, are so far all of Early to Middle Triassic age (Schastlivtseva 1988, Gradinaru & Sobolev 2007, Sobolev & Gradinaru 2008); none of the three possesses any cameral deposits, *Pseudotemperoceras* also shows a much more distinct taper of the phragmocone and swelling of the siphuncle tube between septa, and *Romanorthoceras* (so far not fully described) possesses shorter camerae and is a faintly curved brevicone.

Stipamonotis herangiae Grant-Mackie, n. sp. (Figure 4)

1975. aff. *Bitauniocerас* sp. Grant-Mackie: un-numbered page in Table 2.1., Vol. 2.
2009. aff. *Bitauniocerас* sp., Campbell, in Spencer et al., in Gordon (ed.): p. 224.

Derivation of Name

To honour the late well-known and highly respected Princess Te Puea Herangi, a past leader of the Tainui people of the Waikato, whose wider influence extended to the type area of Kiritehere. In addition, the rarity of *Stipamonotis* is complementary with the widely held opinion that Princess Te Puea was a leader of notably rare quality.

Holotype

C871, partial phragmocone of eight chambers in longitudinal section, showing siphuncle tube and cameral deposits, from collection AU1421, R16/f8625, Arawi Shellbed, cliff and shore platform, 310 m west of southern end of Kiritehere Beach; JAG-M, DCL, 1/61.

Paratypes

C 2137, AU11038; C2138, AU11940; C2139, AU12062; C2140, AU12085; C2141, AU12285; all from the Kiritehere area, southwest Auckland, in the Kawhia Regional Syncline, Murihiku Terrain; and C2136, AU7201 from the Téremba Terrain, New Caledonia (v. Appendix 1).

Additional Material

The new species is represented by 14 additional specimens from Kiritehere localities, and one or two specimens at each of two sites in the Nelson Regional Syncline, Murihiku Terrain, and three on Ile Ducos, Baie de St-Vincent, Téremba Terrain, New Caledonia (all listed in Appendix 1). It has not been recorded from the Southland Regional Syncline of the Murihiku Terrain.

Diagnosis

Unornamented michelinoceratine longicones of small diameter, with straight transverse sutures, long chambers, centrally located orthochoanitic siphuncle lacking endosiphuncular deposits, and mural and episeptal cameral deposits that become campanulate in shape.

Description

Known so far only from conch fragments with no certain apical or apertural portions preserved and unornamented exterior. Fragments up to 46 mm long, straight, longiconic, subcylindrical; up to 17 mm diameter (range from 3.3 mm), with subcentral orthochoanitic siphuncle ~ 0.1-0.12 mm diameter, lacking siphuncular deposits; sutures straight, transverse; chambers relatively long (range 3.1-6.3 mm), generally longer than conch diameter at that stage. Cameral deposits generally obscure due to recrystallisation but better preserved specimens (e.g., Figure 4C) show variation in form according to location along conch, with youngest having no deposits and oldest with episeptal and mural deposits which are bell-shaped, mouth of ‘bell’ being adoral in position; intermediate camerae with deposits thinner than but generally conforming to shape of those of oldest camerae; constriction of bell shape

'behind' mouth of bell located slightly behind mid-height of each chamber. Body chamber not certainly known; no fragment shows evidence of conch curvature. Juvenile portion of shell seems to have been shed in at least some specimens (Figure 4A, B, C, E): earliest septum of holotype (a in Figure 4C) evenly curved, with no indication of septal neck, with septum discontinuous in siphuncular region and with cameral deposits continuous across (these features lost during specimen preparation); next-youngest septum (b in Figure 4C) clearly showed short backwardly-directed septal neck (as thin black lines continuous from septum, now also largely lost in preparation). Two oldest septa very thin, represented by curved black lines; septum no. 3 (c in Figure 4C) twice to 3X thickness of first two (perhaps due to incipient separation along this septum); septa d-f like first two.

Comments

The new species is characterised by its long unornamented cylindrical form, circular section, subcentral empty siphuncle tube, in the campanulate form of its cameral deposits, and in the apparent shedding of the juvenile shell. Although no specimen certainly shows the body chamber, it may be at least partially preserved in one specimen (C2137 - Figure 4D) with a sediment-filled terminal chamber ~ 8mm long which may represent part of the body chamber.

Table 1. Selected dimensions of *Stipamonotis herangiae* n. gen. et sp., listed according to specimen (C …) or collection (AU …) catalogue numbers

Specimen	L	D. max.	D. apic.	No. Chamb.	L. Cham	D. siph.
C871	38,2	5,5	4,4	8	5,6	0,12
C2136	23	4,7	3	6 or 7	6,3	0,1
C2137	14,1	5,5		3	6	
C2138	18,6	5,6	5	4	6	
C2139	32,2	3,5	2	~9	3,1	
C2140	34,4	5,6				
C2141	46,1	7,2	5,1	~10		
AU7201	13,1	3,3	3,2	?6	3	
AU7800	19	3,5				
AU9668a	21,5	4,1		~5	~4	
AU9668b	11	2,5		~3		
AU9668c	13,4	4		~4	3,6	
AU9668d	44	5	3,1	~11	4,1	
AU11038	17,1	4,5				
AU11940	24,3		4,5	>3	5,5	
AU11062	27,2	5,4	3,6	7	5	
AU8367a	19,1	5				
AU8367b	21,1	7,5				
AU8367c	20,8	5				

L = overall length of preserved phragmocone, D. max = maximum diameter of fragment, D. apic = diameter of adapical end of fragment, No. cham = number of preserved chambers, L. cham = length of longest chamber, D. siph = external diameter of siphuncle. In addition to those specimens listed here there are four more in AU8367, with L varying between 19.1 and 21.1 mm and D. max. varying between 4.6 and 17.0 mm. but with no other dimensions obtainable.

Specimen C2141 (Figure 4B) is the only one to show obvious taper, from 4.2 mm to 7.0 mm over 42 mm length. Shorter fragments show less obvious taper.

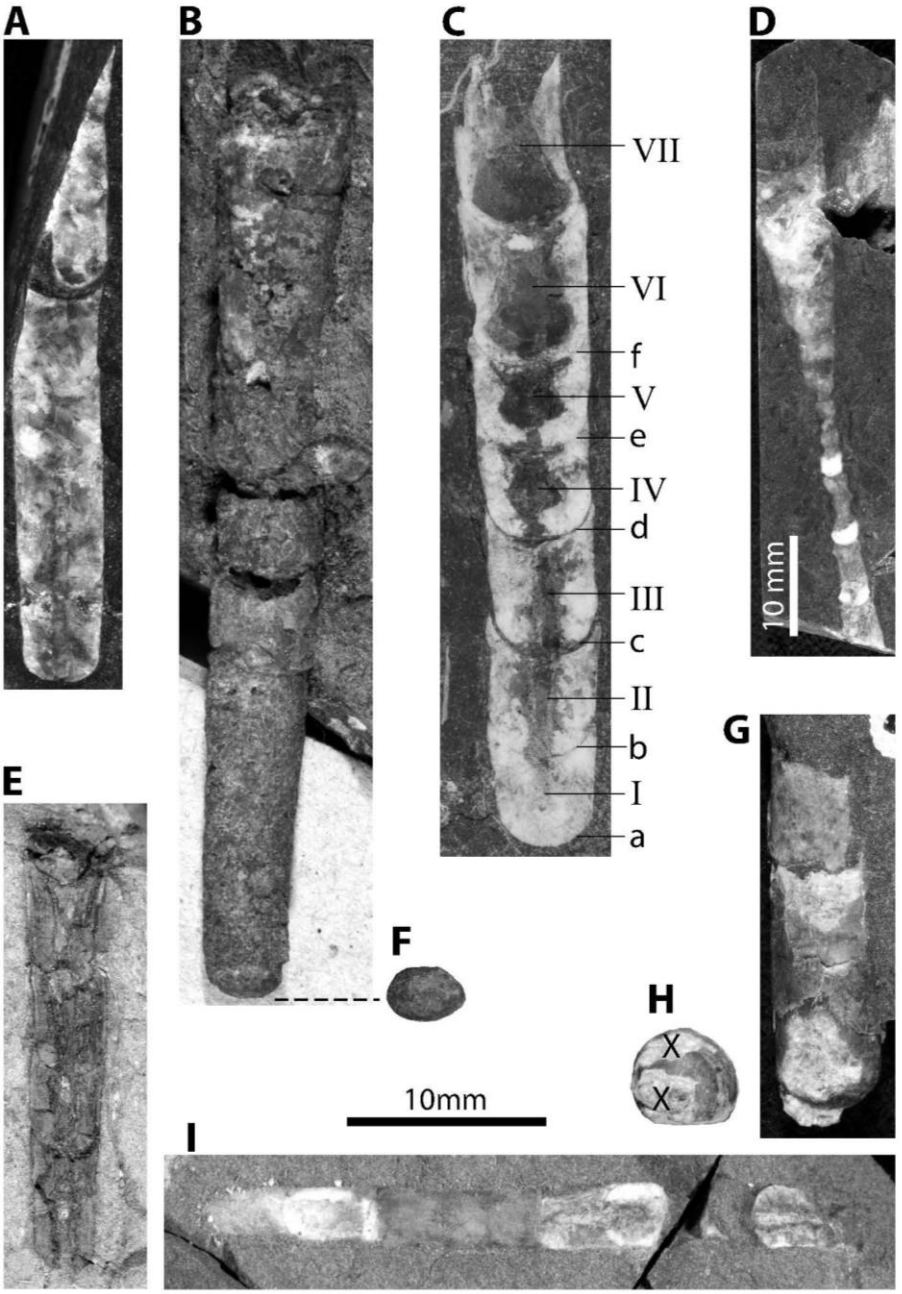

Figure 4(A-I). *Stipamonotis herangiae* n. gen. et sp. All images are X 4, except D, which is X 2. (A) paratype C2140, AU12085, Arawi Shellbed; cut section. (B) paratype C2141, AU12285, Arawi Shellbed; external view. (C) holotype C871, AU1421, Arawi Shellbed; longitudinal section, with septa (a-f) and camerae (I-VII) indicated. (D) paratype C2137, AU 11038, Arawi Shellbed; longitudinal section of bent or broken phragmocone, X 2. (E) paratype C2136, AU7201, Leprédour Shellbed; natural longitudinal section of partial phragmocone. (F) paratype C2141, AU12285, Arawi Shellbed; cast of oldest preserved septum. (G) paratype C2138, AU11940, Arawi Shellbed; partial external longitudinal view. (H) paratype C2138, AU11940, Arawi Shellbed; partial view of fragments of earliest preserved septum (x) and partial cast of remainder. (I) paratype C2139, AU12062, Arawi Shellbed; partial phragmocone showing both external and broken internal views.

The likelihood of the early-formed portion of the phragmocone being dehisced is seen also, with enough specimens showing features suggestive of moulting of the juvenile shell comparable to those described above for the holotype, so it may have been a common event in this species, occurring so as to improve mobility.

Cameral deposits in the holotype specimen vary along the conch in the following way: the first-formed three chambers of the type specimen (I-III in Figure 4C) seem wholly or mainly filled with cameral deposits. In subsequent chambers episeptal and mural deposits are apparent but do not completely fill them; in chambers IV and V internal spaces lacking cameral deposits are filled with sediment between the episeptal deposit and the succeeding septum and each set of deposits has a rounded base conforming to the curvature of the adapical septum, and the abapical septum is about half covered by cameral deposit. In camerae VI and VII cameral deposits are thinner but generally similar in form to those in IV and V. Deposits in the last-formed chamber are less well exposed or preserved but similar to those of VI. Chamber VII is the last-formed amongst those present and, although also poorly preserved, shows deposits similar to those in VI. Other specimens also show aspects of the above features, but less complete and less well preserved than in the type (e.g., C2136, C2137 - Figure 4D, E) but most are thoroughly recrystallized. C2136 shows banding of the cameral deposits indicative of depositional layering (Figure 4E), probably of post-mortem origin.

Species of *Trematoceras* show, inter alia, star- or petal-shaped impressions radiating across the septal surfaces from the septal foramen (Sweet 1964), a feature difficult to see in most specimens but clearly absent from two specimens of the new species (C2138 and C2141 - Figure 4F, H). This emphasises again that this genus seems to be absent from New Zealand Triassic faunas, despite Doguzhaeva's (1994) claim.

Initial superficial study suggested a relationship with *Bitauniоceras* Shimizu & Obata (Grant-Mackie 1975, Campbell 2009), but while this Permian genus does have a cylindrical form, straight transverse sutures and a subcentral siphuncle, it also possesses transverse constrictions and lirae on the phragmocone, both lacking in *Stipamonotis.*

Presently the genus is monotypic. No other Triassic orthocones seen in either GNS Science or University of Auckland collections can be associated with *S. herangiae* n. sp. In particular, most display no cameral deposits, but in addition some have a significantly larger diameter than in that species and/or are of a different age. *Michelinoceras* sp. B (Begg 1981, unnumbered Figure, p. 180) has hyposeptal deposits and other cameral deposits are of a different form from that in *Stipamonotis.* Begg's *Michelinoceras* sp. A (1981, pl. 4, Figure b) has mural and episeptal deposits not comparable with those of the new genus. The coleoid *Prographularia otapiriensis* (Hector), which occurs close by in adjacent Otapirian strata, is also orthoconic but the guard is more strongly tapered and has longitudinal ridges and grooves.

Associated Biota

As the generic name emphasises, the new species is found generally as rare specimens in the monotis beds (v. Appendix 1). It accompanies *Eomonotis rauparaha* (Grant-Mackie), *E. kiritehereensis* (Grant-Mackie), *E. marwicki* (Grant-Mackie), *E. murihikuensis* (Grant-Mackie), *Entomonotis richmondiana* (Zittel), *E. acutecostata* (Trechmann), *Pacimonotis discordans* (Grant-Mackie), *Maorimonotis maniapotoi* (Grant-Mackie), *M. routhieri* (Avias), *M. calvata* (Marwick). There are in addition a variety of other bivalves, diverse but rare brachiopods and occasional cephalopods, etc. (v. Appendix 1).

Age

All occurrences of *Stipamonotis herangiae* so far known are in Warepan strata. Grant-Mackie (1986) has provided a detailed correlation and biostratigraphic subdivision of strata of this age (Figure 3). N28/f7472 in part comes from the Rauparaha Zone at the base of the Warepan, NC/f051, NC/f524 and NC/f546 from the Murihikuensis Zone, N28/f7472 (part) and N28/f7868 from the Acutecostata Zone, R16/f8625, R16/f8726 and R16/f8748 from the Richmondiana Zone, and R16/f8528 from the Calvata Zone. It has thus been recorded in all monotis zones, and the occurrence in R16/f8723 is believed to belong to the Cotterallae zone, pre-Otapirian but lacking monotis, on the grounds that a few of the other packets in the Kiritehere slump (Grant-Mackie & Lowry 1964) were also judged to be from that zone. The time-range of the new taxon is thus the full Warepan Stage.

In 1986 I (Grant-Mackie 1986) proposed subdivision of the Warepan Stage into zones based on their varying content of monotis species. The topmost zone was left un-named, lacking monotis and Otapirian species. With the description of *Makoiamya cotterallae* (Grant-Mackie 2013) we now have a species that can be used to characterise this part of Norian time, so I here define the Cotterallae Zone as that part of Warepan and Marokopan time lacking species of monotis and any indicators of the Otapirian Stage and chacterised by the presence *inter alia* of the bivalve *Makoiamya cotterallae* Grant-Mackie (Figure 3).

ACKNOWLEDGMENTS

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APPENDIX 1

Locality data for specimens of *Stipamonotis herangiae* n. gen. et sp. Full data are given in the first entry and indicators are omitted in subsequent entries, with the same order being preserved for all entries. Collectors, including the author, are shown by their initials (DCL = DC Lowry, FH = F Hasibuan, HJC = HJ Campbell, JAB = JA Bartrum, LX = Li X-C, MM = M Manceñido, SD = S Damborenea, YJ = Yu J-S, ZW = Zhang W-P); the usual initials are used for compass points; Bw = Warepan Stage.

N28/f7472, grid reference N28/195775; collection AU1547, collected by JAB (no date, but pre-1949), Mt Heslington; Richmondiana Shellbed, a mixed Bw fauna, with *Rastelligera mackayi, Clavigera* cf. *bisulcata, Hokonuia* cf. *limaeformis, Eomonotis rauparaha* (of the Rauparaha Zone), and *Entomonotis acutecostata* (of the Acutecostata Zone).

N28/f7868, N28/095691; AU1733, JAG-M, 1/67, farm track N of Ram Ck, 88 Valley; Richmondiana Shellbed, of the Acutecostata Zone, Bw, with *Rastelligera mackayi, Entomonotis acutecostata, Pacimonotis discordans,* Serpulidae indet., *Chondrites* sp.

R16/f8518, R16/586182; AU2997, JAG-M, 2/88; Kiritehere coastal section, 200 m W of S end of beach, "on NE side of point with prominent thrust" (in fact, base of slump); Arawi Shellbed, of the Marokopan Substage, Bw, with *Entomonotis* sp.

R16/f8528, R16/592184; AU1427 JAG-M 7/59; Kiritehere coast, immediately above 'unconformity'; Arawi Shellbed, of the Calvata Zone, Bw, with *Clavigera* sp., *Psioidiella* sp., *'Coenothyris'* sp., *Palaeonucula* sp., *Maorimonotis calvata, Makoiamya cotterellae, Plagioglypta* sp., Crinoidea indet, Echinoidea radiole.

R16/f8625, R16/589186; AU1421, JAG-M, DCL,1/61, cliff & shore platform 310 m W of S end of Kiritehere Beach; Arawi Shellbed, of the Richmondiana Zone, Bw; type locality; with *E. richmondiana, M. routhieri.*

R16/f8723, R16/592184; AU11940, JAG-M, LX, YJ, ZW, 12/88; AU12062, JAG-M, FH, 1/86; Kiritehere coastal section 2-3 m above monotis beds; Ngutunui Siltstone, Cotterallae Zone, Bw, with Nuculacea indet.

R16/f8726, R16/591184; AU12285, JAG-M, SD, MM, 4/90; Kiritehere coastal section 20 m below 'unconformity'; Arawi Shellbed, of the Richmondiana Zone, Bw, with *E. richmondiana, M. routhieri.*

R16/f8748, R16/590184; AU11038, JAG-M, 9/86; AU12085, JAG-M, F H, 1/86; Kiritehere coastal section, in middle of slump deposit; Arawi Shellbed, of the Richmondiana Zone, Bw; with *"Coenothyris"* sp., *E. richmondiana, M. maniopotoi.*

NC/f051, NC/33127248; GS12764, HJC, 10/79; well exposed strata on shore platform, E coast Ile Ducos, NW of île Jacqueline; Leprédour Shellbed, of the Murihikuensis Zone, Bw, with Crinoidea, Gastropoda indet., *Phaenodesmia* sp., *?Mysidioptera* sp., *Eomonotis taringatura, E. kiritehereensis, Hokonuia limaeformis, ?Kalentera* sp., *Proclydonautilus* sp., *?Arcestes* sp., ?Pinacoceratidae.

NC/f524, NC/60945650; AU9668, JAG-M, 10/83; Ile Ducos, S coast of île Jacqueline, 250 m SE of isthmus; Leprédour Shellbed, of the Murihikuensis Zone, Bw, with *Heterastridium conglobatum (s.s.),* conulariid indet, *Pleurotomaria kiritehereensis, Eomonotis marwicki,* wood.

NC/f546, NC/09776501; AU7201, JAG-M, 1979; Ile Ducos, SE coast of île Jacqueline, c. 130 m W of E end of island; Leprédour Shellbed, of the Murihikuensis Zone, Bw, with Ostreacea indet., *Hokonuia limaeformis, Eomonotis murihikuensis.*

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Chapter 47

COMBINED EFFECTS OF OCEAN ACIDIFICATION AND SALINITY ON FORAGING BEHAVIOUR OF INTERTIDAL SCAVENGING GASTROPOD *NASSARIUS FESTIVUS*

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ABSTRACT

Chemoreception is crucial to marine scavengers in food acquisition. The combined effect of salinity (10 psu, 30 psu) and ocean acidification (380 μatm, 950 μatm and 1250 μatm) on food detection at three foraging distances (20 cm, 40 cm and 60 cm) was studied in a marine scavenging gastropod *Nassarius festivus* over an exposure period of seven days. Time and percentage of individuals reaching the bait (excised clams) were recorded. Lower salinity increased food searching time at all distances away from the bait on both Day 1 and Day 7. Higher pCO_2 levels increased food searching time at 20 cm and 60 cm on Day 1, but 40 cm and 60 cm on Day 7. The interactive effect between pCO_2 and salinity was only significant on Day 1 at 20 cm. Lower salinity reduced percentage of individuals engaged in foraging at all distances away from the bait on both Day 1 and Day 7. The effect of pCO_2 was significant at 60 cm on Day 1, but at all distances on Day 7. The interactive effect between pCO_2 and salinity was only significant on Day 1 at 20 cm. In conclusion, food detection ability was negatively affected by low salinity and high pCO_2 with the inhibitory effect of pCO_2 being stronger as the time of exposure increased.

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INTRODUCTION

Animal behaviour is complicated and generally includes how organisms search for food, store their food resources, exhibit aggression and defense, protect their territories, locate their mating partners, etc. All these kinds of behaviour are adaptations to a variable environment and shaped by evolution (Tuomainen and Candolin, 2011). Since behaviour mediates interactions between an individual and the environment, the ability of an organism to respond appropriately under new conditions is crucial for determining their immediate success or failure in the face of environmental changes.

Recent studies have shown that animal behaviour is affected by low oceanic pH that is predicted to occur in the future with sensory impairment such as auditory and olfactory functions. These disturbances eventually lead to problems in decision making. For example, the ability to respond to olfactory cues has been lost in clownfish for navigation (Mundy et al., 2009) and predator detection (Dixson et al., 2010; Ferrari et al., 2011). Munday (2010) also found that the larvae of damselfish *Pomacentris wardi*, when exposed to 850 μatm pCO_2, seem to have lost the ability to differentiate predators and showed 5-9 times higher mortality than the control group without predators. Similar results were reported by Ferrari et al. (2011) regarding the coral reef fish *Pomacentris chrysurus* around 1000 μatm pCO_2. High pCO_2 levels could harm the auditory sense of marine fish (Simpson et al., 2011) and induce the lateralization capacity (Domenici et al., 2012). Ocean acidification could also impair the visual recognition and risk assessment. For example, anti-predation responses to the spiny chromis damselfish *Acanthochromis polyacanthus* were observed in juveniles of the Ambon damselfish *Pomacentrus amboinensis* upon exposure to 440 – 700 μatm pCO_2; this ability, however, was lost when the pCO_2 level increased to 850 μatm (Ferrari et al., 2012).

Food detection is crucial to animals' survival and success. This is particularly true for scavengers owing to sporadic food supply and intense competition (Morton and Yuen, 2000). Better food detection ability not only allows individuals to have access to carrion earlier, but they can also enjoy its most nutritious part. For example, marine gastropod *N. festivus* would choose soft tissues that are of higher energy content (Cheung et al., 2006). In a marine scavenging community, ocean acidification can be a selective force, as responses to ocean acidification are species-specific. Even closely related species may respond differently to acidification (Dupont et al., 2010; Logan, 2010). More sensitive species with greater impairment of chemosensory functions will be less competitive and eventually outcompeted by less sensitive ones.

Salinity has a significant effect on marine animals as they can only survive within a salinity range which is species-specific and life stage-specific (Willmer et al., 2000). Reduced salinity, in particular, affects ion and acid-base regulation and modifies responses to ocean acidification (Dickinson et al., 2012). While Dickinson et al. (2013) showed that moderate hypercapnia increased the shell and tissue growth of the hard shell clam, *Mercenaria mercenaria*, low salinity tended to abolish such growth response. The synergistic effect of salinity and ocean acidification was found in the eastern oyster, *Crassostrea virginica*, with lower salinity weakening the shell and enhancing oxygen consumption under high pCO_2 (Dickinson et al., 2012). In addition, the larval mortality of nassariid gastropods *Nassarius festivus* and *N. conoidalis* was greatly affected by high pCO_2 level (1250 μatm) when salinity was reduced from 30 psu to 10 psu (Zhang et al. 2014). Ko (2013) observed that the early

growth phase from hatching to the 5-day-old veliger stage of the Portuguese oyster, *Crassostrea angulata* showed high tolerance to pH 7.6 at 27 psu, but the larval shell area was significantly smaller at pH 7.4 when the salinity was reduced to 24 psu. Although low salinity (10 psu) reduced the scope for growth in *N. festivus* at elevated pCO_2 levels for 31 days, elevated pCO_2 levels had no effects in isolation on all other physiological parameters and only weak interactions with salinity for excretion and scope for growth (Zhang et el., 2016).

Nassarius festivus is the most competitive scavenger on the sandy shores of subtropical Hong Kong (Morton and Yuen, 2000). Its success relies on its excellent chemoreception, rapid feeding rate, and high tolerance to environmental stresses including starvation, temperature, salinity, and hypoxia. According to Cheung (1997), the salinity tolerance of *N. festivus* ranged from 15 psu to 35 psu. Morton and Yuen (2000) also showed that *N. festivus* is able to detect and move fast towards carrion from a distance of > 80 cm in laboratory conditions. In this study, the foraging behaviour of *N. festivus* was investigated upon exposure to the combined effects of ocean acidification and low salinity.

MATERIALS AND METHODS

Study Organisms

Nassarius festivus (shell length 13 – 15 mm) were collected from Starfish Bay (22º48′N, 114º24′E), Hong Kong, in July 2014 and acclimated to laboratory conditions for two weeks before experimentation (temperature: 24°C, salinity: 30 psu, light-dark cycle: 12 hours: 12 hours). Excised clams *Ruditapes philippinarum* were offered as food twice a week at intervals of three to four days (Cheung, 1994), and seawater was changed immediately after feeding to avoid waste accumulation.

Experimental Setup

A full factorial experiment (3 × 2) with three pCO_2 levels (low: 380 μatm, medium: 950 μatm, and high: 1250 μatm) and two salinities (low: 10 psu, high: 30 psu) was set up, i.e., six treatments with different combinations of pCO_2 level and salinity. 10 psu represents the lowest salinity *N. festivus* experiences in summer due to rainfall, and 30 psu is the normal salinity in Hong Kong waters (Environmental Protection Department, 2012). Since carbon chemistry conditions vary with space and time, for experiments to be ecologically relevant, local scenarios should be taken into account (McElhany and Busch, 2013). The pCO_2 level of 380 μatm (LC) was the estimated current global level, while 950 μatm (MC) and 1250 μatm (HC) are the respective predicted levels for the years 2100 and 2300 according to the IPCC (Intergovernmental Panel on Climate Change, PCR8.5 scenario, Collins *et al*., 2013). Although a large fluctuation in pH is commonly observed on sandy shores, the pH levels *N. festivus* exposed to in this experiment (7.25 – 8.26) were beyond the range experienced in the field. According to Hong Kong government's report, the sampling site's pH varied between 7.75 and 9.00 (Environmental Protection Department, 2012), while pH values below 7.50 were recorded only 3 times in the last 17 years (1986 - 2013). Various CO_2 partial pressures

were prepared by mixing air and industrial CO_2 gas (with a purity of 99.5%, Hong Kong Oxygen & Acetylene) at certain ratios (Findlay et al., 2008). Gases, well-regulated by digital flow meters (GCR-B9SA-BA15, Vogtlin, Sweden), were mixed in sealed bottles containing water, and then dried in conical flasks with silica gel balls. The gas mixture was bubbled into different treatments accordingly (Zhang et al., 2015). The control group was bubbled with ambient air only, while its temperature was kept at 24°C, with salinity at 30 psu. A carbon dioxide online analyzer (LI-260, Li-Cor Company, Switzerland) was used to measure the real time pCO_2 levels. Temperature, pH (NBS scale), pCO_2, and salinity were recorded on a daily basis. The software CO2SYS (Stumpp et al., 2011) was used to calculate the saturation state of calcite (ΩCa) and aragonite (ΩAr), total alkalinity (At), and the relationship between these parameters. Total alkalinity was also double checked every week using an alkalinity titrator (HANNA, HI 84431, Germany). Table 1 shows the environmental parameters of the 6 treatments.

Table 1. Environmental parameters of the six treatment groups (mean ± SD) (L: low, M: medium, H: high; C: pCO_2 level, S: salinity)

	Temperature (°C)	Salinity (psu)	pCO_2 (μatm)	pH	At (μmol L^{-1})	ΩCa	ΩAr
LC-LS	15.2±0.2	10.1±0.4	392±46	7.80±0.18	90.3±15.4	0.58	0.33
LC-HS	15.1±0.3	30.0±0.7	392±46	8.05±0.09	199.3±11.9	4.62	2.93
MC-LS	15.4±0.1	10.1±0.3	934±83	7.50±0.07	97.7±0.6	0.33	0.19
MC-HS	15.2±0.2	30.3±0.2	934±83	7.75±0.02	201.1±19.4	2.14	1.36
HC-LS	15.3±0.1	10.3±0.4	1260±76	7.34±0.09	97.3±5.5	0.21	0.12
HC-HS	15.5±0.3	29.8±0.4	1260±76	7.52±0.04	197.9±6.1	0.98	0.62

Food Detection Experiment

Each treatment had three replicates, with 120 individuals of *N. festivus* randomly assigned to each replicate. As pCO_2 has only a short-term effect on the ingestion rate and scope for growth in *N. festivus* (Zhang et al., 2016), therefore, the experiment lasted 7 days. The food detection experiment was conducted in a plastic swimming pool with a radius of 60 cm. Individuals were arranged in a circle at three distances (20, 40, and 60 cm) away from the bait (an excised short-necked clam *Ruditapes philippinarum*) at the centre of the pool. To achieve similar density at different distances, 20, 40, and 60 individuals were placed at 20 cm, 40 cm, and 60 cm, respectively.

The experiment was performed three times with different durations: 2 hours (acute exposure), 1 day, and 7 days. The hunger level was standardized by offering food every 3 days. For instance, in the acute exposure experiment (2 hours of exposure) gastropods were fed on Day 1. They were then exposed to the experimental conditions on Day 4, and food detection was studied 2 hours after exposure.

A video camera was set up above the swimming pool to record gastropod activities. The number of individuals foraging and the time each individual spent searching for food were recorded. Individuals that did not reach the bait within 60 minutes were identified as non-feeding and were not included in the analysis. A plastic sheet was used to cover the

swimming pool to ensure there was no gaseous exchange between the experimental setup and outside. An air tube was used to supply the desired concentration of CO_2 to each treatment. Salinity, pH, and temperature were kept constant during the experiment.

STATISTICAL ANALYSIS

Two-way ANOVA was performed with distance from bait and time of exposure to pCO_2 as fixed factors. When there was an interaction between the two factors, the effects of salinity and pCO_2 were analyzed separately at each level of the other factor by one-way ANOVA, followed by the multiple comparison Tukey test. For percentage of individuals engaged in feeding, percentage data were arcsine-transformed before the analysis. Normality and equal variance of the data were checked using Shapiro–Wilk and Hartley tests separately before ANOVA tests were conducted. All statistical analyses were performed using statistical software SPSS 20.0.

RESULTS

Food Detection Time

At 20 cm from the bait, no individuals of *N. festivus* exposed to 10 psu approached the bait after 2 hours of exposure, irrespective of pCO_2 level and distance from the bait (Figure 1). In contrast, food detection time increased significantly with pCO_2 ($p < 0.01$) at 30 psu. After one day of exposure, food detection time varied significantly with salinity ($F = 156.40$, $df = 1$, $p < 0.001$), pCO_2 ($F = 8.78$, $df = 2$, $p < 0.01$), and the interaction between salinity and pCO_2 ($F = 4.25$, $df = 2$, $p < 0.05$) with the food detection time increased at lower salinity and higher pCO_2 (Table 2). On Day 7, the effect of elevated pCO_2 became insignificant ($F = 3.31$, $df = 2$, $p = 0.07$) but a longer detection time was observed at 10 psu ($F = 104.90$, $df = 1$, $p < 0.001$) (Figure 1). There was no interaction between pCO_2 and salinity ($F = 1.88$, $df = 2$, $p = 0.20$).

Table 2. Results of the pairwise Tukey's test of food detection time at 20 cm away from the bait after 1 day of exposure to the combined effect of salinity and ocean acidification. Values in the same row with different letter designations indicate that they are statistically significant ($p < 0.05$)

	df	MS	F	p	Tukey's test		
10 psu	2	30.79	6.47	< **0.05**	380 µatm^{a}	950 µatm^{ab}	1250 µatm^{b}
30 psu	2	2.03	7.31	< **0.05**	380 µatm^{ab}	950 µatm^{a}	1250 µatm^{b}
380 µatm	1	62.73	49.46	< **0.005**	10 psu^{a}	30 psu^{b}	
950 µatm	1	146.03	31.84	< **0.01**	10 psu^{a}	30 psu^{b}	
1250 µatm	1	206.51	121.36	< **0.001**	10 psu^{a}	30 psu^{b}	

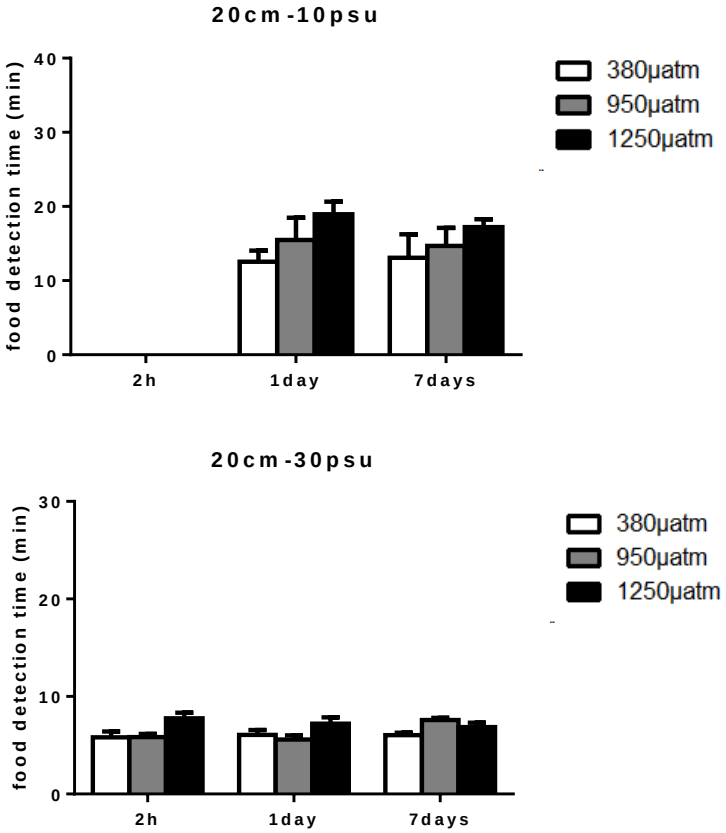

Figure 1. *N. festivus.* Food detection time after 2 hours, 1 day, and 7 days of exposure to the combined effects of pCO_2 (380 µatm, 950 µatm, and 1250 µatm) and salinity (10 psu and 30 psu) at a distance of 20 cm from the bait.

At 40 cm from the bait, no *N. festivus* individuals exposed to 10 psu approached the bait after 2 hours of exposure, whereas at 30 psu elevated pCO_2 levels had no effect on the food detection time ($p = 0.052$). Food detection time increased at the lower salinity on both Day 1 and Day 7 (Figure 2) (Day 1: $F = 71.48$, df = 1, $p < 0.001$; Day 7: $F = 34.11$, df = 1, $p < 0.001$) but an increase in food detection time at higher pCO_2 was only observed on Day 7 (Day 1: $F = 3.61$, df = 2, p 0.06; Day 7: $F = 22.34$, df = 2, $p < 0.001$) with that at 1250 µatm being significantly longer than that at 380 µatm ($F = 6.54$, df = 2, $p < 0.01$).

At 60 cm from the bait, no individuals exposed to 10 psu approached the bait after they were exposed to the experimental conditions for 2 hours, whereas at 30 psu, elevated pCO_2 levels had no effect on the food detection time ($p = 0.428$). On both Day 1 and Day 7, food detection time increased at both the lower salinity (Day 1: $F = 37.16$, df = 1, $p < 0.001$; Day 7: $F = 29.85$, df = 1, $p < 0.001$) and higher pCO_2 (Day 1: $F = 8.35$, df = 2, $p < 0.005$; Day 7: $F = 13.21$, df = 2, $p < 0.001$) but there was no interactive effect between salinity and pCO_2 (Day 1: $F = 1.62$, df = 2, $p = 0.24$; Day 7: $F = 0.65$, df = 2, $p = 0.54$) (Figure 3). The food detection time at 1250 µatm was significantly longer than that at 380 µatm on both Day 1 ($F = 2.39$, df = 2, $p < 0.05$) and Day 7 ($F = 4.39$, df = 2, $p < 0.05$).

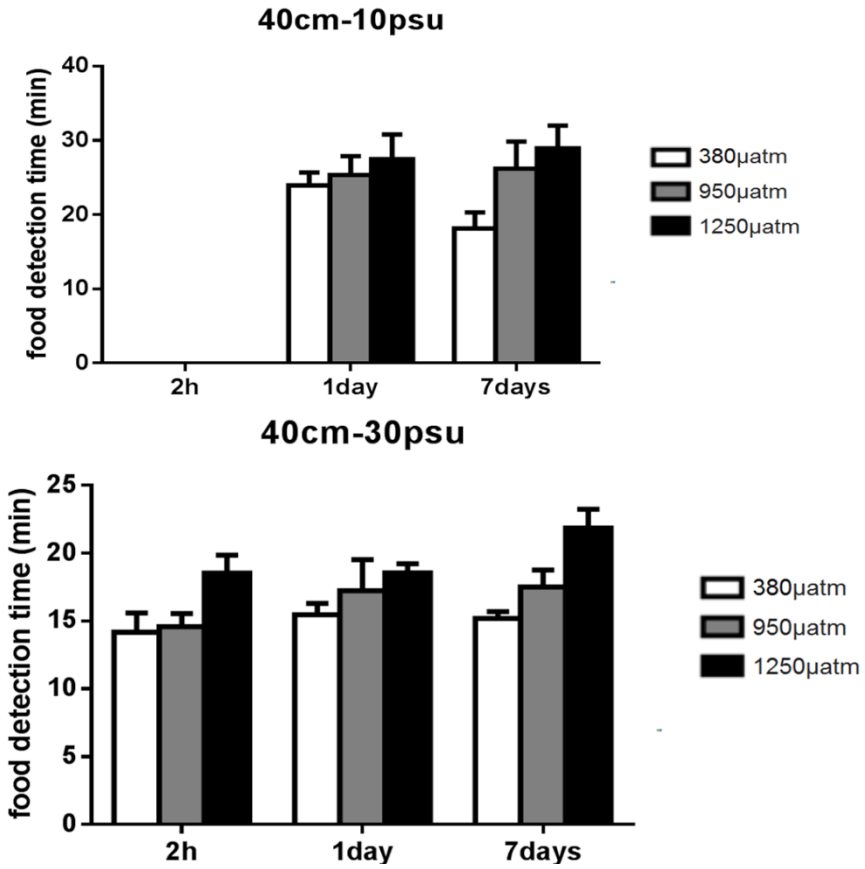

Figure 2. *N. festivus.* Food detection time after 2 hours, 1 day, and 7 days of exposure to the combined effects of pCO_2 (380 µatm, 950 µatm, and 1250 µatm) and salinity (10 psu and 30 psu) at a distance of 40 cm from the bait.

Percentage of Individuals Engaged in Foraging

Individuals exposed to 10 psu for 2 hours did not engage in foraging irrespective of distance from the bait, so only data from 30 psu were analyzed. The level of pCO_2 had no effect on the percentage of individuals engaged in foraging after they were exposed to the experimental conditions for 2 hours.

At 20 cm from the bait, both salinity ($F = 488.00$, $df = 1$, $p < 0.001$) and pCO_2-salinity interaction ($F = 4.74$, $df = 2$, $p < 0.05$) had significant effects on foraging after 1 day of exposure, with a much lower percentage of individuals engaged in feeding under 10 psu and/or at elevated pCO_2 levels (Table 3). After 7 days of exposure, lower pCO_2 ($F = 8.93$, $df = 2$, $p < 0.005$) or salinity ($F = 424.90$, $df = 1$, $p < 0.001$) significantly reduced the percentage of individuals engaged in foraging, but there was no interaction between pCO_2 and salinity ($F = 0.93$, $df = 2$, $p = 0.42$). The percentage engaged in foraging at 380 µatm was significantly higher than that at 950 µatm and 1250 µatm (Figure 4).

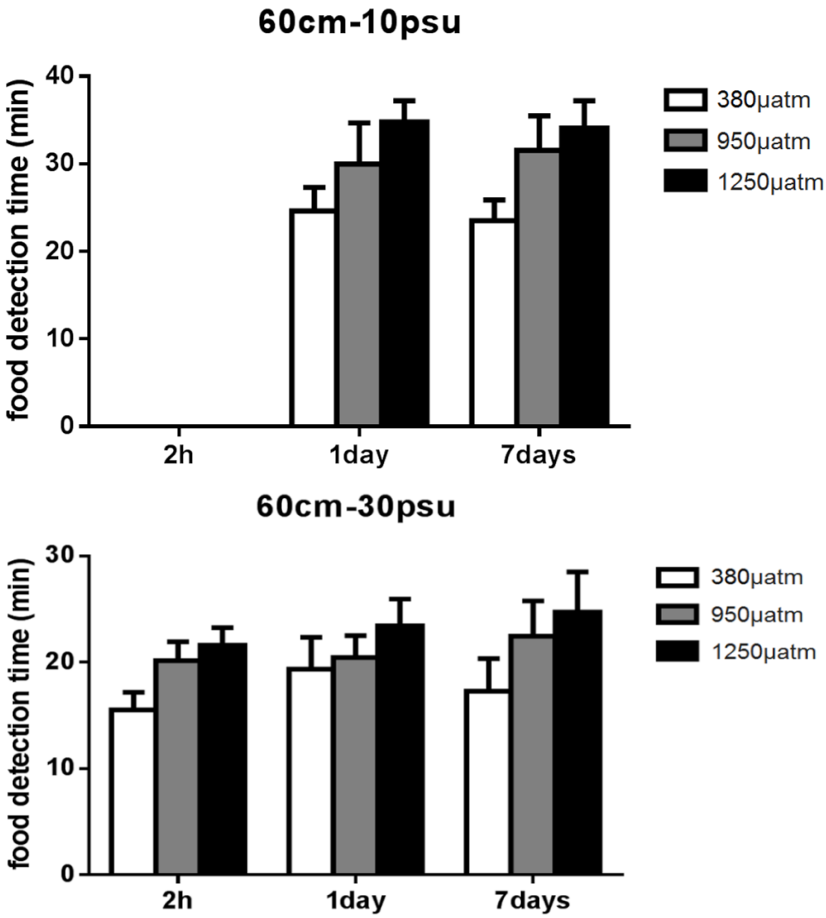

Figure 3. *N. festivus.* Food detection time after 2 hours, 1 day, and 7 days of exposure to the combined effects of pCO_2 (380 µatm, 950 µatm, and 1250 µatm) and salinity (10 psu and 30 psu) at a distance of 60 cm from the bait.

At 40 cm, the percentage of individuals engaged in foraging (at 30 psu) was not affected by pCO_2 after 2 hours (p = 0.824) or 1 day (F = 3.17, df = 2, p = 0.078) of exposure but a lower percentage was observed after 7 days (F = 27.15, df = 2, p < 0.001) with that at 380 µatm being significantly higher than that at 950 µatm and 1250 µatm. Salinity reduced the percentage of individuals engaged in foraging on both Day 1 (F = 376.04, df = 1, p < 0.001) and Day 7 (F = 470.45, df = 1, p < 0.001), but there was no interaction between salinity and pCO_2 (Day 1: F = 0.91, df = 2, p = 0.43; Day 7: F = 0.05, df = 2, p = 0.95) (Figure 5).

At 60 cm from the bait, the percentage of individuals engaged in foraging was reduced significantly after 2 hours of exposure to 950 µatm (p < 0.050) and 1250 µatm pCO_2 (p < 0.05). Both elevated pCO_2 and low salinity reduced significantly the percentage engaged in foraging after 1 day (pCO_2: F = 27.72, df = 2, p < 0.001; salinity: F = 133.89, df = 1, p < 0.001) and 7 days (pCO_2: F = 36.27, df = 2, p < 0.001; salinity: F = 98.46, df = 1, p < 0.001) of exposure, but there was no interaction between salinity and pCO_2 (Day 1: F = 3.50, df = 2, p = 0.06; Day 7: F = 3.55, df = 2, p = 0.06). The percentage was significantly higher at 380 µatm than at 950 µatm and 1250 µatm (Figure 6).

Table 3. Results of the multiple comparison Tukey's test on the percentage of individuals engaged in foraging at 20 cm from the bait upon exposure to the combined effects of salinity and ocean acidification for 1 day. Values in the same row with different letter designations indicate that they are statistically significant ($p < 0.05$)

	df	MS	F	p	Tukey's test	
10 psu	2	144.44	3.06	0.121		
30 psu	2	102.78	2.64	0.150		
380 µatm	1	4537.50	99.00	< **0.001**	10 psu[a]	30 psu[b]
950 µatm	1	8816.67	211.60	< **0.001**	10 psu[a]	30 psu[b]
1250 µatm	1	8066.67	193.60	< **0.001**	10 psu[a]	30 psu[b]

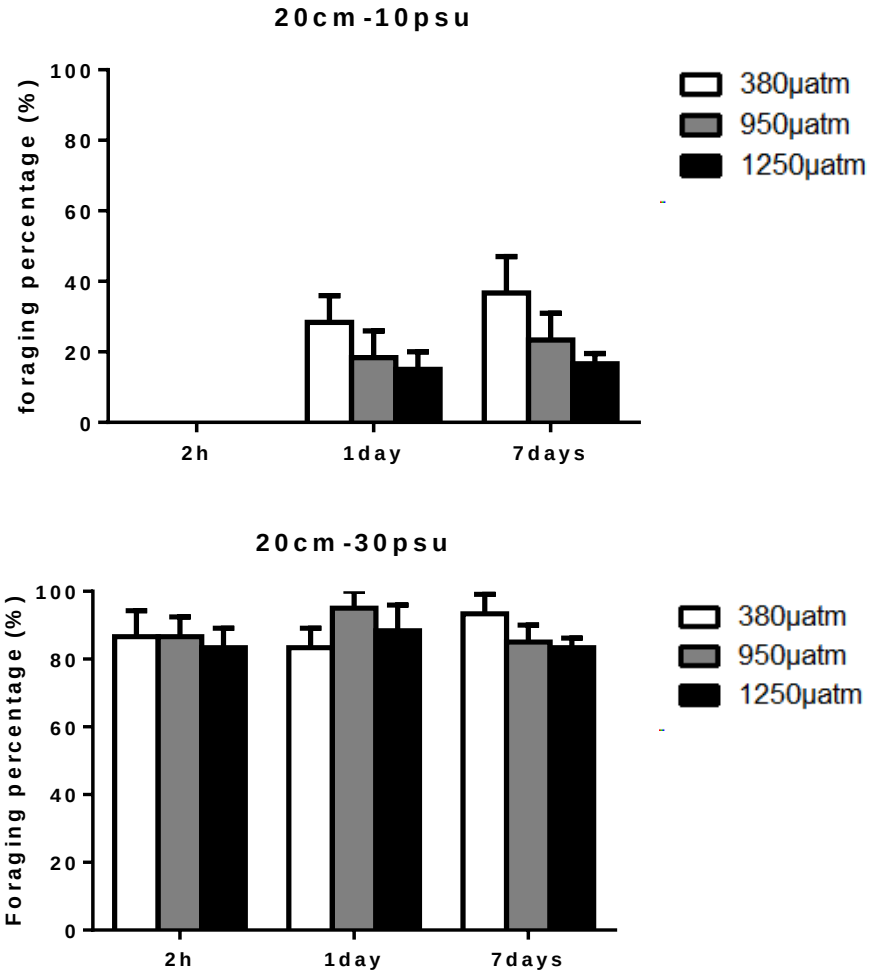

Figure 4. Percentage of individuals engaged in foraging after 2 hours, 1 day, and 7 days of exposure to the combined effects of pCO_2 (380 µatm, 950 µatm, and 1250 µatm) and salinity (10 psu and 30 psu) at a distance of 20 cm from the bait.

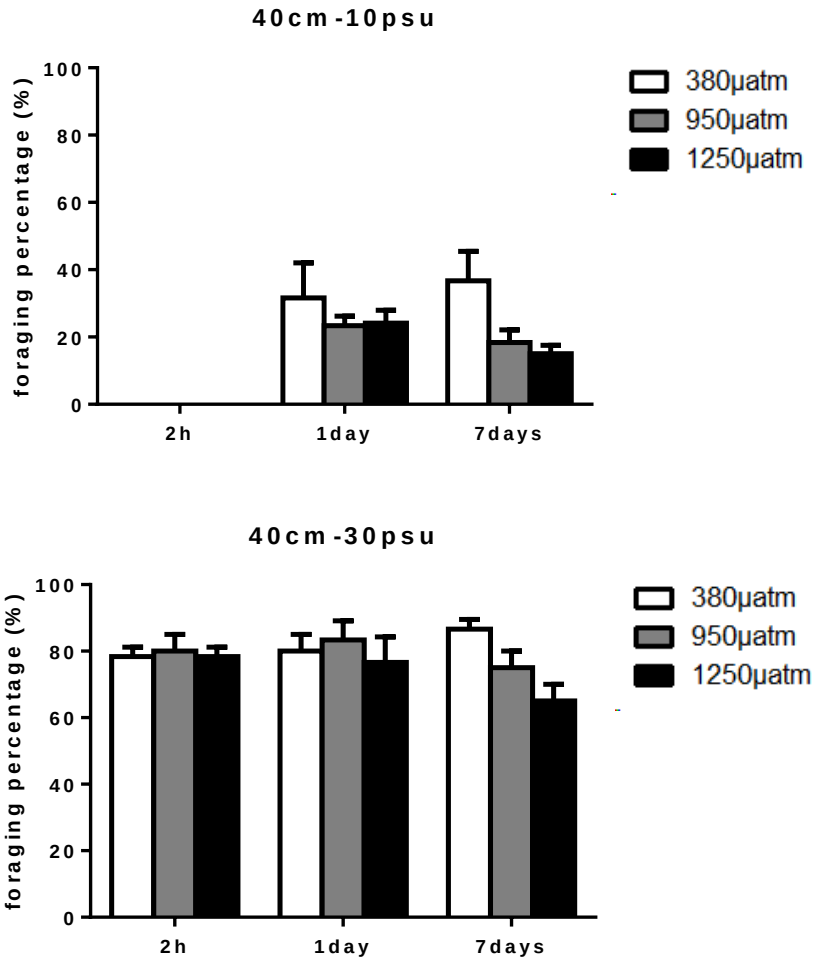

Figure 5. Percentage of individuals engaged in foraging after 2 hours, 1 day, and 7 days of exposure to the combined effects of pCO2 (380 µatm, 950 µatm, and 1250 µatm) and salinity (10 psu and 30 psu) at a distance of 40 cm from the bait.

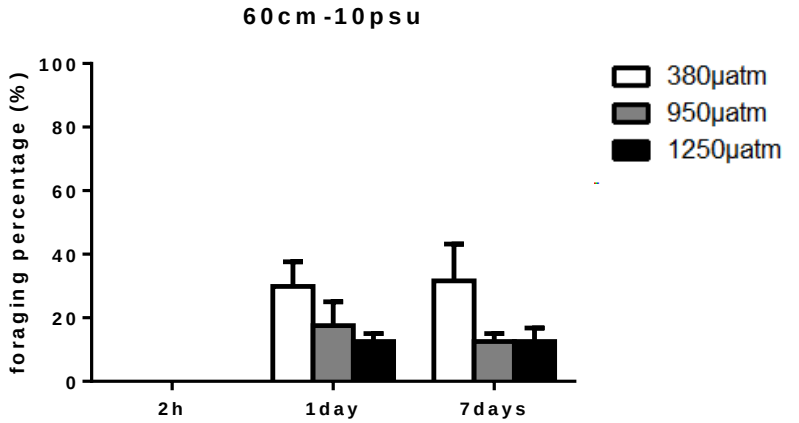

Figure 6. (Continued).

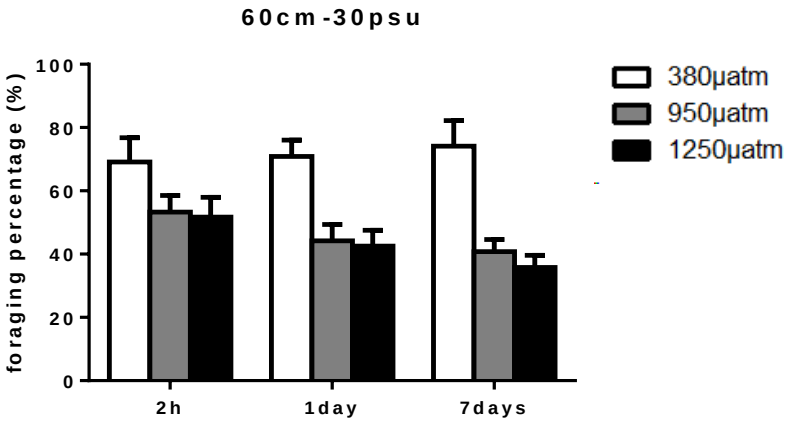

Figure 6. Percentage of individuals engaged in foraging after 2 hours, 1 day, and 7 days of exposure to the combined effects of pCO_2 (380 µatm, 950 µatm, and 1250 µatm) and salinity (10 psu and 30 psu) at a distance of 60 cm from the bait.

DISCUSSION

The present study has demonstrated that elevated pCO_2 and low salinity reduced the percentage of individuals engaged in foraging and prolonged the food detection time in *N. festivus*. Behaviour or performance may be disrupted by reduced functional mechanisms (Briffa et al., 2012). For instance, significant physiological costs for coping with extra copper ions in ambient sea water could reduce the metabolic rate and therefore constrain activities with high energy demand, for instance, aggression (Rovero et al., 2000). The alteration of energy allocation in marine organisms by ocean acidification is well-known (Pörtner et al., 2004; Orr et al., 2005). As a consequence of internal hypercapnia compensation, energy will shift to maintaining an acid-base balance and constrain other behaviours. Dissanayake and Ishimatsu (2011) found that a shallow-water decapod *Metapenaeus joyneri* showed a 30% reduction in swimming activities at a high pCO_2 level (1 Kpa), which might be the result of reduced metabolic scope. As detection of carrion in *N. festivus* requires rapid movement which is energy demanding, a shift in energy allocation towards maintaining an acid-base balance will inevitably affect foraging.

Behaviour and chemoreception restrained under low salinity have been reported in various taxa. For instance, the movement of the marine isopod *Gnorimosphaeroma oregonensis* was reduced significantly at a salinity of 20 psu when compared to that at 30 psu. The Dungeness crab, *Cancer magister*, did not feed when exposed to 3.5 psu (Curtis et al., 2010). The dendrites of olfactory receptor neurons are responsible for receiving odour cues. Exposing the dendrites to an external environment in which ion concentrations are low, however, presents a challenge because low salinity seawater cannot provide the appropriate ionic and osmotic transmembrane gradients required to sustain neural function (Gleeson et al., 2000).

The second reason why the behaviour of animals in polluted environments might change is due to a disruption in the ability to gather (‘perception’) and assess information for making decisions (‘cognition’). Exposure to elevated CO_2/reduced pH caused a shift in the predatory

reef fish *Pseudochromis fuscus* from preference to avoidance of the smell of injured prey, with CO_2 (950 μatm)-treated predators spending approximately 20% less time in a water stream containing prey odour compared with controls (Cripps et al., 2011). The impairment to the nervous system of larvae of the coral reef fish *Neopomacentrus azysron* under ocean acidification (880 μatm) may give support to the cognition impairment theory (Domenici et al., 2012). The impaired olfactory ability of the Clownfish (*Amphiprion percula*) larvae at high CO_2 was restored by an antagonist of the GABA-A receptor (Nilsson et al. 2012), a major neurotransmitter receptor in both vertebrates and invertebrates (Tsang et al., 2007). This indicates that the alteration of chemosensory functions is associated with the interference of neurotransmitter function. Under normal circumstances, opening of the GABA-A receptor causes an inflow of Cl^-, leading to hyperpolarization and inhibition of the neuron. Upon exposure to high CO_2, marine organisms regulate their acid–base balance to avoid acidosis by accumulating HCO_3^- with compensatory reductions in Cl^- which render some GABA-A receptors excitatory, thereby impairing sensory functions (Nilsson et al., 2012). Chung (2014) also found that a pCO_2 level around 900 μatm can influence chemoreception, auditory sense, and visual sense of the damselfish *A. polyacanthus* through interference of the GABA-A neurotransmitter. In addition to changing the charge distribution at odour molecule receptor sites, reduced pH could disrupt chemosensory behaviour by causing a change in the ionic state of the odour molecules themselves (Brown et al., 2002; Leduc et al., 2004), making them unrecognizable or preventing them binding with the receptor sites.

Reduced ability in foraging decreases the chance of success in energy acquisition in *N. festivus* as the occurrence of carrion is sporadic and the competition among scavengers is intense, resulting in long-term effects on population growth. A longer food detection time may indicate a weak sensory ability or confusion in processing olfactory cues. This explains why individuals much further away from the bait (60 cm) were greatly affected by pCO_2 as the concentration of chemical cues from the bait was reduced. When food detection time increased, the chance that the prey was exposed to the predator also increased, a phenomenon commonly found in marine animals (Stein and Magnuson, 1976; Milinski and Heller, 1978). Nevertheless, this could be partially compensated for by a lower percentage of individuals engaged in foraging. This might be a strategy to avoid stress as shown in many other species (Briffa et al., 2012). The epaulette shark (*Hemiscyllium ocellatum*) tended to hide in corals in an attempt to escape from ocean acidification and its associated harmful effects (Heinrich et al., 2014). When animals cannot evaluate the potential risk due to cognitive defect, their behaviour will become circumspect and cabined (Nilsson et al., 2012).

Nassarius festivus and the hermit crab, *Diogenes edwardsii*, are scavengers sympatric on sandy shores in Hong Kong and undergo interspecific competition for carrion with *N. festivus* outcompeting *D. edwardsii* and dominating the feeding clusters. This is achieved by having an extendible proboscis allowing feeding at a distance, and chemoreceptors that permit long distance food detection (Morton and Yuen, 2000). However, the meta-analysis of Wittman and Pörtner (2013) has shown that in general molluscs are more sensitive to ocean acidification than crustaceans. Therefore, whether the competitive dominance of *N. festivus* in the scavenging community will change under ocean acidification deserves further investigations.

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Chapter 48

OXIDATIVE EFFECTS IN AQUATIC ORGANISMS EXPOSED TO LIPOPHILIC MARINE BIOTOXINS

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Harmful algal blooms (HAB) on the coast of Chile are an ongoing problem and a challenge for producing seafood products from natural beds and from farming centers. Lipophilic marine biotoxins (LMB) are one of the groups of marine toxins, which in recent years, have been consistently identified in shellfish; its main characteristics are to have a latitudinal variability and species-specific assimilation/retention for each species.

Shellfish in the aquatic environment represent the best bio-indicator model to allow establishing levels of toxicities related to LMB and also represent an important tool for the constant monitoring of water pollution on the coasts of Chile. Antioxidant enzymes play an important role in the cellular antioxidant defense systems of all organisms and whose main task is to protect against oxidative damage caused by reactive oxygen species (ROS).

Bivalves from rocky strata, sandy bottoms and gastropods were extracted from areas exposed to lipophilic marine biotoxins (Aysén Region) and unpolluted areas (Los Ríos Region) to determine the levels of activity of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), lipid peroxidation (LP), glutathione peroxidase (GPx) and glutathione reductase (GR), and to relate it to the concentrations of LMB in the visceral (hepatopancreas) and non-visceral tissues (mantle, gills, adductor muscle and foot) in shellfish.

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Oxidative damage was detected in all evaluated species and significant differences were established with respect to those obtained from areas not polluted with LMBs. The species with the greatest oxidative damage were those related to the sandy bottom habitat (the Pacific clam and clam). LMB profiles detected in bivalves corresponded to okadaic acid (OA), dinophysistoxin-1 (DTX-1) and pectenotoxin-2 (PTX-2) in a ratio of 3:6:1, respectively. The variation of oxidative damage was related to decreased LMB toxicities in each species.

This study shows that variations in the activity of antioxidant enzymes are directly related to the concentration levels of LMB in shellfish, which could be used to explain the levels of mortality of some species (bivalves) during HAB events in the coast of Chile.

OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE

Climate change has tended to transform seawater into a warmer and more saline environment in certain areas of the Earth, and the constant anthropogenic pollution is added to it. The increase in temperature on the planet tends to produce changes in the metabolic pathways in almost all species of sea creatures and it induces species, particularly those with shorter lifespans, to change their life cycle, thereby contributing to changes in the patterns of distribution of marine species (Ielmini et al., 2014). One of the most relevant effects of climate change at sea is the occurrence of events associated with algal blooms, in particular the proliferation of harmful algal blooms (HABs) associated with the production of toxins which are persistent in marine waters and that may enter into the trophic chain of hydrobiological organisms (EFSA, 2009; Glibert et al., 2014; Botana, 2016).

Hydrological changes, hydromorphological degradation and invasive species tend to contribute to the set of factors that generate stress in the marine environment (Amado et al., 2006). Marine, coastal and estuarine environments are subjected to changes due to natural (algal blooms) and non-natural chemical contamination associated with industrial production and high levels of urbanization. In this context, chemical monitoring programs have commonly used both marine organisms and sediments to identify chemical contaminants in these affected environments. Within these monitoring programs, shellfish are the organisms most widely used as bio-indicators due to their high filtration capacity, which allows them to accumulate and tolerate high concentrations of natural or non-natural contaminants in different geographical areas (Fernández et al., 2010).

However, the concentration of contaminants in their tissues does not provide any information on the biological importance and harmful effects that environmental pollution can cause in biological systems. Likewise, environmental surveillance allows for the protection of biological and ecological systems. With this purpose, general biological effects of exposure to potentially harmful substances in the environment are studied, such as the responses measured at subcellular level (oxidative stress and DNA adducts) and the determination of responses of the whole organism, for example, growth variables and disease occurrence indexes, which may indicate a relationship between pollutants and ecological damages produced (Thain et al., 2008; Fernández et al., 2010).

Acute or chronic exposure to environmental pollutants can produce a variety of alterations in aquatic organisms. Even though aquatic organisms can tolerate a certain amount of biological alteration induced by short-term pollutants, long-term exposure may deplete

repair and defense mechanisms, generating a negative impact on cellular organization, known as stress.

The sequence of biological events produced by stress and subcellular changes can provide evidence of damage before irreversible effects are evident in the organism. In this way, subcellular measurements can clarify the compromised physiological state of exposed organisms. Thus, some cellular components show exposure to a specific group of pollutants, while others, such as oxidative stress, can be used to indicate and establish the cumulative effects of exposure to complex mixtures of pollutants, such as those found in fjords or coastal waters (Gill et al., 2014).

Cell damage is the consequence of the accumulation of damage by oxidation on biomolecules caused by the high reactivity of free radicals and reactive oxygen species (ROS) produced in cells as a result of the necessary use of oxygen. Since oxygen is primarily used in respiration to support the life-sustaining metabolic processes, mitochondria, and specifically deoxyribonucleic acid (DNA), are the first target of oxidation. All aerobic organisms must cope with the need of dealing with reactive oxygen species (ROS).

ROS are chemically reactive molecules that contain oxygen, formed as a natural byproduct of normal oxygen metabolism, and play an important role in cell signaling and homeostasis. The generation of reactive oxygen species (ROS) is a condition inherent to aerobic life (Pamplona and Costantini, 2011). ROS have a dual role since they are involved in protective mechanisms of the cell and, at the same time, act as messengers in cellular signaling pathways (Cooke et al., 2003; Pamplona and Costantini, 2011). Reactive oxygen species (ROS) are unstable atoms or molecules that try to release electrons from other molecules to achieve greater stability, thus creating new radical species and causing chain oxidations (Vinagre et al., 2012). These include the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\bullet}$), nitrogen oxide ($NO^{\bullet}$) and hydroxyl radical ($HO^{\bullet}$) (Winterbourne, 2008). Some ROS contain unpaired electrons (free radicals) while others are non-radical species (e.g., H_2O_2) (Shehan and McDonagh, 2008) (Figure 1).

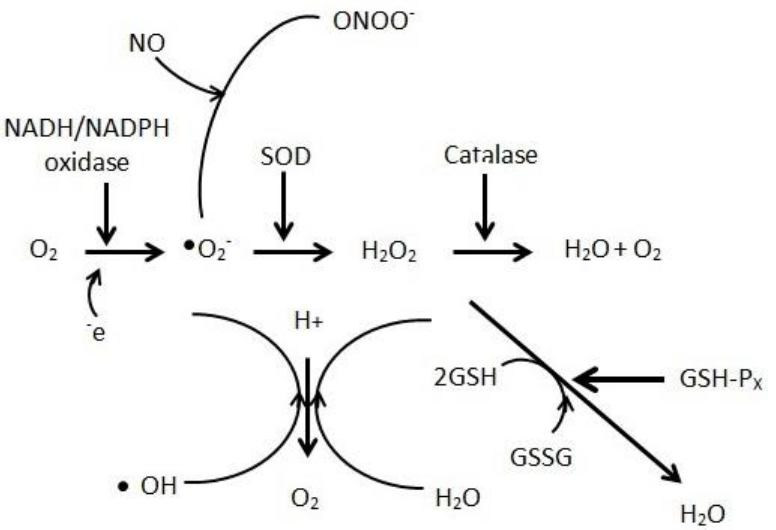


Figure 1. Metabolism of reactive oxygen species (ROS) (Guerreiro et al., 2014).

Many pollutants can exert harmful effects by catalyzing the ROS production. Nevertheless, the imbalance between pro-oxidants and antioxidants can induce harmful effects on macromolecules such as proteins, lipids and DNA (Rosenfeldt et al., 2013). It is well known that many xenobiotics can not only cause an overproduction of ROS, but also alter the levels of antioxidants, which can lead to an oxidative stress condition (Manduzio et al. 2005; Amado et al., 2009). In spite of this, we must consider that oxygen is essential for life and that ROS, in certain quantities, are necessary for many physiological processes that are essential for our survival (Vinagre et al., 2012).

Oxidative stress is an important component of the response to stress in marine organisms which are exposed to a wide variety of environmental stressors at varying time and space scales, for instance, variations in temperature, ultraviolet radiation and anthropogenic pollution (Vinagre et al., 2012). As such, oxidative stress occurs when the rate of production of reactive oxygen species (free radicals) exceeds the rate of clearance produced by endogenous antioxidant molecules (Adeyemi, 2014; Otunola et al., 2014). Therefore, during exposure to environmental stress, ROS levels may increase dramatically. Thus, when production and accumulation of ROS is beyond the ability of the body to treat these reactive species, the so-called oxidative stress is produced, which damages lipids, proteins and DNA (Vinagre et al., 2012).

A biomarker is defined as a variation occurring in cellular or biochemical components, measurable in a biological system and which may be indicative of xenobiotic exposure. In a broader perspective, an entire organism, population, community or ecosystem can be considered as a biomarker (Lam, 2009). Likewise, aquatic organisms present in rivers, lakes and sea are potentially useful as biomarkers of environmental pollutants (Ielmini et al., 2014).

Lipid Peroxidation (LPO) corresponds to the process in which reactive species (RSs) attack the polyunsaturated fatty acids of the cell membrane, inducing a chain reaction with lipid hydroperoxides as intermediates (Halliwell, 1987). This process can also result in the destabilization and disintegration of the cell membrane which causes serious problems to the biological system of the species (Valko et al., 2006). At the beginning of the LPO, peroxyl free radicals (ROO·s) are formed which tend to be rearranged through an endoperoxide cycling reaction, producing or overproducing, as well as other end products of this process, such as malondialdehyde (MDA).

Malondialdehyde (MDA) is a natural biomarker produced in the lipid peroxidation reaction (LPO), which can be quantified and used for the evaluation of this process. MDA can be toxic to cells, causing an inhibition of protein synthesis, where some adducts may be formed with the DNA, leading to genotoxic, mutagenic and carcinogenic processes (Halliwell, 2007). Therefore, reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical ($^{\cdot}OH$), can cause damage to DNA, proteins and fatty acids, causing apoptosis and changes in cytoskeletal proteins (Melegari et al 2012).

The high levels of oxidative damage can result not only from oxidative stress, but also from the limitation of the cellular repair system, so that this dysfunction may cause a deregulation of the cellular defense system leading to cell death (Halliwell, 2007; Paskerova et al., 2012).

Biochemical modulations indicating the onset of oxidative stress are very complex, especially under exposure to an environment such as harmful algal blooms (HABs) (Shahraki et al., 2013; Pamplona and Costanini, 2011). The effects that may be observed are dependent

on the species, age, organ, type of exposure, type of material, duration of the dose and external environmental factors (Paskerova et al., 2012). Therefore, our bodies' functions are based on a perfect balance between ROS levels and those of antioxidants. The loss of this balance, due to an excess in ROS production or an insufficient availability of antioxidants, leads to oxidative stress underlying ROS-related diseases and cell damage (Figure 2).

Antioxidants are substances that retard and/or prevent the oxidation of the cellular substrate at low concentrations. Antioxidants are widely distributed in living organisms and are extremely important as representatives in the direct elimination of free radicals (Halliwell and Gutteridge, 2007). The main enzymatic antioxidants correspond to superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and enzymes involved in the process of glutathione metabolism glutathione reductase (GR), glutathione transferase (GST) and glutathione peroxidase (GPx), which exert the primary defense in the destruction of ROS (Valko et al., 2006). Non-enzymatic antioxidants, such as reduced glutathione (GSH), tocopherol (vitamin E) and ascorbic acid (vitamin C), among others, help to combat reactive oxygen species (ROS) and reactive nitrogen species (Figures 1 and 2) (Halliwell and Gutteridge, 2007).

Among antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPxs) act together to destroy ROSs within the cell (Manduzio et al., 2005).

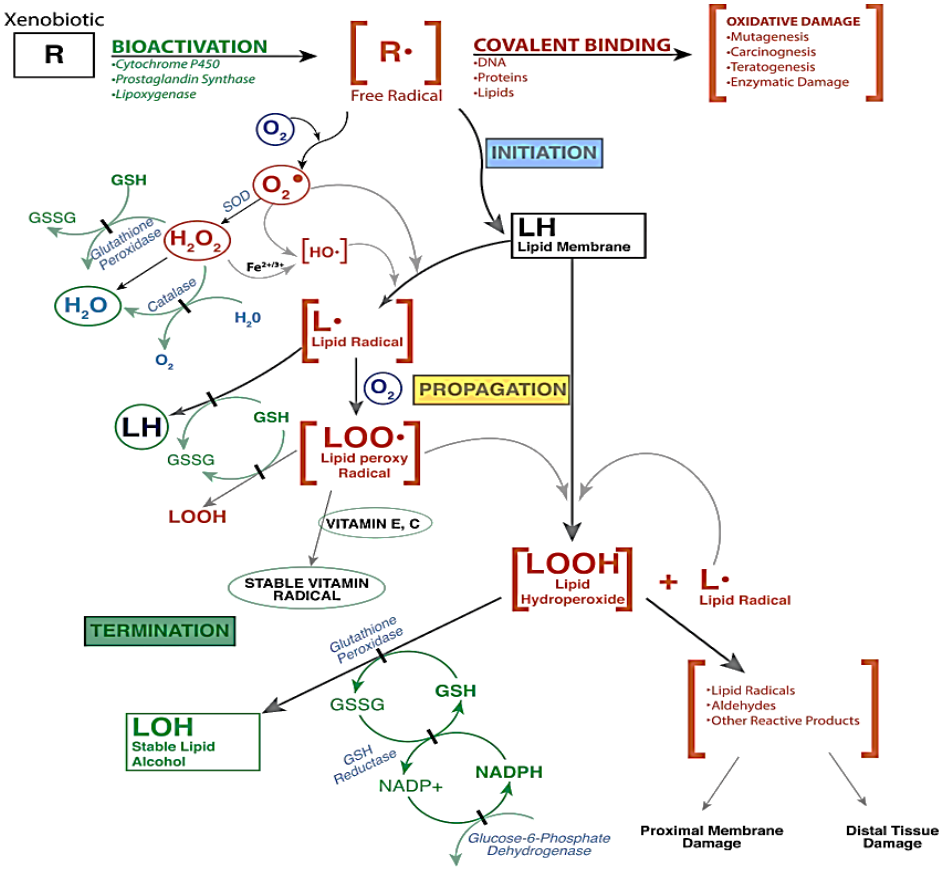


Figure 2. Major generative and eliminative reaction of free radical toxicity (Dickinson & Chang, 2011).

Glutathione-S-transferases (GSTs) represent an important group of detoxification isoenzymes whose natural substrates range from molecules of external origin to byproducts of cellular metabolism. GSTs mainly catalyze the conjugation of reduced glutathione (GSH) to various electrophilic compounds, but they can also act as isomerases or as binding proteins that sequester hydrophobic molecules (Figure 1)

Glutathione reductase (GR) does not play a direct role in the elimination of oxygen radicals; it can be considered as an essential antioxidant enzyme, since it reduces oxidized glutathione (GSSG) and maintains GSSG/GSH balance which is essential for cellular homeostasis and the functioning of other enzymes (Winston and Di Giulio, 1991, Figure 1). GSH plays a central role in the detoxification of ROS and also participates in the metabolism and detoxification of endogenous and exogenous substances (Yan et al., 1997). Therefore, the GR enzyme, which catalyzes the reduction of oxidized glutathione (GSSG), is essential for the maintenance of the GSH/GSSG ratio and the cellular redox state, protecting the cells against oxidative damage (Box et al., 2007).

Furthermore, SOD is a primary defense against oxygen toxicity by catalyzing the conversion of the superoxide anion into oxygen and hydrogen peroxide which can be sequentially removed by CAT or by the GSH-dependent pathway through GPxs (Manduzio et al., 2005, Figure 1). The positive correlation detected between SOD and GPxs indicates that GPxs are an important pathway of degradation of hydrogen peroxide and that the coordinated action of both enzymes acts against the oxidative attack induced by this compound (Bebianno et al., 2005).

The enzymes catalase (CAT) and glutathione peroxidase (GPx) are responsible for degrading or eliminating H_2O_2. The CAT reacts with H_2O_2 molecules to form water and molecular O_2, using one molecule of H_2O_2 as oxidizing molecule and another as reducing molecule (Hayes et al. 2007; Zamocky et al., 2008; Figure 2).

In addition, thiols correspond to compounds containing sulfhydryl groups (-SH) attached to a carbon atom. They are endogenous molecules that help aerobic cells maintain a reducing state, despite an oxidizing environment. They are efficient antioxidants that protect cells against the consequences of free radicals-induced damage. Thiol groups are found in all cells of the body and are indispensable for life (Da Costa et al., 2006).

In this way, biomarkers can provide an early warning indicator of exposure to toxic compounds, allowing changes in biological systems to be identified before the effects are evident at the community level (De machado et al., 2014).

The development of new strategies and methods for the discovery of biomarkers is essential to define early warning signals for the design of interventions and prevention strategies. Techniques such as genomics, transcriptomics, proteomics and metabolomics can provide new insights to develop tools for the identification of threshold values and exposure ratios against environmental pollutants (Lemos et al., 2010; Letendre et al., 2011).

HARMFUL ALGAL BLOOMS AND LIPOPHILIC MARINE BIOTOXINS

The largest supply of nutrients in the sea for the development of life is the phytoplankton (biomass), which is beneficial for living organisms such as shellfish and fish (whether endemic or farmed). However, the contributions of nitrogen (N) and phosphorus (P) from

anthropogenic resources (agricultural farmed and/or fish farmed) can alter the nutrients ratio (16:1) to very high levels (70:1), by altering the marine ecosystem (Hattenrath-Lehmann et al., 2015). This is how the organic imbalance (N:P), which is produced in some areas, creates a global variability in the communities of marine phytoplankton, resulting in an exponential growth of these species in many cases, which, although still a nutritional contribution to the many species of marine organisms, it generates a high trade and human impact since they are associated with the production of toxins capable of killing people (Figure 3) (Gerssen et al., 2010).

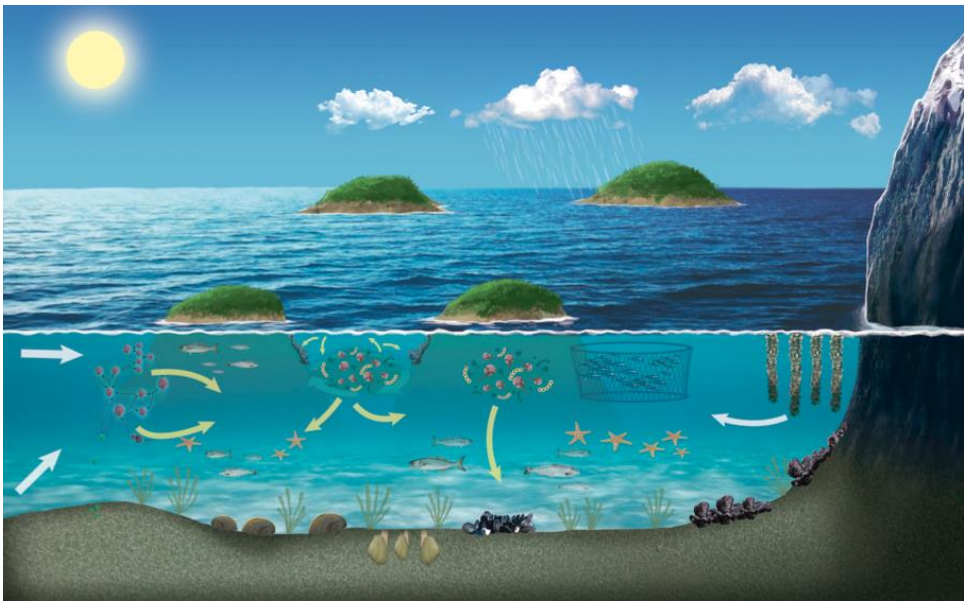


Figure 3. Summary of environmental impacts of Harmful Algal Blooms (HABs) on shellfish and miticulture (mussels) from the South Pacific Ocean.

To date, 5,000 phytoplankton species have been described, from which, under certain circumstances, 300 species have a high proliferation rate resulting in a high density of microalgae in the sea, a condition called “bloom”. The circumstances or factors triggering these “blooms” are still not completely known, but what is certain is that specific changes of climatic and hydrobiological conditions play an important role in the conditions leading to a “bloom” (Hallegraeff, 2010; Picot et al., 2011). About 80 species, out of the 300 already mentioned found in phytoplankton, are dinoflagellates and diatoms that, under certain circumstances, produce phycotoxins (marine toxins). The abundance of this kind of toxic phytoplankton ranges from a few hundred of thousands to several million per liter in the sea, reaching maximum levels called “Harmful Algal Blooms” (HABs) (Anderson et al., 2012; Hallegraeff, 2014).

In order to determine the total toxicity of these toxin-producing dinoflagellates, both the absolute toxin concentration and the relative composition of them should be determined. These factors are directly related to local or regional parameters on which a bloom is developed (light, temperature, nutrients and salinity) (García et al., 2013; Nielsel et al., 2013). Thus, for a proper identification of a bloom, it is necessary to carry out two key processes:

plankton monitoring (stratification in the water column), and toxin monitoring (HPLC and LC-MS/MS) (Hess 2010; May et al., 2010).

In areas where toxic blooms occur, the main bodies affected are shellfish, which, by having high levels of water filtration (clearance rate), may accumulate high concentrations of nutrients and toxic phytoplankton in their tissues (visceral and non visceral tissues) (Navarro and Contreras, 2010; García and Lagos, 2013). This assimilation of toxins in shellfish leads to a variation in the assimilated toxic profiles (dinoflagellates) through different routes of biotransformation (enzymatic and non-enzymatic) present in the different species of shellfish affected by HABs, thus acting as powerful vectors (Rossini and Hess, 2010; Zamorano et al., 2013; García et al., 2015). The toxic variability produced can be enhanced through the transfer of toxins through the food chain, thus allowing for the accumulation of toxins in various organisms in the ocean (zooplankton, fish, sea lions and whales). This has been essential in determining the potential biotransformation forms and degrees of purification occurring in different marine species affected in a toxic bloom (Deeds et al., 2008; Lopes et al., 2013).

Thus, contamination of shellfish by HAB and other marine organisms generate annual economic losses for seafood, which are estimated to be over € 700 million per year on miticulture in Europe (James et al., 2010; Glibert et al., 2012; Reguera et al., 2014). For this reason, in order to prevent intoxications caused by eating shellfish contaminated with phycotoxins, and to mitigate their harmful effects, international agencies have produced regulations, legislation and monitoring programs (EU, 2011). Several phycotoxin detection methods have been developed, many of which allow detection of phycotoxins within shellfish far below dangerous levels. Nevertheless, the challenge of each method is the detection and analysis of the available toxins from different biological matrices (shellfish tissues) and, at the same time, the identification of new toxic analogues that may result from the biotransformation of toxins (Picot et al., 2011; Paredes et al., 2011).

In the past 10 years, one of the most important group of blooms and identification of toxins has been the so-called lipophilic toxins. These are a group of marine toxins formed by the Okadaic acid-group (OA-group), Azaspiracid-group (AZA-group), Pectenotoxin-group (PTX-group) and Yessotoxin-group (YTX-group). It is noteworthy that in the mid-nineties, these toxin groups were part of a single group called diarrhetic shellfish toxins (DSTs). This, largely because the evaluation processes in shellfish (bioassay) and phytoplankton caused a simultaneous extraction of lipophilic toxins, therefore causing a tendency to associate the symptoms detected in bioassays or in human poisonings solely with the DST group. Thus, toxins that make up the AZA-group, PTX-group and YTX-group were excluded from DST group (Gerssen et al., 2010; Dominguez et al., 2010). It was further established in 2011 that the only suitable method for the identification of all lipophilic toxins was the LC-MS/MS (EU, 2011; Orellana et al., 2014; García-Mendoza et al., 2014).

On this basis, the European Union (EU) has established guidelines for maximum toxin lipophilic levels in shellfish for human consumption. The OA, DTXs and PTXs (combined or alone), in edible parts of seafood cannot exceed 160 μg kg^{-1} OA equivalent in shellfish, while maximum levels of YTX cannot exceed 3,75 mg kg^{-1} YTX equivalent in shellfish and 160 μg kg^{-1} AZA equivalent in shellfish for AZAs (EU, 2013; EURLMB, 2015).

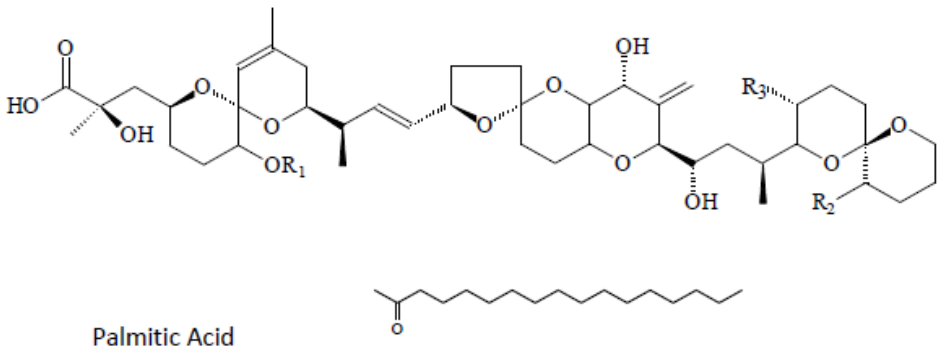
Currently, it has been established that the distribution of most dinoflagellates producers of lipophilic toxins is found worldwide. OA-group toxins have been identified in Europe (U.K., Ireland, Denmark, Sweden, Norway, France, Spain, Italy, Portugal, Holland and

Belgium); Canada; South America (Chile and Argentina), Japan, Australia and Africa (Morocco) (Gerssen et al., 2010; Rodrigues et al., 2010; García et al., 2015); AZA-group toxins have been identified in Ireland, UK, Norway, France, Portugal, North Africa (Morocco), Chile and USA (García and Contreras, 2014; Chevallier et al., 2015); PTX-group toxins have been identified in Ireland, Croatia, New Zealand, Portugal, Norway, Japan and Chile (Dominguez et al., 2010; Fux et al., 2011; Mackenzie et al., 2012) and YTX-group toxins, in Japan, Norway, Italy, New Zealand, Chile, Russia, Canada, United Kingdom and Spain (Tubaro et al., 2010; Dominguez et al., 2010; Pistocchi et al., 2012).

OKADAIC ACID-GROUP (OA-GROUP)

Diarrhetic Shellfish Poisoning (DSP) is a gastrointestinal disease caused by ingestion of shellfish with accumulated fat-soluble polyether toxins (OA-group) produced by dinoflagellates of the *Dinophysis* and *Prorocentrum* genera. Dinoflagellates of the *Dinophysis* genre associated with the OA-group are: *Dinophysis acuminata*, *D. acuta*, *D. caudata*, *D. fortii*, *D. miles*, *D. ovum*, *D. sacculus*, *D. rotundata* and *D. tripos* (Naustvoll et al., 2012; Reguera et al., 2014).

In the South Pacific (Chile), blooms associated with the OA-group are usually produced in the spring-summer period (October to March) (García et al., 2013). The first record of poisoning associated with the OA-group dates from 1970 at the estuary of Reloncavi in Los Lagos Region (García and Contreras, 2014). The main groups that produce such type of toxins are: Okadaic acid (OA), dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2) (Zamorano et al., 2013, Figure 4).



Name				
Okadaic Acid	OA	R1=H	R2=H	R3=CH_3
Dinophysistoxin-1	DTX-1	R1=H	R2=CH_3	R3=CH_3
Dinophysistoxin-2	DTX-2	R1=H	R2=CH_3	R3=H
Dinophysistoxin-3	DTX-3	R1=Acyl	R2=CH_3	R3=CH_3

Figure 4. Chemical Structures Okadaic acid group (OA-group) and their chemical analogues identified in bivalve molluscs.

The variability of the toxic profile produced by dinoflagellate producers of OA-group depends on the species involved and the region in which the bloom occurs. As an example, the variability of the content of toxins in cells of *Dinophysis acuta* is 0-40 OA pg cell^{-1}/0-0.02 DTX-1 pg cell^{-1}/0.3-0.6 DTX-2 pg cell^{-1}, while for *Dinophysis acuminata* it is 0-160 OA pg cell^{-1}/0-7.8 DTX-1 pg cell^{-1}/0-169 DTX-2 pg cell^{-1} (Lindahl et al., 2007). Within this group of toxins, toxic analogues resulting from the biotransformation of toxins in the digestive glands of bivalves have been identified; such biotransformation corresponds to the acylation of hydroxyl groups (-OH) with fatty acids of varying length and saturation (C-14 to C-22). The most identified is the 7-0-palmitoyl-dinophysistoxin-1 group (dinophysistoxin-3, DTX-3) (Rossignoli et al., 2011; Konoki et al., 2013, Figure 4).

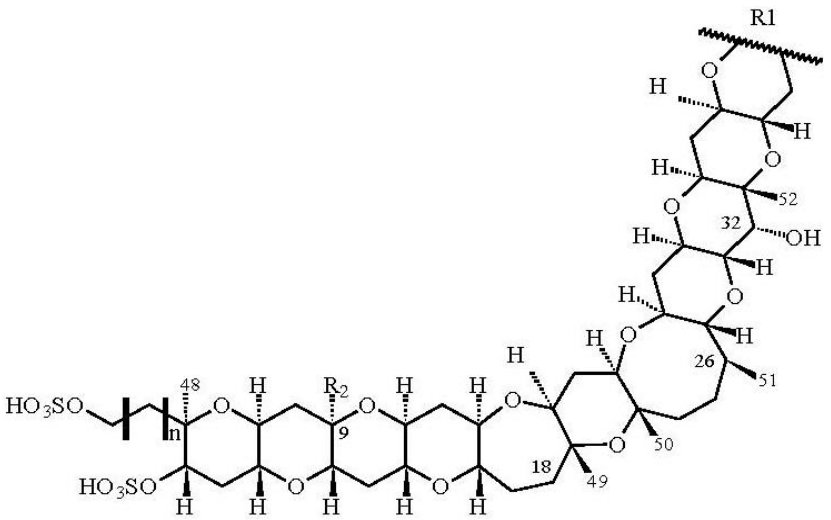
The minimum dose required of OA and DTX-1 to produce toxic symptoms in people, has been estimated at 40 and 36 μg, respectively (Ferron et al., 2014; García et al., 2015). Death of intoxicated patients has not been reported, but okadaic acid (OA) and methyl-okadaic acid (DTX-1) have been shown to be potent tumor promoters in animals (Fujiki et al., 2013). Therefore, they might increase the risk of cancer among regular consumers (Lopez-Rodas et al., 2006; Valdeiglesias et al., 2013). This group also stands out as powerful cytotoxins that inhibit various types of serine/threonine phosphoprotein phosphatases 2A (PP2A) (OA-IC_{50} 2.87 ng/ml and DTX-2-IC_{50}5.96 ng/ml) (Smienk et al., 2013; Ehara et al., 2015).

YESSOTOXINS-GROUP (YTX-GROUP)

Yessotoxins (YTXs) are a group of disulfated polycyclic polyether toxins. Dinoflagellates that are producers of this type of toxin are the species *Protoceratium reticulatum*, *Lingunodinium polyedrum* and *Gonyaulax spinifera* (Paz et al., 2008; Paz et al., 2013).

Most YTXs come from the *Protoceratium reticulatum* dinoflagellate; the first analogue identified was 45, 46, 47-trinor-YTX (norYTX); later, other toxic forms such as homo-YTX, noroxo-YTX, 40-epi-41-keto- YTX and 41-keto-YTXenon were identified (Dominguez et al. 2010). In molluscs, analogues of YTX have been identified, standing out the analogues of 45-hydroxy-YTX, 45, 46, 47-trinor-YTX, homoYTX, 45-hydroxyhomo-YTX, carboxy-YTX and carboxyhomo-YTX (Ciminiello et al., 2007; Dominguez et al., 2010, Figure 5).

The amounts of YTXs produced by each strain are variable, with values ranging from 0 to 71 pg cell^{-1} (Paz et al., 2008). Therefore, the production of YTX toxins is directly influenced by the culturing, growth phase, nutritional and environmental conditions, as well as the type of extraction and/or analysis methods used for determining the toxicity profile (Guerrini et al., 2007). YTXs do not cause diarrhea in bioassays in mice nor cause inhibition of PP2A (Tubaro et al., 2010). Although YTX is known as a potent phosphodiesterase activator (PDE), the precise mode of action currently remains unknown (Paz et al., 2008; Korsnes, 2012). Symptoms caused by intoxication with YTX in humans are relatively unknown because no human poisoning has been reported to date (Franchini et al., 2010; Visciano et al., 2013). Their toxic evaluation in bioassays has determined that an intraperitoneal injection of approximate concentrations of 150 μg kg^{-1} produces dispersed mobility, dyspnea, jumps, tremors and cramps. These symptoms start 4 hours after the injection (Tubaro et al., 2008; Aasen et al., 2011).



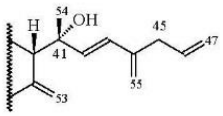
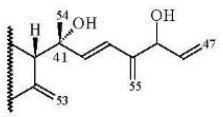
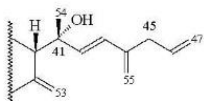
Name	R^1	R^2	n
Yessotoxin (YTX)		H	1
45-Hydroxy-YTX		H	1
Homo-YTX		H	2

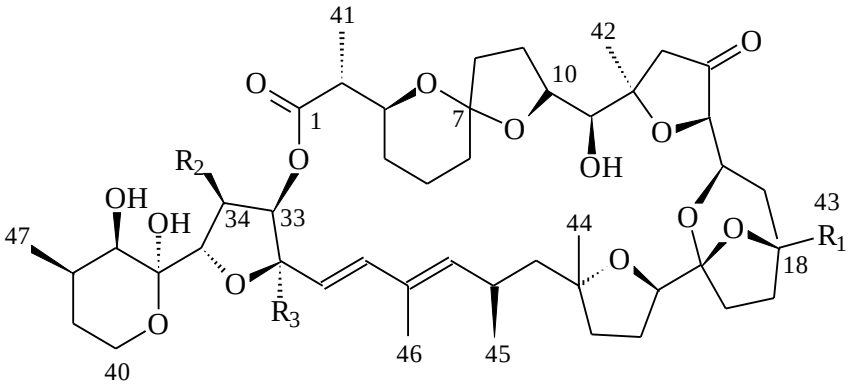
Figure 5. Chemical Structure of Yessotoxins-group (YTX-group) and their chemical analogs identified in dinoflagellates and bivalve molluscs.

PECTENOTOXINS-GROUP (PTX-GROUP)

Pectenotoxins (PTXs) are a group of polyether macrolide toxins produced by dinoflagellates such as *Dinophysis fortii*, *D.* acuminata, *D.* acuta, *D. caudata*, *D. rotundata*, *D. norvegica* and in heterotrophic dinoflagellates such as *Protoperidinium divergens*, *P. depressum* and *P. crassipes* (Dominguez et al., 2010; Fux et al., 2011). Toxic levels of PTX-2 in Dinophysis cells identified in the fjords of Chile are ≈ 180 pg cell^{-1} of PTX-2 (Blanco et al., 2007), while in New Zealand and in Norway, *Dinophysis acuminata* has been reported to be a producer of PTX-2 with levels ≈ 25 pg cell^{-1} (Vale et al., 2008).

New isomeric forms of PTX-2 such as PTX-4, PTX-6 and PTX-7 have been identified in new natural blooms. Said varieties, when filtered and accumulated by shellfish, allow metabolization of toxins, producing new analogues of PTXs (Figure 6). Thus, to differentiate the nomenclature of toxic forms from dinoflagellates and those obtained from shellfish, the

term PTX2-seco acid (PTX2-sa) has been assigned to the latter varieties. Among them, Pectenotoxin-2 seco acid (PTX2sa), 7-epi- Pectenotoxin-2 seco acid (7-epi-PTX2sa), 37-O-acyl-esters-PTX-2-SA, 11-O-acyl-esters-PTX-2-SA and 33-O-acyl-esters-PTX-2-SA stand out. Consequently, this way of toxic accumulation is an important element in the bioconversion of PTXs (Dominguez et al., 2010; Domenech et al., 2014).



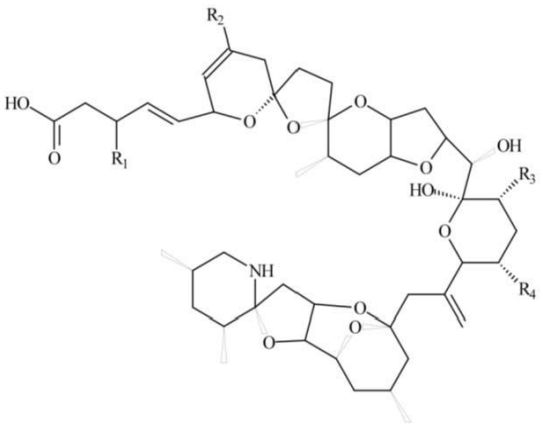
	R^1	R^2	R^3	C-7
PTX-1	CH_3OH	H	H	R
PTX-2	CH_3	H	H	R
PTX-2b	CH_3	H	H	S
PTX-3	CHO	H	H	R
PTX-4	CH_2OH	H	H	S
PTX-6	COOH	H	H	R
PTX-7	COOH	H	H	S
PTX-11	CH_3	OH	H	R
PTX-11b	CH_3	OH	H	S
PTX-13	CH_3	H	OH	R

Figure 6. Chemical Structure of Pectenotoxin-group (PTX-group) and their chemical analogs identified in dinoflagellates and bivalve molluscs.

To date, no case of intoxication by PTX-type toxins has been reported, although biotransformation of PTX-2 has been found in some shellfish species (MacKenzie et al., 2012). So, based on the association of the symptoms and the mussels' toxic capacity, it has been determined that the amount sufficient to produce a toxic condition is 48 μg PTX-2 for a 60 kg person (Domínguez et al., 2010). PTX toxicity after intraperitoneal (i.p.) administration and after oral ingestion in mice is considered comparable. For example, hepatic damage in mice after i.p. administration and oral ingestion seem to be the same (Alfonso et al., 2014). In addition, PTX-2 has been found to be potentially citotoxic in the carcinogenic cell lines of the lungs, colon and kidneys. This effect cannot necessarily be extrapolated to its toxic forms, such as PTX-2-SA and 7-epi-PTX2-SA, demonstrating the importance of the initial structure of toxins that initiate citotoxic effects (Alfonso et al., 2014).

AZASPIRACIDS-GROUP (AZA-GROUP)

Azaspiracids (AZAs) are toxins produced by the species *Azadinium spinosum*. This species has been identified by its production of Azaspiracid-1 (AZA-1), Azaspiracid-2 (AZA-2) (Twiner et al., 2014, Figure 7). Although AZA-1 is the predominant toxin in the shellfish samples, other analogues such as AZA-3, AZA-4, AZA-5 and hydroxyl analogues AZA6-11 have been identified (Kilcoyne et al., 2014; Kilcoyne et al., 2015). However, every year, new analogues of AZAs have been detected in different countries in Europe, which results in varying toxic profiles, depending on the species that accumulates them and their degree of biotransformation (De la Iglesia et al., 2014; Rúbies et al., 2015).



	R1	R2	R3	R4
AZA1	H	H	CH_3	H
AZA2	H	CH_3	CH_3	H
AZA3	H	H	H	H
AZA4	OH	H	H	H
AZA5	H	H	H	OH
AZA6	H	CH_3	H	H

Figure 7. Chemical Structure of Azaspiracid-group (AZA-group) and their chemical analogs identified in dinoflagellates and bivalve molluscs.

The oral toxicity of AZA-1 at high doses (900 μg/kg) in mice produces significant damage to the small intestine, while a 500 μg kg^{-1} dose produces only hepatic damage, evidenced by a volume increase of the organ (38% post-ingesta increase). These data have established that chronic exposure of AZA can cause tumors in tissues (Initiator), and is also a great risk in simultaneous blooms with the OA-group (tumor promoter)(Ito, 2008; Aune et al., 2012). Parallel toxicological studies have established that the lowest observed effect level (LOEL) for AZA lies between 23 and 86 μg kg^{-1} by person, even when levels of 80 μg kg^{-1} AZA eq in shellfish could not produce AZA related intoxication symptoms. However, doses of 30 μg kg^{-1} AZA eq in an ingested 400 g shellfish have produced intoxication syndromes in humans.

LEVELS OF OXIDATIVE STRESS IN MARINE ORGANISMS EXPOSED TO LIPOPHILIC MARINE BIOTOXINS

Fjords and channels of southern Chile (Region of Aysen) are oceanically considered as a marine or estuarine transition system where fresh water from rivers and glacier melt is mixed with deep ocean waters, rich in nutrients and with a greater salinity (>30 psu) (Iriarte et al., 2010). Primary productivity is high (1-3 g C m^{-2} d^{-1}), with diatoms predominating throughout the year (Alves de Souza et al., 2008 a, b; Iriarte et al., 2010).

These characteristics have turned the area into an optimal zone for the culture of high added-value species such as Salmon Coho *(Oncorhynchus kisucht)*, Atlantic salmon (*Salmo salar*) and Rainbow Trout (*Oncorhynchys mykiss*), and have also responded to the sanitary crisis of the ISA virus, occurred in 2007 in the region of Los Lagos, and to the aquaculture industry by relocating the vast majority of farming centers established in the region of Los Lagos towards the regions of Aysén and Magallanes (Saavedro-Gallo, 2013; Gustafson et al., 2014; Sernapesca, 2014).

It should be noted that the highest percentage of salmon cultures is currently found in the region of Aysén, standing out for its demonstration of HABs associated with lipophilic toxins (Zamorano et al., 2013; García et al., 2015). These blooms show seasonal peaks, mainly between January and March; although, it is possible to observe vegetative cells throughout the year (Seguel and Sfeir, 2010). Such blooms would only affect shellfish coming from natural banks (e.g., mussels) (Zamorano et al., 2013; García et al., 2015), since all the reports associated with mortality of endemic fish and in farming centers (*S. salar*) worldwide, and which have not been associated with ichthyotoxins (*Karenia brevis, Prymnesium parvum* and *Hetrosigma akashiwo*)(Shen et al., 2010; Blossom et al., 2014), are only related to STX-group toxins (Kwong et al., 2006; Bakke and Horsberg, 2010; Lage and Reis-Costa, 2013). The STX-group has been identified in the region and its bloom is associated with the dinoflagellate *Alexandrium catenella*; although these blooms have a seasonality of between 3 and 4 years (blooms of years 2009 and 2016) (Seguel and Sfeir, 2010; Varela et al., 2012), they do not significantly affect the salmon farming centers present in the region of Aysén.

The establishment of these farming centers with high added-value resources in areas with constant harmful algal blooms, and given the stages of development of cultured fish (periods from 12 to 14 months), allow for them to be exposed to at least 2 blooms associated with lipophilic toxins before the final product (*S. salar*) reaches its average harvest weight (3.5 to 4.5 kilograms). It is a relevant factor if we consider that HABs could not only affect filtered bivalves, carnivorous gastropods and crustaceans, but also predatory fish, wild fish and salmon in cultures. Therefore, through the feeding pathway and the constant exposure to lipophilic toxins produced by HAB, fish in cultures (*S. salar*) may accumulate lipophilic-toxins (OA-group, PTX-group, AZA-group and YTX-group) in liver and muscle tissues, thus, affecting their basal metabolism, causing genetic damage and favouring a high retention of the lipophilic toxins in their tissues, which are characterized by having high contents of fatty acids in their tissues (muscles).

It is worth mentioning that numerous species of dinoflagellates associated with the production of lipophilic toxins have been identified in the región of Aysén, such as *D. acuta, D. acuminata, D. tripos, D. caudata, D. hastata* and *D. rotundata.* The first two species are associated with the production of lipophilic toxins (OA-group and PTX-group) and *P.*

Reticulatum is identified for producing YTX-group toxins (Seguel and Sfeir, 2010; García et al., 2013; García et al., 2015). Such species may even expand their bloom zones in the region through cultured fish since their production involves the relocation of live fish (wellboat) from different geographic areas (within the region), favoring the dissemination of toxic dinoflagellates described in the area (*Dinophysis sp., P. Reticulatum*) or, possibly, a new toxic species (*A. Spinosum*)(Zamorano et al., 2013).

Likewise, evaluations of enzymatic antioxidants and oxidative damage in fish (*S. salar*) and shellfish (*Mytilus chilensis*) have determined that, in the study area (region of Aysén), there is more relevant damage for filter-feeding species than for cultured fish.

The evaluation data on oxidative damage in tissues of bivalves (*Mytilus chlensis*) and Atlantic Salmon (*Salmo salar*) show that significant changes in the MDA from bivalves and fish exposed to blooms associated with *Dinophysis acuta* are evidenced (≈ 100 cel l^{-1}) if compared to the data obtained from the control group (region of Los Ríos). These data are correlated in all the bivalve's tissues studied (hepatopancreas, Figure 8). In addition, a longer exposure time (10 days) causes a significant increase of this biomarker in the hepatopancreas of bivalves and fish.

Regarding SOD activity, no significant changes were detected in any of the tissues evaluated in mussel and Atlantic salmon (Figure 9). Also, prolonged exposure does not establish any significant change in SOD activity in relation to the control group.

As for the basal level of CAT activity in the hepatopancreas of bivalves, this was higher than in the other evaluated tissues (mantle, hepatopancreas, foot and mantle), but its activity does not show significant changes during the evaluation period, while in fish, no alteration of its activity was detected (Figure 10).

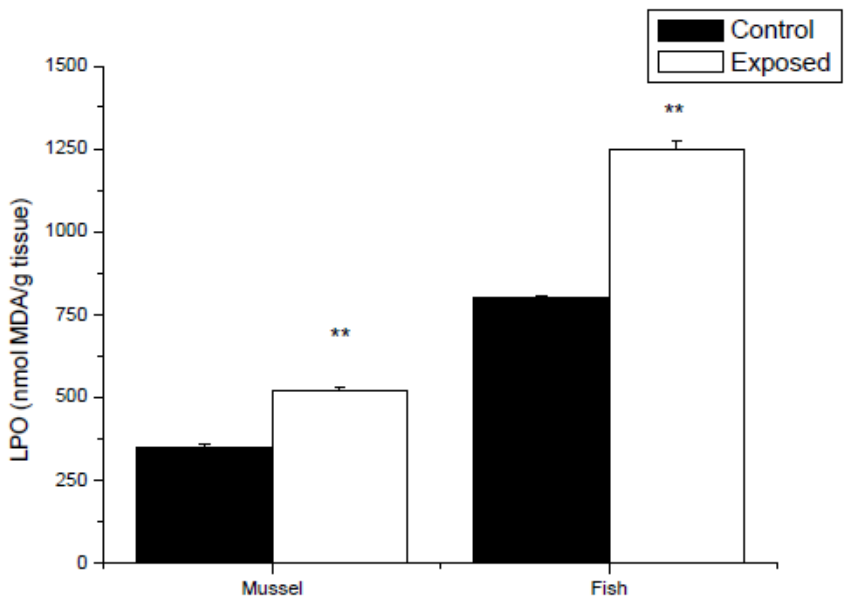


Figure 8. Lipid peroxidation values in the fish gills and the hepatopancreas in the mussel of control and exposed to LMB cells (*Dinophysis acuta* ≈100 cel l^{-1}). The values are expressed as mean ± S.E. (n = 5). The significance levels observed are ** $p < 0.01$ in comparison to control group values.

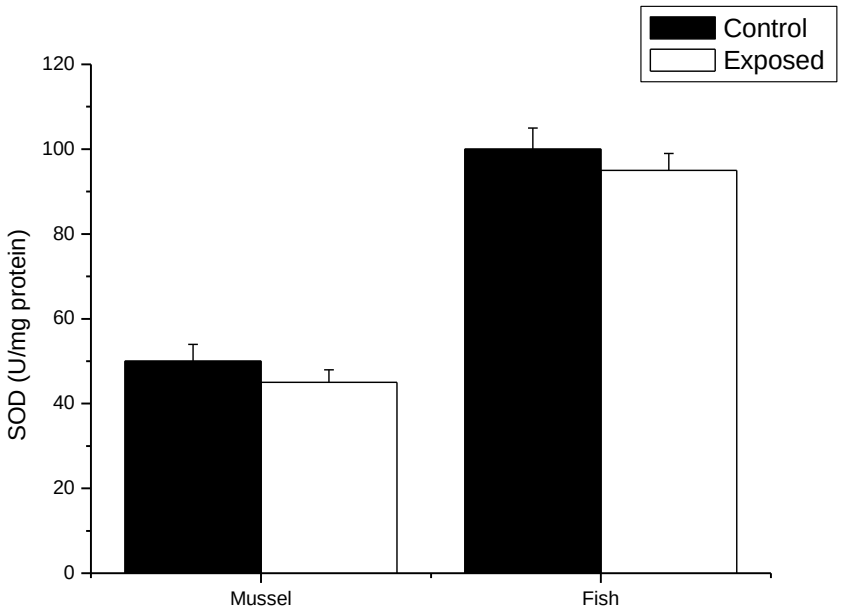


Figure 9. Superoxide dismutase activity in the fish gills and the hepatopancreas in the mussel of control and exposed to LMB cells (*Dinophysis acuta* ≈100 cel l^{-1}). The values are expressed as mean ± S.E. (*n* = 5). SOD activity is expressed as U/mg protein.

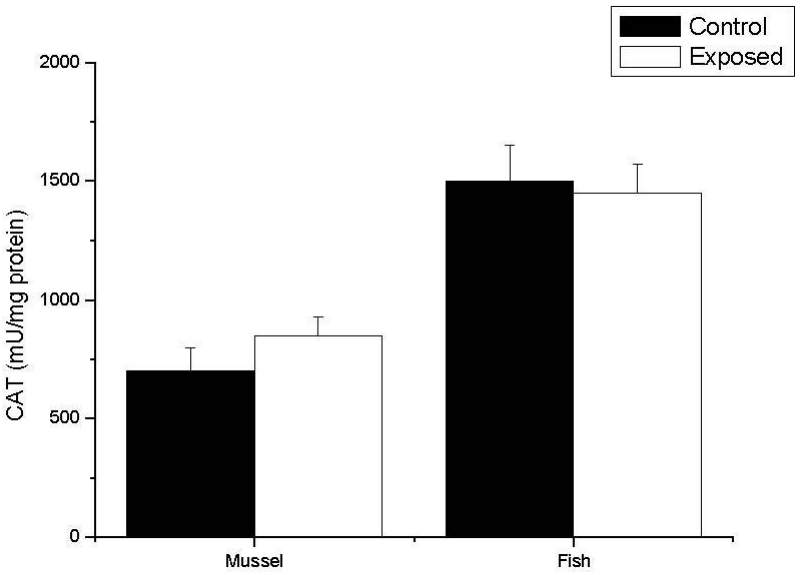


Figure 10. Catalase activity in the fish gills and the hepatopancreas in the mussel of control and exposed to LMB cells (*Dinophysis acuta* ≈100 cel l^{-1}). The values are expressed as mean ± S.E. (*n* = 5). SOD activity is expressed as U/mg protein.

GPx activity maintains a basal level once the exposure period in fish is established, while after exposure, a significant induction of GPx activity in the hepatopancreas and mussel mantle was recorded (Figure 11). In turn, GR behaves just like the previous biomarkers and

does not show any significant change in any of the species (mussel and Atlantic salmon) or in any of the assessed tissues (mantle, hepatopancreas, foot and mantle, Figure 12).

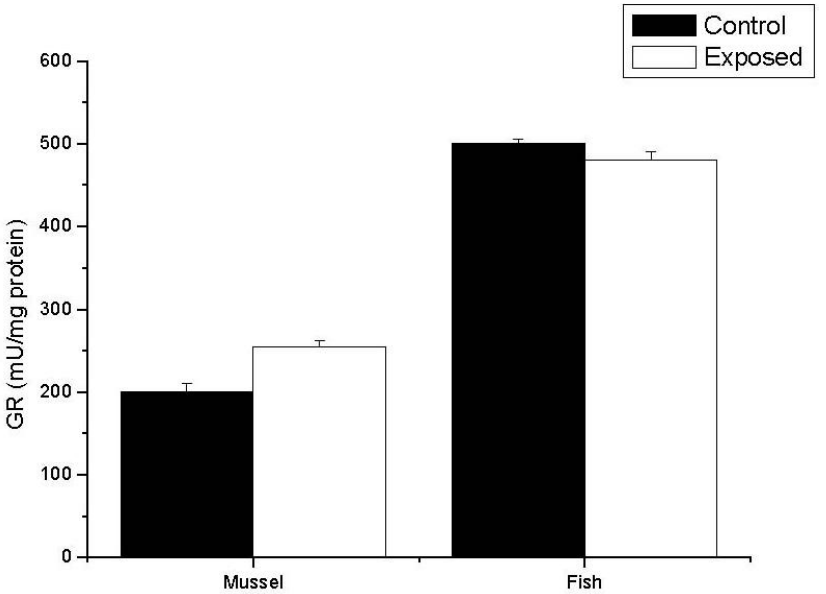


Figure 11. Glutathione peroxidase activity in the fish gills and the hepatopancreas in the mussel of control and exposed to LMB cells (*Dinophysis acuta* ≈100 cel l^{-1}). The values are expressed as mean ± S.E. (n = 5).

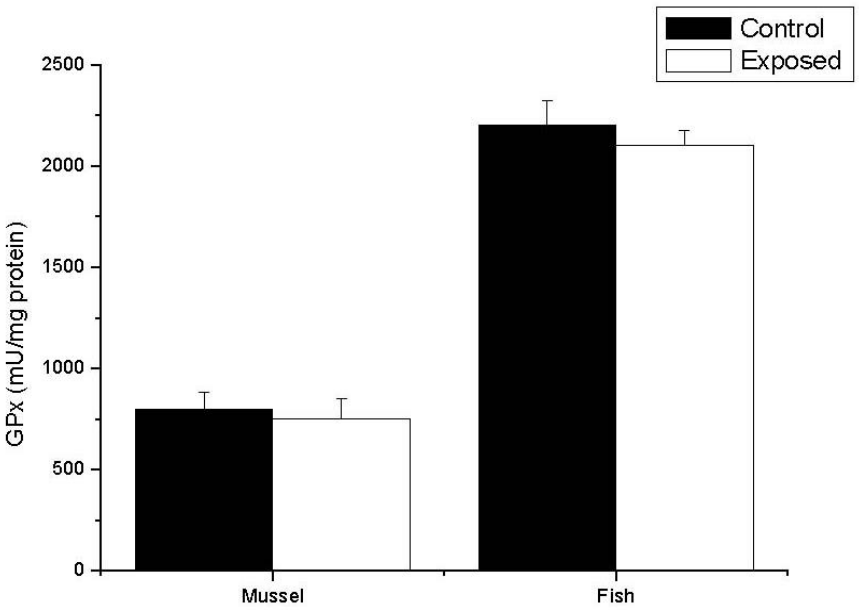


Figure 12. Glutathione reductase activity in the fish gills and the hepatopancreas in the mussel of control and exposed to LMB cells (*Dinophysis acuta* ≈100 cel l^{-1}). The values are expressed as mean ± S.E. (n = 8).

In light of these results, we must consider that the aquatic environment is subject to temporal and spatial variations of quality. Indiscriminate dumping and release of waste containing hazardous substances can cause environmental imbalances that are considered a source of stress for the biotic community (Figure 3). Furthermore, toxicity in aquatic organisms caused by river and sea pollution tends to produce an increase in the production of "reactive oxygen species" (ROS), which causes the so-called oxidative stress. In the aquatic environment, in spite of the presence of constitutive antioxidant defense systems, higher levels of oxidative damage tend to be produced in organisms exposed to pollutants that stimulate the production of ROS (Figure 1). Thus, the use of biochemical or physiological analyses as indicators of toxicity is in constant development due to these analyses have the advantage of being able to outline the effects prior to the manifestation of diseases (Kurutas et al., 2009).

The toxicity of chemicals in the aquatic environment depends not only on their chemical and physical-chemical characteristics, but also on the biological processes in which they are involved. The bioavailability and spatial distribution of pollutants are highly regulated by hydrodynamics, biogeochemical processes and environmental conditions (e.g., redox, pH, salinity and temperature) prevailing in the ecosystem. Moreover, the accumulation of chemical substances depends on the chemical properties of the compound (e.g., polarity, lipophilicity and molecular weight) as well as on various biological processes that regulate food availability and metabolism and excretion efficiency (Dachs and Mejanelle 2010). Also, there are numerous factors that can influence the response of the antioxidant system in fish and bivalves to exposure to pollutants. This is due to the high seasonal variability in which aquatic organisms are frequently found as a result of their biotic regularities related to the environment, such as the reproductive and metabolic status of fish and environmental conditions such as food availability, oxygen level, temperature of water, salinity and photoperiod, among others (Da Rocha et al., 2009).

Nevertheless, the difference in antioxidant responses between species (fish and bivalves) depends on the quantitative distribution of the antioxidant defenses in the different tissues and subcellular compartments. Organ-specific responses to toxic levels of ROS may be related to anatomic location, pathways of exposure and distribution of pollutants, as well as to the defense capacity (Ahmad et al., 2006, Da Rocha et al., 2009), conditions of fasting (Ferreira et al., 2005) and location in the food chain (Solé et al., 2009). The differences of species in the efficiency of antioxidant defenses may partly explain the prevalence of oxidative damage observed in bivalve molluscs (Stoliar et al., 2012).

The main pathways of absorption of pollutants in marine fish and invertebrates (bivalves) correspond mainly to the gills and liver or digestive gland/hepatopancreas in bivalves. Through these pathways, chemicals cross cell membranes through passive or facilitated diffusion and active transport (Turja et al., 2015).

As LMBs are produced by dinofagellates, dominating a certain area in a maritime zone as a result of abiotic stimuli that promote a natural contamination, they act like an exogenous source of oxidative stress, producing ROS that will damage different aquatic species through the LPO of biological membranes. Therefore, as they are produced inside the cells, ROS are able to react with all types of biomolecules, organelles (mitochondria) and enzymes (cytochrome P450) (Inoue et al., 2003). Xenobiotics, such as LMBs, not only can cause ROS overproduction, but also alter the antioxidant levels which can lead to an oxidative stress condition (Amado et al., 2009). Some studies indicate that dinoflagellates, such as *Dinophysis*

sp., cause alteration of antioxidant enzymes and promote the induction of oxidative stress in different tissues of different bivalves species as well as in some fish (Clemente et al., 2010). Thus, LMBs can be bioaccumulated throughout the food chain by decreasing the activity of superoxide dismutase (SOD), GST, glutathione peroxidase (GPx) and causing oxidative stress in different biological structures such as proteins and DNA. However, this event would be likely associated with a more obvious damage in bivalves since the average blooms of *Dinophysis* sp. (100,000 – 200,000 cel l^{-1}), to which fish are exposed in cultures, would allow an interaction with the toxins released from the dinoflagellates in the average range of ≈ 5.0 μg l^{-1}, which does not produce any harmful effect on fish (*S. salar*).

Bivalve molluscs tend to be excellent biomonitors and bioindicators of pollution given their special characteristic as sessile benthic filter feeders, so they can accumulate and tolerate high concentrations of chemical pollutants, allowing them to provide reliable measurements over time of the bioavailable portion of total contaminants.

It should be noted that bivalve molluscs play a key role in the functioning of estuarine and coastal ecosystems since they are present in large geographic areas and, in general, are important commercial species worldwide (García et al., 2012; De machado et al 2014).

At a physiological level, exposure to harmful algal blooms that generate accumulation of toxins (LMB) and ROS toxins for bivalve molluscs implies a variability of the closure of shells, adductor muscle paralysis, retraction of the mantle and production of mucus (Perovic et al., 2000; Hegaret et al., 2012). Facing this event of algal exposure and LMBs, it has been possible to determine a positive correlation detected between SOD and GPxs, which indicates that GPxs are an important pathway of degradation of hydrogen peroxide and that the coordinated action of both enzymes acts against the oxidative damage induced by LMBs. Although, in the case of fish, there is a relationship between the temperature of water and antioxidant responses in the gills that can be detected (high levels of MDA). This is probably related to the fact that the gill epithelium is in direct contact with water and is, therefore, highly sensitive to its fluctuations, which tend to produce variable damages by ROSs. In fact, gills are more susceptible to changes in environmental parameters and more prone to oxidative stress than other tissues, which also implies that the antioxidant mechanisms have to be faster and more efficient in this tissue than in others (Fernandez et al., 2010).

Furthermore, it is described that the distribution of pollutants in organs of bivalves varies as a result of the differences in the surfaces in contact with pollutants, that is to say, by different affinities of pollutants with the binding sites and different rates of accumulation and excretion of the different bivalves species against pollutants (Yap et al. 2008; Fernandez et al., 2010). Thus, in some bivalves species inhabiting in areas constantly exposed to HABs, it is possible to observe a reduction of the antioxidant capacity and, therefore, a greater susceptibility to oxidative stress (Gill et al., 2014). In addition, the high summer temperatures cause increased values related to ROSs in the sea water (Keller et al., 2004). Therefore, antioxidant defenses of marine invertebrates and fish may vary according to their level of aerobic metabolism, exposure to cold environments and UV radiation (Maciel et al., 2004; Gouveia et al., 2005).

Nevertheless, because the oxygen dissolution is higher at lower temperatures, the response to oxidative stress could be expected to be inversely correlated with temperature, due to the increased oxygen availability to the ROS-generating processes at lower temperatures. Even though numerous studies show that the response to oxidative stress is not

directly correlated with temperature, ROSs are lower at the optimum temperature and increase at temperatures outside the upper and lower thermal limits than that constantly inhabited by the species (Vinagre et al., 2012).

In this way, the aquatic environment is an ideal medium for many environmental pollutants as they can be absorbed by aquatic organisms, altering the antioxidant/pro-oxidant balance in fish and bivalves (Lushchak, 2011). Moreover, depending on the source of the pollutant, the steady-state ROS concentration may be transiently or chronically increased, disrupting cellular metabolism and its regulation and damaging cellular constituents (Lushchak, 2011). Thus, some pollutants are substantially accumulated in specific tissues with no toxic effects recorded, while others demonstrate a high toxicity even at low levels (Jourmi et al. 2012).

Finally, stress responses induced by xenobiotics can be broadly classified as signs of intoxication and detoxification. Signs of intoxication may demonstrate debilitating phenomena, while detoxification signals are adaptive in nature and provide protection to biological systems when confronted with toxic xenobiotics (Bhattachary, 2001). This could explain why mortality is only related to some types of marine species affected by HAB or by effects of climate change, such as the El Niño Southern Oscillation (ENSO).

CONCLUSION

The results of this study show that exposure to HABs associated with *Dinophysis* sp. does not cause significant damage in cultured fish (*Salmo salar*) considering the damages detected in the filter-feeding species *Mytilus chilensis*, even though both species were exposed to an equal number of cells (≈100 cel l^{-1}) during the same period of time (≈10 days). Thus, it is established that the endogenous antioxidant defense system of both species is altered in a manner characteristic for each species, and independent of time. Simultaneously, the level of lipid peroxidation was the marker with the greatest alteration detected in the hepatopancreas of mussel, but it was not detected in fish. These results suggest that oxidative stress plays an important role in the *in vivo* toxicity that lipophilic toxins can produce in bivalves, which could explain, in some cases, the death of some species in areas with high cellular levels and high variables of abiotic conditions (variability of salinity and temperature) tending to produce oxidative stress to these filter-feeding species.

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Chapter 49

THE JUMBO FLYING SQUID (*DOSIDICUS GIGAS*) OFF OF COSTA RICA

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ABSTRACT

The fishery biology of jumbo flying squid (*Dosidicus gigas*) was studied based on squid resource surveys made by Chinese squid jigging vessels off the Costa Rica Dome in 2009 and 2010. The daily catch of *D. gigas* in two survey cruises ranged from 0 to 5.5 t and was mostly obtained from the areas bounded by 6°N－9°N and 91°W－94°W and by 6°30′N－7°30′S and 96°W－97°W. The sea surface temperature in the areas yielding the most catch ranged from 27.5 to 29°C. The sex ratio of the total catch was 3.75: 1 (female: male). Squid sizes ranged from 205 to 429 mm dorsal mantle length (ML) with ages no more than 10 months for females and 8 months for males. In the relationship of the mantle length (mm) and body weight (g) of the squid, there was no significant difference between sexes. The size (mantle length) and age at the first sexual maturity were 297 mm and 195 days in females, and less than 211 mm and 130 days in males. Growth in ML was best described by a linear function for both the sexes, while growth in body weight (BW) was best quantified by an exponential function for females and a power curve for males. The maximum absolute daily growth rates and instantaneous growth rate in ML were reached during 181-210 and 151-180 days for females and males, respectively. The Costa Rica Dome and its adjacent waters were considered to be a potential spawning ground. Most of the sampled stomachs (70.6%) had no food remains. The major prey of the squid were fish, cephalopods and crustaceans, with the most abundant being *Myctophum orientale* and *D. gigas*. The prey in more than 65% of the non-empty sampled stomachs evidenced cannibalism of *D. gigas*. The results improve current understanding of the fishery biology of *D. gigas* off the Costa Rica Dome, and may facilitate the assessment and management of related fishery resources.

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INTRODUCTION

Over the last two decades, the jumbo flying squid, *Dosidicus gigas* (d'Orbigny, 1835), has evolved as one of the most important species of cephalopod fisheries in the eastern Pacific. *D. gigas* is widely distributed in the eastern Pacific in the north from California (37°N), to the south in Chile (47°S) and east up to 125°W (Nesis, 1983; Nigmatullin et al., 2001). The highest abundance of *D. gigas* usually occurs off the Peruvian and Chilean coast in the southern hemisphere as well as in the Gulf of California and off the western coast of Baja, California in the northern hemisphere (Markaida and Sosa Nishizaki, 2003; Liu et al., 2010). Additionally, *D. gigas* is distributed in the water off the Costa Rica Dome where there is a strong upwelling. Japanese squid jiggers targeting *D. gigas* in this area obtained a high catch during the autumn of 1997 (El Niño) but poor catches during the fall of 1999 (La Niña) (Ichii et al., 2002) in the waters off the Costa Rica Dome.

Many studies have been conducted on the fisheries biology of D. gigas in the Gulf of California, off the Peruvian coast (Masuda et al., 1998; Arguelles et al., 2001; Morales-Boj´orquez et al., 2001; Nigmatullin et al., 2001), and the high seas of the southeast Pacific Ocean (Ye, 2002; Ye and Chen, 2007; Liu et al., 2010; Chen et al., 2011). Three distinct groups were identified for *D. gigas* based on their difference in size at maturity (Nigmatullin et al., 2001): a small-sized group (mantle length (ML) 140-340 mm for females and 130-260mm for males), a medium-sized group (ML 280-600 mm for females and 240-420mm for males), and a large-sized group (ML 550- 650 to 1000-1200 mm for females and >400-500 mm for males). D. gigas grow fast and do not live for more than 2 years with an average life span of ~1 year (Masuda et al., 1998; Arguelles et al., 2001; Chen et al., 2011).

At present, little information is available on the fishery biology of *D. gigas* in the waters off the Costa Rica Dome. Based on two scientific surveys of *D. gigas* by Chinese squid jigging vessels in the waters off the Costa Rica Dome undertaken in 2009 and 2010, the present study aimed to identify the environmental variables that influence the spatial distribution of *D. gigas* and to provide information on the population structure, maturity, and feeding characteristics of this squid species, further improving the understanding of the biology of *D. gigas* off the Costa Rica Dome. The information derived from this study is critical for quantifying the population dynamics of this squid and is of guiding significance to the assessment and management of this important marine resource.

RESULTS

1. Fishery

1.1. Catch and Its Relationship with Environmental Factors

Ichill et al., (2002) reported that a fishery operation for *D. gigas* was conducted in waters of 7°-9° N and 92°-100°W from June through November in 1997, and the monthly average CPUE (tonnes per vessel per day) was 19.4 tonnes in August, 15.2 tonnes in September, and 8.5 tonnes in October of that year. In 1999, the fishery was only operational in the same fishing areas from August to September and the monthly average CPUE was low, 3.1 tonnes in August and 2.3 tonnes in September of that year. The abundance of jumbo flying squid

within the 200 nautical miles exclusive economic zone of the Costa Rica Dome was unknown because fishery operation was not permitted.

The Chinese Government conducted two surveys off the Costa Rica Dome during 2009 to 2010 (Figure 1). The daily catch (CPUE) of *D. gigas* ranged from 0 to 5.5 t with no bycatch. The total catch was 95.5 t and the average CPUE reached 0.72 t. Overall, 75.7% of the fishing days had a CPUE lower than 1 t, and 17.4% of the fishing days had CPUE more than 2 t. The catch mainly came from the areas bounded by 6°-8°N and 95°30'-97°W, and by 6°-9°N and 91°- 95°W (Figure 2). The high density of *D. gigas* appeared in the above two areas under different environmental conditions (Table 1).

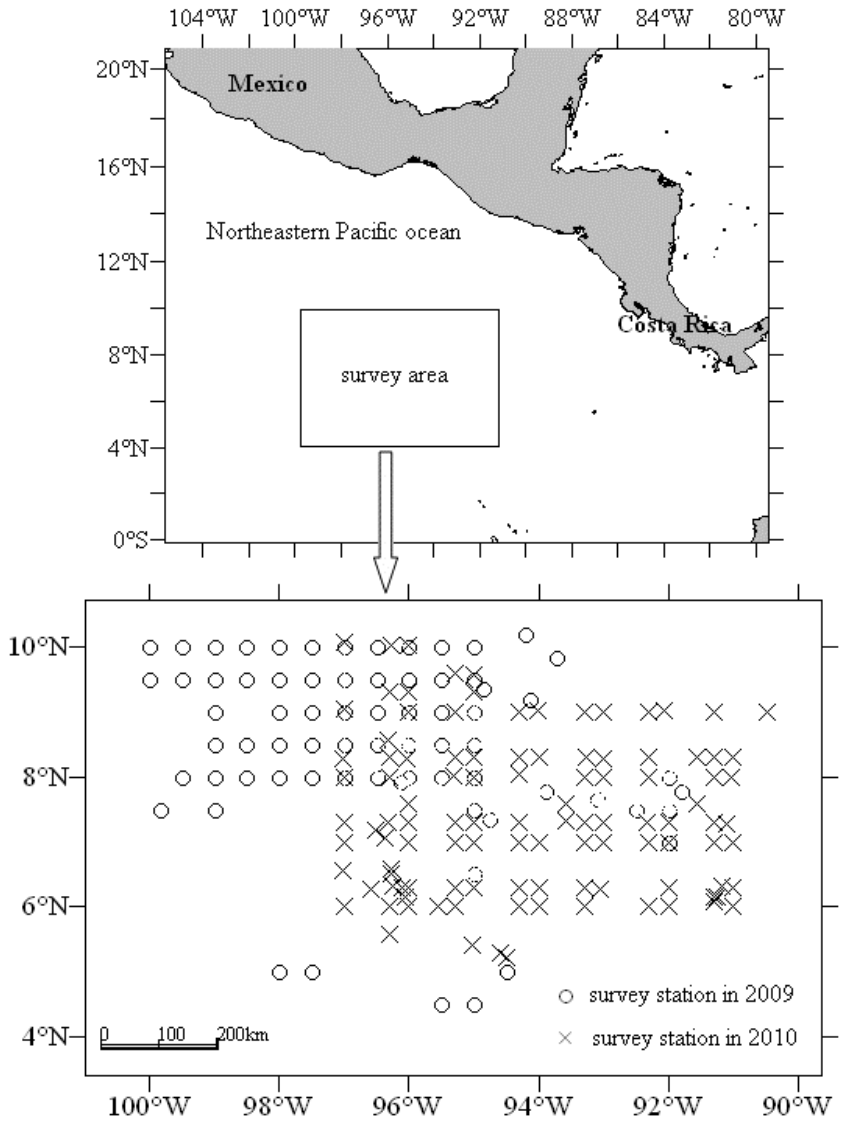


Figure 1. The two survey areas covered by the Chinese squid jigger vessels off the Costa Rica Dome in 2009 (a) and 2010 (b).

In the fishing area of 6°-8°N and 95°30'-97°W and during the period from July to September 2009, surface water temperature ranged from 27.5 to 29.0°C, at a depth of 50m the temperature ranged from 14.0 to 15.0°C and at a depth of 200m the temperature ranged from 13.0 to 13.7 °C. The corresponding water salinity ranged from 33.4 to 33.9 psu, from 34.8 to 34.9 psu and from 34.8 to 34.9 psu, respectively (Table 1). In the fishing area of 6°-9°N and 91°-95°W and during the period from February to March 2010, water temperature and salinity were 28.0–29.0°C and 33.8-33.9 psu on the surface, 24.0–25.0°C and 34.3–34.4 psu at the depth of 50 m, and 15.0–17.0 °C and 34.7–34.8 psu at the depth of 200 m, respectively (Table 1).

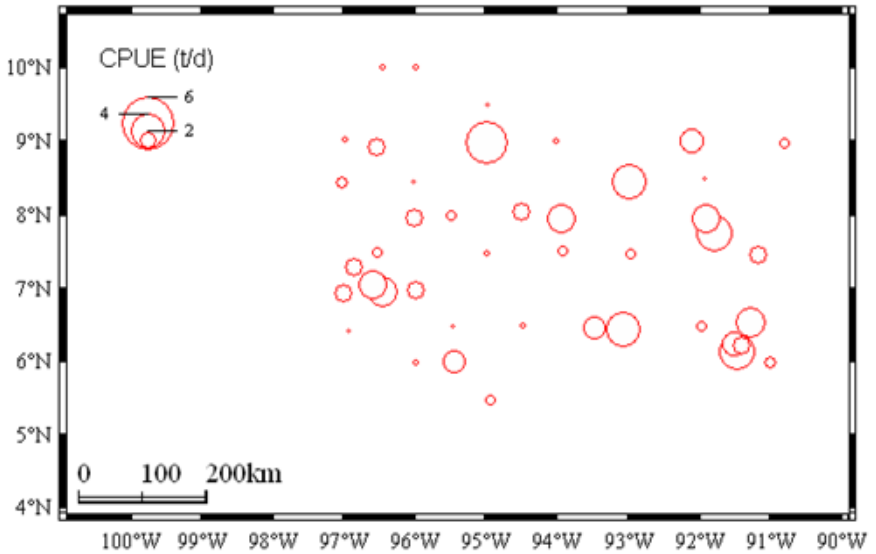


Figure 2. The spatial distribution of daily catch of *D. gigas* at 0.5°×0.5° latitude and longitude off the Costa Rica Dome in 2009 and 2010.

Table 1. Summary of the survey areas and sample collection of *D. gigas*

Date	Vessel	Survey area	Planned station	Fishing station	No. of samples	Range of ML (mm)
July - August 2009	Fenghui No 16, Zhe Yunyu No 807	5° - 10°N, 91° - 100°W	121	115	215	204-429
Feb. –Mar. and Aug.-Sep. 2010	Fenghui No 16, Zhe Yunyu No 807	5°21′N - 10°06′N, 90°49′W - 97°03′W	106	110	350	204-426

1.2. Fishing Technology

The fishery was predominantly operated by automated jigging using night lights to attract the squid. Experimental data showed when using high pressure sodium lamps the squid were attracted to shallower waters, which improved fishing efficiency, and the average product per hour was 1 to 6.5 times greater than in previous catches.

2. Hard Structure

2.1. Statolith

Statolith length (SL) ranged from 1247 to 2249 µm with a mean of 1846 ± 140 µm and statolith weight (SW) ranged from 1247 to 2249 µg with a mean of 816 ± 161 µg. The relationship between statolith length and weight was best fitted by an exponential curve (Figure 3) showing no significant difference between sex (ANCOVA, $F_{1,176} = 0.472$, $p = 0.493 > 0.05$). The relationship between SL and Mantle length (ML) was best fitted by a logarithmic curve (Figure 3） showing no significant difference between sex (ANCOVA, $F_{1,176} = 1.996$, $p = 0.160 > 0.05$).

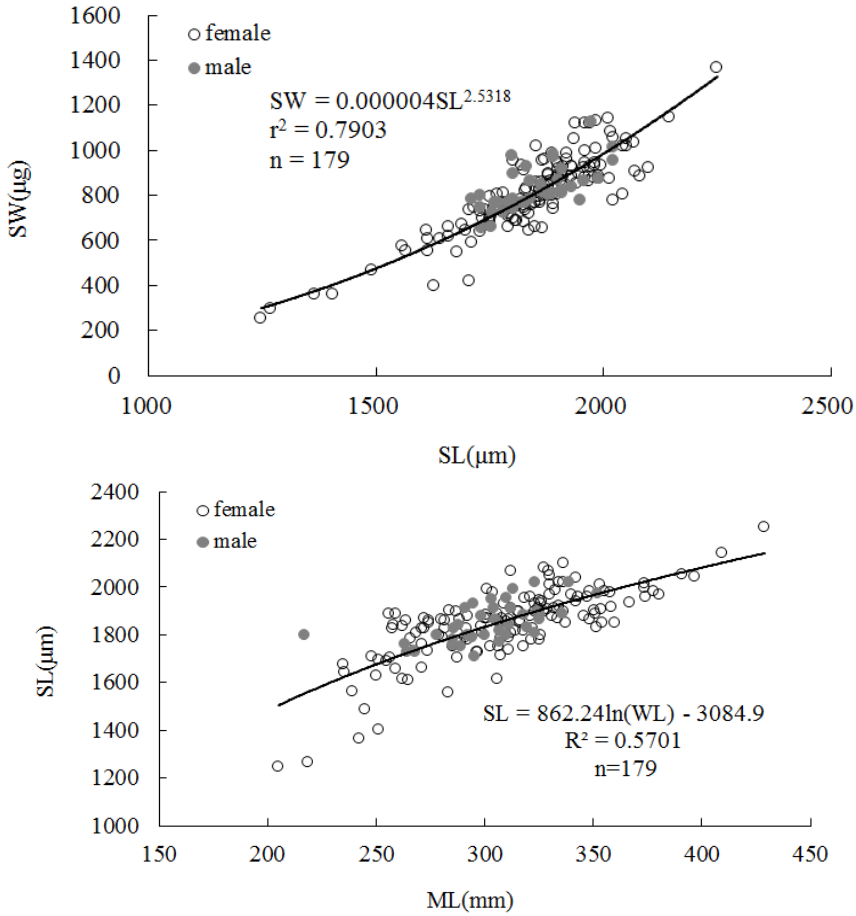


Figure 3. The relationship between statolith length and statolith weight and between statolith length and mantle length.

Three distinct growth zones, namely the postnuclear zone (PN), dark zone (DZ) and peripheral zone (PZ), could be identified based on incremental width from the focus to the edge of the dorsal dome on the statolith microstructure of all the individuals examined (Figure 4). Growth increments in the DZ were much wider than those in the PN and PZ. The first complete check is formed at the time of hatching and is referred to as the “natal ring” (NR;

Figure 4A). The second complete check usually lies in the transitional area between the PN and DZ marking the end of paralarval stage (check 1; Figure 4A). The complete check on the outer boundary of the DZ (check 2; Figure 4A, B) and checks within the PZ were found in some statoliths (check 3; Figure 4A). The number of increments within the PN was 26.2 ± 3.7(n = 120) and within the DZ was 86.1 ± 11.5(n = 120). Incomplete checks were occasionally observed in the lateral dome (See Figure 4 A check4). Second-order bands with fortnightly increments were observed within a few statoliths (check 5; Figure 4C). An additional center and rings were also revealed in several statoliths (See Figure 4D, E).

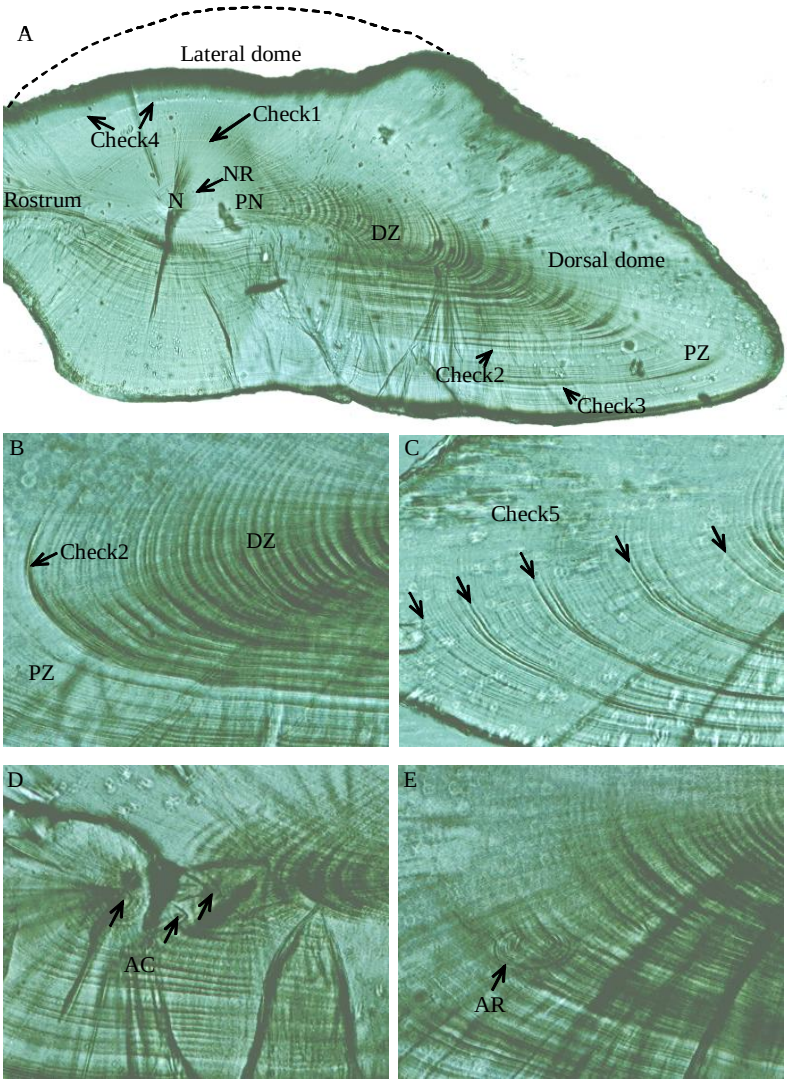


Figure 4. Light micrograph of *D. gigas* statolith caught off the EEZ of Costa Rica (A) an immature female of 179 days old and 251 mm ML; (B) a mature female of 216 days old and 310 mm ML; (C) an immature female of 165 days old and 246 mm ML; (D) a mature female of 205 days old and 331 mm ML; (E) a mature male of 197 days old and 325 mm ML; N is nuclear; NR is natal ring; PN is postnuclear zone; DZ is dark zone; PZ is peripheral zone; AC is additional center; AR is additional rings; Broken line shows the predicted normal outline of the lateral dome if the statolith does not have a check 4).

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) analysis showed that calcium (Ca), sodium (Na) and strontium (Sr) are the major elements in the statolith of *Dosidicus gigas*. The Sr/Ca ratio is highest at the embryonic phase (Figure 5a) and declines with age, and its relationship with Sea surface temperature (SST) shows a reciprocal ratio. There is no significant difference of Sr/Ca ratio between different hatching months in every life stage. The Ba/Ca ratio shows a "U" form during the larva to adult stage (Figure 5c), and this could be seen as an indicator of vertical movement with larvae living on the surface and adults living in the deep layer. The nucleus is higher than the peripheral zone in the Mg/Ca ratio (Figure 5d) which presents a positive correlation with SST, and might possibly be due to the gradual decrease of growth rate in the statolith. *Dosidicus gigas*, in its embryonic phase, uses its own vitellicle as a nutrient substance, so the trace elements in this phase are related to the genetic factors of the parent rather than the water environment.

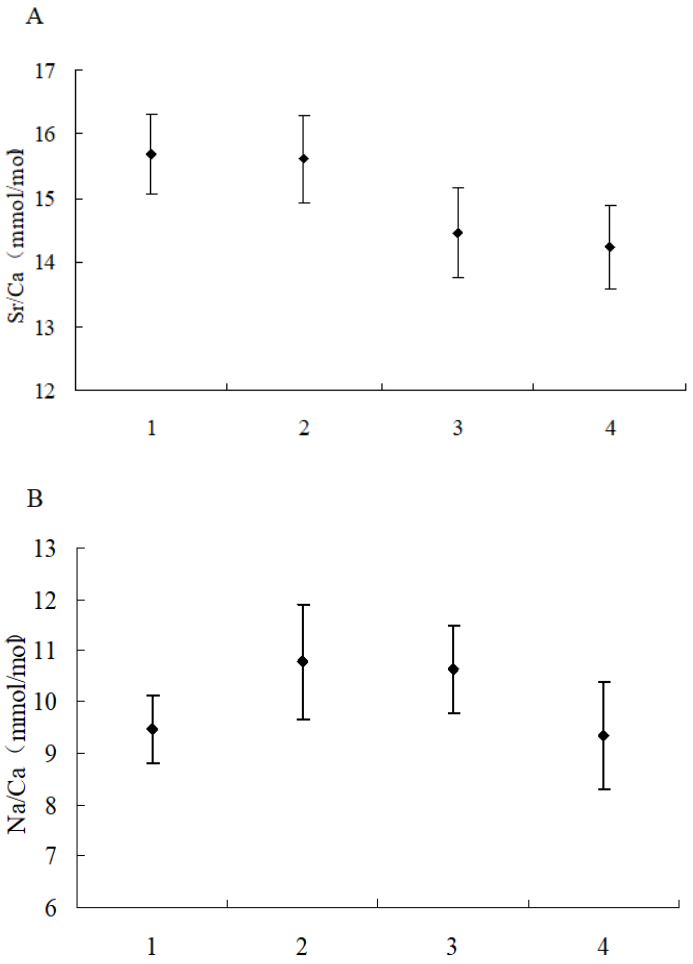


Figure 5. (Continued).

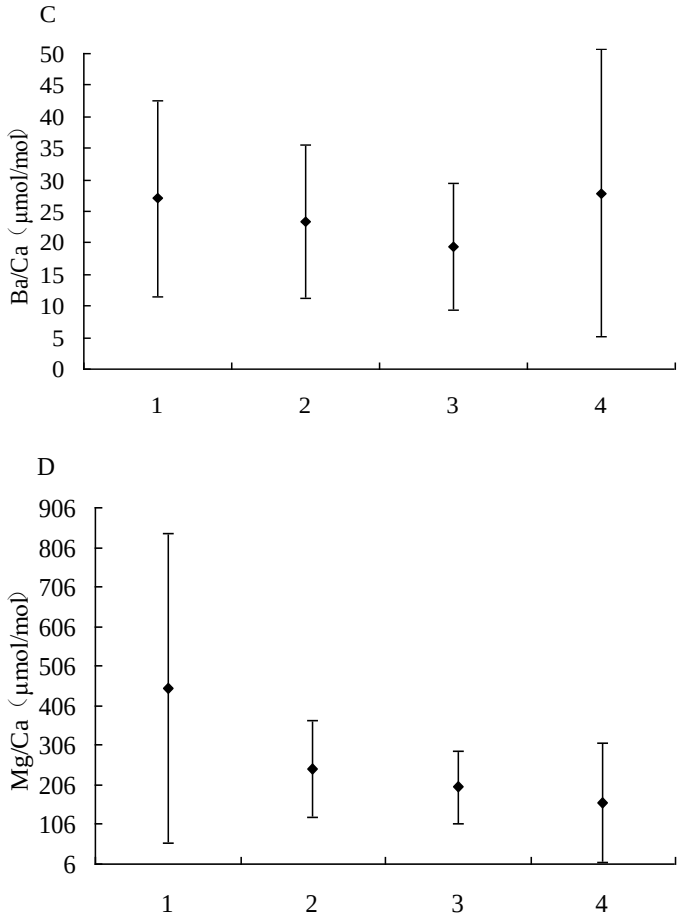


Figure 5. Element/Ca ratios measured with LA-ICP-MS in different growth zones of D. gigas statoliths from the nucleus to the edge. (1-4 represent: nucleus; postnuclear zone; dark zone and peripheral zone)

2.2. Beak

D. gigas' beak dimensions are best described by linear functions with mantle length but show a positive correlation by exponential equations with body weight. With squid ontogenetic growth, the size of each part of the beak becomes larger with the hood and crest growing faster but the rostrum and wing growing more slowly. The hood and crest of the upper beak grow faster than that of the lower beak.

After the upper beaks of *D. gigas* were regularly cut and ground, a pattern of bands could be seen on the medial surfaces of the rostrum sagittal section (RSS) across the rostrum tip to the joint of the hood (dorsal part) and crest (ventral part) (Figure 6). The anterior portion of the bands in both parts meet at the internal rostral axis in the shape of a '<', and the posterior portions are parallel to the rostrum edges (Figure 6). These bands consist of a wider light increment and a thinner dark increment (Figure 7A, B), which in the hood is more discernable and clearer than in the crest (Figure 7C, D). The increments at the tip of the rostrum are narrowest (Figure 7A), gradually becoming wider at the anterior joint (Figure 7B), reaching to the widest at the medial part of the rostrum (Figure7C), and then become slightly narrower again at the posterior joint (Figure 7D).

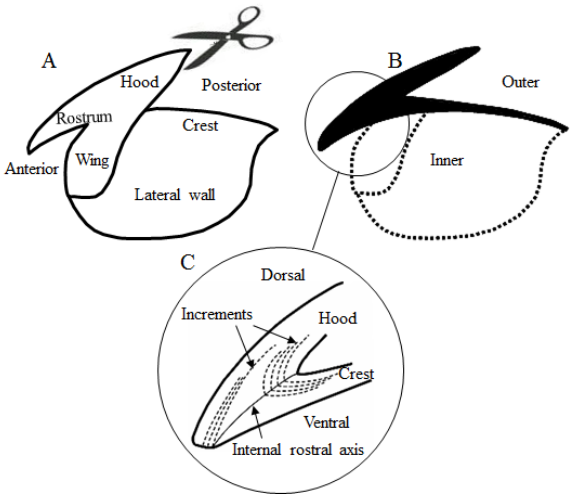


Figure 6. Diagrams of upper beak of *D. gigas*. A: terms of upper beak; B: medial surface (dark) after sagittal sectioning; C: RSS show growth increments in the dorsal (hood) and ventral (crest).

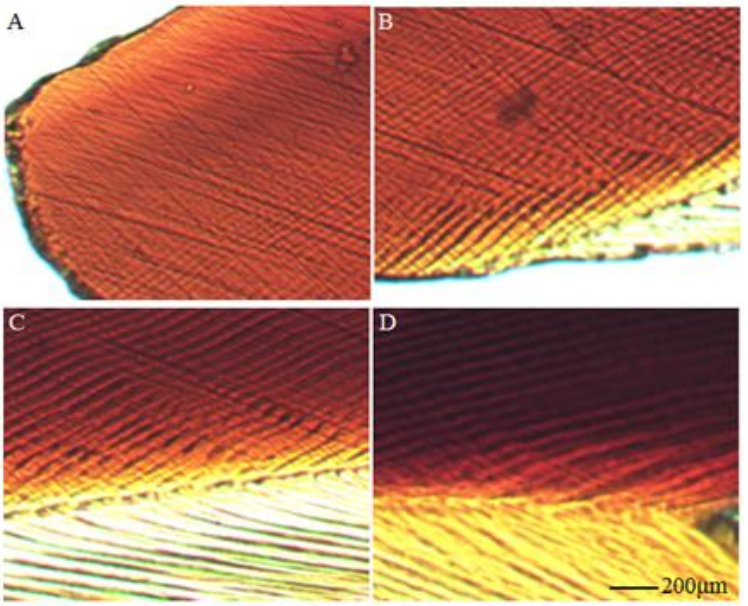


Figure 7. Increments in the tip part of rostrum (A), the anterior joint part (B), medial part of rostrum (C) and posterior joint part (D) for one *D. gigas* showing the difference in the width of increments alone counting direction.

There was no significant difference in either $\delta^{13}C$ and $\delta^{15}N$ values between the upper and lower beak ($P > 0.05$), although the values in the lower beak were higher than in the upper beak. The $\delta^{13}C$ in the upper and lower beaks had a relatively small range from -18.67 to -17.02‰ (-18.05 ± 0.36‰) and -18.36 to -16.69‰ (-17.81 ± 0.35‰) respectively, but the range for $\delta^{15}N$ was much larger at 5.14 to 9.84‰ (6.77 ± 1.09‰) and 5.94 to 10.04‰ (7.28 ± 1.02‰) respectively. Both $\delta^{13}C$ and $\delta^{15}N$ values in the upper and lower beaks increased with mantle length and age.

2.3. Gladius

For *D. gigas*, a strong linear correlation was detected between gladius length and mantle length (r = 0.987, mantle length = 0.952 gladius length + 0.190, Figure 8), while a positive exponential relationship was found with body weight. In the vicinity of the Costa Rica Dome, water of a high temperature and sufficient food availability is appropriate to the longitudinal growth of the proostracum (Figure 9), thus the gladius is adequate to support the growth of mantle and internal organs. Moreover, a relatively short conus already provides sufficient reinforcement for fin locomotion to accommodate the low speed current field adjacent to the Costa Rica Dome. After gladii specimens are washed and wiped dry, the growth increments of the gladius can be observed directly on the edge of the lateral plates (Figure 10). These are roughly symmetrically distributed on both edges and are more discernable on the side of the body than on the dorsal surface.

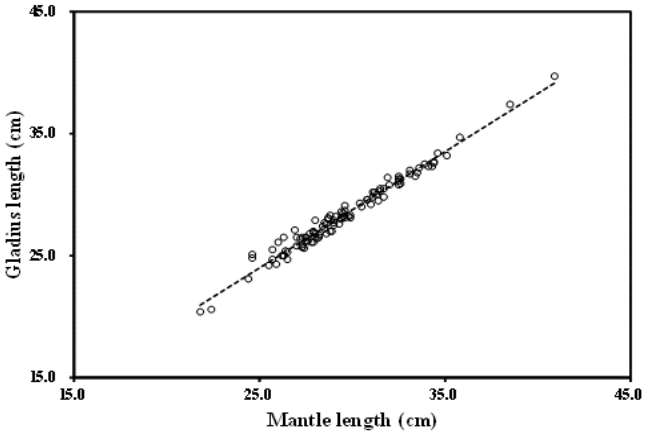


Figure 8. Relationship between gladius length with mantle length of *Dosidicus gigas.*

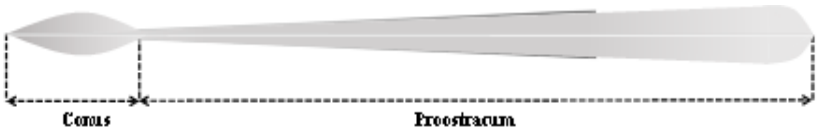


Figure 9. Schematic figure of *Dosidicus gigas* gladius (adapted from Lorrain et al., 2011).

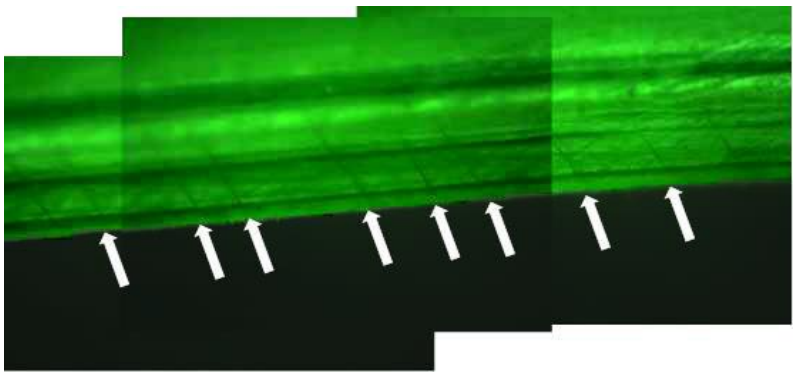


Figure 10. Growth lines of gladius on the edge of lateral plate (marked with white arrows).

3. Biology

3.1. Size, Age and Hatching Time

For f the 281 collected statoliths, led males were all mature except for on specimens re mature, but fore sampled males only on the 281 squid sampled, ML ranged from 205 to 429 mm for females and 212 to 355 mm for males, and individuals with MLs between 290 and 350 mm dominated the sample (Figure 11). Of all the samples, 263 statoliths were successfully read from 211 females and 52 males. Estimated ages ranged from 130 to 289 days with dominant ages from 180 to 240 days, consisting of more than 75% of all the samples (Figure 12). The youngest female obtained was mature at 130 days and 218 mm ML, and the youngest male was mature at 130 days and 212 mm ML. The oldest female was mature at 289 days and 429 mm ML, and the oldest male was mature at 240 days and 352 mm ML. The hatching dates for squid collected during July and August in 2009 in this study ranged from November 2008 to April 2009 with a peak between January and February (winter), accounting for 72.2% of the total sample (Figure 13).

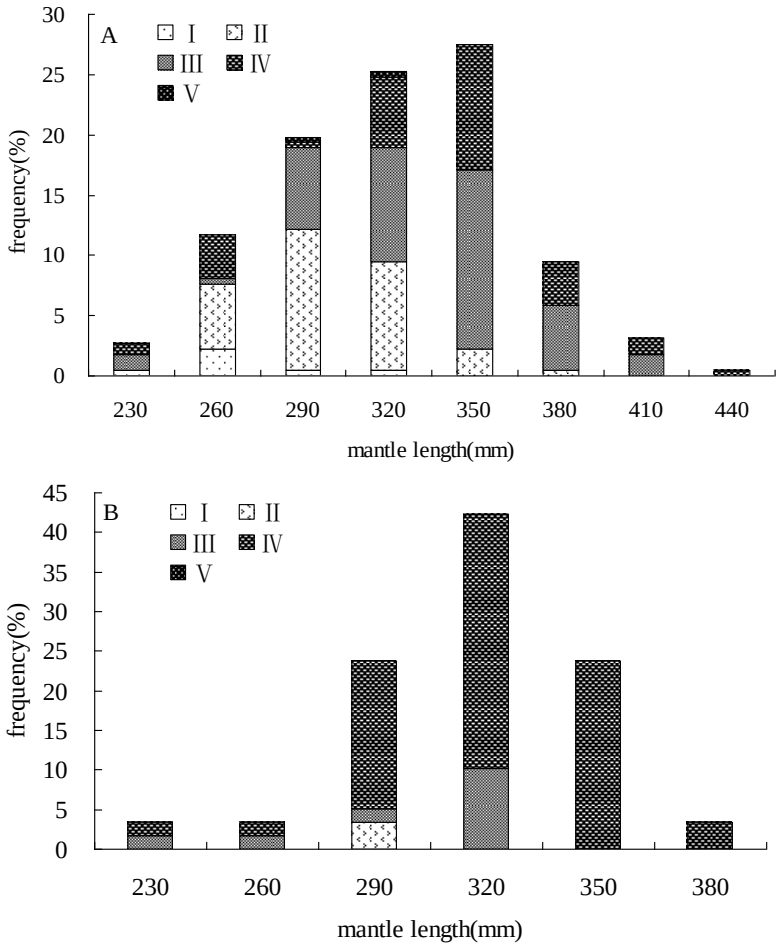


Figure 11. Mantle length (ML) frequency distribution by sex and maturity stage of *D. gigas* off the EEZ of Costa Rica Dome.

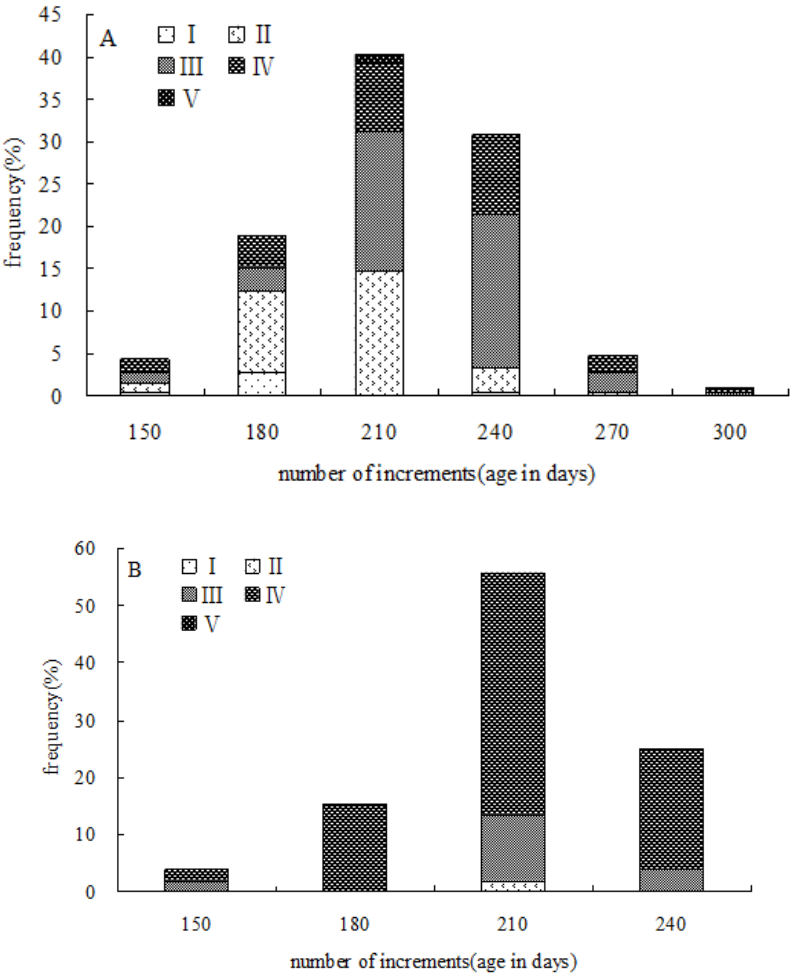


Figure 12. Age frequency distribution by sex and maturity stage of *D. gigas* off EEZ of Costa Rica Dome.

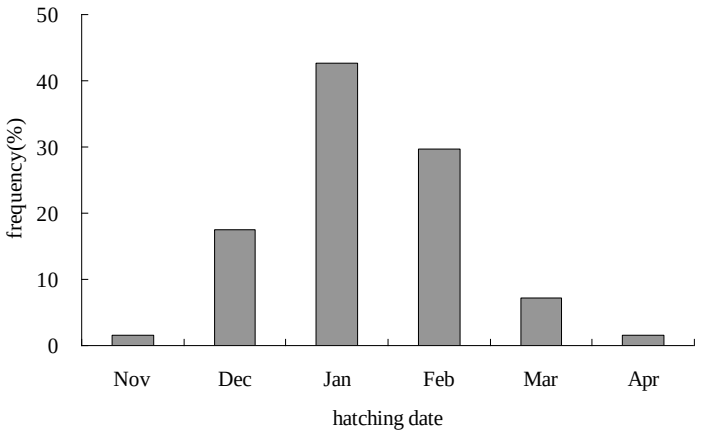
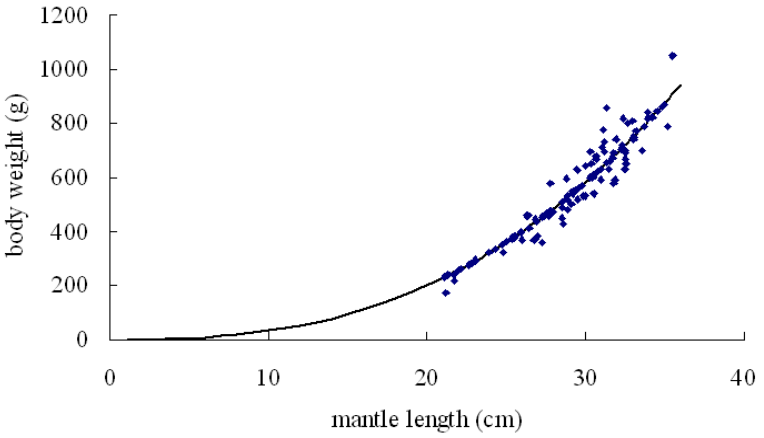


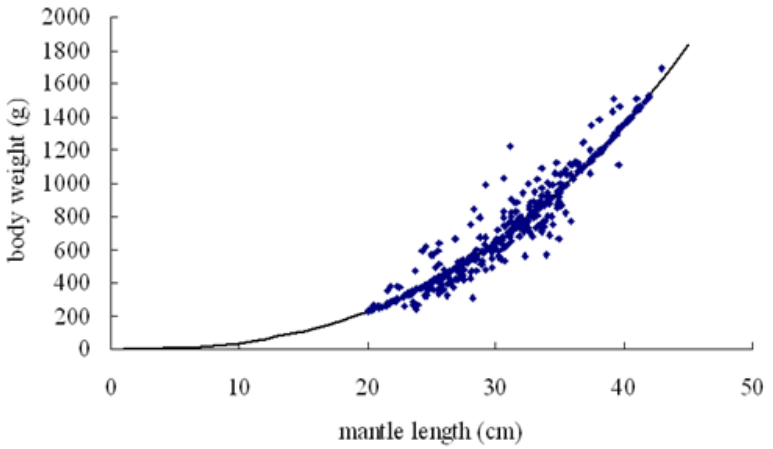
Figure 13. Back-calculated hatching frequency by month for *D. gigas* off EEZ of Costa Rica Dome.

3.2. The Relationship between ML and BW

The ML (cm)-BW (g) relationship of the samples collected were estimated as $BW = 0.076186ML^{2.62945}$ ($R^2 = 0.8868$, n = 119) for males (Figure 14a), and $BW = 0.100846ML^{2.5745}$ ($R^2 = 0.89233$, n = 446) for females (Figure 14b). There was no significant difference in the BW-ML relationship between sexes ($P > 0.05$).



(a)



(b)

Figure 14. The relationship between body weight and mantle length of male (a) and female (b) squid.

3.3. Maturity

In total, the sex ratio of the catch was 3.75: 1 (female: male), which was significantly higher than the expected (1: 1; $P > 0.05$). The maturity of the squid was different between sexes (Table 2). In males, 81.4% of the total were at stage IV, 15.2% at stage III, and 3.4% at stage II. In females, 42.4% of the total were at stages I–II, 40.1% at stage III, and 26.6% at stage IV (Table 2). Only 0.9% of the females were at stage V (Table 2). For a given size, males were more likely to be mature than females (Table 2).

Table 2. Composition of the sexual maturity stages of female and male *D. gigas*

Sex	N	Sexual maturity (%)				
		Immature		Maturing	Mature	Spent
		I	II	III	IV	V
Females	446	3.6	28.8	40.1	26.6	0.9
Males	119	0	3.4	15.2	81.4	0

Estimated female $ML_{50\%}$ was 297 mm and $Age_{50\%}$ was 195 days. The change in the proportion of mature females with size and age (Figure 15) is described by the following equations: $p_i = \frac{1}{1+e^{-0.02499\times(ML_i-297)}}$ (R^2=0.964) and $p_i = \frac{1}{1+e^{-0.04035\times(Age_i-195)}}$ (R^2=0.985). For males, the relationship between the proportion of maturity with size and age could not be quantified with logistic models because there were too few immature males, so $ML_{50\%}$ or $Age_{50\%}$ were not estimated. However, we believe that the size and age at first maturity of males should be smaller or younger than those of the smallest specimen in this study (i.e., 211 mm and 130 days old) because only 2 immature males were found in the catch.

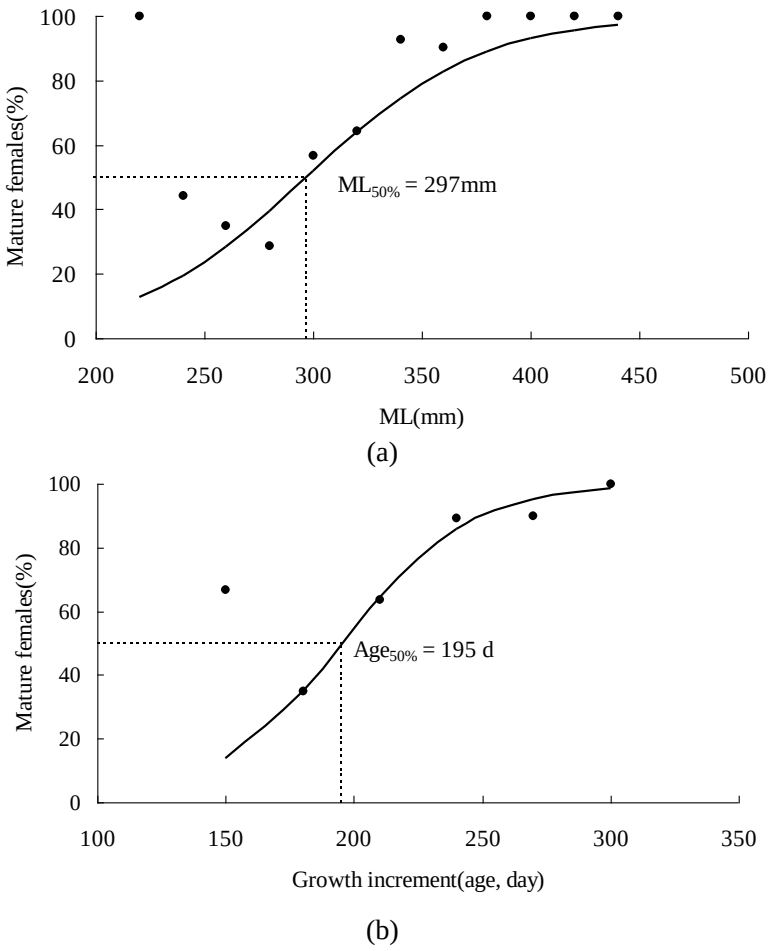


Figure 15. The size (a) and age (b) at first maturity of female squid.

3.4. Growth and Growth Rates

The age-ML data are best described by linear curves based on the AIC (Figure 16A) and no significant difference was found between females and males (ANCOVA, $F_{1,260} = 0.004$, P = 0.951). The age-BW data are best described by exponential curves for females, but by power curves for males based on the AIC (Figure 16B)

For females, the maximum DGR (1.46 mm d^{-1}) and G (0.52) in ML occurred in 181-210 days, while the maximum values (2.07 mm d^{-1} for DGR and 0.85 for G) for males were reached at 151-180 days although small sample sizes in some age classes might influence the reliability of the estimated growth rates (Table 3). The DGR in BW for females increased with age and the maximum G (1.25) was reached in 181-210 days, but those for males decreased with age (Table 3).

Table 3. Absolute daily growth rates (DGR) and instantaneous growth rate (G) for mantle length (ML) and body weight (BW) for female and male squid off the Costa Rica Dome

Sex	Age class(d)	Sample size	Mantle length			Body weight		
			Average ML(mm)	DGR(mm $30d^{-1}$)	G($30d^{-1}$)	Average BW(g)	DGR(g $30d^{-1}$)	G($30d^{-1}$)
Female	121-150	9	226.2	/	/	341.1	/	/
	151-180	40	259.5	1.11	0.46	454.7	3.79	0.96
	181-210	85	303.2	1.46	0.52	661.2	6.88	1.25
	211-240	65	338.1	1.16	0.36	896.4	7.84	1.01
	241-270	10	375.5	1.25	0.35	1220.0	10.79	1.03
	271-300	2	419.1	1.45	0.37	1600.0	12.67	0.90
Male	121-150	2	214.5	/	/	295.0	/	/
	151-180	8	276.5	2.07	0.85	460.3	5.51	1.48
	181-210	29	302.7	0.87	0.30	591.9	4.39	0.84
	211-240	13	329.0	0.88	0.28	737.7	4.86	0.73

3.5. Diet

The stomachs analyzed rarely contained food remains and on average 70.6% of the stomachs were empty. Regarding the fullness stages, 19.9% and 8.1% of the samples were graded to stage 1 and 2, respectively, while only 1.4% of the total samples were graded to stage 3.

The stomach contents included three major prey groups: fish, cephalopods and crustaceans, which represented 55.8%, 38.1%, and 6.1% of the stomach contents by weight, respectively. The species remains in the stomach contents were identified as Myctophidae, Sardinella, and *D. gigas*. Approximately 65% of the stomachs showed evidence of cannibalism. However, cannibalism was much greater for those squid caught in the light field around the survey vessel, and small squid (ML <10 cm) were often attacked by large-sized *D. gigas* around the squid jigging vessels in the fishing area of 6°-8°N and 95°30'-97°W during August to September in 2010.

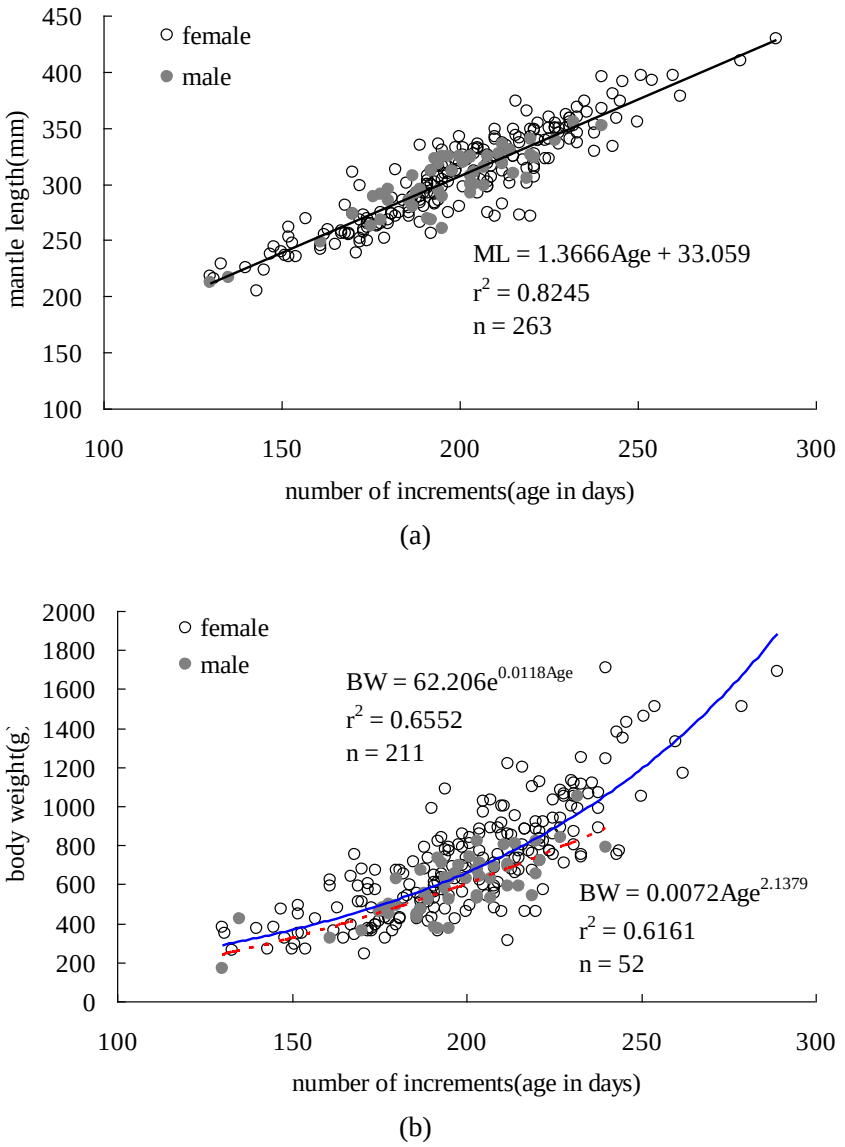


Figure 16. Relationships between age and ML and between age and BW of *D. gigas* off the EEZ of Costa Rica Dome.

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Chapter 50

SPATIAL DISTRIBUTION AND SPECIES COMPOSITION OF ZOOPLANKTON IN THE EASTERN TROPICAL PACIFIC OCEAN WATERS OFF COSTA RICA

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Shanghai, China

ABSTRACT

An analysis of zooplankton species composition, abundance and spatial distribution was undertaken based on investigation data of zooplankton carried out by Chinese squid jigging vessels in the waters (4°30′N-10°24′N, 91°20′W-100°00′W) of the eastern tropical Pacific Ocean off Costa Rica from July to August in 2007 The analysis showed there to be 23 species 17 genus 10 family 8 order in the Coelentera; 74 species 43 genus 30 family 8 order in the Crustacea; 4 species 1 genus 1 family 1 order in the Chaetognatha; 5 species 5 genus 3 family 3 order in the Urochordata; 6 species 5 genus 3 family 1 order in the Annelida; and 3 species 3 genus 1 family 1 order in the Mollusca and other species, including paralarvae, juvenile fish and cephalopod, and pelagic larvae. The species of zooplankton with high abundance were Copepoda and Sagittoidea, while the species with high occurrence frequency were Copepoda, Sagittoidea, Macrura, paralarvae, juvenile fish and cephalopod. The average biomass and amount of zooplankton were 124.78 ± 176.83 mg/m^3 and 848 ± 1219 ind/m^3 respectively, in which Copepoda was the highest at 727 ind/m^3 and the next was Sagittoidea with an abundance of 373 ind/m^3.

Keywords: zooplankton, species composition, abundance, spatial distribution, eastern tropical Pacific Ocean waters off Costa Rica

1. INTRODUCTION

The upwelling of the eastern tropical Pacific Ocean waters off Costa Rica is rich in species of zooplankton and biomass is high (Ferández-Álamo and Färber-Lorda 2006). Tuna, dolphins, blue whales and other large predators perennially prey on smaller fish and cephalopods in this area (Fiedler 2002). Zooplankton is an important part of the marine ecosystem, and the number and type of species directly impacts on the abundance of fish stock. Local studies have been undertaken to analyze the composition and distribution of zooplankton species in the northwestern Pacific, the *Ommatrephes bartramii* fishing ground and the Indian Ocean in the northwest ocean, and the results show that species composition and the distribution of zooplankton are important factors in the formation of fisheries (Xu et al. 2004; Qian et al. 2006). Wangelin and Wolff (1996), Bednarski and Morales-Ramírez (2004) studied species composition, biomass and spatial distribution of zooplankton in the Pacific coast bay of Costa Rica. The EASTROPAC program reported the distribution of zooplankton in the Costa Rican Pacific high seas. From July to August 2009, Chinese squid fishing vessels conducted a survey on the resources of the jumbo flying squid (*Dosidicus gigas*) in the eastern Pacific Ocean off Costa Rica, and collected zooplankton samples. Based on the survey data, this paper considers and analyzes the composition, biomass and spatial distribution of zooplankton species in the sea surface area, and provides basic data for further research on the formative mechanisms of cephalopod resources.

2. RESULTS

2.1. Total Biomass and Abundance

The biomass of each site was 2.2 ~ 1138 mg/m3 with an average value of 150.3 ± 184.5 mg/m3, and abundance ranged from 18 ~ 7773 ind/m3 with an average value of 1030 ± 1273 ind/m3. About 40% of the site had a biomass greater than 100 mg/m3 and an abundance greater than 1000 ind/m3. These areas were mainly concentrated at 8° 00'N ~ 9° 30'N 98° 00'W ~ 100° 30'W, 9° 00'N ~ 10° 00'N 96° 00'W ~ 97° 00'W, 7° 30'N 95° 00'W and 8° 00'N, 92° 00'W (Figure 1, Figure 2).

2.2. Composition of Zooplankton

There was a variety of species in the collected zooplankton. The Cnidaria included 8 orders, 10 families, 17 genera and 23 species, and all of these were jellyfish. The Crustaceans included 8 orders, 30 families, 43 genera and 74 species (6 of which were identified only to the family) and were mainly copepods. The Chaetognatha comprised 1 order, 1 family, 4 genera, and these were all Sagittoidea. The Tunicata included 3 orders, 3 families, 5 genera 5 species and were mainly cysticercosis. The Annelida comprised 1 order, 3 families, 5 genera and 6 species. The mollusks included 1 order, 1 genus, 3 genera and 3 species. Other zooplankton included the larvae and eggs of cephalopods, the larvae of fish, and long tail,

short tail and oral foot type of floating larvae, etc. of which there were more than 15 (see Table 1).

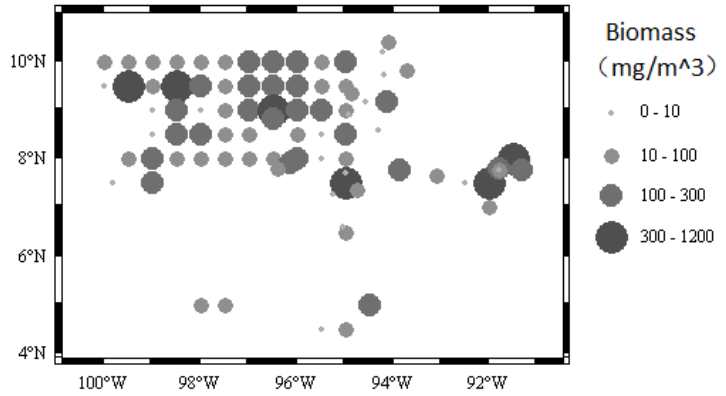

Figure 1. The spatial distribution of zooplankton total biomass.

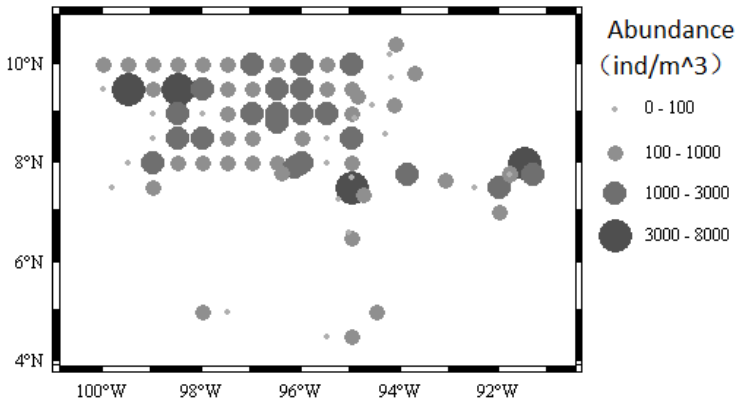

Figure 2. The spatial distribution of zooplankton total abundance.

2.3. Distribution and Frequency of Zooplankton

The copepods and Sagittoidea were the most widely distributed species in the whole surveyed area. The second most widely distributed were the juvenile of cephalopods, paralarvae, and the juvenile and larvae of fish, Amphipoda, Brachyura and Oikopleuridae (Figure 3 - Figure 10). The average abundance of copepods was the highest with a value of 727 ind/ m^3, followed by Sagittoidea with a value of 373 ind/m^3 and an abundance of other species below 100 ind/m^3. There was no obviously dominant distribution area for each group, although the spatial distribution of zooplankton abundance was different among the sampling sites (Figure 3 - Figure 10).

The copepods distributed in almost all surveyed waters and they were one of the dominant species with an occurrence of 82.7% (Table 1). Their abundance was about 22 ~ 4789 ind/m^3 with an average value of 727 ind/ m^3. The sites where abundance was greater

than 500 per/m^3 accounted for more than 50% of the total and were mainly distributed in 7° 00'N ~ 8° 00'N and 9° 00'N ~ 10° 00'N (Figure 4).

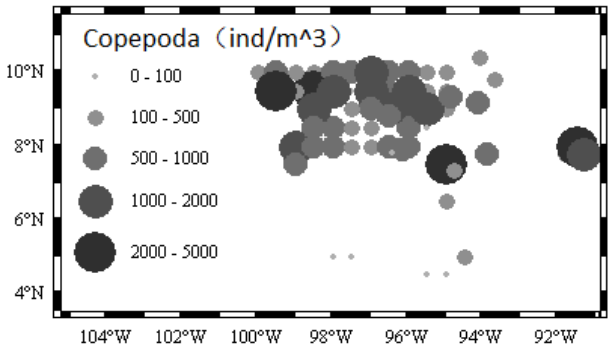

Figure 3. The abundance distribution of Copepoda.

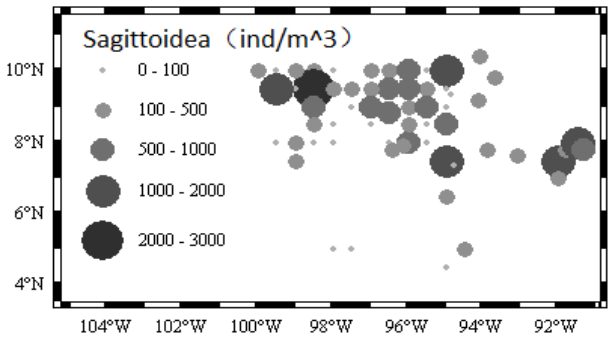

Figure 4. The abundance distribution of Sagittoidea.

Sagittoidea were also distributed in most of the surveyed waters, and were one of the dominant species with a frequency occurrence of 82.7% (Table 1). Their abundance was about 6 ~ 2002 ind/m^3 with an average value of 373 ind/m^3. The sites where abundance was more than 500 ind/m^3 accounted for about 25% of the whole area, and these sites were mainly distributed in 9° 00'N 98° 30'W ~ 99° 30'W, 7° 30'N ~ 10° 00'N 95° 00'W ~ 97° 00'W and 8° 00'N around 92° 00'W (Figure 5).

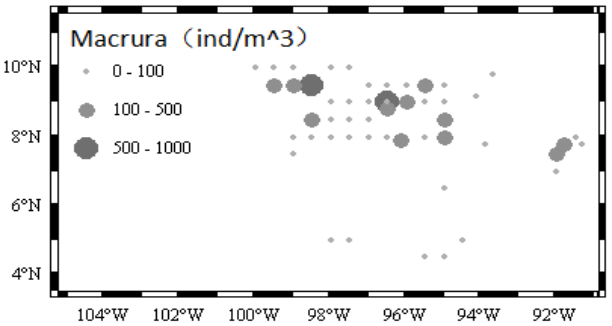

Figure 5. The abundance distribution of Macrura.

Table 1. The species composition of zooplankton

species	Amphipoda	Larvae of Brachyura	Sagittoidea	Copepoda	Juvenile of Cephalopoda	Paralarvae and Juvenile	Larvae of Macrura	Oikopleuridae
	18	9	62	62	47	38	58	5
(%)	24.0%	12.0%	82.7%	82.7%	62.7%	50.7%	77.3%	6.7%

Macrura were widely distributed in the study area with an occurrence of 77.3% (Table 1). Abundance in most sites was low, and there were only two sites where the abundance was about 500-1000 ind/m^3, which accounted for 3.4% of the total area of the survey (Figure 6).

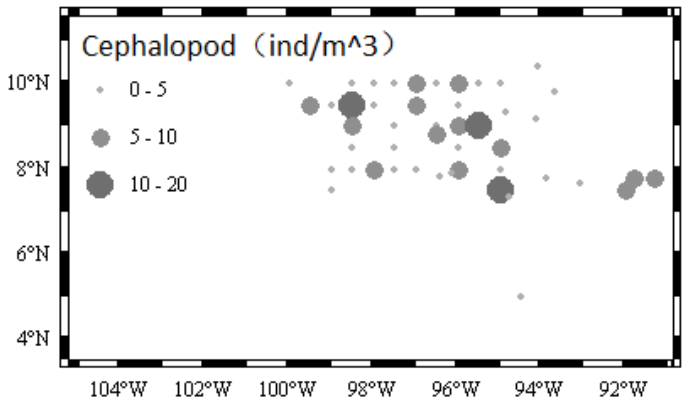

Figure 6. The abundance distribution of Cephalopod.

Cephalopods were also widely distributed in the study area with an occurrence of 62.7%. Abundance was 2 ~ 16 ind/m^3 with an average value of 5 ind/m^3. The abundance in most sites was less than 5 ind/m^3 (Figure 7).

The larvae of fish were widely distributed in the study area with an occurrence of 50.7%. Abundance was 2 to 52 ind/m^3 with an average value of 12 ind/m^3. The abundance in most sites was less than 10 ind/m^3. The larvae of fish in 8° 30'N and 96° 30'W had the highest abundance (Figure 8).

Amphipoda distributed in a few sites with a frequency of 24.0%. Abundance was 4 ~ 239 ind/m3 with an average value of 43 ind/m^3. The species living in 3.9° 30'N, 99° 800'N had the largest abundance at 200 ~ 300 ind/m^3 (Figure 9).

Brachyura only distributed in 9 sites with an occurrence of 12.0%. Abundance was 2 ~ 16 ind/m^3 with an average value of 8 ind/m^3 (Figure 10).

The distribution of Oikopleuridae was very small, and appeared in only 5 sites, with a frequency of 6.7%. Abundance was 6 ~ 54 ind/m^3, with an average value of about 25 ind/m^3.

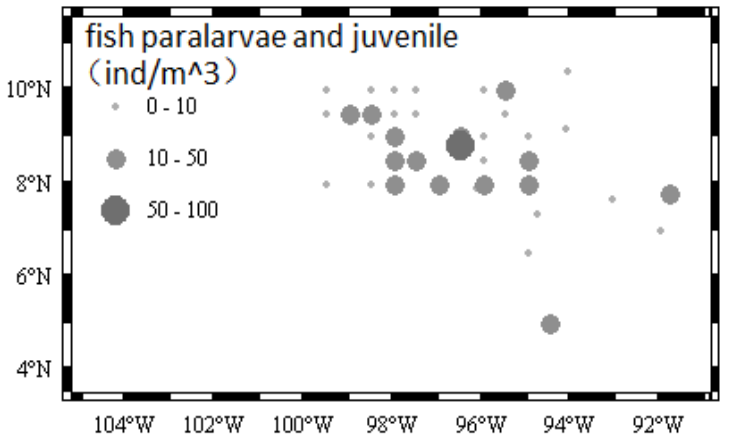

Figure 7. The abundance distribution of fish paralarvae and juvenile.

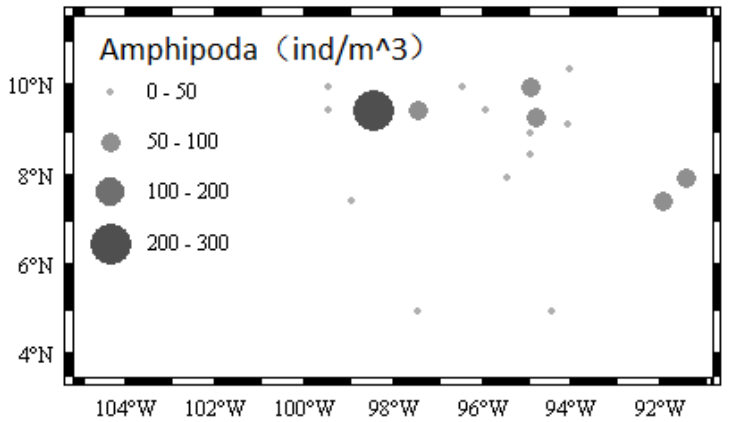

Figure 8. The abundance distribution of Amphipoda.

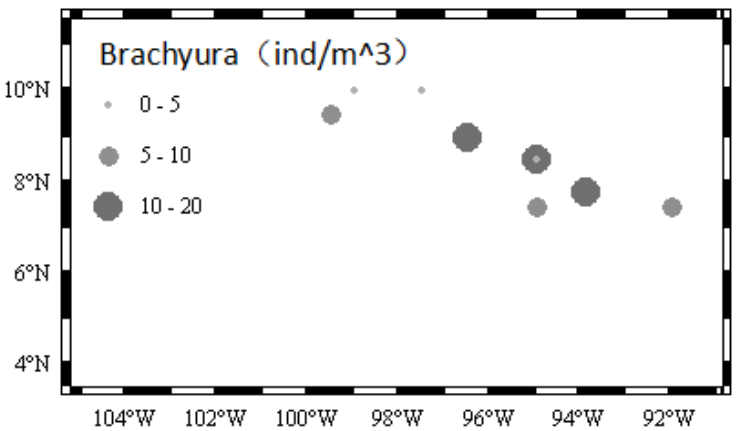

Figure 9. The abundance distribution of Brachyura.

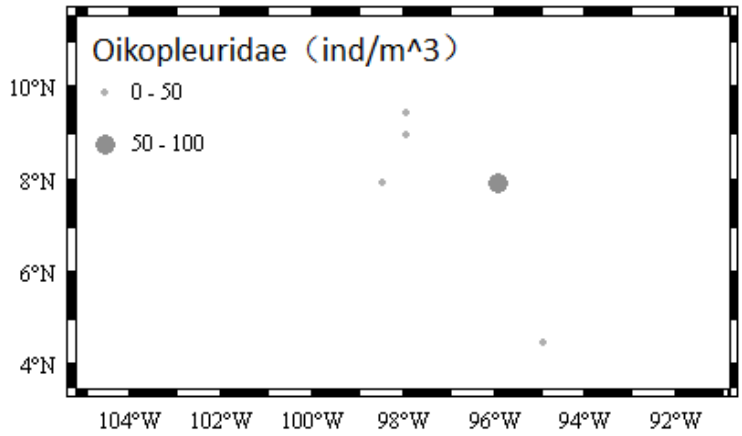

Figure 10. The abundance distribution of Oikopleuridae.

APPENDIX: ZOOPLANKTON SPECIES LIST

1. Coelentera
   - Hydroidomedusa
     - Filifera
       - Cytaeididae
         - *Cytaeis tetrastyla*
   - Proboscoida
     - Phialucidae
       - *Phialucium carolinae*
   - Conica
     - Malagazzia
       - *Octophialucium indicum*
     - Eirenidae
       - *Eutima levuka*
   - Trachymedusae
     - Rhopalonematidae
       - *Rhopalonema velatum*
       - *Aglaura hemistoma*
   - Narcomedusae
     - Aeginidae
       - *Solmundella bitentaculata*
       - *Aeginura grimaldii*
   - Siphonophorae
     - Calycophorae
       - Diphyidae
         - *Diphyes dispar*

- *Diphyes bojani*
- *Diphyes chamissonis*
- *Lensia challenger*
- *Lensia subtiloides*
- *Lensia fowleri*
- *Lensia conoides*
- *Lensia multicristata*
- *Muggiaea atlantica*
- *Chelophyes appendiculata*

- Abylidae
  - *Abyla trigona*
  - *Enneagonum hyalinum*

- Scyphomedusae
  - Semaeostomeae
    - Pelagiidae
      - *Pelagia noctiluca*
      - *Chrysaora helvola*
  - Rhizostomeae
    - Cepheidae
      - *Cephea conifera*

2. Crustacea
   - Ostracoda
     - Halocyprida
       - Halocyprididae
         - *Comchoecia macrochoeire*
         - *Porroecia porrecta*
         - *Porroecia spinirostris*
   - Copepoda
     - Calanoida
       - Calanidae
         - *Neocalanus robustior*
         - *Calanus pacificus*
         - *Undinula darwinii*
       - Eucalanidae
         - *Eucalanus pseudattenuatus*
         - *Eucalanus attenuatus*
         - *Eucalanus incrmis*
         - *Rhincalanus cornutus*
       - Clausocalanidae
         - *Clausocalanus arcuicornis*
         - *Clausocalanus paupulus*

- Aetideidae
  - *Aetideus armatus*
  - *Euchirella amoena*
  - *Euchirella galeata*
- Euchaetidae
  - *Euchaeta marina*
  - *Euchaeta concinna*
  - *Euchaeta tenuis*
- Phaennidae
  - *Phaenna spinifera*
- Scolecithricidae
  - *Scolecithrix danae*
- Temoridae
  - *Temora discaudata*
  - *Temora longicornoides*
- Centropagidae
  - *Centropages furcatus*
- Augaptilidae
  - *Haloptilus setuliger*
- Candaciidae
  - *Candacia tenuimana*
- Pontellidae
  - *Labidocera eucha*
  - *Labidocera detruncate*
  - *Labidocera lubbocki*
  - *Pontellina morii*
  - *Pontellina plumata*
- Paracalanidae
  - *Paracalanus parvus*
  - *Paracalanus cauleatus*

- Cyclopoida
  - Oithona
    - *Oihona similis*
  - Oncaeidae
    - *Lubhockia aculeate*
    - *Oncaea venusta*
  - Sapphirinidae
    - *Sapphirina augusta*
    - *Sapphirina iris*
    - *Sapphirina nigromaculata*
    - *Copilia mirabilis*

- Corycaeidae
  - *Corycaeus speciosus*
- Harpacticoida
  - Aegisthidae
    - *Aegisthus mucronatus*
- Malacostraca
  - Amphipoda
    - Vibiliidae
      - *Vibilia longicarpus*
      - *Vibilia gibbosa*
    - Hyperiidae
      - *Lestrigonus schizogeneios*
      - *Lestrigonus halmus*
      - *Themisto sp.*
      - *Phronimopsis spinifera*
      - *Hyperoche mediterranea*
      - *Hyperoche sp.*
    - Phronimidae
      - *Phronima pacifica*
      - *Phronima sedentaria*
      - *Phronimella elongate*
    - Lycaeidae
      - *Brachyscelus rapax*
    - Oxycephalidae
      - *Oxycephalus latirostris*
      - *Rhabdosoma minor*
      - *Rhabdosoma brevicaudatum*
      - *Streetsia porcella*
      - *Streetsia mindanaonis*
      - *Streetsia challengeri*
    - Platyscelidae
    - Parascelide
  - Mysida
    - Mysidae
      - *Siriella gracilis*
      - *Siriella aequiremis*
  - Euphausiacea
    - Euphausiidae
      - *Euphausia pacifica*
      - *Euphausia tenera*
      - *Stylocherion carinatum*

- *Stylocheiron affine*
- Decapoda
  - Sergestidae
    - *Sergestes brevispinatus*
    - *Sergestes geminus*
  - Luciferidae
    - *Lucifer typus*

3. Chaetognatha
   - Sagittoidea
     - Aphragmophora
       - Sagittidae
         - *Sagitta enflata*
         - *Sagitta pacifica*
         - *Sagitta bedoti*
         - *Sagitta bierii*
4. Urochordata
   - Appendiculata
     - *Caudata*
     - *Oikopleuridae*
     - *Oikopleura dioica*
   - Thaliacea
     - Cyclomyaria
       - Doliolidae
         - *Dolioletta gegenbauri*
         - *Doliolum denticulatum*
     - Hemimyaria
       - Salpidae
         - *Salpa fusiformis*
         - *Thalia democratica*
5. Annelida
   - Polychaeta
     - Phyllodocida
       - Alciopidae
         - *Naiades cantrainii*
         - *Alciopina parasitica*
         - *Rhynchonerella gracilis*
       - Tomopteridae
         - *Tomopteris kefersteinii*
         - *Tomopteris pacifica*
       - Typhloscolecidae
         - *Sagitella kowalevskii*

6. Mollusca
    - Gastropoda
        - Thecosomata
            - Cavoliniidae
                - *Diacria quadridentata*
                - *Creseis virgula*
                - *Cavolinia longirostris*
7. Other zooplanktons
    - Cephalopod larvae and juvenile
        - *Sthenoeuthis oualaniensis*
        - *Onychoteuthis banksi*
        - *Bathyteuthis abyssicola*
    - Fish egg, larvae and juvenile
        - *Idiacanthus antrostomia*
        - *Stomia columbrinu*s
    - planktonic larva

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Chapter 51

TURBOT AQUACULTURE IN SPAIN: AN OVERVIEW

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ABSTRACT

Turbot (*Psetta maxima*) is one of the main species of farmed flatfish. Spain is by far the largest turbot producer in Europe and is the world's second-largest producer, with more than 99% of Spanish production concentrated in the Galicia region. Turbot aquaculture began in Scotland in the 1970s, after which it was introduced to France and Spain in the early 1980s. Spain expanded its production volume and number of farms, where improvements in juvenile production motivated construction of new installations. By the early 1990s, there were 16 turbot producers in Spain. This chapter addresses Spanish turbot aquaculture from 1983 – when the first industrial turbot farming company in Spain was set up in Galicia – to 2016. In that year, the turbot became the first vertebrate to be genetically sequenced in Spain; this breakthrough opened the way for further investigation into its resistance to various diseases and for the development of more efficient genetic selection projects to improve production. Here, the production and economic performance of Spanish turbot are analyzed in the local, European, and global context. Spain is the dominant market in Europe, but turbot farming was recently introduced to China and production levels are high there. In the coming years, Spanish turbot farming will face challenges that include advances in genetic engineering, a higher standard of research on fish meal substitutes, the development of vaccines for diseases to which turbot are prone, and preservation of the environment.

1. INTRODUCTION

Flatfish have long been of interest to European aquaculture. Turbot (*Scophthalmus maximus* or *Psetta maxima*), sole (*Solea solea*), and halibut (*Hippoglossus hippoglossus*) are

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species viewed as candidates for commercial culture, but turbot is the only flatfish species to have made a significant step forward on that score (Varadi et al., 2001). In 1968, Alan Jones was the first researcher to attain turbot larvae metamorphosis after 68 days of nursing. The potential for farming of Atlantic turbot has been under investigation since the early 1970s; researchers in Lowestoft (United Kingdom) reported on the first successful attempts to rear larval turbot in captivity in 1973. Significant research and development continued in the United Kingdom and France in the late 1970s, followed by more recent work on turbot cultivation in Spain, Holland, and (to a lesser extent) Chile.

Turbot farming began in the 1970s in Scotland, and it was then further developed in France and Spain. This farming required intensive culture systems with turbot juveniles supplied from the wild or by marine hatcheries. Starting in the late 1970s, the supply of juveniles from hatcheries was the industry's strategic bottleneck. While problems with hatcheries were being investigated, juvenile turbot continued to be obtained from the wild. This wild juvenile supply had traditionally come from northern Europe (United Kingdom, Germany) and been sent to production units on the Atlantic coast, usually in southern Europe. Hatcheries became effective suppliers of juvenile turbot by the mid- to late 1980s, and wild juvenile collection ceased at that time. Over subsequent decades, the production of hatchery-reared juvenile turbot has expanded rapidly, with most of the increase due to improvements in hatchery techniques for the large-scale rearing of turbot fry. Whereas 1984 saw the production of 4 tonnes of juvenile turbot, 5,000 t. were produced in 2004; here "t." abbreviates "tonnes" (a.k.a. "metric tons," 1 t. = 1,000 kg).

Turbot is currently produced in Scotland, France, Spain, Germany, Denmark, Ireland, the Netherlands, and Portugal. The main production centers are in southern Europe, especially Spain, because of the more suitable sea temperatures there. However, investment in recirculated turbot systems in northern Europe is continuing with mixed commercial success. Other technological improvements include feeds development and vaccines to combat disease. Most of the turbot produced in the European Union (EU) is also consumed there. The fish are generally sold whole, although they are gutted before sale in some countries. To satisfy market demand, Spain has begun selling filleted turbot. Predictions are for a gradual expansion of turbot production throughout Europe (Bjørndal and Øiestad, 2011).

Worldwide, Spain is the second largest producer of turbot (after China), and in 2016 it produced about 7,100 t. The waters and the environment make Spain, especially Galicia (northwestern Spain), an ideal location for turbot production. The temperature and environmental conditions of Galicia's estuaries are the main facilitators of turbot farming in the region. The Spanish Institute of Oceanography played an important role in the research and development that made possible one of the world's most thriving turbot industries.

2. BIOLOGY AND HABITAT OF THE TURBOT

Turbot (*Scophthalmus maximus*, also known as *Psetta maxima*) is a flatfish species of the family Scophthalmidae. It is a demersal marine fish widely distributed in the eastern Atlantic Ocean, from Iceland and Norway to northern Africa – including the Baltic Sea, the North Sea, the Mediterranean, and the Black Sea. It has an asymmetric and almost round body, with both eyes on its left side. The skin is scaleless but with bony and irregularly distributed

protuberances. Turbot has a demersal lifestyle; thus it lives on sandy and muddy bottoms ranging in depth from shallow waters, where younger individuals tend to live, to 150 meters. It is carnivorous but with a narrow spectrum of prey: juveniles feed on mollusks and crustaceans, and adults feed mainly on other bottom-living fish (e.g., sandeels, gobies) and cephalopods yet also consume crustaceans and bivalves. Females reach maturity at 3 years of age and males at 2 years (and at respective lengths of about 46 cm and 30 cm). The maximum length is about 100 cm; the maximum published weight is 25 kg, and the maximum reported age is 25 years. Turbot exhibits one of the most rapid growth rates observed in flatfish (about 10 cm per year).

Spawning usually occurs from February through April in the Mediterranean and from May through July in the Atlantic. The natural sequence of spawning is every two to four days, and more than 5 million eggs are produced each spawning season. The eggs are pelagic in the Atlantic Ocean and demersal in the Baltic Sea. They are smooth and spherical, measure 1.1 mm in diameter, and contain a single oil globule (of diameter 0.18 mm). The larvae are initially symmetric but, at the end of the metamorphosis (day 40–50, 25 mm), the right eye moves to the left side and so loses its initial bilateral symmetry. The egg size is 0.9–1.2 mm, and the larval length at hatching is 2.7–3.1 mm (Fishbase, 2000).

3. TURBOT FARMING

The turbot's rearing cycle begins in a hatchery, where the breeding specimens are housed. Turbot do not spawn spontaneously in captivity, so gametes are routinely hand-stripped. Starting in the third year of age, the breeders start laying gametes, which are carefully fertilized. Turbot females may produce 1–10 million eggs during the season, depending on fish size. The resulting spherical, pelagic eggs are incubated for four to five days at a controlled temperature. Once hatched, turbot larvae are more than 2.7 mm long. Larval rearing is typically intensive, with densities of about 20 million larvae per liter. They are fed daily with live food: rotifera, artemia, and phytoplankton. The ideal temperature for larvae nursing is from 18° to 20° (Celsius). Metamorphosis ends after 40–50 days, when larvae are about 25 mm long.

The 5–10-g turbot juveniles are then transported to a nursery section and stored in square- or round-shaped tanks (10–30 m^2) with an open-circuit system for pumping seawater. Oxygen is often added to the tanks. The fry are fed manually or automatically with balanced dry feed. This stage of the rearing process, known as nursing, spans the fish's development from 10 g to 100 g and lasts for approximately five months. Once turbot juveniles weight 200–400 g, they are transferred to permanent land-based grow-out tanks that typically have a water surface area of 60–120 m^2 and a depth of 0.5–1.0 m. The initial density of 20 kg/m^2 will increase to 50–70 kg/m^2 for market-sized turbot. An open-circuit seawater system is used here, too, although some fisheries have experimented with closed-circuit systems.

The turbot are fed with extruded pellets, which are distributed manually or automatically. Water temperature is the key factor for optimal development. The ideal growth temperature is 14° to 18°, although turbot can tolerate temperatures as extreme as 8°–24°. The rearing period until the fish are ready to go to market, with an average weight of 1.8 kg, is 18 to 24 months (Fernández, 2013). Some 10% to 25% of the larvae survive to metamorphosis, and the

proportion of juveniles without deformities – and thus selected for continued cultivation – usually exceeds 90%. All juveniles are vaccinated against predominant fish diseases (Bjørndal and Øiestad, 2011).

Bjørndal and Palmieri (2008) used data from several fishing farms in Spain and Portugal to analyze the cost of producing turbot. Their base-case company has an annual output of 133 t., and its average cost is estimated to be €7.54 per kilogram of turbot. If that company expanded its production capacity to an annual output level of 400 t., then it would enjoy a nearly 33% reduction (to €5.07) in the average cost per kilogram. A turbot farm with production capacity of about 133 t. per year requires an investment totaling about €4.3 million; tripling the production capacity (to 400 t.) would require another €1.8 million. Thus the additional investment costs are considerably less than the increase in output, which indicates that economies of scale are present. In addition, higher levels of output entail a more efficient use of both labor and management, which can be expected to reduce production costs even more (Bjørndal and Palmieri, 2008).

4. SPANISH RESEARCH ON TURBOT AQUACULTURE

4.1. Farming Research

The research on turbot farming carried out since the early 1980s by the Instituto Español de Oceanografía[1] (IEO, the Spanish Institute of Oceanography), and specifically the IEO center in Vigo (Galicia), was fundamental for implanting and developing this industry in Spain. The first research project in Vigo that was funded by the IEO began in 1982 and is entitled "Research on the viability of flatfish culture." The objective was to identify species most suited to being cultivated in Galicia. After the relevant biological, commercial, and market factors were analyzed, the turbot was selected as the first marine species to be cultivated on an industrial scale (Iglesias, 2009).

Subsequently, in the period from 1984 to 1986, the first subsidy from the CAICIYT (Comisión Asesora de Investigación Científica y Técnica) for a turbot culture research project in Galicia was obtained. This project focused on two main types of activities. The first type involved obtaining fertilized eggs in captivity, maintaining (in IEO facilities) a stock of mature females, and using males from the Vigo fish market to obtain sperm. Here the incubation and larval culture processes were started by artificially fertilizing (in "dry" conditions) the mature eggs. In Galicia, the first turbot eggs fertilized in captivity were obtained in April 1984; these results were formally communicated to the *International Council for the Exploration of the Sea* in 1985 (Iglesias et al., 1985).

The second type of activity addressed by this project was developed in the nursery Puerta Oviedo (Baiona), where 300 turbot juveniles were housed in order to determine the growth rate of this species in Galicia – that is, until reaching commercial size. The first results on turbot growth in Galicia obtained by an official research center were published in the journal *Marine Biology* (Iglesias et al., 1987); these data were the essential reference source, on the

[1] Advisory Commission for Scientific and Technical Research (1958–1987), a now defunct public extinct institution of the Spanish government.

cultivation of this species, used by later studies and initiatives of private companies carried out in Galicia.

In parallel with this project, two pioneers of the turbot industrial sector – Alfredo Fernández and Sergio Devesa – proposed in 1983 and 1984 that the IEO monitor the maintenance of the first turbot juveniles to be fattened on an industrial scale. These juveniles were initially conditioned in the Plan Marisquero de Galicia[2] facilities (Vilanova de Arousa) and were sampled monthly until being moved to Insuiña S.L. (O Grove), the original Galician turbot farming company.

The Vigo IEO centre proposed that the other turbot farm then installed in Galicia, Cultipec S.L. (also in O Grove), become the first company to handle the entire turbot production cycle – that is, including the hatchery and nursery phases. The plant was designed based on guidelines provided by IEO technicians, and the company's first biologists were also the beneficiaries of IEO scholarships.

Thereafter, the IEO coordinated successive projects related to turbot culture development. Examples include a study of the nutritional benefits of larvae (FAR-UE Project), a study of pathologies (with the University of Santiago de Compostela), the use of a dry feed in a study undertaken with the company Ewos (CDTI Project), the design of floating cages in Baiona Bay, and a genetics project for the production of sterile turbots and all-female populations (which have the highest growth rates).

In light of the surplus turbot juvenile production at the IEO plant in Vigo, a European project began in 1989 to establish the basis for future repopulation programs: "Evaluation of stock enhancement of marine flatfish" (Støttrup et al., 1998). The project consisted, on the one hand, of learning about the distribution, survival rate, and feeding of cultivated turbot juveniles released in the Vigo estuary; this information could then be used to determine sustainable recapturing percentages. On the other hand, this project analyzed the minimum optimal age at which to release specimens and compared the mortality of cultivated specimens with that of wild individuals in the natural population. All these data, published in accesible scientific journals (Iglesias et al., 1994; Iglesias et al., 2003), served as a reference for the turbot repopulation plan of the Xunta de Galicia (Galician Local Government) Fisheries Department.

After the *Prestige* oil spill off the coast of Galicia (2002), the Xunta de Galicia initiated stocking programs for turbot (among other species) toward the end of restoring their populations. Stocking began in 2005, and the program lasted five years until 2009; releases took place at two Atlantic sites on the Galician coast (5,000 and 10,000 fish released per year) and came from a mix of broodstock of wild origin (25%) and from aquaculture companies (75%). Most of these programs were carried out using juveniles (0+ or 1+ year class), which were released in shallow coastal habitats. Released turbot exhibit a mortality rate similar to that of wild turbot. Although there are differences between wild and hatchery turbot with regard to prey and feeding behavior, released hatchery turbot can evidently adapt to a natural diet within a few weeks. Releases of hatchery turbot have been proposed to enhance fisheries recruitment when integrated with a resource management program of sustainable fisheries and habitat restoration. Of course, such releases should proceed with great care becasue both positive and negative ecosystem effects are anticipated at the species, community, and ecosystem level (AquaTrace, 2014).

[2] Currently CIMA (Centro de Investigaciones Marinas).

4.2. Vaccine Research

In 2000, researchers at the University of Santiago de Compostela (USC) patented an anti–*Vibrio anguillarum* vaccine (GAVA-3) for the prevention of vibriosis in turbot and *Salmonidae* fish; they also patented the process for producing that vaccine. The GAVA-3 can be administered by short bath, prolonged bath, or injection, and it gives both turbot and salmon remarkable levels of protection against vibriosis.

In 2008, the first vaccine for turbot parasites was registered by USC researchers. The efficacy of a vaccine against the fish pathogen *Philasterides dicentrarchi* was evaluated in four groups of turbot by measuring the production of specific antibodies and the length of protection (Sanmartín et al., 2008).

4.3. Genetic Research

In 2007, the turbot was genetically mapped by researchers of the Veterinary Faculty of Lugo (University of Santiago de Compostela). This study represented the first linkage map in the turbot, and it is suitable for the identification of productive traits in this species and for further evolutionary studies in fish and other vertebrate species (Bouza et al., 2007). The 2007 genetic mapping was concluded in 2016.

The turbot is the first vertebrate genetically sequenced in Spain. The results of the turbot's complete genome sequencing – carried out by scientists from the Spanish National Research Council (CSIC), the University of Santiago de Compostela, and Spain's National Centre for Genome Analysis (Barcelona) – were published in the magazine *DNA Research* in March 2016 (Figueras et al., 2016). According to this study, the turbot has a much more refined visual system than other fish because it has evolved to adapt to the seabed's shortage of light. In addition, the fat in the cell membranes is double that of other species, which enables turbot to withstand the low water temperatures of their habitat. These results could be used in the future design of genetic selection programs or of vaccines. This work was funded by the Spanish Government and the Xunta de Galicia Local Government.

Researchers compared the turbot genome with model fish genomes to investigate teleost chromosome evolution. They identified gene family expansions and positive selection of genes associated with vision and metabolism of membrane lipids, which suggests adaptation to (respectively) a demersal lifestyle and cold temperatures. The data indicate a quick evolution and diversification of flatfish to adapt to benthic life, and they provide clues for understanding the origin of these species (which is a subject of controversy). Scientists have also investigated the genomic architecture of growth, gender determination, and disease resistance, which are key traits for understanding local adaptation and thereby boosting turbot production. The genomic architecture of these productive traits has allowed for the identification of candidate genes and has enriched pathways that could yield information useful for future, genetic marker–assisted selection of turbot.

The turbot, with both eyes on its left side, underwent a process of metamorphosis during its development, which is when it began to develop a body distribution atypical of flat fish. It lives on the seabed because of this circumstance, so it has been forced to adapt to an environment of very little light and chillier waters. Many of the genes involved with sight (mainly, those that carry pigment codes), and others involved in forming the eye crystalline,

are repeated in this vertebrate – that is, more so than in other fish. The implication is that the turbot has evolved, refining its sense of sight as an adaptation to the low light levels in its environment. In order to tolerate the low temperatures, the turbot has a number of genes related to fatty acids in the repeated cellular membranes, which are found in lower numbers (or not at all) in organisms that live at higher temperatures. The lipid composition of these membranes is a key factor when it comes to withstanding cold.

Scientists have managed to identify the most important genes involved in growth, sexual differentiation, and disease resistance, including which specific parts of the genome affect these production traits. This information is essential to the development of more efficient genetic selection projects, which aim to identify the breeding fish with the best production traits. Another of the challenges facing this sector is how to shorten the time required for fish to reach a marketable size. This problem can be addressed by selecting for the genes involved in growth and sexual differentiation – given that females grow at a much faster rate than do males.

The study concludes that the genome sequence of turbot is among those with highest coverage within fish (and similar, in that regard, to that of the tongue sole) as well as one of the most compact sequences among vertebrates. These findings contribute immensely to our understanding of the organization and evolution of fish genomes. The integration of the turbot genetic and physical maps and their comparison with model fish genomes are powerful tools for future studies in turbot, fish, and vertebrates. This genome has shed light on the genetic basis of relevant productive traits, and it represents a milestone for boosting turbot production through more efficient, marker-assisted selection programs.

5. THE SPANISH TURBOT INDUSTRY

The first industrial turbot farming company in Spain, Insuiña, was set up in 1983 by three entrepreneurs in O Grove (Galicia). The first production of turbot was sold in 1985 at a profitable €15 per kilogram (Fernández, 2013). Galicia was the only Spanish region producing turbot until 1986, when Cantabria and the Basque Country regions commenced their turbot production; Asturias also produced turbot during the period 2003–2007. The Basque Country ceased production in 2013, so Galicia and Cantabria are now the only two Spanish regions producing turbot. There are currently about 20 turbot-rearing farms and five nurseries in Galicia, but there is only one rearing farm in Cantabria (property of Rodecan, S.L.).

In 1988, Galician turbot producers joined to create AROGA (Asociación de Productores de Rodaballo de Galicia). This association now consists of seven companies whose most important production centers are located in Galicia: Acuidoro, S.L.; Aquacria Arousa, S.L.; Insuiña, S.L.; Luso Hispana de Acuicultura, S.L.; Piscicola del Morrazo, S.A.; Punta Moreiras, S.L.; and Stolt Sea Farm, S.A. The last-named company, Stolt Sea Farm, is the world's leading producer of farmed turbot. The company produces a million juveniles annually in two hatcheries in Galicia, which in turn supply five grow-out facilities in the region. Stolt Sea Farm is, moreover, the leader of a group including two other companies in France and Portugal. In was also during 1988 that moist feed was first used by Galician turbot farms.

In 1989, a crisis motivated creation of the Mar Novo Galician Turbot Cooperative, which centralized all marketing activities as well as the procurement of fry and other supplies. That year, the first vaccine for *Listonella anguillarum* (also known as *Vibrio anguillarum*) was used in Galicia. Production was limited by an unstable source of juveniles owing to high mortaly rates during the larval stages. Vibriosis has been – and still is – one of the major disease problems of the aquaculture industry. *Vibrio anguillarum*, *V. salmonicida*, *V. ordalii*, and *V. vulnificus* are among the pathogens responsible for the greatest losses in aquaculture worldwide (Sandlund et al., 2010).

Spanish turbot aquaculture faced a crisis in 1992, before the sector's commercial structure had become firmly established. More than 1,000 t. of turbot were offered on the market that year – a 52% increase in production – but the industry then lacked a consolidated commercial marketing network. Fish farms were small and had high production costs. So whereas 1991 production costs were €7.50/kg, the 1992 sales price peaked at €5.42/kg. Company losses could not be averted, and a restructuring of the industry ensued. This crisis led to the closure of six farms. Reorganization of the sector commenced shortly thereafter, which ended up increasing not only the volume of production but also the number of countries in which turbot is farmed (Fernández, 2013).

In 1993 there emerged the first reports of zoonosis (human–vertebrate disease transmission) associated with Galician turbot production. The resulting greater mortality of juveniles reduced production volumes in 1996 and 1997 as compared with predictions based on the growth trends in previous years; see Table 1 and Figure 1 (in Section 6.1). Also in 1993, the use of additional oxygen in the commercial cultivation of turbot began (Fernández, 2013).

The Galician Aquaculture Cluster was born in 2000 from the association of eleven Galician companies dedicated to the growing of turbot. Its main objective is to consolidate the competitive position of the Galician aquaculture sector by generating and fostering its members' advantages through research and development. Other objectives include increased cooperation among association members and the promotion of a "business innovation" culture. The Aquaculture Cluster in Galicia has become an international repository of information about the farming of flatfish, turbot, and sole. Its members, which manage facilities throughout Europe, represent 85% of the European production of flatfish (turbot and sole). The Aquaculture Cluster's operating model has inspired the creation of other clusters and similar networks in Spain and Europe.

Under the Aquaculture Cluster's tutelage, the Galician Aquaculture Technological Centre (CETGA) was designed as a pilot aquaculture factory dedicated to experiments. The CETGA researches and develops critical, high-impact technology and scientific projects whose success is instrumental in the *sustainable* development of Galician aquaculture.

In 2002, the OPP 59 (Organización de Productores del Rodaballo) was created; also, the Asociación de Productores de Rodaballo was included – as a national-level fishery producers organization – in the General Register of Fishing Producers Organizations of the General Direction of Fisheries Management. However, that recognition was removed in December 2016 at the association's own request.

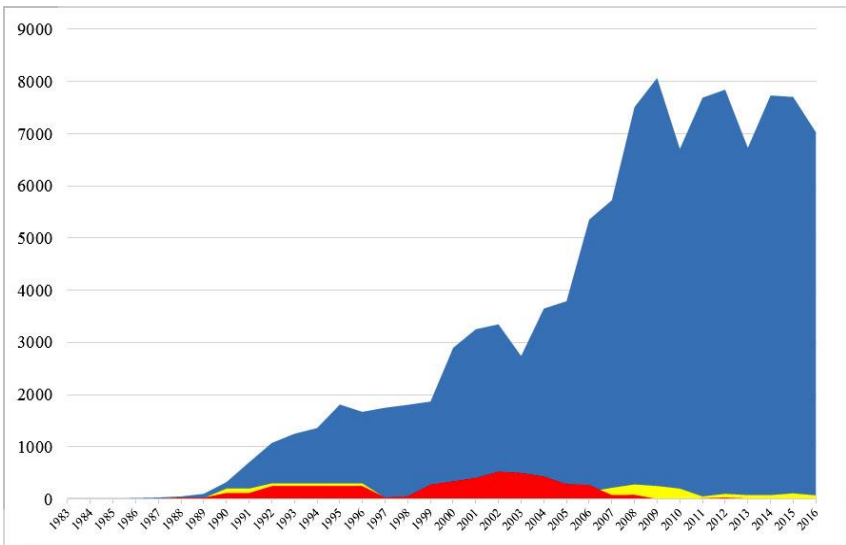
In 2008, the Plataforma Tecnológica Española de la Pesca y la Acuicultura (PTEPA) was established. This "Fisheries and Aquaculture Spanish Technology Platform" is a nonprofit association whose main purpose is promoting technological development and innovation – to include the processing and marketing of products – in the fisheries and aquaculture sector. In

general terms, the platform provides members with valuable information about innovation while facilitating their elevation to a more privileged position within the national aquaculture and fisheries sector. One aim of PTEPA is bring together all stakeholders in the fisheries and aquaculture sector for the purpose of coordinating actions and information about the various technologies being employed. Thus, a common national strategy for research, development, and innovation is proposed to secure a competitive position for national aquaculture and fisheries companies.

6. TURBOT PRODUCTION IN SPAIN

6.1. Production Volume

The increase in Spanish turbot production – from 15 t. in 1983 to 7,100 t. in 2016 – was nearly continuous, interrupted only by health-related problems and fry shortages. Spain's greatest turbot production (8,320 t.) was reported in 2009; subsequent years have seen relatively stable production, with annual volumes averaging about 7,500 t. Galician turbot production now represents nearly 99% of the total national volume, while Cantabria accounts for the remaining 1%; in 2015, their respective production volumes were 7,706 t. and 109 t. (see Figure 1 and Table 1).



Source: APROMAR.

Figure 1. Turbot production (tonnes) in Spanish regions, 1983–2016: Galicia (blue), Basque Country (red), and Cantabria (yellow).

The substantial increase in turbot production during the early decades of this time span can be attributed mainly to improvements in the technology of fry production, which increased the quantity and quality of the fish. As a consequence, the supply of juveniles was no longer a limiting factor in the development of aquaculture (Rodríguez and Fernández-Casal, 2008). The five nurseries in Spain (all in Galicia) achieved their maximum production in 2014: a total of 22.3 million juveniles (see Table 2).

Table 1. Spanish turbot production (tonnes), 1983–2016

Year	Galicia	Asturias	Cantabria	Basque Country	Total
1983	15	0	0	0	15
1984	15	0	0	0	15
1985	15	0	23		38
1986	20	0	20		40
1987	30	0	20		50
1988	50	0	47		97
1989	100	0	171		271
1990	325	0	315		640
1991	705	0	120		825
1992	1,075	0	547		1,622
1993	1,250	0	289		1,539
1994	1,360	0	450		1,810
1995	1,811	0	363		2,174
1996	1,670	0	519		2,189
1997	1,750	0	49		1,799
1998	1,805	0	105		1,910
1999	1,868	0	100	275	2,243
2000	2,897	0	110	340	3,347
2001	3,254	0	100	405	3,759
2002	3,348	0	125	525	3,998
2003	2,740	80	120	500	3,440
2004	3,650	50	125	431	4,256
2005	3,790	50	150	285	4,275
2006	5,355	50	140	270	5,815
2007	5,725	65	215	75	6,080
2008	7,510	0	280	80	7,870
2009	8,070	0	250	0	8,320
2010	6,710	0	200	0	6,910
2011	7,690	0	50	15	7,755
2012	7,845	0	100	25	7,970
2013	6,729	0	75	10	6,814
2014	7,733	0	75	0	7,808
2015	7,706	0	109	0	7,815
2016 [a]	7,029	0	71	0	7,100

[a] Preliminary data.
Sources: APROMAR, Fishstat Plus.

Table 2. Number of juveniles produced in Spain, 1998–2015

Year	**Number of juveniles**
1998	1,100,000
1999	1,300,000
2000	1,500,000
2001	1,500,000
2002	2,200,000
2003	4,450,000
2004	4,550,000
2005	4,915,700
2006	7,550,000
2007	12,269,700
2008	13,040,000
2009	12,540,000
2010	13,498,000
2011	17,127,000
2012	18,950,000
2013	13,168,000
2014	22,300,000
2015	9,700,000

Source: APROMAR.

Within the European Union, Spain is the main producer of farmed turbot; in 2015, its 67.6% of the EU total amounted to 7,815 t. Portugal, with 3,144 t. (27.2% of the EU total) produced, is the second-leading country. Note, however, that this rank reflects the 2009 opening – by the Galician company Pescanova – of a plant in Mira that is now the world's largest turbot farm. Substantially smaller production (altogether 5.2% of the EU total) occurs in France, the Netherlands, and Iceland (see Table 3).

Table 3. Spanish, European Union, and global turbot production (tonnes), 1985–2015

Year	Spain	EU	World
1985	38	53	53
1986	40	50	50
1987	50	65	65
1988	97	112	112
1989	271	287	287
1990	640	656	656
1991	825	925	925
1992	1,622	1,725	1,725
1993	1,539	1,693	1,693
1994	1,810	2,399	2,399
1995	2,174	2,978	2,978
1996	2,189	2,571	2,571
1997	1,799	3,001	3,001
1998	1,910	3,087	3,087
1999	2,243	4,103	4,103
2000	3,347	4,785	4,786
2001	3,759	4,829	4,856
2002	3,998	5,258	5,269
2003	3,440	5,331	5,377
2004	4,256	5,962	6,010
2005	4,275	6,792	6,908
2006	5,815	7,703	7,803
2007	6,080	8,105	8,205
2008	7,870	9,521	9,573
2009	8,320	9,168	69,557
2010	6,910	8,396	68,890
2011	7,755	10,800	75,413
2012	7,970	12,842	77,117
2013	6,814	7,721	76,998
2014	7,808	11,067	71,851
2015	7,815	11,555	N/A

Note: N/A = not available. Sources*:* APROMAR, Fishstat Plus.

In global terms, the farmed production of turbot was almost exclusively EU based until 2008; the only two exceptions were South Africa (since 2000) and Iceland (since 2001). In recent years, however, production has increased dramatically on other continents. Farming was developed in China with broodstock imported from Europe. Data compiled by the FAO (FAO, 2016) indicates that, since 2009, China has produced about 60,000 t. of farmed turbot annually. In addition, Chile is producing some 300 t. per year.

6.2. Production Value

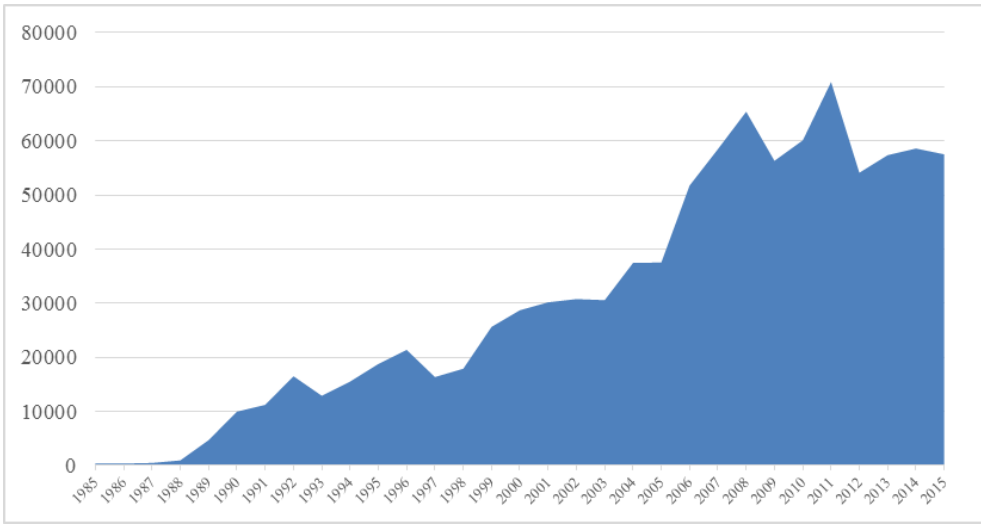
The production value of Spanish farmed turbot was generally increasing (similarly to the production volume trend) until 2011, when a maximum of €70.9 million was attained (86.6% of the EU total production value; see Table 4). The value of production has been stable in recent years, averaging €57 million for the period 2012–2015 (see Figure 2 and Table 5).

Table 4. European Union turbot production volume, production value, and first-sale price, 1985–2015

Year	Production volume (t.)	Production value (€)	First-sale price (€/kg)
1985	53	467,500	8.82
1986	50	475,200	9.50
1987	65	629,900	9.69
1988	112	1,131,500	10.10
1989	287	4,960,800	17.29
1990	656	10,247,600	15.62
1991	925	12,623,700	13.65
1992	1,725	17,718,900	10.27
1993	1,693	14,410,300	8.51
1994	2,399	20,849,500	8.69
1995	2,978	26,680,300	8.96
1996	2,571	24,847,700	9.66
1997	3,001	26,924,800	8.97
1998	3,087	27,186,500	8.81
1999	4,103	N/A	N/A
2000	4,785	39,454,600	8.25
2001	4,829	39,360,400	8.15
2002	5,258	40,933,600	7.79
2003	5,331	40,060,200	7.51
2004	5,962	45,281,400	7.60
2005	6,792	51,575,500	7.59
2006	7,703	60,373,600	7.84
2007	8,105	64,250,800	7.93
2008	9,521	N/A	N/A
2009	9,168	N/A	N/A
2010	8,396	N/A	N/A
2011	10,800	81,918,400	7.59
2012	12,842	63,599,200	4.95
2013	7,721	75,600,000	9.79
2014	11,067	74,600,000	6.74
2015	11,555	N/A	N/A

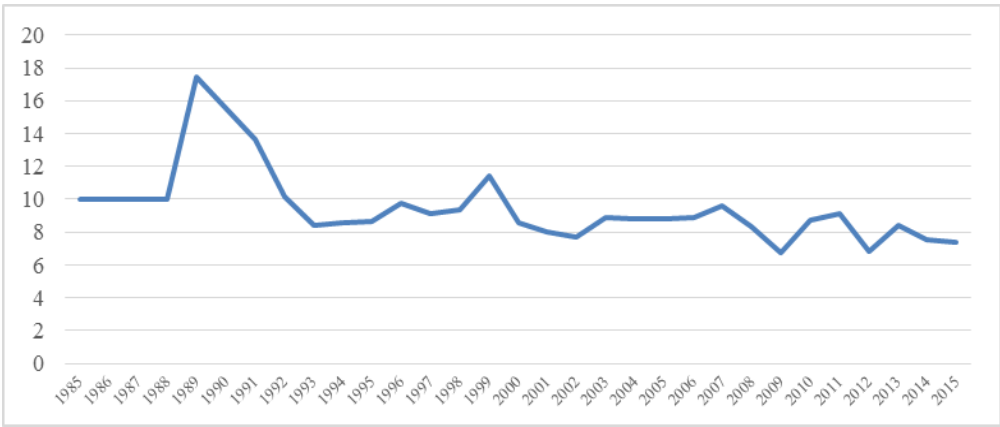
Note: N/A = not available.
Sources: APROMAR, Fishstat Plus.

Notwithstanding some severe oscillation in earlier years, turbot's annual average first sale price has remained relatively stable over the last two decades even as production levels have increased. The average first-sale price for the period 1995–2015 was €8.60/kg. Recent years have evidenced a slight decline in that price – to €7.36/kg in 2015 (see Figure 3 and Table 5).



Sources: APROMAR, Fishstat Plus.

Figure 2. Value of Spanish turbot production (€ thousands), 1985–2015.



Sources: APROMAR, Fishstat Plus.

Figure 3. Spanish turbot: annual average first-sale price (€/kg), 1985–2015.

In some years (2009, 2012) there have been significant price reductions – to below €7/kg. One possible explanation is that most turbot is harvested at a weight of 1.5–2.0 kg per fish and that larger fish fetch a higher price *per kilogram* than do smaller fish. Some companies that experienced financial problems during the Great Recession were forced to sell product at lower-than-optimal weights simply to maintain cash flow. These developments also put downward pressure on the prices that other companies can charge (Bjørndal and Øiestad, 2011).

For large farms in Galicia, the cost of turbot production ranges from €5.00 to €5.50 per kilogram. Packaging and transport amount to an additional €1.00/kg. Smaller farms do not benefit from economies of scale and so have higher production costs (Bjørndal and Øiestad, 2011).

Table 5. Spanish turbot production volume, production value, and first-sale price, 1985–2015

Year	Production volume (t.)	Production value (€)	First-sale price (€/kg)
1985	38	380,000	10.00
1986	40	400,000	10.00
1987	50	500,000	10.00
1988	97	970,000	10.00
1989	271	4,720,800	17.42
1990	640	9,977,600	15.59
1991	825	11,226,700	13.61
1992	1,622	16,518,900	10.18
1993	1,539	12,910,300	8.39
1994	1,810	15,536,300	8.58
1995	2,174	18,794,400	8.65
1996	2,189	21,403,400	9.78
1997	1,799	16,372,600	9.10
1998	1,910	17,917,900	9.38
1999	2,243	25,641,000	11.43
2000	3,347	28,713,000	8.58
2001	3,759	30,178,800	8.03
2002	3,998	30,776,000	7.70
2003	3,440	30,581,600	8.89
2004	4,256	37,452,800	8.80
2005	4,275	37,534,500	8.78
2006	5,815	51,753,500	8.90
2007	6,080	58,489,600	9.62
2008	7,870	65,399,700	8.31
2009	8,320	56,326,400	6.77
2010	6,910	60,117,000	8.70
2011	7,755	70,880,700	9.14
2012	7,970	54,116,300	6.79
2013	6,814	57,373,880	8.42
2014	7,808	58,600,000	7.50
2015	7,815	57,518,400	7.36

Sources: APROMAR, Fishstat Plus.

6.3. Markets for Turbo Production

Spain is one of the world's largest turbot markets. That national market is served almost entirely by farmed product, since landings of wild turbot are negligible (41 t. in 2014). According to Bjørndal and Øiestad's (2011) analysis of the Spanish national turbot market, in 2009 supermarkets represented the most important retail channel (39% of total sales), followed by hypermarkets (24%) and fishmongers (19%). The relative shares of supermarkets and hypermarkets have evidently increased over time while that of fishmongers has declined. Although turbot is a traditional species in Spain (mainly in the northern area), it is consumed less than other farmed species such as seabream or seabass. Over 2005–2007, fewer than one in five Spaniards (about 18%) consumed turbot; the maximum frequency was about once per

month. Consumers view turbot as fish of good quality that is safe to eat but somewhat expensive (Bjørndal and Øiestad, 2011).

Spain is also, by far, the largest exporter of farmed turbot in Europe. Most of the national production is exported to European markets, which include Italy, France, Germany, and the United Kingdom. In 2014, 69.6% of Spain's total production (5,431 t., valued at €35.4 million) was exported. In 2014, turbot exports fell 2.82% in volume yet rose 3.46% in value: to 5,278 t. and €36.7 million, respectively (ANFACO-CECOPESCA, 2016).

7. FUTURE OUTLOOK AND CHALLENGES FOR SPANISH TURBOT FARMING

Further development of the turbot industry and market depends on factors such as innovative processes and institutional support (Sturrock et al., 2008). Supply will be affected by the following factors: site availability and cost, while considering both physical availability and government regulations; support for innovation, including government and business investment in research and development; and the support of governmental and financial institutions for commercial (technology-based) risk takers (Bjørndal and Øiestad, 2011). On the demand side, turbot is a popular but premium fish species and so prospects for steady growth remain within price-based limits (Bostock et al., 2008).

In Spain, a key factor in the further expansion of turbot farming is the future Aquaculture Law of Galicia. Now in preparation, it will be the first aquaculture law in Europe. The sector is not currently subject to any specific regulations beyond those pertaining to all fish products. Site availability for plants along the coast – and their possible environmental effects – is key aspects to be regulated. The impending legislation seeks to identify suitable areas for aquaculture uses, to establish how plants should be integrated with the surrounding landscape, and even to address the potential consequences for climate change. So that companies will have greater legal certainty, the law intends to clarify the competencies that each administrative body will have for granting concessions to occupy the public domain and for granting aquaculture permissions.

Future challenges faced by the Spanish turbot farming include turbot diseases, turbot nutrition, turbot genetics, environmental management, and government support (Fernández, 2013). As for diseases, several pathogens (bacteria, viruses, parasites) affect the health status of farmed turbot. Although there are effective vaccines and treatments that target a variety of such pathogens, there is no easy solution to the problem of other diseases – especially those induced by viral agents. Neither vaccines nor therapeutic treatments are commercially available for most of the viral diseases affecting fish (Pereiro et al., 2016). More research on fish meal substitutes is also needed if aquaculture is to be sustainable. Several experiments have suggested that turbot requires a high level of dietary protein (50%–60% of the diet). Hence there is interest in replacing some of the fish meal, which accounts for 50%–70% of their diet, with protein-rich plant ingredients (Burel et al., 2000).

Progress in genetic improvement is also essential, and the genome sequencing of turbot is a significant step forward in that regard. It may be necessary to "improve" the genetics of turbot in order for large-scale aquaculture to succeed in a highly competitive world market. The control of inbreeding, avoiding the loss of genetic diversity, the identification of gender-

determining mechanisms for manipulating gender ratios, and the selection of broodstock for disease resistance and growth rate are stepping-stones to improved turbot production (Bouza et al., 2007). Selection of fast-growing and late-maturing fish is another way to increase turbot growth (Person-Le Ruyet, 2002). As regards environmental management, it is crucial to undertake detailed studies of coastal water behavior so that environmental disasters can be prevented. The environmental effects of water recirculating systems for turbot farming – such as the eutrophication and acidification of coastal waters – have been studied (Aubin et al., 2006) but certainly merit further attention. Finally, government support remains vital where legislation, advertising, marketing, and finance are concerned. That support is needed to advance the agenda of an Aquaculture Law in particular and of an aquaculture program more generally, both of which are needed for Spain's turbot farmers to remain competitive industry players.

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Chapter 52

THE GREGARIOUS BEHAVIOR OF MARINE FISH AND THEIR RELATION TO FISHING

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ABSTRACT

Throughout the later years, the interest in studying the features and behavior of fish schools and their relation with fisheries management has increased. Of the total of teleost fish species, 25% might own some type of grouping which has been categorized into three main terms: aggregation, shoal and school. The gregarious behavior, serves to provide benefits to fish in relation to obtaining food, reproduction, protect the group and reduce energy expenditure, however the avoidance of predators being by several authors as the most mentioned advantage, but this will not always be effective, as it is closely related to the predator's ability to increase the rate of consumption on a polarized group of fish. The factors that cause the formation of certain type of group depend on several causes such as species type, age, biotic and abiotic factors, among other and the possible combinations between these. Here are discussed the anatomical adaptations that allow this behavior, as well as the advantages and disadvantages that the natural environment represents for fish organization as a school. On the other hand, humans have used the knowledge of this behavior for the fisheries, taking advantage of the great diversity of fish species that form shoals or schools. When fishes are grouped, they become relatively easy to catch and their abundance makes their extraction economically profitable. The present chapter examines some examples of how the study of gregarious behavior in teleosts is a complex topic that has been necessary for the modernization of fisheries,

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which will allow the modification of capturing methods as well as the fishing gear that is necessary according to the site, season and target species.

Keywords: small pelagic fish, school, shoal, fishing gears

INTRODUCTION

Nowadays, teleost fish represent the vertebrates group with the most diversity of species, which exhibit a great variety of behaviors to ensure their survival. In a strict meaning and according to Collocot & Dobson (1974), behavior includes all the observable and measurable activities that a living animal presents as a response to its interaction with the environment, including contractions and expansions of tissue responsible for movement, color change, glandular secretions, etc.

A type of particular behavior in the teleosts, is the related to the interspecific and intraspecific interactions of the individuals and that can be referred as "gregarious behavior" which according to Shaw (1978), is present at some stage of at least 25% of the described fish's species.

According to Pavlov & Kasumyan (2000), a confusion persists in the applied terminology to refer to the fish groups types, caused by a vague distinction between different terms. This is because the internal organization and the relation patterns among the individuals are widely varied, due to they are subject to diverse factors like the type (or types) of specie, age, diverse biotic and abiotic factors, lifestyle and possible combinations between these.

In accordance with the amount of interaction between fish, three main concepts can be defined for this gregarious behavior:

In the first place, we have the term *"aggregation"* that involves a group of fish gathered time-space, attracted by different factors like diet, temperature and/or shelter (Pitcher, 2001; Helfman et al., 2009). On the basis of this concept, the group can be conformed by individuals of one or various species with a relatively random orientation between them (unpolarized), does not necessarily derive in a social attraction. From this point forward, it is necessary to mention the word "grouping" as referring to fish groups, in spite of any of the three levels of interaction that the group might present, with the purpose of avoiding a confusion with the word aggregation (Figure 1a).

The second concept is named *shoal*, which involves a group of three or more fish on the same ontogenetic phase or not, with well defined social characteristics and where each individual reacts to the movements of adjacent fish, adjusting speed and direction of their swim (polarized) without showing a high level of synchrony in response to external stimulus (Partridge, 1982; Pitcher, 2001). Shoals are usually conformed by individuals of a specific kind. Although there are other fish that can assimilate, these must have swimming aptitudes that are similar to most fish in the group as well as synchrony (Figure 1b).

Finally, *school*, which includes temporary fish groups on similar conditions to the ones of a shoal. In this organization a high relation of mutual attraction exists among the individuals that results on a high level of swimming coordination, which benefits all the individuals of the group (Radakov, 1973; Pitcher et al., 1983; Pitcher & Parrish, 1993; Pavlov & Kasumyan, 2000; Figure 1c). This high degree of coordination produces a discrimination effect among members, allowing fishes of a same species and cohort be part of the group, or individuals of

other species with sizes, ontogenetic phase, and similar swimming and reaction capacities join the group.

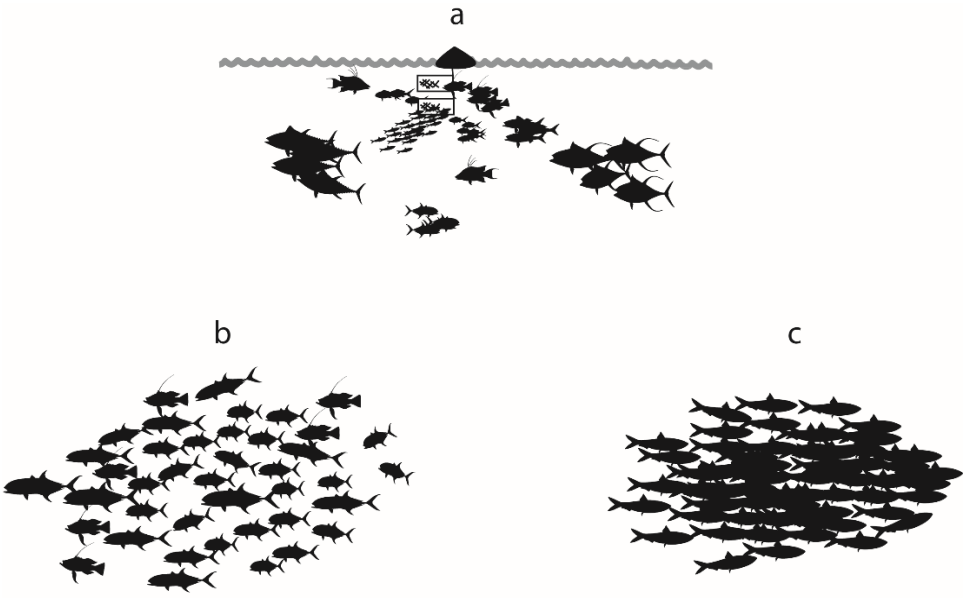

Figure 1. The degree of socialization among members of different groupings determines if we have an aggregation (a), a shoal (b) or a school (c).

One of the advantages of this last term is that schools formed by "familiar" members probably entail the formation of hierarchy with more stable dominances and, therefore, a reduction of aggressive behavior among members of the school (Gómez-Laplaza, 2005), besides, forming schools with familiar individuals eases the evolution of altruistic behavior (Utne-Palm & Hart, 2000), which in turn increases the probability of survival. In addition, this characteristic of familiarity has demonstrated that anti-predator behavior is better than those who do not show this feature (Griffiths et al., 2004). For example, it has been shown that in the salmonid species levels of aggression are lower when these have a familiar relation (Brown & Brown, 1993). Likewise being away from the shoal must be avoided, for lonely fish will lose antipredatory advantages associated to the grouping's lifestyle (Bertram, 1978; Rubenstein, 1978). In the studies made by Tien et al., (2004), it is noted that the degree of cohesion is larger in the presence of a predator, in comparison to swimming activity or search for food (sensu the 'life-dinner' principle [Dawkins & Krebs, 1979]).

ADVANTAGES AND DISADVANTAGES OF GROUPING BEHAVIOR

Advantages

Based on the concept of aggregation it is hard to observe advantages that benefit all the fish that form this type of grouping, given the reduced interaction of all members; however, fish who swim far from the attacking source can be attracted when receiving sensorial stimuli

from other fish making use of a resource and in this way benefit in the face of the detection for possible threats.

On another hand, amongst the main advantages that characterize the groupings as shoal or school we can emphasize: diet, reproduction, protection, vigilance and the reduction of energy expenditure in swimming (Radakov, 1973; Partridge, 1982; Fréon & Misund, 1999).

The most evident benefit of schools and shoals is protection, due to these groupings coexist on the same habitats as their predators, the first step to avoid being hunted is the early detection of possible sources of danger and at this point vigilance starts to become the first antipredatory advantage, which works in the following way: the presence of many detection organs (mainly visual and mechanoreceptor-related) acting in ensemble for the location of possible threats difficult the predator's attempts to come close the grouping without being detected (Figure 2a). This first defense mechanism requires for the visual conditions to be beneficial in most individuals and thus keep a suitable distance from the predator, which can allow an appropriate response for the attack (Cushing & Harden-Jones, 1968; Pavlov & Kasumyan, 2000). This can explain the alteration of the school structure in the face of a predatory threat (e. g. when a flattened school becomes more spherical to permit a better vigilance on different angles; Partridge, 1980; Figure 2a).

If the predator continues to get close the group, the defense will continue to employ mechanisms of confusion (Landeau & Terborgh, 1986; Pitcher & Parrish, 1993). Visual confusion in teleosts that present grey tonalities is very common, this fact difficult for the predator to focus their sight on a bright target of a moving mass that, besides, presents different contrasts of tonalities and light reflections. This is one of the reasons why the attacks on fish that form schools are often more successful at dawn or at dust, where the effectiveness of the glint of the fish scale towards the predator's sight is reduced (Moyle & Cech, 1988).

Regardless of coloring, there is another effect of confusion towards the predator's sight when trying to locate multiple potential targets. Some authors have even mentioned that the possibility of becoming a prey during a predatory attack, inversely declines in relation to the group size (Bertram, 1978; Foster & Treherne, 1981).

The confusion effect can also be aimed to the ear and the mechanoreceptor (octavolateralis system) of the predator. Vibrations of the surroundings, fish swimming, vocalizations and other mechanic disturbances generate gradients of pressure near the already stated source when generating diverse flows in the water. The fin movements of a lonely fish can act as an emission source of punctual-shaped waves, these signs can help the predator fish detect the prey with help of its internal ear and its lateral line. When finding lots of generator sources of densely grouped waves, as it is previously mentioned, they will cause an overlay of the hydrodinamic signs and the sensors of the internal ear of the predator will not be able to decipher the spatial details of the prey, avoiding its detection of the group (Larsson, 2012; Figure 2b).

If the predator gets closely enough to carry out attack tactics, breaking the minimal distance of approximation, the group can perform coordinated maneuvers of escape, for which imitation behavior is extremely important, mostly in the school's configuration, helping to keep the unity within the school during quick and complicated maneuvers when fish are scared (Pavlov & Kasumyan, 2000; Figure 2c).

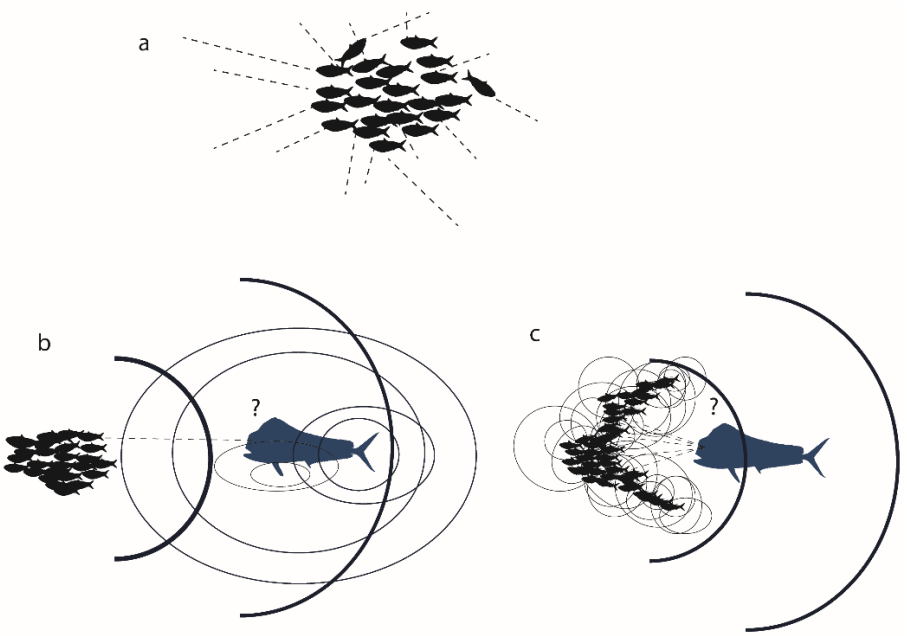

Figure 2. Defense mechanisms start with vigilance (a), when finding a near predator, it will be perceived by a great amount of receptive organs that will alert the fish group (b), and if it breaks minimal distance the grouping could perform escaping tactics (c).

The main tactics of escape were described by Charnov & Krebs (1975), based on four main types of behavior: "Escaping and the minimum approach distance", is based on the distance that is supposed to be between the herd and the predator, as well as the direction of their swim opposite to the attack in order to improve the escape tactics and acceleration. A second tactic is the one called "Fountain effect," where the predator's velocity maximizes, when it is about to attack the school; nevertheless, the school reorganizes itself behind the predator. When the herd is too big, this tactic can even simulate an ameboid behavior of the school or shoal, where the predator gets sucked into a sort of vacuole.

These last two tactics require a high communication and cohesion among individuals, this is why they are only possible in school groupings. The "Trafalgar Effect" is another tactic where in the face of a great number of pounding stimuli, there can be a great number of maneuvers, product of the fast transference of information across the organisms that are part of the school. Finally, the "Bait ball" tactic, that schools of small pelagic fish use, which increases the degree of cohesion in the herd to be able to shelter in a strongly compressed spherical formation where the individuals located in the middle are less likely to be hunted. Hamilton (1971), proposes that this is a "selfish" tactic because all organisms seek to be positioned at the center of the herd, however, this configuration allows to detect approximations from all possible angles and react on time, benefiting all the members of the herd.

It is important to point out that the defensive advantage maintaining the group is not always effective, this will depend on the predator's abilities. If the measure of the predator's attacks increases in proportion to the school's size, the probability of becoming a victim will be the same as a lonely fish (Pitcher, 1986). It is important to observe that when this behavior appears, lots of ichthyophagous predators will be excluded of accessing to one of the

members of the herd, nevertheless the competition of defense and attack, only some species will be capable of avoiding these mechanisms and feed from fish that form schools.

This defense instinct is so important that it persists when the abundance of sardines, sardinellas or anchovies is low, which tend to form mixed schools among them, this due to it is priority being member of a school that has fish with size and shape alike (individuals that are similar targets in the face of potential predators), this distinctive feature called *school trap*, which indicates a strong meaning of survival to remain a member of a school and indirectly displays the strong reluctance of any individual to leave the school voluntarily (Bakun & Cury, 1999).

Another of the documented advantages, is the increase of resistance in a fish's swim, which increases two to six times when it is part of a school; this hydrodynamic difference is due to vortices generated by the flick of tails to find an even flow to save swimming energy expenditure. Under a diamond-shaped configuration, the most advantageous position is the one located behind the middle point between two fish that are at front, this without remaining too far from their vortices (Weihs, 1973).

This swimming advantage makes it easier for migrations with an active swim for hundreds of kilometers to function, maintaining high velocities during this period. It has been observed that in species like *Clupea harengus* the shifting as a school not only makes it easier for fish to find ideal migratory routes, but it also increases their precision (Pitcher & Parrish, 1993). It is likely that the level of motivation attached to the behavior of imitation works as a filter to group those individuals that are prepared to participate in a migration. This effect favors the formation of big schools of clupeoids and gadids when long migrations of spawning and diet begin to happen.

Another necessity for the survival of the species is reproduction and remaining in group turns out to be advantageous for this, not only in the search and shifts to spawning sites, but also with the encounter of couples too. There are coastal fish like the case of gulf corvina *Cynoscion othonopterus*, which makes a migratory circuit inside the delta of the river "Río Colorado" in Mexico (Chao & Musick, 1977) including a period of diet and growth, until they reach maturity. Once they are mature, the individuals penetrate the delta for spawning, which has a duration of approximately two months. Once this is over, the organisms return to the sea to start a state of non-reproductive activity for a year (Solana-Sansores et al., 2012). Within the same region the poorly-studied spawning behavior of the gulf grunion, *Leuresthes sardina*, can be found. It only has a five-day duration. During the spawning period (mainly related to tides), the fish of this species come together throughout the beach zone, the female buries the posterior region of its body, while the male surrounds the female for the spawning stimulation (Carmona et al., 2015), and like these cases, there are lots of teleosts species of commercial importance that present reproductive groupings.

In the pelagic environment where dimensions can be spatially larger, movements from one place to another are often in big distances, and because of this pelagic fish tend to remain short time in the spawning sites and when finding themselves grouped with potential couples the spawning events speed up (Fréon & Misund, 1999). In sardines the reproductive events are performed when they have the right physiological conditions in a great number of grouped fish, due to they have a promiscuous behavior of external fertilization, where the individuals spawn and fertilize gametes without any apparent coordination between both sexes, creating in this way Aneer et al. (1983) a "mass orgy."

Another of the advantages of remaining as a group is that the foraging ability can improve, due to the ability of transference of information over the distribution of food (Pitcher et al., 1982; Ranta & Juvonen, 1993). This can occur latitudinally like the big sardine and anchovy shifts along the coast exploring from bay to bay in search of food. Nevertheless, the same effect can occur with vertical shifts that the big shoals of mesopelagic fish generate, that in their search for food in superficial waters they transfer and redistribute energy and organic matter among the epipelagic and mesopelagic zones as a consequence of their daily migratory behavior (Beamish et al., 1999).

Disadvantages

Despite the great amount of advantages that the formations of fish groupings can have, there can also be a series of disadvantages in this type of behavior. The benefits are closely related to the costs and the survival of the organisms, well there is indeed a bigger competition for resources among the group, a bigger risk of directly catching parasites and diseases, among others. Some studies have shown that the decision making related to grouping will be based on the values of the aforesaid costs against the benefits (e.g., Ashley et al., 1993; Krause & Godin, 1994).

One of the main disadvantages is the cost that has to be paid for belonging to a grouping, like the increase of competition for resources, like food, which can damage the smallest individuals (Booth, 1995). The strongest fish choose their competition with the weakest fish with the purpose of maximizing their own intake of food (Gregson & Booth, 2005).

Another example is that lots of predators individually possess better systems of detection (visual, olfactory, sensorial or acoustic), that together with the attack tactics increase their rate of consumption over grouped fish, generating devastating effects for the survival of a big number of fish when they are in a school. This puts a restrain in the argument that says that the protection of an individual increases when being part of a school, in comparison to dispersed fish; only the average members of the group will be protected if the rate of consumption of the predator is lower when feeding off fish groups (Pitcher, 1986). In the particular case of aggregations, the cumulus of fish in a certain zone does not only attract fish competitors for the resource, also attracts potential predators that are attracted by the fish cumulus, because of the absence of a social behavior among all individuals the success of the predation will have a higher efficacy.

An example of the prior can be observed in ensemble attacks coming from air and in water, which generate a feeding frenzy of different types of predators over fish groupings. A great list of organisms can include: from all different flocks of birds like elegant terns, cormorants, pelicans, diving birds, among others, that generate disturbances in the surface to make fish organizations lose control. In addition to this, in the water, attacks from billfish, dolphins and sea lions create confusion in the fish formations through multiple fast attacks, just as sharks or cetacean that through aiming a direct flick of their tails to the schools they generate the same effect. The humpback whale takes advantage of this group characteristic in a special way, forming bubble clouds to gather a considerable amount of fish, in an area near the surface to at last devour whole schools of sardines.

Finally, another inconvenience that can appear when finding lots of gathered individuals is the possibility of a quick distribution of parasitic infections, which negatively alter the

school's behavior and that can increase the susceptibility of the group in the presence of predators (Barber & Huntingford, 1996). Parasitism produces an energy expenditure in the host, whether because of the direct use of the host's resources for its own growth and development or because of the immune response that results from a high energy cost or when increasing the energetic costs of locomotion (Barber et al., 2000), in addition, other adverse conditions like weight and health loss, erratic energy and swimming, which makes them vulnerable to predators.

GROUPING'S STRUCTURE

The choice of formation for a certain school or shoal does not happen randomly, this behavior is influenced by a whole diversity of factors like body size (Ranta et al., 1992; Krause & Godin, 1994), competitive capacities and abilities (Krause, 1993; Krause & Ruxton, 2002), group size (Krause & Ruxton, 2002), parasitic load (Ward et al., 2005), level of predation (Johannes, 1993; Brown & Warburton 1997) or coloring (McRobert & Bradner, 1998; Modarressie et al., 2006). Besides, the preferences of shoal formations is very linked to the preferences of certain habitats (Pitcher & Parrish, 1993; Brown & Warburton, 1997).

For the specific case of pelagic fish, gregarious behavior in wild conditions has been studied to a lesser extent compared to the attention it has received in laboratories (Pitcher, 1993). To know the size and shape of pelagic schools in the environment different methodologies have been put into practice all around the world. The aerial observations has been one of them, where through registers quick change in shape and structure of compaction and dispersion of sardine schools during feeding or avoid predators events (Hampton et al., 1979; Hara, 1985; Fréon & Misund, 1999).

The school organizations can be defined by size, density and three-dimensional position and location of the fish group in the column of water (Bahri & Fréon, 2000). The group dimensions can thoroughly change, fish of the same species can form very different schools that go from a few meters to hundreds of meters (Tikhonov, 1958).

The vision and octavolateralis system (SOL) development in the fish increase the grade of perception. In fish with size and shape similar, hydrodynamic signals generated have the same intensity, and that together with sensorial increase, can avoid be part of a school with bigger fish. It has been seen that small fish avoid being part of a school with big fish, although big fish do not avoid being part of a grouping of smaller fish (Lachlan et al., 1998). It is through the mechanoreceptor system that discrimination for schools' sizes can happen, generating a more homogeneous shoal or bank with an increase in the group synchrony capacity.

The positioning of an individual in the school can also influence survival (Larsson, 2012). Fish that leads the school can have a better access to food than other members; however, it has been observed that fish leaders are attacked on a higher frequency unlike fish in other positioning in the school (Krause et al., 1998). Some authors like López et al., (2012) suggest that forming schools is regulated by three behavioral basic rules, listed as following:

- The attraction rule, which allows group cohesion due to fish discrimination to produce groupings of individuals with similar physical and physiological conditions. Vision governs this rule (Pitcher & Parrish, 1993).
- The alignment rule, which allows a directed swim of the grouping adjusting direction and velocity of the fish's swimming and is governed both by vision and mechanoreceptor system.
- The repulsion rule, where individuals seek to keep a certain distance between them to avoid collisions. This rule is governed by the mechanoreceptor system (Pitcher & Parrish, 1993).

If there are slight changes in distance among close fish members, they can generate a change in the number of fish by unit of volume, just as any change of polarization has an effect on the school's structure, which can be considered disorganized when fish lack uniform orientation and swim in opposite directions (Fréon & Misund, 1999; Domenici et al., 2010).

According to the theory of compactation and stretching of schools, when the stress is too big the distance among individuals diminishes and the vacuoles forms (spaces within the grouping) in the interior of the school quickly collapses. The generated evasion reaction is quickly transmitted but a great amount of repeated stimuli to compact a whole school is necessary.

In the spherical-shaped defensive method described for schools of pelagic species it is possible to observe a maximum compaction within a spherical structure of fish that takes the repulsion rule to its limits. In the same way the schools that are not in movement tend to be spherical to observe their surroundings in a defensive way (Radakov, 1973).

The group will decompress just as the individual exploratory behavior commences; every fish will chose what fish will stay by their side, with the purpose of maintaining that maximum distance between members, and the small vacuoles will start to appear (Fréon et al., 1992).

INFLUENCE OF THE BEHAVIOR OF GROUPING TYPE IN THE DEVELOPMENT OF FISHING ACTIVITY

From the perspective of fishing, the behavior of fish can be observed from two contexts, as a grouping behavior and as an individual behavior (Howarth, 2016). During the last years, the interest in studying the grouping features and behavior in relation with the management of fisheries has increased. Behavioral ecology has taken advantage as a complimentary knowledge of fishing studies, since some of the aspects of fish behavior like distribution, growth, escaping, etc. are closely related with the implementation of fishing technology (Fréon & Misund, 1999). As it is previously mentioned, some predators have known how to take advantage of the cumuli of prey in a reduced space, to feed off a great number of individuals in each of their attacks; and humans have not ignored this behavior in their search to obtain larger fish captures, developing fishing gears and capturing methods that contemplate grouping behavior.

In species that are commercially exploited, remaining united does not appear to be very advantageous. If, in a conservative way, we defined species that form schools, such as

scombroid, carangid and clupeoid fish these would include up to 50% of annual captures with approximately 35-40 millions of metric tons of fish (Parrish, 1999). The vast majority of these species are important targets in fishing, not because of their individual value, but because when grouped they become relatively easy to capture and their global abundance makes their extraction economically profitable (Parrish, 1999). With the exception of some tunas, where only an individual can represent high commercial value, which will depend on its biomass and the conservation quality.

Humans have developed different fishing strategies and gears depending on the degree of grouping in which fish are. A exploitation of the aggregation can be exemplified with the empirical observation of the attraction of small species that tend to shelter themselves or stick their eggs (e. g. Exocoetidae o Hemiramphidae) on floating objects like trunks, trash, seaweed, among others, in the pelagic system; with which aggregations of different fish species are generated, that according to distance and the degree of dependence among them and the object, they can be classified as intranatant (a distance below 50 cm), extranatant (from 50 cm to 2 m) and circumnatant (further 2 m *sensu*; Parin & Fedoryako (1999)).

Gooding & Magnuson (1967) y Hunter & Mitchell (1967) began to explain causes that determinate aggregate behavior of fish under these floating objects. In that regard, there are various theories on their utilization on behalf of fish, among them the employment of the objects as refugee and food source; besides serving as a transport for dispersion as in the case of objects adrift, there are studies who consider that these are references to restructure schools along migratory routes. However, this behavior depends not only on the species, but also on the development phase and size of the individuals (Castro et al., 2002; Santana-Ortega, 2015).

In addition, it is known that according to peculiar characteristics of a floating object (physical or structural) it will be the type of fish that will be attracted to them (Workman et al., 1985; Bard et al., 1985; Hall et al., 1999).

The prior is used for artisanal, industrial or sport fishing of pelagic fish of major sizes like swordfish, candlefish, dolphin fish and tunas, that are circumnatant predators temporarily associated to the object and that can swim from a great distance of it (Castro et al., 2002).

In the case of fish of the Scombridaec family they move along the pelagic zone in a lonely way or in school in search of food, when locating a region where there are fish aggregations it has been observed that tunas temporarily stay in the area while they eat. This behavior was observed by fishers, who based on their empirical knowledge, started to design devices that would have the same attraction of species of commercial importance. It is here where fishers have taken advantage, reducing search time for shifting schools.

The design of these fish aggregating devices (FAD´s) generates the same effect as natural floating objects in an artificial way, indirectly aggregating individuals susceptible to fishing in a determined area definite by the fleet, saving time and money in the operation. The first devices were installed in Philippines with the purpose of attracting *Thunnus albacares* (yellowfin tuna) and its success has been such, that the tuna industry has employed this knowledge during the last four decades in its operations involving searching, location and capture of the resource (Castro et al., 2002).

Studies referred to the FAD's observed that this devices tend to concentrate a bigger amount of juvenile organisms belonging to a certain number of species. These young organisms can be responsible for the accumulation of species of a higher economical value, as is the case of tunas and dolphin fish. Besides, the studies mention that the annual season can

be the main cause that regulates composition of the ichthyofauna as well as its abundance (Castro et al., 2002; Santana-Ortega, 2015).

These devices can be anchored or adrift (accessible through radio wave broadcasting devices), these being the most used in commercial fisheries, even the most modernized have echo sounder that transmit real time data of fish groupings, as it happens in the Spanish fleet where this data alert near boats when the concentrations of tuna are sufficiently elevated for a commercial capture (Ariz et al., 1992 in: Fréon & Misund, 1999).

Like this, some tuna boats use bait to aggregate schools beneath themselves and in this way, capture them in a more prolonged way, meaning that they act as FAD´s. This technique is known as "patch," which, once it is formed, can be moved to more shallow zones to make capture easier. The boat acts as an aggregating device, once its boat freezer are full of fish, it is replaced by another ship while the first one directs itself to the port to discharge (Santana-Ortega, 2015).

There are some controversies about the employment of the FAD's, on one side, some authors mention that the massive use of these devices could produce overfishing in various groups of fish, because they do not only group adult fish, but because the majority are juveniles, lots of species are also captured as a part of the by-catch, which are discarded afterwards due to its lack of economic value (Fonteneau et al., 2000; Filmalter et al., 2013). In contrast, there are those who considers that FAD's can be used as a sustainable fishing method while they are well regulated (Dagorn et al., 2013).

On another hand, among the methods that fishers recur most on to attract fish and concentrate them on action zones of fishing gear, we can find chemical attraction through odors, which mainly consists in place a site with specific bait (alive or dead) for the target species. The odor emanating from the bait moves in the water in the form of dissolved chemical substances, which makes fish perceive it and makes them feel attracted by them.

Attraction with artificial light is another fish aggregating technique that was very used until halfway through the twentieth century to capture tuna and other pelagic fish. This effect is possible due to lots of groupings of fish possess positive phototaxis, which means that in lighting stimulus these tend to move to the source that generates them. Long time ago fishers used torches to generate artificial light and attract fish and other marine species like squids; further, with the appearance of new illumination systems such as electric lanterns, kerosene and gas, the infrastructure of fishing also got modernized. Nowadays, electric lamps and chemical or bioluminescent devices that emit artificial light are still being used in an industrial way, which mimics with the light that the prey species would generate (Sokimi & Beverly, 2010).

In the fishing of tuna or swordfish bait is usually formed by dead squid, that with submarine chemical or bioluminescent lanterns they attract the resource to the fishing hooks. Some commercial fishing boats employ powerful submerged lights or even from deck to attract small pelagic fish to the surface and be able to perform capturing maneuvers.

The degree of attraction to the light will vary in species depending on age, physiological and environmental conditions (Fréon & Misund, 1999). For example, small sardines of the S*ardinella* genus usually show higher positive phototaxis than big sardines (Ben-Yami, 1976). It has been set out that in the case of sardines artificial illumination allows necessary visual contact to generate the school's behavior near the surface and the advantages that this entails (Fréon & Misund, 1999).

In the same way, a temporary factor of great interest for fishers are the lunar cycles, due to lots of species aggregate more densely during nights with a full moon (Nikonorov, 1973). In Bangladesh, the most important fishing gear to capture *Sardinella spp.* traditionally are the beach seine nets, which are employed while they swim along the coast. Schools are captured in the majority of the year's months in coastal waters mainly during the night, obtaining a maximum capture in nights with a full moon and new moon in the months of November to December and February to April. It has been reported that there is a vertical differential distribution of the sardine species that inhabit fishing zones, both industrial and artisanal fisheries have an impact over the *Sardinella* species due to they are captured between 10 and 50 m deep, while the *Dussumieria* genus that usually distributes between 40 and 60 m deep can only be available for trawlers of the industrial fleet (midwater trawlers) that make fishing operations between 10 and 80 m deep (Jit et al., 2013).

In Japan, different nets have been used historically on a small scale to capture sardines, such as: gillnets, beach seines and traps, the intensity of the sun's light has influence on certain capturing methods; where when fishing during twilight moments of the day fishermen increase their catch, even until the 1980s the use of systems of gleaming flashlights is registered to attract the sardine schools to make their capture easier (Cifuentes et al., 1986; Parrish, 1999; Sonu, 2001).

Either by attraction, exploitation of aggregations, or a combination of both, when encountering organisms in a group the search to capture them diminishes, and their abundance makes their extraction economically profitable. Fish use their habitat differently depending on local conditions, for a predator or a fisher this translates into a different capturability for the target species when conditions change.

As it has been mentioned previously, among the most important groups subject to fishing exploitation and form shoals or schools, we have the small pelagic fish. This artificial group of marine fish of relatively small size (between 10 and 40 cm) is formed by different species depending on the region of the planet. The most distinctive species belong to the Order of the Clupeiforms where the sardines and the anchovies are, as well as some Perciforms of the Carangidae and Scombridae families. Nevertheless, diverse species of the Exocoetidae, Hemiramphidae, Caesionidae, Atherinidae families, among others, can be considered as small pelagic fish.

The worldwide captures of small pelagic make a total of 17 million tons that represented 22% of the total of global captures in 2012 (FAO, 2014). Without doubt, the fishing of these fish on a global scale has been one of the most important and profitable for the abundance that its represents, that is why there are diverse artisanal fishing gears for their extraction, as well as industrial.

Before the dominance of occidental technology, the survival of sardine and anchovy fisherman relied on the knowledge of the location of schools, the local material available to catch and when these were more accessible for fishing, as well as techniques to attract them and concentrate them (Parrish, 1999).

Despite that lots of local knowledge got lost in favor of technological advances with the expansion of the western world (Johannes, 1980 in: Parrish, 1999), on a global scale there are still a wide amount of techniques for capturing sardines and anchovies in artisanal fisheries.

Until before the 1900s the catch of *Sardinella brasiliensis* in Brazil were mainly carried out by traditional fishermen and used as food in coastal communities (Diegues, 1995). This traditional fishing still exists in lots of regions of the country, which is performed using cast

net and purse seine net at bays and estuaries, using knowledge about tide fluctuation to concentrate and catch fish that come in and out some coastal lagoons (Lieber, 1994; Van Marlen, 2003).

Temporary nature is very important for coastal fishing of small pelagic fish. In Ghana, *Sardinella maderensis* is exploited during most of the year, while *S. aurita* is essentially captured during the largest upwelling periods (July-September) extending itself to months with lower upwellings like January, February or March, in such way that the artisanal capture of the country depends on the quality of these seasonal events (Cury & Roy, 1991).

There are regions like the Indian Ocean and the Western Indian Pacific where have the largest amount of clupeid, more than any tropical region, due to the abundance becomes larger in tropical regions in comparison with septentronial or meridional regions with a mild weather (Lavoué et al., 2005). Besides this, fishery in lots of countries of this zone is traditional, performed on a traditional way with beach seine, having in this way a low production (5% of worldwide capture) and only foreign fleets that operate in the area (mainly Korea, Japan, France, Taiwan and Spain) can access the resource farther from the coast (Al-Jufaili et al., 2006; Sherman et al., 2009). A similar case occurs in Western Africa, where even countries like Mauritania have given in to the exploitation of their exclusive economic zone to fleets of industrial countries like Germany or Russia (Cury & Roy, 1991).

INDUSTRIAL FISHERIES OF SHOALS AND SCHOOLS

Inside the industrial fishing sector there are different types of fleets: tuna purse seine, shrimp trawl fleet, coastal purse seine fleet, among others. In Ecuador, the coastal purse seine fleet extracts schools of small pelagic to be used as raw material for the production of fish flour, preserved canned food, as well as fish oil. The main species that are captured are *Sardinops sagax, Scomber japonicus, Opisthonema spp., Cetengraulis mysticetus, Etrumeus teres, Trachurus murphyi* (Aguilar, 1998).

On another hand, we can mention the east coast of the United States as an example of the transition to the fishing modernization of small pelagic simultaneous with knowledge on aggregations. Since the decade of the 1880s there has been fishing of sardines in that region, in places like Monterey's bay, during fishing season from the middle August until halfway through February, fishers would use the first ships with lamps to attract schools during the nights (Shillinglaw & Burnett, 2011). However, towards 1919 the country's marine already used an aerial detection system to support the location of *Sardinops sagax* schools, which consisted in training pilots to locate and report groupings of fish on surface, once detected, any vessel of the marine would give a sign to the group of boats to move to the place and commence fishing maneuvers. The whole operation was performed during dark nights where the gleam of schools underwater were more visible to pilots (characterized by the green interior and the red exterior edges) (Ueber & MacCall, 1992).

Before, the vessels that were employed had a lower autonomy and were built with wooden hulls, with time they were modified until being completely made of steel. Today modern boats for the fishing of these species are self-sufficient, within their infrastructure they contain detection equipment in real time, better storage capacity and fishing gears that allows them to extract great volumes of the resource in only one operation (Itano, 2002).

These fishing gears can be of different types and they are usually classified in two: active and passive. The category is related to the gear's manueverability and the behavior of the target species. In the case of passive gears, a fish usually heads towards the gear (e. g. set nets), while the active gears involve a pursuit aimed to the target species (e. g. trawls, purse seine, etc.).

PELAGIC FISHERIES WITH PURSE SEINE NET

Technified fishing has increased the captures four times larger between 1950 and 1990 (Platt et al., 1995). Nowadays industrial fishing of tuna, sardines and anchovies is dominated by the use of purse seine ships due to their efficacy to catch a great amount of fish, considering that the social configuration for this type of fishing gear are the schools or shoals of pelagic species. As it has been mentioned previously, in a school the individuals have a high mutual attraction among them which allows the cumulus of a great amount of fish in a small area and volume, this is used as advantage by the ships with purse seine nets saving, in this way, time and fishing effort.

Although fishing with purse seine net, was performed with canoes or sailboats while ago, the addition of "power block" to purse seine vessels during the decade of the 1950s was a key factor for the mechanization of capturing methods. This invention provided a lot to the effectiveness of the technique, making it the most efficient gear to captures fish species that grouping in schools or that are near the surface, which caused the substitution of traditional fishing methods in many places (FAO, 2016).

The ships with purse seine net, depend on the detection and location of the fish species that are gathered in schools (Misund, 1997). Before, the search for schools was exclusively done in a visual way, and lots of detection methods still prevail among fishers around the world (Au & Pitman, 1986). A sample of this is the fishing for small pelagic in Mexico, particularly in the Gulf of California where fishing is done essentially at night. The beginning of sardine fishing season is closely related to the behavior of schools in regards to the lunar phases, just like with the beginning of the shift of the first schools of *Sardinops sagax* along the coast of Sonora; it is in this way that fishing begins in October during a period of approximately 22 days, centered on each month's new moon (Sokolov, 1974; Cisneros-Mata et al., 1991; Nevárez-Martínez et al., 1996; Martínez-Zavala et al., 2010). In traditional embarkations of Mexico and other countries, the location of schools is done visually due to these produce a luminous effect that allows them to be seen at 15 or 20 meters far in this dark period, in addition to this, the fleet starts its voyage to fishing sites during sunset which allows them to observe biological activity on the sea's surface such as: flock of birds feeding, unusual movement on the surface of a calm sea, dolphins, among others (Cifuentes et al., 1986; Parrish, 1999; Tesco Real Food, 2016).

The detection in modern embarkations is performed in a complementary way with acoustic instruments. When schools swim bellow 60 m it is not possible to observe traces of them from the surface, which is why in most countries that keep an industrial fleet echo sounders are employed (Cifuentes et al., 1986; SAGARPA, 2006; KinoshitaFishingNet, 2009). At present fishing boats can detect schools any time of day, however, bigger success in their capture depend on the depth of schools distribution, this is why they usually prefer

working at night taking advantage of the behavior that small pelagic have of emerging to more shallow layers (Bahri & Fréon, 2000; KinoshitaFishingNet, 2009).

The fishing capacity depends on the boat's size, for example, there are embarkations with purse seine of until 200 m of keel that allow a potential access to deeper schools during daytime (SAGARPA, 2006), although these diurnal maneuvers will depend on a major degree of factors like the crew's expertise, hydrographic, geographic and meteorological conditions, as well as the season to have a successful catch.

The fishing maneuver can be carried out by one or two embarkations, once that the aggregation has been detected, the net opens around the fish school, forming a circle (whether it is with another embarkation or an auxiliary embarkation), when the net is in the water every sound generated by the boat must be avoided and all the lights go off except the navigation ones, this to avoid sudden movements of the school (Tesco Real Food, 2016; Figure 3b). The net is closed by pulling a seine that goes through loops that are in the inferior with the purpose of generating some type of bag to avoid fish from escaping by swimming under, afterwards the bag is moved closer in a slow way to one of the boat's sides (FAO, 2016; NMFS.NOAA, 2016). When the school is compressed inside the net it is possible to lift it to deck through suction pumps, nets of smaller size in the shape of a spoon or even by directly tying the product like it happens with tuna.

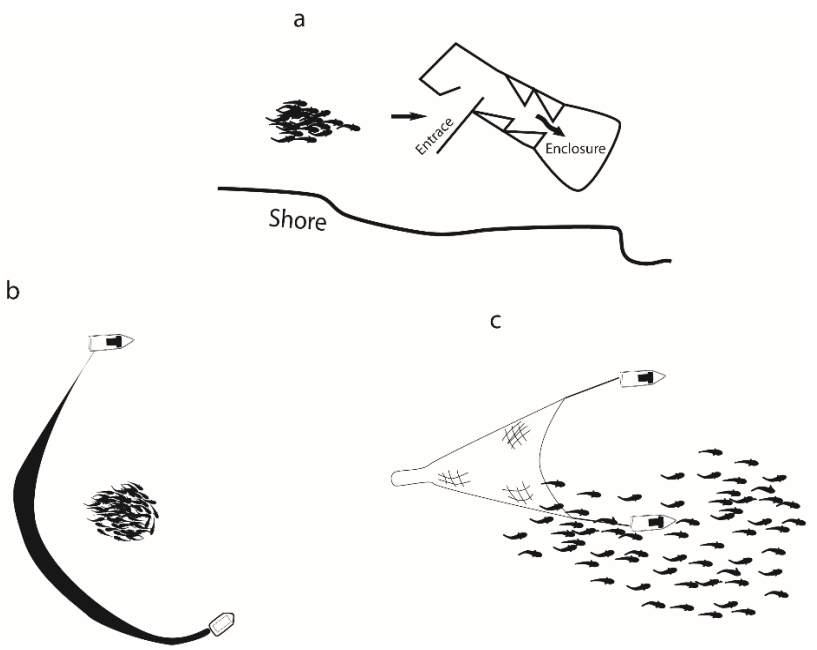

Figure 3. The mechanism of following a leader is a distinctive feature of fish that swim in shoals, schools and that is taken advantage of in set nets (a). The compaction of a great amount of fish in a school is an advantage in the purse seine fishing method (b). When fish are dispersed forming shoals the trawling fishing method can capture a portion of the fish group (c).

As it has been mentioned, in the oceanic system fishers have a distinguishing availability of small pelagic species throughout the day, which is linked to daily vertical migrations (Parrish, 1999).

Besides, it has been described that during nights, the small pelagic groups feed on the surface because in their adult phase they are planctonivore microphages and in this zone there are larger amounts of food. Some of them, like the sardine, are usually selective with their prey, after observing it has come to the conclusion that there is a high percentage of planctonivore organisms swallowed in their stomach, which were found in a low percentage in the water column (Culley & Kerkut, 1971). This selectivity of prey and habitat determines for most fleets for the majority of trawls to be profitable when fish are organized in shoals in superficial layers during the night (Fréon & Misund, 1999). Tied to this, the sardine shows a strong escaping during the day and less during the night, unless fish come in contact with the net (Parrish, 1999).

In New Zealand, there is a great commercial interest for the fishing of S*ardinops sagax*, although *Engraulis australis* (Australian anchovy) is also captured. Since 1993 most captures are performed in oceanic waters with embarkations that use purse seine, however, fishing net lights of similar appearance to the purse seine but smaller are also used, which combines the attraction for light and the enclosing maneuvers since it has lights in the central part. It does not have a rope in the bottom that allows to make a bag, whereas the bottom rope is much shorter than the rope that contain the floats forming a partial floor when the net is near the school, particularly in the central portion. Just like the purse seine, the fishing net light is placed around the school and the ends of the two wings are pulled together, the school shifts to the interior and it is held in the central portion until the net is taken near the boat so afterwards the fish can be taken with a submersible net (Paul et al., 2001).

PELAGIC FISHERIES WITH MIDWATER TRAWL NET

Midwater trawls are one of the most employed gears to capture at open sea, the same with purse seine, the social configuration for this type of gear can be for schools, but are mainly shoals of pelagic species of shallow or profound waters, however, unlike the purse seine, this one does not necessarily require for fish to be gathered in a determined area (FAO, 2016; NMFS.NOAA, 2016).

The ships are equipped with radars and echo sounders that allow the detection of shoals and schools, a single trawl can capture a substantial fraction of a school (Parrish, 1999) (Figure 3c). The net is towed behind one or two vessels and depending on the target species and the net design, the trawl velocity varies from 2.0 to 5.0 km. The wings or doors that are in the cable of the net has generate horizontal opening, while vertical opening is caused by positive floatability that is given by buoys from the superior edge and the counterweight of the inferior edge.

The process in which fish enter and stay in the net involves a complex sequence in their behavior. In the previous zone to the trawl, reaction to sounds of low frequency of the embarkation, can generate an escaping behavior that shows with direction change (vertical movements of fish happen at the beginning of the trawl when the embarkation's noise abruptly increases) and with a change in their swimming's velocity (He, 2010). Both patterns seem to be adaptive from a point of view of predatory escape (Lima & Dill, 1990). In the zone between tables and the net's entrance it has been observed that fish tend to execute the "fountain effect" to keep the threat (in this case the net's entrance) on the verge of their visual

range. Fish that swim to the exterior of the doors will escape, while those that swim inside will immediately enter the bag's trajectory (He, 2010).

Just like purse seine fishing, the capture with midwater trawls of pelagic fish is usually less complicated on nocturnal hours, suggesting that a visual detection on a vigilance schedule allows the precise location of the net and increases the coordinate escape reactions of the species (Fréon & Misund, 1999).

PELAGIC FISHERIES WITH SET NETS

Other fishing gears to capture small pelagic on a big scale are set nets, which are big fixed structures spread out in coastal waters through which fish access and are guided to different spaces where they cannot find an exit, trapping themselves (He, 2010). The social configuration for which this type of gear is designed are shoals and schools of pelagic species, this due mainly to the imitation behavior that fish have, where a leader with a swimming direction can make other members of the school to follow him, in this case, to the fishing nets.

Due to these fishing nets stay fixed, they have a series of advantages: they can function 24 hours of the no-vigilance day; it is not necessary to detect and follow schools; fish usually stay alive until their extraction; it is possible to know the volume of the catch before collecting it, which allows to prepare the amount of ice and the transport to move it properly, without spending excessive energy; the effort to collect the fishing net is minimal, besides that a selective fishing and the liberation of other living organisms of other species is allowed.

Japan is the country that most uses stationary nets, obtaining around 25% of the total of their coastal waters catch in the 1980s. Likewise, another area where these traps are widely used is on the east coast of Canada, where traps receive the name of spillways and that with seine boats they are the most employed fishing methods for the capture of small pelagic like *Clupea spp.*, *Scomber spp.* and *Mallotus spp.*

In some places, traps consist of wooden posts buried in the bottom, laid out in a semicircular pattern which holds on to fishing nets in a way that it resembles a corral, once the sardines enter the area of the main container they are caught and can stay alive until their extraction is required, which can be through a suction pump on the embarkation with smaller nets (Brunswick, 2016).

These traps are passive fishing gears that must be installed on a strategic way all along the coast, on small coves visited regularly by schools. Lots of schools are captured because of the effect of following the leader, when studying this effect with different species it was observed that only a small number of schools entered the traps without their leader, in contrast with the number of schools (76%) that entered with their leader moving towards the interior. Leaders can have an influence of up to 60 m of distance over the school, however, this distance depends on the species and the visibility of the site (He, 2010).

The position and angle of the entrances are placed on a certain way to intercept groupings of fish along their migratory routes (Figure 3a). The previous knowledge of predictions in migration patterns in terms of route and season are extremely important for the success of trapping operations.

It is frequent to find regions where industrial fishing is performed employing more than one capturing method, like in the fishing of *Sprattus sprattus* and *Clupea harengus* (Atlantic herring) in the east of Northern America, from New Scotland (Canada) up to Gulf of Maine (United States) which employs fixed fishing nets to capture sufficient volume of the resource during migration season, the seine nets are usually used to fish in shallow waters or to fish shallow schools, while the other boats with trawls are more effective to fish schools in deeper waters (Brunswick, 2016). In contrast, fishing sectors can also have conflicts trying to capture schools in a same place, as it happens in the Western Indian Ocean, where foreigner fleets tend to destroy set nets from local communities besides reducing the available resources in the area (Sherman et al., 2009).

As it has been mentioned throughout this chapter, the aggregative behavior of fish has played a very important role in technological advance of fisheries, however, it is necessary to establish well backed-up norms which will allow to maintain ecological balance and sustainability.

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Chapter 53

THE INTERACTION OF AQUATIC ORGANISMS (*MYTILUS* SP.) WITH HARMFUL ALGAL BLOOMS: COMPOSITION, DISTRIBUTION AND METABOLISM OF LIPOPHILIC MARINE BIOTOXINS IN THE AUSTRAL PACIFIC FJORDS

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ABSTRACT

For years, marine resources have been an important part of human development due to the high nutritional contribution they possess. *Mussels* sp. are the most consumed species in the world. As a result of requirements and needs exceeding the natural global production, man developed aquaculture in order to regulate the imbalance between nutritional requirements and natural production.

Under natural conditions, there are several and wide ranging varieties of mussels, with multiple sea habitats: sandy, rocky and stratified bottoms in the water column in rocky strata. This allows them to assimilate a varied diet of micronutrients consisting mostly of phytoplankton. Although, depending on their habitat, mussels can filter waste

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from other species such as feces, pseudofeces, microcellular debris or compounds from anthropogenic input.

From the many species of phytoplankton, about 80 of them are known for producing phycotoxins through blooms, *Dinophysis* sp. being the species of dinoflagellates associated with the production of toxins in the Austral Pacific fjords. These dinoflagellates are filtered and accumulated by hydrobiological organisms, such as mussels, that accumulate toxins in the digestive glands. Here they are subjected to numerous reactions of biotransformation mediated by enzymatic pathways, nonenzymatic pathways or by bacteria present in bivalves, which extends the toxic spectrum of the assimilated toxins, an event that is magnified by the interaction of vector organisms that expand the toxic forms through the trophic chain.

Lipophilic marine biotoxins (OA-, AZA-, PTX- and YTX-group), constitute the group of toxins that affect mussels from natural origin and aquaculture the most. These kind of toxins are accumulated in the tissues of mussels where they are retained according to their chemical affinity and/or modified into more toxic forms (AZA-1→AZA-3) and/or thermodynamically more stable forms (PTX-2-SA→7-epi-PTX-2-SA), in some cases allowing accumulated toxins by mussels to become harmless (DTX-1→DTX-3; YTX→carboxy-YTX).

Since blooms associated with lipophilic marine biotoxins may occur simultaneously, different species of mussels and those species related to their habitat can accumulate and/or transform the wide spectrum of toxins. This generates a toxin profile and variable toxicity in their tissues, which lasts according to the exposure time to the bloom and the purification pathway of toxins from mussels.

In this chapter, the main events associated with lipophilic marine biotoxins (OA-, AZA-, PTX- and YTX-group), as well as their interaction in the habitat of *Mussels* sp. in the Austral Pacific fjords are presented.

Keywords: lipophilic marine biotoxins, mussels, metabolism, distribution, Chile

1. INTRODUCTION

Mytilids, internationally known as “mussels”, are bivalves. They are filter-feeding molluscs that belong to the family Mytilidae, bivalvia class, which pump seawater through their gills to filter out food items and other suspended particles. They release filtered water back into the environment. Materials retained by a mucous material on the gills—including tiny organisms, detritus, suspended sediment, and contaminants—are eaten and then assimilated into their tissues or passed through their bodies undigested. By filtering, they remove the phytoplankton and inorganic particles from the water column, thereby reducing turbidity. Therefore, turbidity reduction is directly proportional to the abundance of bivalves [Asmus & Asmus, 1991].

On the southern Pacific coast, mytilids are distributed from Perú to the Strait of Magellan and, on the Atlantic Coast, along the coast of Argentina to Brazil. From the 70 species of mytilids described in the South Pacific Region, three species are economically important resources in Chile: Blue mussels (*Mytilus chilensis*), Giant mussels (*Choromytilus chorus*) and Ribbed mussels (*Aulacomya ater*). There are closures during certain times of the year to control the extraction of these mussels from natural beds and to ensure their availability in the future [Avila et al., 1995].

Under natural conditions, there are diverse and wide ranging varieties of mussel species with multiple habitats at sea, such as sandy, rocky and stratified bottoms in the water column in rock strata together with species such as *Pyurapreaputialis*, *Lessonia nigrescens* and *Perumytilus purpuratus*. This allows them to absorb a varied diet of micronutrients consisting mainly of phytoplankton, and depending on their habitat, waste from other mussel species such as pseudofeces, micro-cellular debris or compounds from anthropogenic inputs. These baseline nutritional intakes are added to the ascending variable produced by climate change that could be a predominant factor in the development of marine resources from natural beds or aquaculture development products.

For years, natural marine resources have been an important part in human development given the high nutritional content they have. Their contribution has been so significant that requirements and needs have exceeded natural contribution worldwide. Previously sustainable resources have become endangered or have been deemed under threat of extinction. In order to reduce the disparity between nutritional requirements and natural production, man developed aquaculture. Through aquaculture, it is possible to reduce naturally occurring damage caused to natural resources and to allow adequate separation between natural resources and those resources coming from farming centers, thus preventing the extinction of more mussel species of great commercial interest. About 85% of the global production comes from aquaculture activities. With the exception of a few countries where the extraction is strictly controlled, natural beds have been overexploited. The supply of this kind of global resource is based on the stability of the activity and the ability of farmers to increase their crops and to improve products [Cortez et al., 2012].

The phytoplankton biomass increase associated with the nutrient-enriched input is beneficial for filter feeder bivalves. Nevertheless, N and P inputs from anthropogenic sources alter the nutrient ratio (16:1) to very high levels (70:1) by nutrients from agriculture through rainwater. In nutrient-enriched systems with high levels of primary production, intra- and interspecific competition for food is minimal when the abundance of bivalves is at low or intermediate levels. However, in less productive systems with limited water circulation and/or high levels of bivalve biomass, intra- and interspecific competition may occur between stocks from natural bivalves and those produced by aquaculture [Cloern, 2001].

Additionally, organic imbalance (N:P) generated in some areas globally, produces a variability in marine phytoplankton communities. In many cases, this generates an exponential growth of these species, which, even though they are still a nutritional source for multiple species of mussels, generate high commercial and human impact, by being associated with the production of toxins capable of killing people [Newells, 2004].

To date, 5,000 phytoplankton species have been described, from which, under certain circumstances, 300 species have a high proliferation rate resulting in a high density of microalgae in the sea, a condition called a “bloom”. The circumstances or factors triggering these “blooms” are still not completely determined, but what is certain is that specific changes of climatic and hydrobiological conditions play an important role in the conditions leading to a “bloom”. These “blooms”, in general, generate beneficial conditions for the development of aquaculture and marine biology. However, about 80 species, out of the 300 already mentioned found in phytoplankton, are dinoflagellates and diatoms that -under certain circumstances- produce phycotoxins (marine toxins). The abundance of this kind of toxic phytoplankton range from a few hundred thousand up to several million per liter in the sea, reaching

maximum levels called “Harmful Algal Blooms” (HABs) [James et al., 2010; Anderson et al., 2012].

HABs are produced by a group of cells (dinoflagellates, cyanobacteria and diatomae) that, due to different natural environmental or anthropogenic conditions, may alter the relative abundance of these species, allowing for them to prevail over other microplankton species. The above mentioned is associated with the production of powerful toxins that dramatically affect marine fauna, mammals and people. Authorities of inhabited zones where HABs are detected, react by closing large sea areas to fishing and halting harvesting activities in zones where farmed and wild molluscs and bivalves are found, triggering a chain reaction of economic damage at industrial, touristic and sanitary levels (Figure 1) [Hallegraeff, 2010; Picot et al., 2011].

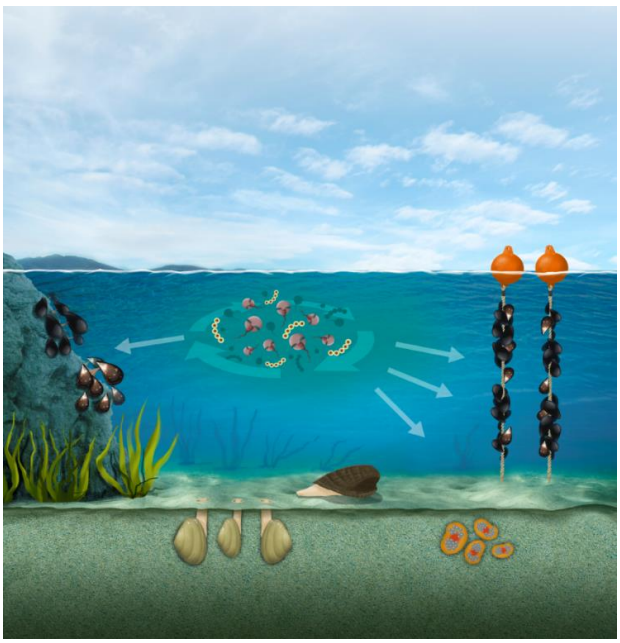


Figure 1. Summary of environmental impacts of Harmful Algal Blooms (HABs) on carnivorous shellfish (mussels) and miticulture from the South Pacific Ocean.

Phycotoxins can be accumulated in several marine species, such as fishes, crabs and every kind of filtering bivalve [Oyaneder et al., 2017]. Mussels, because of their high rate of filtering, can accumulate great amounts of phycotoxins in their digestive glands, with no adverse effects. However, when highly contaminated mussels are eaten by animals or humans, they can produce severe intoxications. The worldwide number of phycotoxin induced intoxications per year is about 60,000 cases. Additionally, there is a strong impact on ecosystems, the environment and the economy, and as a consequence of the negative effect on tourism, recreation and miticulture and aquaculture industries [Gerssen et al., 2010; Kudela et al., 2015].

Visibly occurring in all seasons except summer, mussels coming from aquaculture facilities can alter the nutrients in the water column, where phytoplankton grow faster, not being able to control the phytoplankton biomass. Moreover, water flows increase the seston

flow through the crop sites, thus ensuring an adequate supply of food to bivalves [Nakamura & Kerciku, 2000].

In Europe, annual estimated losses in the tourism sector, as a consequence of HABs, are approximately € 700 million and € 116 million on miticulture [Morgan et al., 2009]. In order to prevent intoxications caused by eating phycotoxin contaminated shellfish and to mitigate their harmful effects, international agencies have produced regulations, legislation and monitoring programs [EU, 2011].

Health risk prevention, as well as the industry need for monitoring phycotoxin presence within several toxin carrying vectors, has made it necessary to develop a potentially toxic microalgae watch program. Several phycotoxin detection methods have been developed, many of which allow detection of phycotoxins within shellfish far below dangerous levels. These methods challenge many difficult issues, such as insertion of toxin within complex carrying matrix and the presence of mixtures of structurally related analogs [Fernandez et al., 1996].

The basic contribution of toxins provided from harmful phytoplankton is varied, depending on the involved species and the areas where the bloom is produced at a global level. It should also be considered that different species of toxin-producing microalgae may produce simultaneous blooms, thus, causing the same species of hydrobiological organisms, from both natural beds or aquaculture facilities, to possibly accumulate more than one variety of toxin resulting in a toxic profile and variety of toxicity which is increased as they get involved in the trophic chain. Nevertheless, mussels do not only accumulate these toxins inside their bodies, but also in their metabolic pathways and pathways mediated by enzymes; they make key modifications in order to decrease the toxicity towards them, which often cannot be extrapolated to humans, for whom these chemical modifications may become more toxic. This impact of natural origin not only affects natural resources, but also resources from the development of aquaculture by adding, economic and social development repercussions in local communities that make a living from this activity [García et al., 2005; García et al., 2016].

By affecting the development of aquaculture, HABs not only contribute to the basal level of toxins for mussels, but there is also a contribution of toxins derived from biotransformation processes caused by mussels and toxins produced by the different stages of the production development process (canned or frozen products). These products, when subjected to different physical-chemical variables, further expand the toxin's forms that mussels possess, transforming from innocuous to toxic forms [McCarron et al., 2008; Blanco et al., 2016; García et al., 2016].

Based on the chemical properties of the toxins found in phytoplankton and shellfish, they have been classified in two classes: hydrophilic and lipophilic toxins. Toxins associated with the Saxitoxin group (STX-group) are hydrophilic and share a common trait: they are of low molecular weight (about 500 Dalton). Toxins associated with lipophilic toxins, the Okadaic acid group (OA-group), the Azaspiracid group (AZA-group), the Pectenotoxins group (PTX-group) and the Yessotoxins group (YTX-group) share two common traits: 1) their molecular weight is between 600 and more than 2000 Dalton; and 2) they have a lipophilic chemical nature [Gerssen et al., 2010].

2. LIPOPHILIC MARINE BIOTOXINS

Lipophilic marine biotoxins are formed by a group of toxins of lipophilic nature corresponding to the Okadaic acid group (OA-group), the Azaspiracid group (AZA-group), the Pectenotoxins group (PTX-group) and the Yessotoxin group (YTX-group). Initially, PTX-, YTX- and the AZA-group had been classified within the OA-group, since a large proportion of this group of toxins was co-extracted along with other toxic groups, during the evaluation processes in mussels and phytoplankton. Thus, the symptoms detected in the bioassays, or those caused by intoxication of people, tended to be associated with the entire group of lipophilic toxins. Subsequently, it was determined that this group was composed of different types of toxins, very different from each other from a chemical point of view and with different toxicological properties. The PTX, YTX and AZA groups were then excluded from the DSP group and classified as independent groups [Paz et al., 2008; Dominguez et al., 2010; Gerssen et al., 2010].

The EU has established guidelines for maximum toxin levels in shellfish for human consumption of OA, DTX and PTX, whether combined or single value whose edible parts of seafood cannot exceed 160 μg OA equivalent/(shellfish kg), while maximum levels of YTX cannot exceed 3.75 mg YTX eq./(shellfish kg) and 160 μg AZA equivalent/(shellfish kg) for AZAs. As of July 2011, the Commission Regulation (EU) Nº 15/2011, amended Regulation (EC) No. 2074/2005, which recognized testing methods for detecting marine biotoxins in live bivalve molluscs and established the EU-RL LC-MS/MS method was established as the reference method for detection of lipophilic toxins and used as a matter of routine, both for the purposes of official controls at any stage of the food chain and in house inspections by food business operators [EU, 2011; EU, 2013].

2.1. Okadaic Acid Group

The Okadaic acid group (OA-group) is a gastrointestinal disease caused by ingestion of shellfish with accumulated fat-soluble polyether toxins produced by dinoflagellates of the *Dinophysis* and *Prorocentrum* genera. The density of cells associated with a bloom of OA-group corresponds to $< 10^4$ cell L^{-1}, with an abundance of $< 10^3$ cell L^{-1}. Intoxications associated with OA-group are a worldwide threat to public health and the shellfish industry [James et al., 2010; Glibert et al., 2012; Reguera et al., 2014].

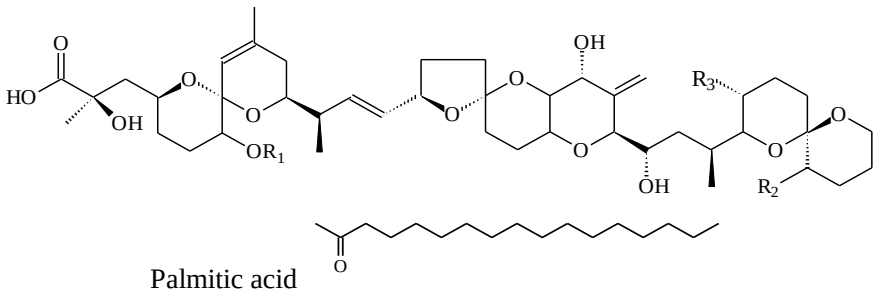
The first documented intoxications with OA-group were recorded in Holland (1961) [Kat, 1979]. To date, high levels of these toxins have been found repeatedly off the coast of Europe (U.K., Ireland, Denmark, Sweden, Norway, France, Spain, Italy, Portugal, Holland and Belgium); Canada; South America (Chile and Argentina); Japan; Australia and Africa (Morocco) [García et al., 2004; Elgarch et al., 2008; Gersenn et al., 2010]. In the South Pacific Ocean, blooms associated with OA-group usually occur in spring-summer periods (October-March) [García et al., 2012].

Okadaic acid (OA) and its isomers dinophysistoxin-1 (DTX-1) and dinophysistoxin -2 (DTX-2) are the main toxins produced by OA-group-producing dinoflagellates (Figure 2) [Yasumoto et al., 1978], which present specific toxicity equivalence factors (TEFs), OA= 1.0 (TEF); DTX-1 = 1.58 (TEF); DTX-2 = 0.48 (TEF) [EFSA, 2008a].

The minimum dose of OA and DTX-1 necessary to induce toxic symptoms in humans has been estimated at 40 and 36 μg, respectively [Hamano et al., 1986]. Death of intoxicated patients has not been reported, but okadaic acid (OA) and methyl-okadaic acid (DTX-1) have been shown to be potent tumor promoters in animals [Fujiki et al., 2013]. Therefore, they might increase the risk of cancer among regular consumers [Cordier et al., 2000; Maneiro et al., 2008].

Toxins associated with OA-group are powerful cytotoxins that inhibit various types of serine/threonine phosphoprotein phosphatases 2A (PP2A) [Huhn et al., 2009; Hayat et al., 2012]. These proteins are a group of enzymes that carry out dephosphorylation of numerous proteins, a very important function which is closely related to many essential metabolic processes of eukaryotic cells [Van den Heuvel, 2002]. OA and DTXs are more potent against PP2A than PP1 (OA-IC_{50} 2.87 ng/ml and DTX-2-IC_{50} 5.96 ng/ml) [Huhn et al., 2009; Hayat et al., 2012].

Depending on the species and natural depuration, OA-group toxins are modified inside the molluscs, where OA, DTX-2 or DTX-1 are esterified in the hydroxyl group of Carbon 7, palmitic acid being the most common fatty acid found in dinophysistoxin-3 (DTX-3) (Figure 2). Normally, palmitic acid amounts to 90% of the total fatty acids linked to the 7-*O*-acyl-derivative of dinophysistoxin-1 to produce DTX-3 in Chilean samples [García et al., 2005]. Although other forms of acyl derivatives, such as C14:0, C16:1, C16:0, C18:1 and C18:0 have also been detected in *M. galloprovincialis* and *D. trunculus* in samples collected along the Portuguese coast [Vale, 2006].



Palmitic acid

Name		R^1	R^2	R^3
Okadaic acid	(OA)	H	H	CH_3
Dinophysistoxin-1	(DTX-1)	H	CH_3	CH_3
Dinophysistoxin-2	(DTX-2)	H	CH_3	H
Dinophysistoxin-3	(DTX-3)	Acyl	CH_3	CH_3

Figure 2. Chemical structures of okadaic acid-group and analogues. **R** indicates the substitution of the corresponding radical in the molecule.

Vectors play an important role in transmitting OA-group toxins through the food chain, causing unknown toxic effects to other organisms [Landsberg, 2002; Torgersen et al., 2008a; Torgersen et al., 2008b].

Periodic OA exposure in mussels results in changes in gene expression, either by specific interactions of OA-group with receptors or indirectly by inducing intracellular signaling cascades, producing a loss of stabilization of focal adhesion and cytoskeletal disorganization [Svensson & Förlin, 2004; Manfrin et al., 2010].

Since mussels are the main vector of OA-group toxins and its contamination causes great harm to mussels coming from natural beds and/or from farming facilities, multiple methods of detoxification have been developed. These methods are required to be quick, efficient, easy to apply and should not alter the organoleptic qualities of products. Among these processes, thermal shock, freezing, evisceration, supercritical CO_2 with acetic acid, ozonation and radiation are highlighted; these processes are characterized by destroying or reducing the toxicity of mussels [Reboreda et al., 2010; García et al., 2016].

From the above mentioned processes, the most widely used corresponds to radiation, because it is able to destroy pests and pathogens in large production processes of different kinds of foods [EFSA, 2008a; EU, 2009]. Thus, mussels containing toxins close to the regulatory limit of 0.16 μg OA equivalents g^{-1} mussel tissue, when subjected to γ - irradiation, show a marked decrease in their toxic content, resulting in products that are suitable for consumption and sale. However, it should also be considered that this procedure can lead to highly toxic or carcinogenic compounds (2-alkylcyclobutanones and radyolitic derivatives of triglycerides) [Raúl et al., 2002].

Another frequently used method corresponds to ozonation; when this method is applied to homogenized mussels, it generates a highly positive and favorable effect for the destruction of OA-group toxins, this is attributable to the penetration capacity of the gas (ozone) on the tissue of the mussel, thus, having the option of directly interacting with the molecules of OA-group. However, when the toxic profiles of mussels are dominated by toxic varieties such as acyl-derivatives (acyl-OA or DTX-3), the ozonation process may cause a decrease in ester forms (DTX-3), producing an OA-group increase in nonacylated forms (OA or DTX-1). Therefore, ozonation may alter the toxin profile of OA-group toxins, with the final product (canned mussels) being dominated by a highly toxic profile of toxins. It must be taken into account that the toxicities of 7-O-acyl-esters forms may have toxicities similar to the OA-group-toxins if the esters are hydrolyzed in the human digestive system [Jørgensen et al., 2005; García, 2015].

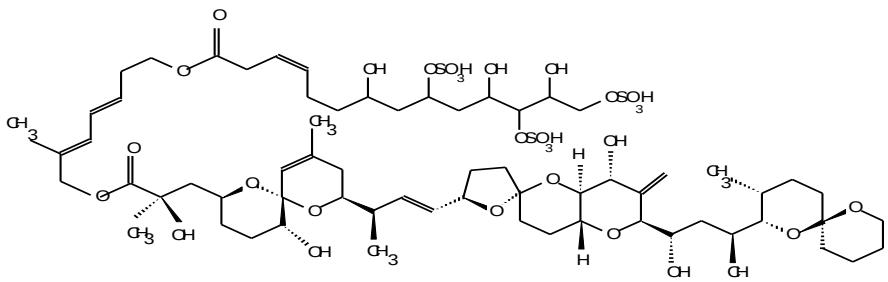
From a toxicological point of view, the toxicity of mussels highly depends on the variability of lipid content in the hepatopancreas (≈ 10 - 20%), which favors the toxins' retention factor [Svensson et al., 2003; García et al., 2006; García et al., 2012]. This explains why these toxins mostly predominate in this tissue, in comparison with the whole body which has a lipid content of only 3%.

Despite the high levels of exposure to blooms associated with OA-group toxins, mussels do not show mortality effects of species from natural beds or culturing centers during their adulthood and this may be attributed to a protective effect of mussels against blooms. *In vitro* studies have established that PP1 and PP2A from mussels are inhibited by OA-group toxins like those from any organism. However, *in vivo* studies have demonstrated that the toxic effects of OA-group toxins on cytosolic enzyme (glycogen synthase) are lower, suggesting that mussels have a mechanism that prevents the accumulation of OA-group toxins in ambient cytosolic. This response against the intracellular effects of OA is mainly due to multixenobiotic resistance (MXR) in the blood cells of mussels, which by being exposed to OA are more resistant against the toxin's cytotoxic effects [Chambers et al., 1993]. This route of resistance is related to the over-expression of a plasma membrane phosphoglycoprotein (p-gp) which is particularly in the gill, mantle tissue, digestive gland membrane vesicles and blood cells and which functions as an ATP-dependent drugs efflux pump [Cornwall et al., 1995; Kurelec, 1995].

Another protective mechanism associated with mussels exposed to OA-group toxins is related to the uptake and storage of OA/DTX-1 in the lysosomal system and whose organelles are known by concentrating foreign compounds, such as lipophilic xenobiotics. Thus, contaminants such as OA/DTX-1 would be captured and carried through vesicles to lysosomes, predominantly favoring it, because toxins filtered by mussels (OA/DTX-1) are associated with degraded algae fragments, so they are captured by endocytosis to intracellular digestion in lysosomal compartments. This would finally explain why there is no accumulation of OA-group toxins in the cytosol in mussels and, therefore, no effect on the activity of cytosolic enzyme regulated by PP1 and PP2A [Svensson et al., 2003].

Since OA-group is a serious problem for aquaculture worldwide, it has been established that its predominance in the aquatic environment further responds to the ability that toxins, of which they are composed, have to inhibit the growth of a variety of microalgae in a concentration-dependent manner, thereby suggesting an allelopathic role of toxins. In dinoflagellates, cells mainly produce toxic forms such as OA-diol-ester and dinophysistoxin-4 (DTX-4) which are rapidly hydrolyzed to produce OA or DTX-1 (Figure 3). In the early stages of the production of toxins, DTX-4 is the majority toxin and, therefore, the route of excretion of toxins from cells, which is rapidly hydrolyzed to OA-diol-ester and then to OA by enzymes, and it may even be effective against predators that break their cells during feeding. Through *in vitro* studies, it has been determined that DTX-4 is 50 times less effective than OA on PP2A inhibition, where the OA-diol- ester toxic form is relatively inactive [Hu et al., 1995a; Hu et al., 1995b].

DTX - 4



DTX – 5

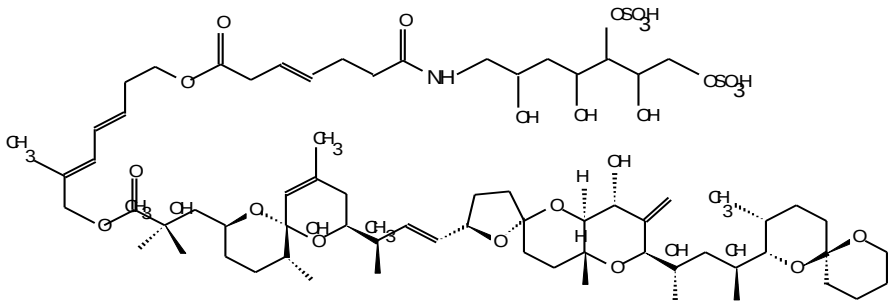


Figure 3. Toxins that compose the toxicity profile of OA-group producing dinoflagellates and that are part of the initial stage prior to being disposed of in the sea or filtered by bivalves. Dinophysistoxin-4 (DTX4); Dinophysistoxin-5 (DTX-5).

An important factor is related to the fact that since OA is a lipid-soluble polyether, its carboxylic group can be ionized at physiological pH, which would make diffusion difficult through the membrane. Thus, since pre-toxic forms such as OA -diol-ester have no ionizable groups, they can move through the membrane with relative ease, allowing it to mobilize into the cell and being inwardly hydrolyzed acquiring toxicity 1,000 times higher than its predecessor.

2.2. Yessotoxins Group

The Yessotoxin group (YTX-group) corresponds to a group composed of sulphated polyethers (Figure 4), which were isolated for the first time from the Japanese oyster *Patinopecten yessoensis*. Yessotoxin (YTX) is produced by the marine phytoplanktonic microalgae *Protoceratium reticulatum* (Gonyaulax grindley) [Satake et al., 1997], *Lingulodynium poliedrum* (Gonyaulax polyedra) [Tubaro et al., 1998; Paz et al., 2008] and *Gonyaulax spinifera* [Rhodes et al., 2006; Paz et al., 2008; Sosa et al., 2013]. At present, this group of toxins have been identified in different countries worldwide such as New Zealand, Italy, Spain, Norway, Russia, Canada, United Kingdom, Japan, Argentina and Chile [Paz et al., 2008; Alvarez et al., 2011; Paz et al., 2013; Akselman et al., 2015]. Blooms associated with YTX-producing dinoflagellates are characterized by a concentration of cells of 10^3 cells L^{-1}, where the production of YTX toxins in dinoflagellates corresponds to an average of 34 pg $cell^{-1}$, in which homo-YTX and YTX are the principal toxins that compose the profile in variable concentrations and depending on the area where they had been identified [Paz et al., 2008].

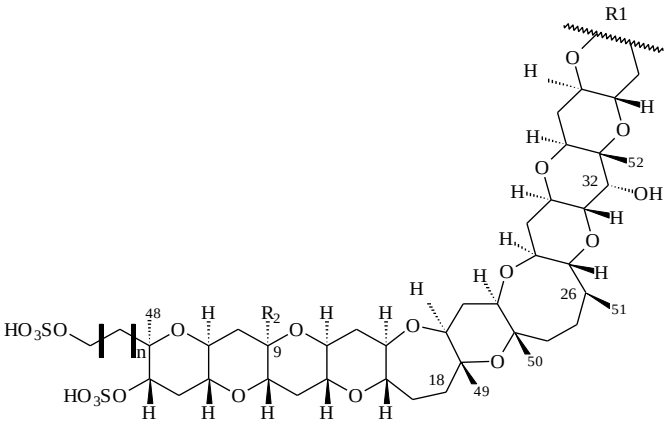
Historically, YTXs have been classified within the DSP-toxins group. However, these toxins do not induce diarrhea in mouse bioassays, nor do they produce protein phosphatase inhibition. This is reason enough to classify them in a separate group [Ogino et al., 1997; Tubaro et al., 2003]. The precise mode of action currently remains unknown, although YTX is known as a potent phosphodiesterase activator [Alfonso et al., 2003].

Symptoms caused by poisoning with YTX in humans are relatively unknown because no human intoxication case has been reported to date [Dominguez et al., 2010]. YTX toxic evaluation, in bioassays, has determined that after an intraperitoneal injection of approximate concentrations of 150 μg kg^{-1}, symptoms that begin 4 hours after the injection is given are: reduced motor coordination, dyspnea, tremors and cramps [Aune et al., 2002; Alfonso et al., 2003]. EFSA has established that toxic effects may not occur if the concentrations do not exceed the levels of 3.75 mg YTX-equivalent kg^{-1} shellfish [EU, 2013].

Currently, 100 natural derivatives of YTX have been identified and characterized by NMR and detected by liquid chromatography coupled with mass spectrometry (LC-MS). Some YTXs are directly produced by dinoflagellates (norYTX, 41-keto-YTX and 41a-homo-YTX) while shellfish metabolize through different oxidation pathways, such as hydroxylations, carboxylations, desulfations, methylations and amidation to other YTX analogs [Ciminiello et al., 2003; Miles, 2006; Paz et al., 2008].

The main vector of YTXs corresponds to scallops and mussels, which can accumulate a large amount of toxins in their tissues due to the high filtration capacity they have. The hepatopancreas is the main tissue of accumulation and according to chemical modifications caused by mussels, they can spread to other tissues such as mantle, gill and adductor muscle

[Aasen et al., 2005]. Thus, YTX-toxins have been found in gastric glands of mussels, associated with several enzymatic metabolism derived toxic forms, of which new forms have been identified so far such as 45-hydroxyYTX, 1a-homoYTX, 45-hydroxy-YTX and 45-hydroxy-1-homoYTX (Figure 4) [Suzuki & Quilliam, 2011]. Each of them present a specific toxicity equivalence factor (TEF), YTX= 1.0 (TEF); 1A-homo-YTX = 1.0 (TEF); 45-OHYTX = 1.0 (TEF) and 45-OH-1ª-homo-YTX = 0.5 (TEF) [EFSA, 2008b].



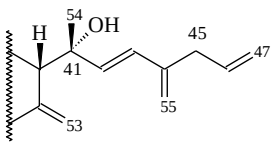
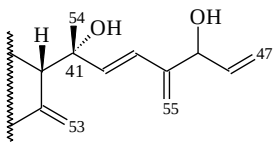
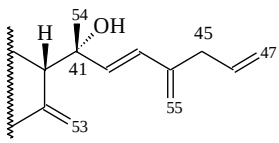
Name	R^1	R^2	n
Yessotoxin (YTX)		H	1
45-Hydroxy-YTX (45-OH-YTX)		H	1
Homo-YTX (1a-Homo-YTX)		H	2

Figure 4. Chemical Structure of the Yessotoxins-group and their chemical analogs identified in dinoflagellates and bivalves. **R** indicates the substitution of the corresponding radical in the molecule.

When mussels are exposed to this type of bloom composed of yessotoxin-producing dinoflagellates, they absorb algae and the YTX-toxic content is rapidly oxidized via enzymatic action- to 45-OHYTX to then be slowly transformed into carboxy-YTX, an isomer which is more typical in the depuration phase of mussels [Ciminiello et al., 1999; Ciminiello et al., 2000; Ciminiello et al., 2001].

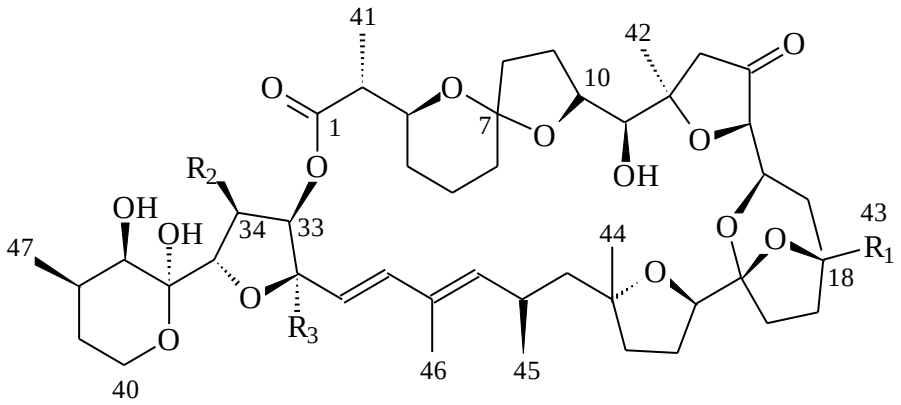
2.3. Pectenotoxins Group

The Pectenotoxins group (PTXs group) are a family of polyether macrolide toxins (Figure 5) which are produced by the same species that produce the toxic forms of OA-group, namely: *Dinophysis fortii*, *Dinophysis acuta*, *Dinophysis acuminata*, *Dinophysis caudata* and *Dinophysis norvegica* and in heterotrophic dinoflagellates such as *Protoperodinium divergens*, *Protoperodinium depressum* and *Protoperodinium crassipes* [Miles et al., 2004; Li et al., 2010]. Their names come from the name of the shellfish were each of them was first found. For example, Pectenotoxin-2 (PTX-2) was isolated from the Japanese scallop *Patinopecten yessoensins* [Yasumoto et al., 1985]. To date, however, numerous PTX analogs have been found in algal cultures, shellfish and blooms associated with dinoflagellates that produce this kind of toxin.

Several new isomeric forms of PTX-2, such as PTX-4, PTX-6 and PTX-7, have been found in natural blooms (Figure 5). Also, when filtered by shellfish and metabolized in the hepatopancreas (digestive glands) they undergo biotransformation. PTXs have been reported in Ireland, Croatia, New Zealand, Portugal, Norway, Japan and Chile [Krock et al., 2009a; Dominguez et al., 2010; García et al., 2012]. To differentiate isomeric forms of PTX-2 found in dinoflagellates from those found in shellfish, the nomenclature coined the term PTX-seco acid (PTX-SA) [Dominguez et al., 2010]. Toxic isoforms such as Pectenotoxins-2 (PTX- 2), Pectenotoxins-2 seco acid (PTX2sa) and 7-epi-Pectenotoxins-2 seco acid (7-epi PTX2sa) are the predominant toxic forms found in Europe [Vale & Sampayo, 2002]. To date, 15 forms of toxic analogs of PTX have been described. The last identified isomers esterified forms of PTX-2A-SA (37-O-acyl-ester-PTX-2-SA, 11-O-acyl-ester-PTX-2-SA and 33-O-acyl-ester-PTX-2-SA) were found only in shellfish but not in PTX producing dinoflagellates [Miles, 2006; Aasen et al., 2006].

The coextractability of PTXs with diarrhetic toxins of okadaic acid group led PTXs to be included with okadaic acid analogues in the OA group toxins [Suzuki et al., 2005; Miles, 2007]. However, PTXs are now classified as a separate toxin group, on the basis of their toxicity, thus for example, PTX1 and PTX6 do not inhibit PP2A. Consequently, based on the association of symptoms and the toxic ability of mussels, it has been determined that the amount sufficient to cause toxic symptoms is 2.0 μg PTX-2-SA/Kg for a 70 kg person [Burguess & Shaw, 2001].

Available information on PTXs is still insufficient with just a few biological results. These few results are from *in vitro* and *in vivo* experiments, aiming to provide information about the risks to human health that ingestion of these toxins may pose [Dominguez et al., 2010]. PTX toxicity after intraperitoneal (*i.p.*) administration and after oral ingestion is considered comparable. For example, hepatic damage in mice after *i.p.* administration and oral ingestion seem to be the same. However, diarrhea was never reported in those experiments [Miles et al., 2004]. In addition, PTX-2 has been found to be potentially citotoxic in lung, colon and kidney carcinogenic cell lines, an effect that cannot necessarily be extrapolated to its toxic forms, such as PTX-2-SA and 7-epi-PTX2-SA. This shows the importance of the initial structure of toxins that initiate citotoxic effects [Jung et al., 1995], where 7-epi-PTX-2-SA is the result of interconversion of PTX-2-SA to thermodynamically more stable forms and may be understood as a protective detoxification effect by mussels [Suzuki et al., 2001; Miles et al., 2004].



Name	R^1	R^2	R^3	C-7
Pectenotoxin-1 (PTX-1)	CH_3OH	H	H	R
Pectenotoxin-2 (PTX-2)	CH_3	H	H	R
Pectenotoxin-2b (PTX-2b)	CH_3	H	H	S
Pectenotoxin-3 (PTX-3)	CHO	H	H	R
Pectenotoxin-4 (PTX-4)	CH_2OH	H	H	S
Pectenotoxin-6 (PTX-6)	COOH	H	H	R
Pectenotoxin-7 (PTX-7)	COOH	H	H	S
Pectenotoxin-11 (PTX-11)	CH_3	OH	H	R
Pectenotoxin-11b (PTX-11b)	CH_3	OH	H	S
Pectenotoxin-13 (PTX-13)	CH_3	H	OH	R

Figure 5. Chemical Structure of Pectenotoxins-group and their chemical analogs identified in dinoflagellates and bivalves. **R** indicates the substitution of the corresponding radical in the molecule.

To date, no case of intoxication by PTX-type toxins has been reported. However, biotransformation of PTX-2 has been found in some shellfish species and these toxic forms have been detected with increasing frequency [Miles et al., 2004].

Studies from various toxins have determined a difference in the profiles from producing dinoflagellates and in the bivalve profiles, establishing that the metabolic transformation occurs in mussels after ingestion of toxic algae [Suzuki & Quilliam, 2011]. Through standard incubation periods (24 h/ digestive gland), it is shown that the biotransformation of PTX-2 toxin has two stages:

1. Assimilation and biotransformation
2. Distribution of biotransformed toxins to the different tissues.

Based on the results obtained, it has been determined that this process occurs exclusively in the digestive gland at pH 7.5, which is related to the environmental conditions of its habitat (the south of Chile). In addition, it has been shown that the biotransformation kinetics of PTX2 is low (23.98%) and that, therefore, sandy bottom-dwelling species (*Venus antiqua*) do not represent a health risk, since the final product is only associated with non-visceral tissues (foot), in which no biotransformation of the PTX-2 toxin is produced and whose identification is only related to biotransformed toxins coming from the distribution from the digestive gland of the bivalve [Oyaneder et al., 2014].

2.4. Azaspiracid Toxins Group

The AZA-group corresponds to toxins produced by toxic dinoflagellates, *Protoperidinium crassipes*, *Azadinium spinosum* and the sponge, *Echinochlathria* sp. [James et al 2004; Tillman et al., 2009; Potvin et al., 2012; O'Driscoll et al., 2014], although direct evidence between the high level of cells that produce AZAs and the concentration of toxins in shellfish that filter them, has not been yet provided [Krock et al., 2009b]. The first intoxication cases related to this type of toxin happened in Holland in 1965, where the symptoms were initially linked to the presence of OA and DTXs.

To date, 24 different forms of Azaspiracids (AZAs) have been found, the most common forms being Azaspiracid-1 (AZA-1), Azaspiracid-2 (AZA-2) and Azaspiracid-3 (AZA-3) (Figure 6) [Rehmann et al., 2008]. All of them are characterized by having specific toxicity equivalence factors (TEFs) (AZA-1 = 1; AZA-2 = 1.82; AZA-3 = 1.43) [Botana et al., 2017].

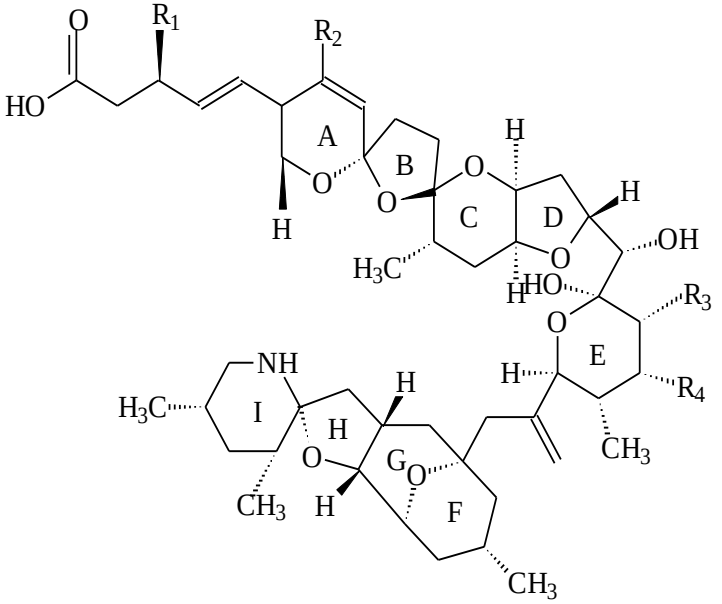
Identification of AZAs in shellfish has been reported in Ireland, UK, Norway, France, Portugal, North Africa (Morocco), Chile and U.S.A. [Amzil et al., 2008; Twiner et al., 2008; Hess et al., 2015]. However, new forms of AZAs are detected every year by the monitoring programs in countries of the EU, which translates to variable toxic profiles, depending on the species that accumulate the toxins and the extent of their biotransformation in the hepatopancreas. These last isoforms of AZAs have only been observed with LC-MS/MS analysis, which has been implemented as an alternative technique in sanitary controls of shellfish [James et al., 2008; McCarron et al., 2011].

Action mechanisms of this type of toxin have not been clearly determined. However, preliminary *in vitro* studies in mammal cells show alterations in the cytoskeletal structure and in the E-cadherin system, responsible for cell to cell interaction [Twiner et al., 2005; Ronzitti et al., 2007]. These experimental results could somehow explain symptoms associated with the described intoxications; gastrointestinal disorders, abdominal pain and diarrhea [James et al., 2002]. Bioassays in mice with shellfish extracts with low concentrations of AZA do not induce diarrhea [Ito & Satake, 2002], however after a while animals show paralysis and respiratory disorders leading to death about 1.5 hours post injection of the extract [Satake et al., 1998]. Further studies have demonstrated that the lethal *i.p.* dose in mice depends on the toxic form present in the shellfish extracts. AZA-1 has a lethal dose of 200 μg kg^{-1}, while isomers AZA-2 and AZA-3 are significantly more toxic with lethal doses of 110 μg kg^{-1} and 140 μg kg-1, respectively [Twiner et al., 2008].

Biotransformation may play a significant role in the number of toxic variations within shellfish. Toxic variations AZA-4 and AZA-5 were found in mussels that originally only had AZA-2 and AZA-3 (Figure 6) [O'Driscoll et al., 2011; Jauffrais et al., 2012]. These biotransformed variations, although less toxic, constitute a source of concern from a public health standpoint [Twiner et al., 2008; Furey et al., 2010].

On the other hand, oral toxicity of AZA-1 at high doses (900 μg kg^{-1}) in mice, produce significant damage to the small intestine, while a 500 μg kg^{-1} dose will only produce hepatic damage, evidenced by a volume increase of the organ (38% post-ingestion) [Ito et al., 2000]. Parallel toxicological studies have established that the lowest observed effect level (LOEL) for AZA lies between 23 and 86 μg kg^{-1} per person, even when levels of 80 μg AZA eq kg^{-1} of shellfish could not produce AZA related intoxication symptoms. However, doses of 30 μg AZA eq kg^{-1} (shellfish kg) in 400 g of ingested shellfish have produced intoxication syndromes in humans. These apparently contradictory data may be explained when

considering the biotransformation produced towards AZA, which generates different toxic forms (analogues) during their assimilation in the mussel (*Mytilus chilensis*) [Hess et al., 2015].



Name	R^1	R^2	R^3	R^4
Azaspiracid-1 (AZA-1)	H	H	CH_3	H
Azaspiracid-2 (AZA-2)	H	CH_3	CH_3	H
Azaspiracid-3 (AZA-3)	H	H	H	H
Azaspiracid-4 (AZA-4)	OH	H	H	H
Azaspiracid-5 (AZA-5)	H	H	H	OH
Azaspiracid-6 (AZA-6)	H	CH_3	H	H

Figure 6. Chemical Structure of Azaspiracids-group and their chemical analogs identified in dinoflagellates and bivalves. **R** indicates the substitution of the corresponding radical in the molecule.

Based on both the toxic information in humans and the biotransformations determined in mussels, EFSA has established that toxic effects in humans may not occur if the concentrations of AZAs do not exceed the levels of 30 μg AZA-1-equivalent kg^{-1} shellfish [EFSA, 2009].

Proteomic studies on the hepatopancreas of *Mytilus edulis* exposed to AZA, indicate that they show an increase in the protein levels associated with the defense mechanisms of mussels, thus establishing that the hepatopancreas plays an important role in the metabolism of toxins [Nzoughet et al., 2009]. The toxicity in mussels contaminated with AZA persists for an average period of five months, which, in some cases, is explained by the high distribution of toxins in the different tissues of the mussel in relation to the digestive gland [James et al., 2002]. Even when AZAs are usually concentrated in the hepatopancreas, these toxins may migrate to other tissues of the mussel (gonads and adductor muscle) given their chemical qualities of zwitter-ion [James et al., 2002; Magdalena et al., 2003].

Under production conditions, mussels contaminated with AZA which are subjected to thermal processes for a short time and in the absence of water, experience high levels of AZA-3, a situation that is repeated by subjecting mussels to low temperatures (4°C) for a prolonged time. In both instances, the toxic precursors AZA-1 and AZA-2 have no considerable variations to the originally evaluated levels, even when the levels of AZA-3 correspond directly to those provided by AZA1/AZA-2. A plausible answer is that toxic contributions come from other sectors of the matrix of mussels or also by bioconversion from unidentified toxins such as AZA-17, which is decarboxyled when subjected to high temperatures, generating AZA-3. A similar situation occurs with decarboxylation of AZA-19, AZA-21 and AZA-23, which tend to form AZA-6 [McCarron et al., 2009]. These important factors are emphasized when considering that multiple production processes of mussels may damage the walls of their internal bodies in order to facilitate the distribution of toxins to other compartments.

3. Composition and Biotransformation of Lipophilic Marine Biotoxins in Mussels

Assessment and identification of lipophilic marine biotoxins (OA, YTX, PTX and AZA groups) and of their described and identified toxic forms resulting from metabolism processes (acyl-derivatives) in shellfish, whether these come from rocky strata or soft bottom habitats, demonstrate that the profiles are always characterized by the predominance of DTX-1 (65% average among species), followed by a toxic variability dependent on the involved species. Hence, the sequence of predominance of toxins in *Mytilus chilensis* is composed by DTX-1>OA>PTX-2>YTX>AZA-1, where each toxin is followed by the toxic forms which have resulted from the metabolic transformations occurred in the tissues of the mussels (Acyl-DTX-1>PTX-2SA>45-OHYTX>7-epi-PTX-2SA>homoYTX>AZA-2>Acyl-OA>AZA-3).
For its part, Ribbed mussels (*Aulacomya ater*) are characterized as having a profile dominated by DTX-1>PTX-2>YTX>AZA-1>OA and their toxins resulting from biotransformation by PTX-2SA>Acyl-DTX-1>45-OHYTX>7-epi-PTX-2SA>Acyl-OA>homoYTX>AZA-2>AZA-3 (Figure 7). Meanwhile, the profile of dominance of toxins in bivalves in soft bottom species (sand), which are part of the infauna, are likewise different. The dominance criteria for *Venus antiqua* is DTX-1>YTX>PTX-2>OA>AZA-1, where Acyl-DTX-1>45-OHYTX>AZA-2>homoYTX>AZA-3>Acyl-OA>PTX-2SA >7-epi-PTX-2SA dominate in its biotransformed toxins (Figure 7).

The main toxic forms of the biotransformation of PTX-2 correspond to PTX-2-SA/7-epi-PTX-2-SA, which may dominate in the variety of PTX in mussels after one month. This dominance is related to the chemical stability of metabolic forms and to the high retention affinity in the mussels' tissues, where this transformation pathway is also related to the protective effects towards mussels. This toxic profile may present strong toxic stratification in mussels from farming facilities because the availability and assimilative capacity of food may vary as the depth increases or decreases.

The identification of AZA-1 in mussels was dominant and also directly related to their interaction with the other aquatic organisms in the related habitat. The presence of AZA-

2/AZA-3 isomers, known toxins structures that result from the bioconversion of AZA in mussels is also established (Figure 7).

The variability in the accumulation of lipophilic toxins in shellfish can be largely explained by the different degrees of hydrophobicity that toxins have in the different species. Their direct relationship in the lipid content must also be considered, where Blue mussels (*Mytilus chilensis*) and Ribbed mussels (*Aulacomya ater*) are dominant species, known for having high and varied concentrations of lipids [Freites et al., 2002; Pazos et al., 2003; Svensson & Förlin, 2004, García et al., 2006; Rossignoli & Blanco, 2010].

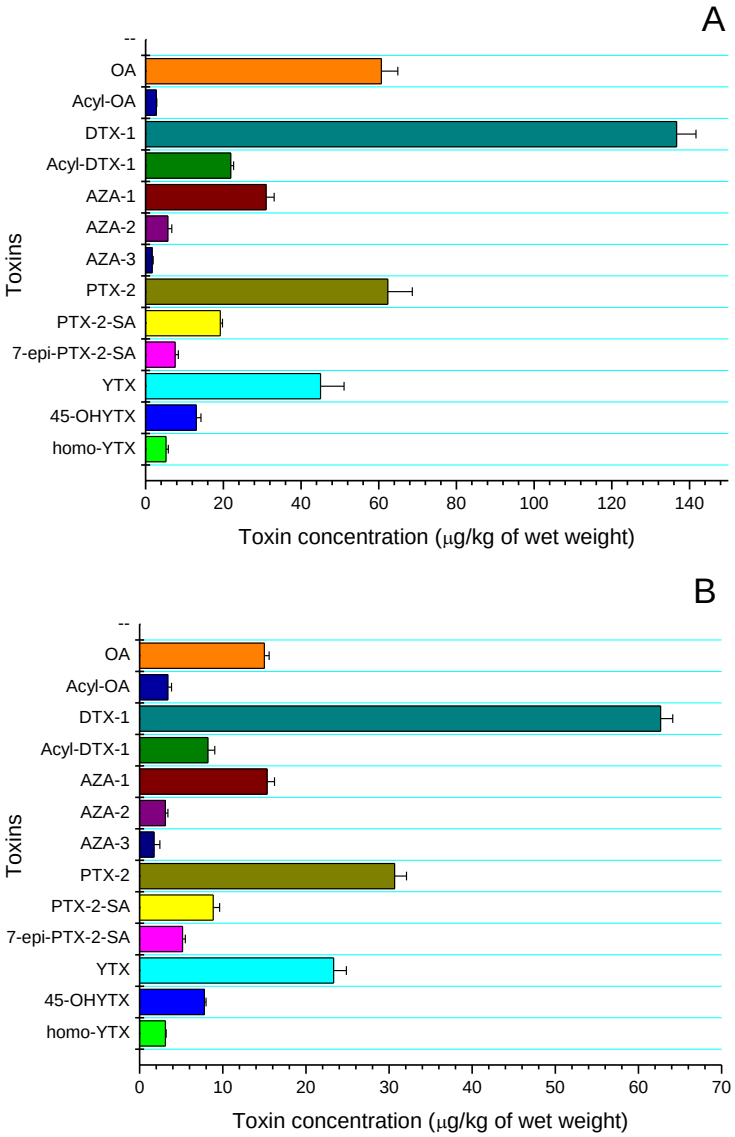


Figure 7. (Continued).

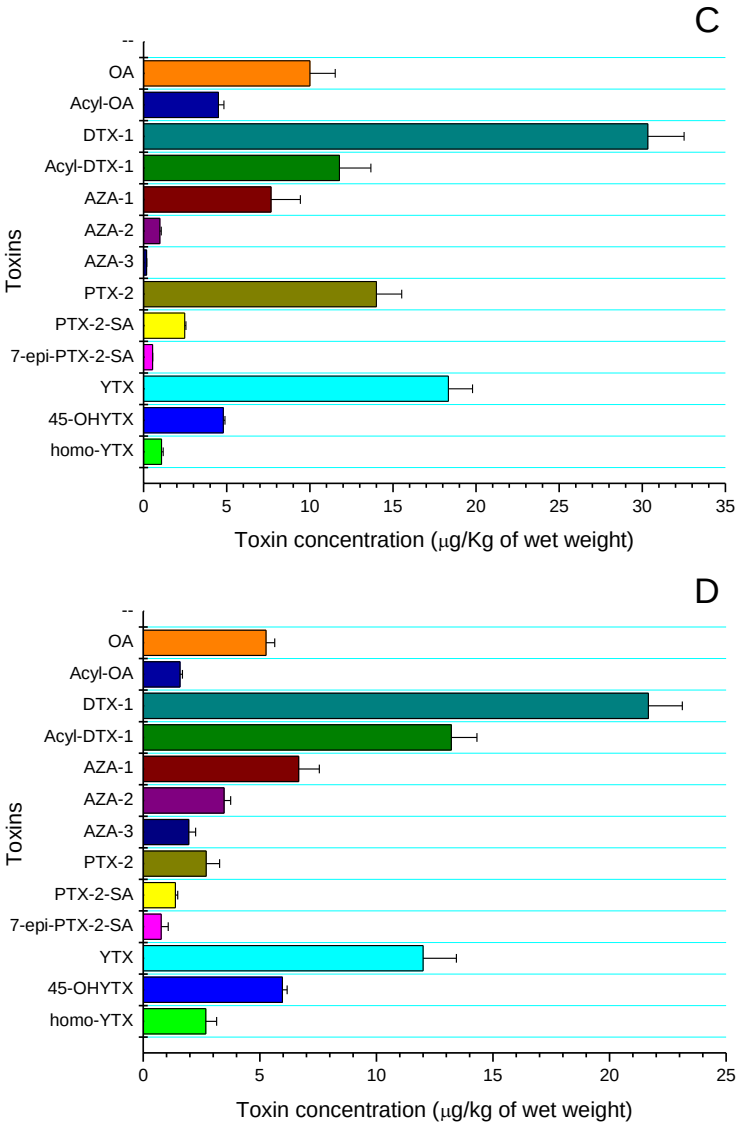


Figure 7. Comparison of lipophilic toxin profiles of bivalve species: A) Blue mussels (*Mytilus chilensis*); B) Ribbed mussels (*Aulacomya ater*); C) Razor clam (*Tagelus dombeii*) and D) the carnivorous gastropod Loco (*Concholepas concholepas*) from the South Pacific Ocean.

The detection of toxic forms resulting from biotransformation, evidences the conversion capability that mussels and habitat-associated species have to modify lipophilic toxins, where the transformation ratio is again associated with the degree of compartmentalization of toxins provided in the bloom and where each of them will be chemically modified according to the degree of exposure of the species to the bloom and also to the degree of retention or purification by the aquatic organism. Such factors may also vary according to the availability of food by filter-feeding organisms, where the diet may be also subjected to food from feces and pseudofeces more directly exposed to the water column, producing a contribution of toxins which were already subjected to metabolic or biotransformation processes by other

species present in the water column (Figure 8A) [Suzuki & Mitsuya, 2001; Miles, 2006; Torgersen et al., 2008a].

The main assimilation tissue of lipophilic toxins in mussels is the digestive gland (hepatopancreas), in a ratio of 7:3 with respect to non-visceral tissues (gonads, gills, mantle and adductor muscle) (Figure 8B-E). This distribution is variable over time due to: a) the modification the profile in each tissue; b) the biotransformation processes; and c) the affinity variables of toxins in the tissues of mussels. The transformation of DTX-1 by mussels favors the accumulation of DTX-3 principally in mantles, which can reach an accumulation level of 90% depending on the period of evaluation after the bloom (≈ 5 months).

Toxic profiles of carnivorous species such *Concholepas concholepas* were characterized as having profiles directly related to the typical diet (*Mytilus chilensis*) and by the high biotransformation capacity they have.

The precise mechanisms of purification of these kinds of toxins in the assessed species are unknown, although the literature suggests a relationship between quality and quantity of available food, together with additional elements such as salinity and temperature conditions, factors that could affect the purification levels of bivalves, and which periods may last as long as 44 days after the bloom occurs [Svensson & Förlin, 2004; Duinker et al., 2007]. However, parameters regarding the chemical configuration of some toxins and their purification levels in bivalve species, establish that esterified forms from OA, DTX-1 and DTX-2 toxic forms are purified faster than free forms (nonesterified forms), depending largely on the involved fatty acid, which directly influences the rate of purification of toxins (≈10-17 days) [Torgersen et al. 2008a].

From the point of view of the contribution of lipophilic marine biotoxins to different species, variability can be explained on the basis of the predominance in the bloom of dinoflagellates species that provide this type of toxin and the profile of toxins provided from cells to bivalves. For instance, the cell concentration of *Dinophysis* sp. in the water column may represent a small fraction of it, since according to the oceanographic conditions, this species is capable of experiencing vertical migration generating growth according to the nutritional needs [Maestrini, 1998; García et al., 2015]. The latter is an equally important point, considering that according to the abundance of food, in this case of toxic dinoflagellates, the filtration rate of the different species of bivalves may experience a decrease in their physiological needs for food on a regular basis [Sampayo et al., 1990].

Furthermore, the determination of the levels of lipophilic toxins has determined the different retention levels that bivalves and gastropods have. Similarly, some species have a higher retention capacity or a greater degree of insensitivity to these toxins, because if they are subjected to repeated blooms, early stages of development of some species could be affected due to the low effectiveness of their detoxification enzymatic processes, thus decreasing bivalves or gastropods populations.

It should be pointed out that these blooms may affect various larval stages of the different studied species (mussels) by affecting the oxygen uptake rate by larvae, which causes a reduction in their capacity for locomotion. This generates a negative effect since it directly affects the migration of different larval stages involved in vertical migration in the water column. This may cause the different larval stages to be exposed to considerable populations of predators (chaetognaths, fishes and/or hydrozoans) affecting the balance of the adult population in a zone of great impact on economically profitable resources and with some endemic cases in the country [García et al., 2013].

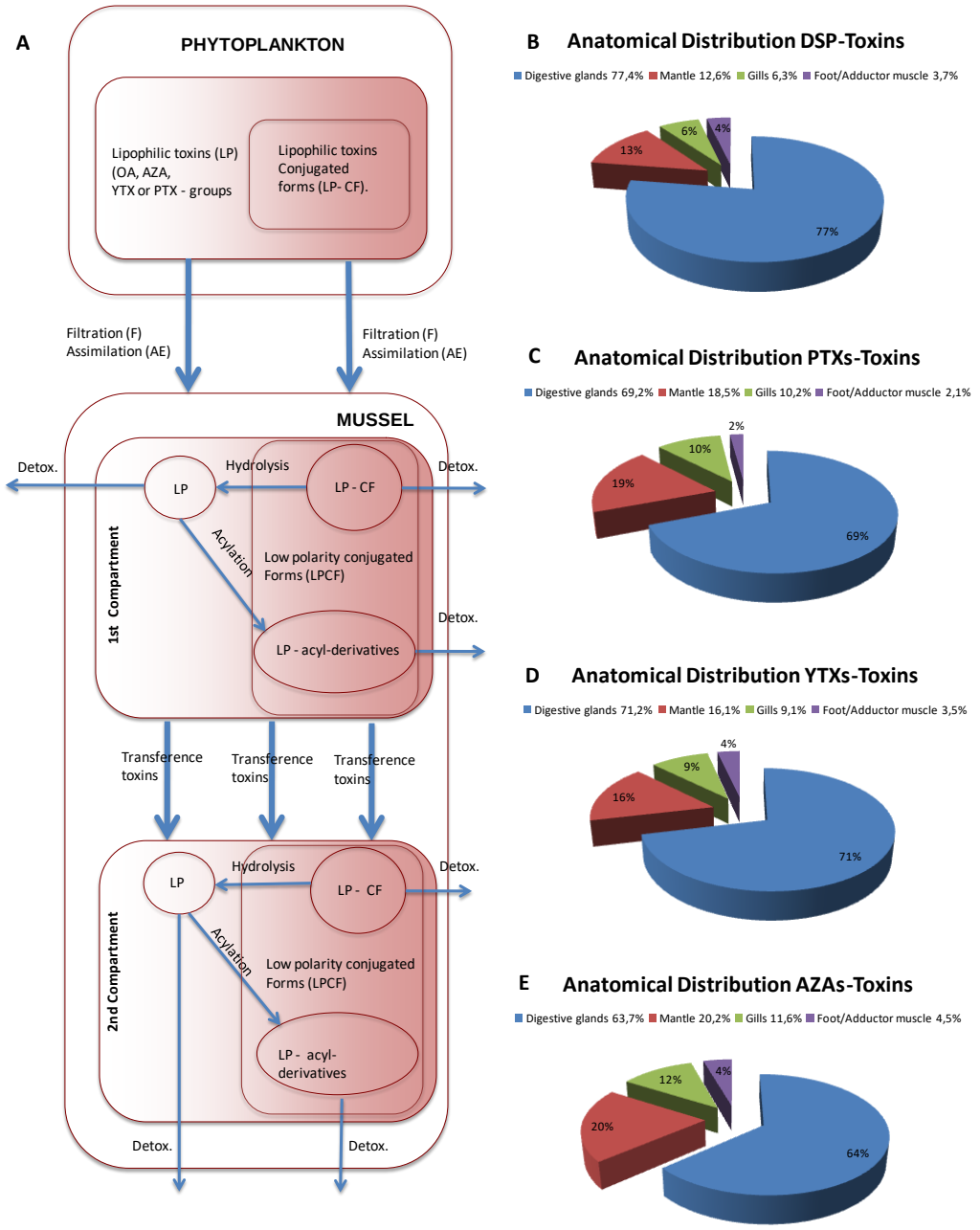


Figure 8. Schematic representation of the distribution, accumulation and biotransformation of lipophilic-toxins from phytoplankton to the mussels' tissues (modified and updated by Moroño et al., 2003). Anatomical distribution (B) OA-group toxins in visceral and non-visceral tissue in mussels; (C) PTX-group toxins in visceral and non-visceral tissue in mussels; (D) YTX-group toxins in visceral and non-visceral tissue in mussels; (E) AZA-group toxins in visceral and non-visceral tissue in mussels.

4. IMPACT OF LIPOPHILIC MARINE BIOTOXINS ON AQUACULTURE DEVELOPMENT IN THE SOUTH PACIFIC FJORDS

The *Mytilus chilensis* samples collected in the area are exclusively destined for aquaculture development in the South Pacific Ocean (Chiloe Island and Austral fjords). Given the constant conditions of annual blooms, these tend to occur in summer periods (October – April), where mussels tend to present variable levels of lipophilic marine biotoxins, which may exceed international standards. The different amounts of each type of detected toxin are associated with the location of the sample in the water column, since each sample is collected at different depths of the shellfish aquaculture lantern and, therefore, exposed to different densities of toxic dinoflagellates. Mussels that are at the lowest depth (10 meters) and in the most superficial positions (1-3 meters from the surface) show the lowest amount of DSP toxins, 67.2 and 33.5 – 36.7 nanograms of DTX-1/gram of digestive gland, respectively. Similar results are obtained in STX-group toxin analysis [Zamorano et al., 2013].

These data show a clear stratification of the lipophilic marine biotoxins in the shellfish samples in the water column. This stratification measured in the mussel extracts obtained from mussels from aquaculture at different water column depths can only be explained by the stratification of the toxic dinoflagellates in the water column (Figure 9).

Another important factor to be considered is that the exploitation of these resources generates production batches that may come from different areas of the same culturing area and also from different depths. In the first instance, this may cause errors in the determination of the profile and toxicities of the exploited mussels, tending to qualify products as free from STX-group-toxins and/or lipophilic-group-toxins.

These data become relevant, considering that 90% of Blue mussels that Chile exports (from Austral fjords) are farmed mussels, whereas exported Ribbed mussels are harvested from wild banks. The notorious differences in toxin levels found are directly related to the filtration capacity of each species [García et al., 2016].

In recent years, the production of mussels has been subjected to the determination of previously unidentified lipophilic marine biotoxins (AZAs and YTX). This impact, that is more relevant during the spring–summer season in this region, may be attributed to the presence of algae, mainly due to the favorable luminescence and nutrient conditions during this time of the year. We must also consider the Chaitén Volcano eruption (Chiloe Island surroundings, 2008), the Aysén Volcano eruption (Austral fjords, 2010), the Calbuco Volcano eruption (Los Lagos region, 2015) and the earthquake–tidal wave in the year 2010. These phenomena could explain the population dynamic change in marine microalgae that would allow the development of dinoflagellates that produce different types of toxins (YTX, AZA and PTX) in areas of higher production of mussels from aquaculture [Zamorano et al., 2013].

In addition, the latitudinal distribution of phycotoxins along the coasts of Chile in the Austral fjords, in which some studies have clearly detailed the presence of isomers of YTX, OA and AZAs groups, has been defined [Trefault et al., 2011]. However, geographical definitions associated with the presence of lipophilic marine biotoxins are an off-shore assessment in certain points but they cannot be considered a representation of the behavior of HABs in Austral fjords, areas where farming centers of mussels are preferably set up. It has been determined that *Dinophysis* sp. may exhibit preferences for certain sea and habitat conditions, which could explain the regional toxic differences in the fjords of the same

species and that result in different initial toxic profiles prior to the consideration of a contribution of a toxins' biotransformation by bivalves [García et al., 2004; Elgarch et al., 2008; García et al., 2010]. Comprehensive assessments on the complex oceanography and geography of the Austral fjords show very different behavior in the presence of HABs, in which local zones (estuaries), that make up the fjord's geography, determine very different levels of *Dinophysis* sp. blooms' behavior in different points [Díaz et al., 2011]. Therefore, individual (oceanographic) characteristics of each fjord make the HABs' behavior very different and unique, or difficult to characterize, among others, by particular physio-chemical parameters in some cases.

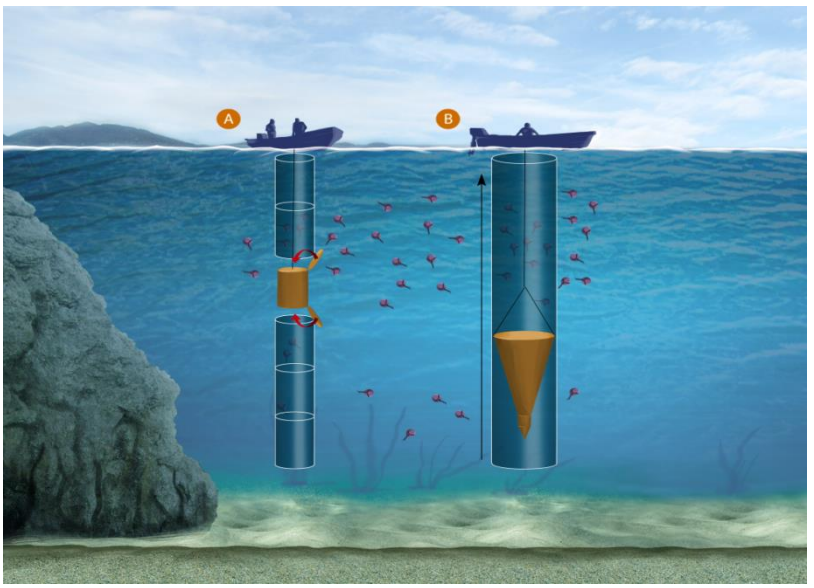


Figure 9. Procedure for identifying harmful algal blooms (HABs). A) Plankton monitoring and B) Toxin monitoring.

The distribution of different *Dinophysis* species (*Dinophysis acuta* and *Dinophysis acuminata*) has been defined as variable and it is directly associated with oceanographic conditions, determining the simultaneous presence of species, with a variability associated with the water column. The characterization of a region composed by multiple fjords is delimited to the transitions from upwelling to downwelling in fjords at different times of the year (inland seas) [Escalera et al., 2012]. Therefore, defining a characteristic condition of a profile or predominance of *Dinophysis* sp. or a toxin in particular, responds directly to the methodological and sampling inadequacy in space-time with which HABs are related in this zone of Austral fjords.

Additionally, lipophilic marine biotoxins act as an exogenous source of oxidative stress, producing reactive oxygen species (ROS) that will damage different aquatic species through the lipid peroxidation (LPO) of biological membranes. Therefore, as they are produced inside the cells, ROS are able to react with all types of biomolecules, organelles (mitochondria) and enzymes (cytochrome P450) [Inoue et al., 2003]. Xenobiotics, such as lipophilic marine biotoxins, can not only cause ROS overproduction, but also alter the antioxidant levels which can lead to an oxidative stress condition [Amado et al., 2009]. Some studies indicate that

dinoflagellates, such as *Dinophysis* sp., cause alteration of antioxidant enzymes and promote the induction of oxidative stress in different tissues of different bivalve species as well as in some fish [Clemente et al., 2010]. Thus, lipophilic marine biotoxins can be bioaccumulated throughout the food chain by decreasing the activity of superoxide dismutase (SOD), GST, glutathione peroxidase (GPx) and causing oxidative stress in different biological structures such as proteins and DNA.

Bivalves tend to be excellent biomonitors and bioindicators of pollution given their special characteristic as sessile benthic filter feeders, so they can accumulate and tolerate high concentrations of chemical pollutants, allowing them to provide reliable measurements over time of the bioavailable portion of total contaminants [Glibert et al., 2014; Oyaneder et al., 2017].

On a physiological level, exposure to harmful algal blooms that generate accumulation of lipophilic mairne biotoxins and ROS toxins for bivalves implies a variability of the closure of shells, adductor muscle paralysis, retraction of the mantle, and production of mucus [Perovic et al., 2000; Hegaret et al., 2012]. Facing this event of algal exposure and lipophilic marine biotoxins, it has been possible to determine a positive correlation detected between SOD and GPxs, which indicates that GPxs are an important pathway of degradation of hydrogen peroxide and that the coordinated action of both enzymes acts against the oxidative damage induced by lipophilic-toxins.

CONCLUSION

Mussels and bivalves exposed to HAB accumulate filtered microalgae in their digestive glands (hepatopancreas) where 70% of the total amount of the detected toxins is present in this organ. From these glands, toxins are subjected to biotransformation mechanisms by three types of pathways:

(a) By bacteria such as *Pseudomonas* sp. and *Vibrio* sp. which are an important part of their environment.
(b) By aquatic organisms which are characteristic of diverse habitats.
(c) By enzymes identified in bivalves which play an important role in esterifying and metabolizing toxins into chemically more stable, easy to purify isomers by mussels.

The mechanisms of biotransformation (hydrolysis and acylation) of toxins present in bivalves lead to the formation of new pseudo-toxic forms. They are directly associated with the stage of excretion of toxins by bivalves where these new pseudo-toxic forms are often not detected by conventional detection methods (bioassays). Their intraperitoneal and oral toxicities are not related, which generates naturally contaminated products as toxin free products, often causing these nontoxic forms to again be transformed into toxic forms during production processes of processed seafood or by enzymatic action in the digestive processes of the human body.

This lipophilic marine biotoxin biotransformation process is relevant because it cannot be detected by conventional monitoring methods (such as mouse bioassay), which then may

classify the tested individuals as toxin free bivalves or could lead to an erroneous definition of the bloom effect depuration processes.

The need to determine the profile of toxins in contaminated bivalves and their biotransformation levels during or after an exposure to a HAB has become extremely necessary in order to define the levels of toxic and chemical modifications of toxins in bivalves subjected to industrial manufacturing processes due to two essential factors:

1. the high variability and transformation probability to which toxins are subjected to enzymatic processes in bivalves which impedes the collection of adequate information about the evolution of toxic events and/or purification processes in bivalves.
2. the difficulty in identifying new conjugated or biotransformed forms by conventional methods such as bioassays, since there is no reference material available in most cases which impedes the identification and quantification of new toxic forms.

Knowledge of the interactions among contaminated shellfish and the evolution of the transformation of toxins in this study may allow for an a efficient risk management of resources to be performed. This will reduce adverse effects from an economic standpoint, as shellfish extraction is more efficient and extended closures as a result of toxic blooms can be avoided.

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Chapter 54

THE EFFECT OF THE NITROGEN-SOURCE MODIFICATION AND THE ADDITION OF CO_2 ON THE GROWTH AND COMPOSITION OF LIPIDS IN *NANNOCHLOROSPIS* SP.

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ABSTRACT

Nannochloropsis genus includes marine unicellular organisms with spherical shape, a parietal chloroplast, and a characteristic pigment composition (with only chlorophyll and high amount of violaxanthin). This genus is used in aquaculture as live feed for fish larvae, rotifers, bivalves, and other organisms, because of its nutritional quality. Recently, some researchers have proposed that this genus can produce triacylglycerols, which can be used to obtain biodiesel based on the fact that under stress conditions, the lipid content in the dry biomass of *Nannochloropsis* can reach 50% and because fresh water is not required for its culture. *Nannochloropsis* usually obtains nitrogen from nitrate, nitrite, ammonium, or urea. The provision of nitrogen from different sources is a strategy used to modify the biomass composition. In this chapter, the effects of the modification of nitrogen source and addition of CO_2 on the growth and lipid content of *Nannochloropsis* sp. are analyzed. Nitrate is the preferred nitrogen source and gives the highest biomass concentration and specific growth rate when compared with urea and ammonium. This effect is higher when the cultures are bubbled with CO_2-enriched air because

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Nannochloropsis does not have an external carbonic anhydrase and depends on the flux of bicarbonate, which is very low when only air is insufflated. Both CO_2 enrichment and nitrogen source modification cause slight modifications in the specific lipid content. The lipid productivity strongly depends on the biomass concentration and the growth rate of the culture. If the objective is the mass culture of *Nannochloropsis*, nitrate is the best nitrogen source. However, it is important to highlight the ability of *Nannochloropsis* sp. to use ammonium, because this permits it to obtain nutrients from wastewater, while delivering high value products (like biomass and lipids).

1. INTRODUCTION

Microalgae are photosynthetic, eukaryotic or prokaryotic, microorganisms that are distributed in diverse environments, such as oceans, lagoons, rivers, and estuaries [1]. The microorganisms in this group grow faster than plants, and do not require fertile lands and potable water for their cultivation. Thus, they can be potentially used for the production of high value compounds, such as carbohydrates, proteins, pigments, and polysaccharides [2]. Their use is favored by the higher biomass production vis-à-vis the plants, with some reports indicating biomass close to an order of magnitude higher than those of crops, like corn and rape, etc. This could cause a huge reduction in the land and infrastructure required for the production of same quantities of biomass [3–5]. The tendency for the production of lipids (oils) is also similar, with higher production compared to that obtained from oleaginous seeds [6–8].

Nannochloropsis is a genus of preferably marine oleaginous microorganisms that have been investigated in the last few years, with lipid contents around 30%; under stressful conditions the content of non-polar compounds (bio-oils) can reach upto 60% [9].

1.1. Microalgae

The term “microalgae” is commonly used to refer to a huge group of microorganisms with the capability of carrying out oxygenic photosynthesis; these organisms do not have a common origin, and are, therefore, considered as a polyphyletic group. They are very similar to plants in terms of their metabolism and accumulate the same compounds as carbon reservoirs. The autotrophic microalgae obtain their energy by the process involving absorption of light, reduction of CO_2, and oxidation of water molecules [1]. Despite the metabolic similarity with plants, microalgae are more efficient in the conversion of solar energy because they have a simple cellular structure. The fact that they are suspended in liquid permits an efficient nutrient supply (mainly of nitrogen, phosphorus, and CO_2) [1, 10].

The size of microalgae varies from 0.2–2.0 μm, and they can grow either submerged or partially exposed to the atmosphere. The aquatic microalgae are distributed in waters that are fresh or salty, and are tolerant to wide fluctuations in pH, temperature, turbidity, and O_2 and CO_2 concentrations [1].

1.2. Eustigmatophyceae

The Class Eustigmatophyceae was appeared upon careful study of the microorganisms that were previously included in the Class Xanthophyceae; differences were observed in the metabolism and pigment composition of some members of Xanthophyceae, based on which researchers decided to include them in a new Class, which was named as Eustigmatophyceae [11–14].

Some members of Eustigmatophyceae have been isolated from samples collected from salty and marine ecosystems as well as from soil samples (Hibberd 1990). At microscopic level, the members of Eustigmatophyceae are coccoid, yellowish-green in color, and almost all the genera have spherical shape with diameters ranging from 2 to 8 μm [12–14]. Their small size and similarity with other genera makes it difficult to classify them only on the basis of microscopic observations, and identification using genetic tools is, therefore, preferred [15].

1.3. *Nannochloropsis*

As of date, six species are included in the genus *Nannochloropsis*. This genus includes isolated elliptical cells (3–4 μm), with parietal chloroplast and a pyrenoid visible under the optical microscope. The vegetative cells have a small red-colored body that increases its size in aged cultures. The organisms included in this genus have a particular pigment composition: chlorophyll a, which is characterized by a high content of violaxanthin, and a low vaucheraxanthine: violaxanthine ratio (compared to that in the members of the Class Xanthophyceae); few species also contain α–carotene [8, 11–14, 16].

The majority of the members of *Nannochloropsis* grow in salty or marine environments. For this reason, the artificial media used for their culture have salinities ranging from 28 to 44‰. These microorganisms use nitrate, nitrite, ammonia, or urea as nitrogen source; normally, the growth is similar when nitrate and ammonia are used and it is usually lower when urea is used. Inorganic phosphate (sodium or potassium) is used as the source of phosphorus [16, 17]. The growth of *Nannochloropsis* remains unchanged in the pH range from 7.0 to 8.5 [18–20].

The carbon absorption in the members of *Nannochloropsis* is carried out by active transporters because there is no extracellular carbonic anhydrase and the absorption of carbon is in the form of HCO_3^-. This process is dependent on light and can be maintained by brief period afterlight exposure (for some minutes) [21]. This system of bicarbonate transport increases the concentration of inorganic carbon inside the cells and the intracellular carbonic anhydrase maintains the equilibrium between CO_2 and HCO_3^- [22, 23].

The microorganisms of the genus *Nannochloropsis* are used in aquaculture, especially in the production of fish larvae, rotifers, and bivalves, as live-feed because of their small-size and nutritional qualities. In the last three decades, the benefits of using these microorganisms as live-feed have been demonstrated, and their use is recommended over microorganisms of other genera, as *Scenedesmus* and *Chlorella* [18, 24]. Some of the most important characteristics that make these microorganisms useful as live-feed are high protein content (which could be higher than 30% of the dry biomass in some strains), presence of all the essential amino acids, high lipid content, and the fact that almost 50% of the fatty acids are

polyunsaturated, with eicosapentaenoic acid (EPA) (20:5 ω–3) being the predominant polyunsaturated fatty acid [25].

Few years ago, microorganisms of the genus *Nannochloropsis* were proposed to be feasible sources of bio-oil for the production of biodiesel owing to their high lipid content and because the majority of their lipids could be esterified [26, 27]. However, the accumulation of lipids is a specific aspect and can vary among different strains. Moreover, the accumulation of carbon sources (lipids or carbohydrates) only occurs when the specific growth rate diminishes, that is, during the stationary phase [15, 28–30].

The members of *Nannochloropsis* preferably accumulate non-polar compounds (lipids) during the stationary phase [6]. The term lipid includes the fraction extracted from the biomass using non-polar solvents, such as hexane and chloroform. The amount of lipid extracted depends of both the solvent employed and the pretreatment (lyophilization, grinding, etc.) that the biomass is subjected to [31]. The lipids of *Nannochloropsis* include mono, di, and triacylglycerols, as well as phospho and glucolipids [6]. Under stressful conditions, the lipid content can reach almost 60% of the dry biomass [15, 28–30]. This chapter describes the analysis of the effect of CO_2 addition and modification of nitrogen source on the growth, lipid content, and productivity of *Nannochloropsis* sp.

2. Effect of Nitrogen-Source Modification and CO_2 Addition on *Nannochloropsis* sp.

2.1. Effect of CO_2 Addition on *Nannochloropsis* Cultures

Normally, the biomass production of *Nannochloropsis* is carried out in airlift photobioreactors (PBR), with either tubular or flat panel configurations. In these kinds of PBRs, the mixing is caused by bubbling air and this permits accumulation of biomass to concentrations ranging from 1 to 2 g L^{-1} [18, 19, 29, 32–34]. In these PBR configurations, the cells are exposed to low shear stress and the cultures are mixed well [35]. However, the CO_2 concentration in air is very low and its supply is not enough to maintain exponential growth for several days for achieving high biomass concentrations. Thus, addition of CO_2 to the gaseous stream favors higher biomass concentrations. Several researchers recommend the enrichment of the gaseous stream with at less 1% CO_2; there have been reports where pure CO_2 was used [29, 36, 37]. For mass culture, the strategy that is most frequently employed is to link the CO_2-supply with the pH value; usually, the introduction of CO_2 initiates when pH reaches 8–8.5 and is interrupted when it is 6.5–7 [30, 32]. This strategy helps in diminishing the loss of CO_2 to the atmosphere.

Under some culture conditions, the effect of CO_2 addition is immediate and very strong. The effect of enrichment of the air bubbled through a culture of *Nannochloropsis* sp. with 3% CO_2 is shown in Figure 1. The enrichment with CO_2 caused an increase in the biomass by approximately 20% in the first 2 days of culture; however, the increase in the biomass was much higher (being 200–400% of the biomass obtained from the culture in which only air was bubbled) from the third day to the end of the experiment. This effect was because of the mechanism used by *Nannochloropsis* for CO_2 absorption. As stated earlier, this microorganism lacks extracellular carbonic anhydrase and the supply of inorganic carbon

depends on the concentration of bicarbonate, which has to be added continuously [22, 38]. The effect is more important considering the fact that the uptake of bicarbonate is activated by light [21]. Several researchers have reported increases in both biomass production and productivity by the addition of CO_2 to *Nannochloropsis* cultures (Table 1).

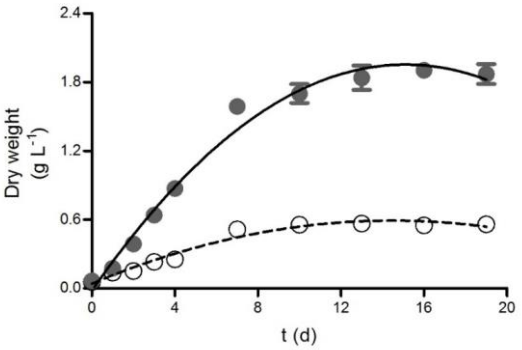

Figure 1. Effect of CO_2 addition in the insufflated stream on *Nannochloropsis* sp. cultures. Closed circles indicate the cultures bubbled with CO_2-enriched air (3% v/v) [76].

Table 1. Biomass production/productivity under different CO_2 conditions in *Nannochloropsis* cultures

Strain	Culturing condition	Biomass production/ productivity	Reference
N. oculata NCTU-3	Air without enrichment	0.268 ± 0.022 (g L^{-1})	[50]
	Air enriched with 2% v/v CO_2	1.277 ± 0.043 (g L^{-1})	
Nannochloropsis sp.	Air without enrichment	~350 (mg L^{-1})	[51]
Nannochloropsis sp. UTEX2379	Air enriched with 2% v/v CO_2	2.1 ± 0.371 (g L^{-1})	[15]
Nannochloropsis sp.	Air enriched with 2% v/v CO_2	40.6–67.3 (g L^{-1})	[44]
Nannochloropsis sp. (CCALA 804)	Air enriched with 3% v/v CO_2	1.36 ± 0.13 (g L^{-1})	[29]
Nannochloropsis sp. (PP983)	0.35 mL CO_2 L^{-1}	457 ± 15.1 (mg L^{-1})	[77]
Nannochloropsis sp. (PP983)	2.80 mL CO_2 L^{-1}	633 ± 27.1 (mg L^{-1})	
Nannochloropsis sp. (PP983)	2.80 mL CO_2 L^{-1}	403 ± 15.2 (mg L^{-1})	[36]
Nannochloropsis sp. (CCALA 804)	pH control from 7.5 to 8.5	183.1 (mg L^{-1} d^{-1})	[32]
Nannochloropsis sp.	pH control	~400 (mg L^{-1} d^{-1})	[20]
Nannochloropsis sp.	pH control at 7.8	450 a 760 (mg L^{-1} d^{-1})	[64]
Nannochloropsis sp.	pH control at 7.6–7.8	~800 (mg L^{-1})	[78]
Nannochloropsis sp.	pH control from 7.6 to 7.8	561 ± 36.5 (mg L^{-1})	[46]

As mentioned above, in mass cultures of *Nannochloropsis*, the most commonly used strategy for CO_2 supply is to link CO_2 addition with pH because the concentrations of bicarbonate, carbonate, and CO_2 are directly related to the pH of the culture medium. This is because of the fact that CO_2 dissolves in water to produce carbonate and bicarbonate ions and carbonic acid, all of which remain in dynamic equilibrium; the proportion of these moieties depends on the pH. In the case of *Nannochloropsis*, the optimum pH is 8.5 because at this pH

bicarbonate is the predominant ion, which provides the inorganic carbon inside the cell in this microorganism; this guarantees both higher growth rate and productivity [20, 32, 39, 40].

2.2. Effect of the Nitrogen Source on *Nannochloropsis* Cultures

The supply of CO_2 is very important to ensure high biomass concentration in *Nannochloropsis* cultures. Nitrogen source is another parameter that modifies the biomass concentration, growth rate, productivity, and the biomass composition. The *Nannochloropsis* species are capable of using different nitrogen sources, most common of which is nitrate; however, the use of ammonia, urea, as well as commercial fertilizers has also been reported [17, 41–49]. In all the cases, the biomass concentration and productivity are related with the strain and the culture conditions that are employed.

Normally, the highest biomass concentration is obtained when nitrate is used as a nitrogen source (Figure 2). However, the effect of nitrate is almost negligible when air is used as a carbon source; the effect is more evident when the insufflated air is enriched with CO_2. This is because the cultures supplied with nitrate and ammonia grow faster and make the differences higher. As shown in Figure 2, the cultures supplied with nitrate and ammonia insufflated with CO_2-enriched air (3%) reached 100 and 200% higher biomass concentrations than the cultures that were insufflated with air. In the comparison between cultures that were supplied with different nitrogen sources (along with enriched air), the culture in nitrate containing medium reached a biomass concentration that was ~80 and ~200% higher than the concentrations obtained in the cultures supplemented with ammonia and urea, respectively. Several studies have shown the capability of *Nannochloropsis* to use different nitrogen sources present in the culture medium. The values for the production/productivity of biomass in the presence of different nitrogen sources are listed in Table 2.

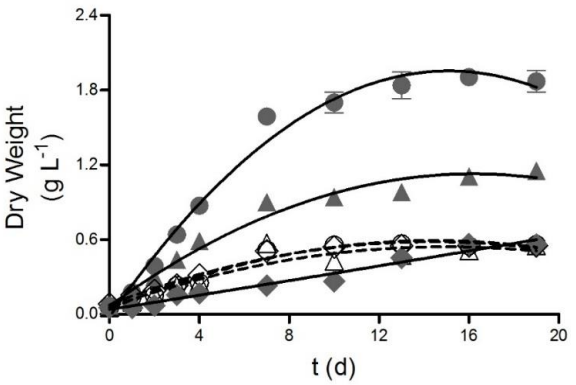

Figure 2. Effect of modification of nitrogen source on *Nannochloropsis* sp. cultures. (●)Nitrate, (▲) Ammonia, (♦) Urea. The closed symbols denote results for cultures supplied with CO_2-enriched air (3% v/v) [76].

Usually, *Nannochloropsis* strains are cultivated in media with nitrate as the nitrogen source [15, 29, 32, 44, 50, 51]. However, recent studies have proposed the use of residuals as the source of nutrients [52–54]. These residuals have diverse composition and in some cases may contain very high levels of ammonia, which can have negative effects on the growth of

the microalga. When wastewater is used as nitrogen sources, it is important to highlight the toxicity of ammonia to the photosynthetic organisms. Tolerance to ammonia toxicity depends on the strain used as well as on the culture conditions [55, 56]. The toxicity of the ammonia is due to the fact that it decouples the electron transport chain in the chloroplast [57].

Table 2. Biomass production/productivity using different nitrogen sources in *Nannochloropsis* cultures

Strain	Nitrogen source	Biomass production/ productivity	Reference
N. oculata NCTU-3	Nitrate	1.277 ± 0.043 (g L^{-1})	[50]
Nannochloropsis sp.	Nitrate	362 (mg L^{-1})	[51]
Nannochloropsis sp. UTEX2379	Nitrate	2.1 ± 0.371 (g L^{-1})	[15]
Nannochloropsis sp.	Nitrate	40.6–67.3 (g L^{-1})	[44]
Nannochloropsis sp. (CCALA 804)	Nitrate	1.36 ± 0.13 (g L^{-1})	[29]
Nannochloropsis sp. (PP983)	Nitrate	633 ± 27.1 (mg L^{-1})	[77]
Nannochloropsis sp. (PP983)	Nitrate	403 ± 15.2 (mg L^{-1})	[36]
Nannochloropsis sp. (CCALA 804)	pH Control from 7.5 to 8.5	183.1 (mg L^{-1} d^{-1})	[32]
Nannochloropsis sp.	Nitrate	~6 × 10^6 (cells mL^{-1})	[79]
	Ammonia		
	Ammonia + Nitrate		
N. salina *(strain 1776)*	Nitrate	~4.5 × 10^6 (cells mL^{-1})	[80]
	Urea	~7.5 × 10^6 (cells mL^{-1})	
	Ammonium chloride	~3 × 10^6 (cells mL^{-1})	
	Nitrate + Ammonia + Urea	~4 × 10^6 (cells mL^{-1})	
N. gaditana (CCMP 527)	Nitrate	~900 (mg L^{-1})	[81]
	Nitrate + Urea	~100 (mg L^{-1} d^{-1})	
	Urea	~50 (mg L^{-1} d^{-1})	
N. salina (CCAP 849/6)	Effluent from anaerobic digestion	~100 (mg L^{-1} d^{-1})	[52]
N. salina (849/6)	Effluent from anaerobic digestion	~400 (mg L^{-1})	[53]
N. salina (CCAP 849/6)	Effluent from anaerobic digestion	91.9 ± 2.1 (mg L^{-1} d^{-1})	[54]
Nannochloropsis sp. F&M-M26	Nitrate	210 (mg L^{-1} d^{-1})	[28]
Nannochloropsis sp. F&M-M27	Nitrate	200 (mg L^{-1} d^{-1})	
Nannochloropsis sp. F&M-M24	Nitrate	180 (mg L^{-1} d^{-1})	
Nannochloropsis sp. F&M-M29	Nitrate	170 (mg L^{-1} d^{-1})	

Sometimes, the bioprocesses of microalgal biomass production are evaluated with regard to the final biomass concentration. However, ideally the evaluation must be carried out by comparing the values of biomass productivity, because this parameter considers not only the biomass produced but also the time used in the process [29]. Usually, the biomass productivity values achieved in *Nannochloropsis* cultures range from 100 to 300 mg L^{-1} d^{-1} (Table 1).

It is important to highlight the fact that biomass productivity is not linear or constant; the maximum biomass productivity is attained in the middle of the exponential growth phase (Figure 3); thereafter, it diminishes with the beginning of the deceleration phase. The biomass productivity depends on the specific growth rate (which is maximum in the exponential

phase) that decreases when nutrients (carbon, nitrogen, phosphorus, etc.) and light become limiting [29, 58, 59].

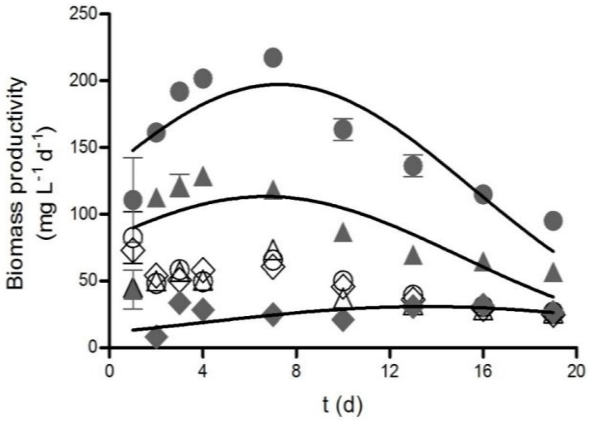

Figure 3. Effect of nitrogen source modification on the biomass productivity in *Nannochloropsis* sp. cultures. (●)Nitrate, (▲) Ammonia, (♦) Urea. The closed symbols denote results for cultures supplied with CO_2-enriched air (3% v/v) [76].

2.3. Effect of Nitrogen-Source Modification and CO_2 Addition on the Content and Profile of Lipids

Several studies have demonstrated that the specific content of lipids (accumulation) is directly related to the growth rate [29] and that the nitrogen source can modify the biomass composition, because the incorporation rate of nitrogen is different for each source [60]. Under standard culture conditions, *Nannochloropsis* has specific lipid content close to 25%; but some stress conditions can cause an increasing in the amount of lipids and the specific content can reach around 55% of the dry biomass (Figure 4 and Table 3). Irrespective of the nitrogen source used, the content of lipids does not change significantly. Only under nitrogen starvation conditions, a significant effect in the percentage of lipids was observed (Figure 4). The starvation phase can be eliminated by the providing stress conditions and the percentage of lipids can be increased. The specific contents of lipid achieved by employing different induction methods in previous studies are shown in Table 3.

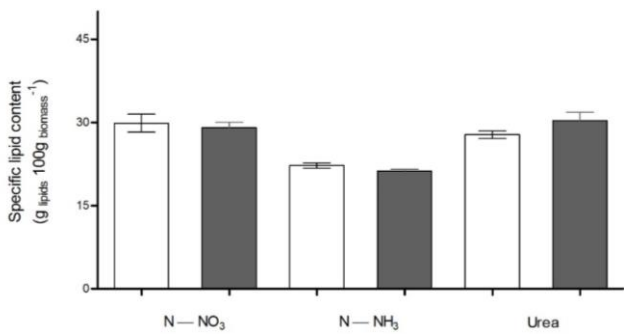

Figure 4. Effect of nitrogen source modification on the specific content of lipids in *Nannochloropsis* sp. cultures. Closed bars indicate the results for cultures bubbled with $C0_2$-enriched air (3% v/v) [76].

Table 3. Effect of stress conditions on the specific lipid content in *Nannochloropsis* cultures

Strain	Nitrogen source and CO_2 supplied	Stress condition	Lipid specific content	Reference
N. oculata NCTU-3	Nitrate and CO_2-enriched air (2% v/v)	Exponential growth phase	~30%	[50]
		Early stationary growth phase	~40	
		Stationary growth phase	~50	
Nannochloropsis sp. UTEX2379	Nitrate and CO_2-enriched air (2% v/v)	40 g L^{-1} of NaCl	~35%	[15]
Nannochloropsis sp. (CCALA 804)	Nitrate and CO_2-enriched air (2% v/v)	13.5 g L^{-1} of NaCl	~30%	[29]
		27 g L^{-1} of NaCl	~30%	
		54 g L^{-1} of NaCl	~45%	
N. gaditana (CCMP 527)	Nitrate and CO_2-enriched air (5% v/v)	Exponential growth phase	~40%	[81]
N. salina (CCAP 849/6)	Effluent from anaerobic digestion	Nutrient starvation	30–35%	[52]
N. salina (CCAP 849/6)	Effluent from anaerobic digestion	Nutrient starvation	20–35%	[54]
Nannochloropsis sp.	Nitrate and pH control at 7.8	Outdoor cultivation	~21%	[64]
Nannochloropsis sp. PTCC 6016	Nitrate and pH control at 7.5	Open pond (200 L)	~50%	[82]
Nannochloropsis sp. F&M-M24	Nitrate, pH control, Green Wall PBR, outdoor conditions	4 days under nitrogen starvation	~60%	[28]
Nannochloropsis sp.	CO_2-enriched air (15% v/v) and 50% of municipal wastewater	High light	~50%	[83]
		High light and nitrogen starvation	~60%	
Nannochloropsis sp. F&M-M24	Nitrate, pH control, Green Wall PBR, outdoor conditions	6 days under nitrogen starvation	~60%	[19]
Nannochloropsis sp.	Nitrate	Mixotrophic culture with glucose	38.7 ± 2.1	[84]
Nannochloropsis sp.	Nitrate	350 µE m^{-2} s^{-1} and medium without nitrogen	~45%	[85]
N. oculata	Nitrate	Green light (520 nm)	~35%	[86]
N. oceánica		Green light (520 nm)	~35%	
N. salina,		Green light (520 nm)	~35%	
N. oculata	Nitrate	30°C	~25%	[87]

Different stress conditions, such as increase in the salt concentration of the medium, temperature, and quality of the light supplied, favor an increase in the lipid content (Table 3). However, such conditions are not useful in the development of a large-scale process because the lipid productivity values reached are very low [15, 29, 61]. Several researchers have reported lipid productivities ranging from 4 to 450 mg lipids L^{-1} d^{-1} depending on the strain employed [15, 20, 29, 61–64]. Nevertheless, the processes with highest lipid content had lower lipid productivity because under stress conditions, the cellular division and the production of new biomass is very low [15, 29]. This is the result of an inverse relationship between lipid accumulation and growth rate [29]. Because of this, recently, some researchers

have proposed intensive lipid production in two stages: the first stage involves the production of biomass under optimal conditions (even in a continuous culture) and in the second stage, the accumulation of lipids is induced by imposing a stress for a short duration. This strategy favors an acceptable biomass and lipid productivities in the process [29, 65–69].

2.3.1. Fatty Acid Profile

Usually, fresh water microalgae (mostly green) have a lipid profile that is rich in saturated and monounsaturated fatty acids but the marine microalgae, such as *Nannochloropsis* species, typically have high amounts of polyunsaturated fatty acids [3, 70]. The lipid profile can be modified by changing the operation conditions of the process. However, in *Nannochoropsis*, modification of the conditions is not much important and the amount of unsaturated fatty acids remains almost constant. The typical fatty acid profiles of different *Nannochloropsis* strains as reported in some studies are shown in Table 4. It is important to highlight the fact that the fraction of saturated fatty acids in all the strains ranges from 19 to 39% whereas the proportion of unsaturated fatty acids is close to 55%.

Table 4. Fatty acid profile reported in different *Nannochloropsis* strains

Fatty acid	Percentage of the total fatty acids (%)						
	Nannochloropsis sp.	*N. salina* CS-190	*N. oculata* CS-216	*N. oculata* CS-179	*N. oculata* CS-170	*Nannochloropsis* sp. UTEX2379	*Nannochloropsis* sp. (CCALA 804)
14:0	7.16	5	3.3	4.6	5.4	6.5	7.9
16:0	23.35	27.8	17.8	14.2	26.0	32.6	27.0
16:1	26.87	31.8	26.6	29.4	22.0	26.1	22.4
18:0		1	0.9	0.6	1.2		4.1
18:1	13.20	8.3	7.7	6.3	5.8	7.7	5.9
18:2	1.21	1.5	2.9	2.0	3.3	2.6	2.1
18:3 (n3 + n6)		0.6	0.7	0.4	1.4		1.3
20:4	2.74	4.0	7.1	8.8	5.5	3.1	3.1
20:5	14.31	24.2	28.4	28.8	24.9	17.8	23.9
Others	11.16		4,6	4.9	4.5	3.6	2.3
Saturated	30.96	33.8	22.0	19.4	32.6	39.1	39.0
Monounsaturated	40.07	40.1	34.3	35.7	27.8	33.8	28.3
Polyunsaturated	18.26	30.3	39.1	40.0	35.1	23.5	30.4
Reference	[88]	[89]		[90]		[15]	[76]

Owing to the presence of high amounts of neutral lipids, recently *Nannochloropsis* strains were proposed to be potential sources of fatty acids for the production of biodiesel [71]. Biodiesel can provide sustainable energy supply, which is needed urgently. It is possible to obtain biofuels, such as hydrogen, bioethanol, and biodiesel from microalgae [72, 73]. In particular, biodiesel can be obtained through esterification of the triacylglycerols present in the biomass [6, 7]. The production of biodiesel from *Nannochloropsis* is not easy because the

fatty acid profile of the strains is not appropriate for its production. *Nannochloropsis* contains large amounts of polyunsaturated fatty acids, mainly EPA, which can reach 20–25% of the total lipid content under some conditions. The biodiesel produced from oil containing high amounts of polyunsaturated fatty acids does not fulfill the quality parameters required by the international organizations [70].

For the production of biodiesel from oil, it is necessary to perform transesterification of fatty acids, irrespective of the source of oil (wasted oil, vegetal oil, or lipids obtained from microalgae). The fatty acids methyl esters (FAMEs), typically methyl, ethyl, and propyl esters, formally constitute the biodiesel, the quality of which can be modified by changing the characteristics of the oil used for its production [6]. Some of its characteristics can be altered by changing the proportion of the polyunsaturated fatty acids; the most important characteristic is the oxidation stability because the double bonds are reactive sites susceptible to be attacked by free radicals. The higher is the number of unsaturated bonds, the easier it is to degrade the biodiesel [70]. The standard EN 14214 of the European Committee for Standardization refers to the maximum proportion of unsaturated fatty acids (measured as linolenic acid methyl ester, 18:3) being lower than 7.5%. All the reports included in Table 4 show that the amount of unsaturated fatty acids in *Nannochloropsis* is higher than 18%, because of which the biodiesel produced from the lipids of *Nannochloropsis* does not reach the European normativity.

It is important to highlight the fact that the lipids of *Nannochloropsis* can be used for biodiesel production if a hydrogenation process is included before the transesterification reaction [72]. Although the lipids of *Nannochloropsis* are not ideal for biodiesel production, this microalgae has continuously been proposed as an option because it grows in salty and marine water and could be an endless source compared to the microalgae that flourish in fresh water [6, 7, 72]. The oil obtained from *Nannochloropsis* can also be used as a source of EPA in animal feedstock as well as for human nutrition [74]. The potential of *Nannochloropsis* as a source of fatty acids is very important but the production of biomass must be performed in a biorefinery with the objective of obtaining the highest benefits (in terms of the production of carbohydrates and pigments, polysaccharides, etc.) from the biomass, favoring economic feasibility of the entire process [75].

CONCLUSION

Nannochloropsis is a genus of microalgae, capable of growing in marine water, and has high amounts of lipids when cultured under standard conditions. For the mass culture of these organisms, a sufficient amount of CO_2 is necessary because the entry of inorganic carbon inside the cells is not efficient. For large culture volumes, the most useful strategy for CO_2 supply is to link pH to the operation of the CO_2-valve. The pH must be from 7 to 8.5 to ensure an optimal condition and a sufficient quantity of carbon in the medium.

This genus can achieve specific lipid content, higher than 50% of the dry biomass, under stress conditions imposed on the culture. However, under such conditions, the productivity (biomass or lipids) of the process is very low. For the development of a large scale process, it must be performed in two stages. The first stage should be for biomass production under optimal conditions and sufficient supply of nutrients (even in continuous cultures), which

should be followed by the induction stage, employing a stress condition, over a short period of time, that could cause an increase in the lipid content. According to some recent reports, this stress condition could be an increase in salinity achieved by the evaporation of the water if open systems are employed for the cultivation of *Nannochloropsis*.

The oil obtained from the biomass of *Nannochloropsis* can preferably be used in animal feedstock because it contains almost 25% EPA. However, when the objective is the production of biodiesel, the lipids must be hydrogenated before their transesterification for attaining the international standard and for increasing the oxidation stability, consequently prolonging the useful life of the biodiesel.

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Chapter 55

POTENTIAL OF *NANNOCHLOROPSIS* IN BETA GLUCAN PRODUCTION

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ABSTRACT

Microalgae are an important source of beta glucans (βG). Its content varies from 0.96 to 411.9 pg·cell-1. It has been proposed to microalgae, for example *Nannochloropsis*, as potential candidates for the production of these compounds at industrial levels for the following: the molecular structure of their βG are similar to so called biological response modifiers, industrial scale microalgae biomass can be obtained through photobioreactors and it can modify the composition of microalgae via increasing carbohydrates fraction. Therefore, in the present work the βG content was evaluated in three strains of *Nannochloropsis* (Ochrophyta, Eustigmatales) and a commercial

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microalgae concentrate of *N. oculata* (Nanno 3600®, Reed Mariculture Inc). Cultures of the three strains (Keys: NN-X-1, CIB76 and NpUNAM) were performed in batch systems (n=9) and cell division rates (μ) were registered to identify the stationary phase (Σμ) of growth curves (NN-X-1: 14d, CIB76: 18d & NpUNAM: 22d) which was the reference for obtaining lyophilized biomass that was used to determine the concentration of βG through the enzymatic method (Megazyme®; K-YBGL 12/16). *Nannochloropsis* strains had 23-31% dry weight as carbohydrates, of which 14-21% were βG. The individual content of βG is ~1 pg·cell-1, with a maximum volumetric productivity of 81.55 mg·l-1. However, although the presence of βG in *Nannochloropsis* cells has been demonstrated, molecular characterization is not yet available. Thus, common methods for characterization of βG from diatom and non-diatoms microalgae that can be applied for this purpose are described. In conclusion, *Nannochloropsis* (particularly NpUNAM) has great potential for the industrialized production of βG and because of its probable molecular characteristics, it can be estimated that there are potential applications as an immunostimulant. Future research is necessary to characterize βG isolated to *Nannochloropsis*, optimize its production and assess its capacity as an immunostimulant.

Keywords: microalgae, carbohydrates, beta glucans, immunostimulants

1. INTRODUCTION

Microalgae are highly diverse photosynthetic microorganisms that play an important role as primary producers in aquatic food chains. These organisms convert inorganic substances into organic matter rich in lipids, proteins, carbohydrates and other molecules, so they are potential sources of natural components with biotechnological and therapeutic applications [1–3].

An important group of microalgae are the *Nannochloropsis* spp. (*Nannochloropsis*) species. This genus belongs to the Eukaryota empire, Chromista kingdom, Ochrophyta division, Eustigmatophyceae class, Eustigamatales order and family Monodopsidaceae. At present, there are 12 nominal species, of which only 5 are recognized as valid species: *Nannochloropsis australis*, *Nannochloropsis granulata*, *Nannochloropsis limnetica*, *Nannochloropsis oceanica* and *Nannochloropsis oculata*. These non-motile unicellular microalgae are components of marine and freshwater picoplankton. Its cells are spherical, slightly oval (2-4 μm in diameter) or ciliary (3-4 x 1.5 μm), with chloroplast of green-yellow color [4]. Chlorophyll a is the only chlorophyll present in this genus, and its main accessory pigment is violaxanthin [5].

Two important characteristics in *Nannochloropsis*, its growth in wide salinity ranges [6] and its high nutritional value due to its high content of proteins and polyunsaturated fatty acids, especially eicosapentaenoic acid [7, 8]. It is for these qualities that *Nannochloropsis* are widely used by industry [3, 9]. For example, in aquaculture they have been widely used as food for molluscs, zooplankton and for early stages life of crustaceans and fish [10].

However, although the nutritional utilization of carbohydrates from biomass *Nannochoropsis* (and is generally applied to microalgae) have not been as essential, they have been considered as the preferred substrate for significant biofuel production (such as bioethanol, biobutanol, Biohydrogen, etc.) through biotechnological conversion technologies [11]. But the greatest potential of microalgal carbohydrates lies in their application as

immunostimulants [9, 12], since they are mostly presented as β-1,3 glucans that can be found both in the cell wall [13] and in cytoplasmic vacuoles [14]. Similar beta glucans (βG) are considered to act as reserves in several classes of microalgae. For example, species of eustigmatophyte class belong to *Nannochloropsis* [13].

The BG are polysaccharides constituted per heterogeneous groups of glucose polymers that have different molecular weights and branching degrees [15]. The name of βG comes from its structure. The suffix "*an*" describes a molecular structure consisting of a single carbohydrate (Glucose), while the prefix "*Gluc*" means polyglucose. Thus, a polyglucose (polyglucopyranose) is called a "glucan." Moreover, β-1,3 glucans, is the most common term for homopolysaccharides link β-1,3 in backbone, but may also possess β-D-glucosidic bonds in position 6 and 4 [15, 16]. These polysaccharides can also be found in a variety of plants, fungi, yeasts and algae [17].

Otherwise, the immunostimulation capacity is due to the fact that molecular structures of βG are characteristic of microbial communities and are therefore called "Pathogen-associated molecular patterns," PAMPs [15, 18]. PAMPs play a role as alarm molecules that activate the immune system, because they are identified through specific pattern recognition receptors (PRRs), such as Dectin-1, TLR2, C3 and Scavengers, present in macrophages, neutrophils and natural killer cells [19]. BG can activate the inflammatory response through binding to their receptors, which activate signaling pathways that culminate in the transduction of intracellular signals and transcription factors [20]. This effect is decisive in the transcription of genes, synthesis and release of preinflammatory cytokines, as well as for activation of different immunological responses associated with the presence of pathogens [15].

1.1. Carbohydrate Metabolism in Microalgae

In microalgae, carbohydrates act as structural components of cell walls and as storage components within cells. Carbohydrates, as storage compounds, provide the energy required for metabolic processes and allow, if required, temporary survival in adverse conditions [21].

Microalgae transform solar energy into chemical energy through photosynthesis [22]. There are two main types of photosynthetic reactions, light and dark. In clear reactions, solar energy is absorbed through pigments of photosynthetic antennas and is used to divide water into protons, electrons and oxygen [11]. Electrons and protons are used to generate energy carriers (NADPH and ATP), which support the metabolic needs of the cell. In dark reactions, carbon dioxide is reduced to carbohydrates via Calvin Cycle inside the chloroplast, using energy derived from NADPH and ATP [23]. In Calvin Cycle, the first step is assimilation of carbon dioxide, which is catalyzed per ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco). Carbon dioxide is used for carboxylation of five-carbon compounds (ribulose-1,5-bisphosphate) in two three-carbon compounds (3-phosphoglycerate), one of which is used as a substrate for formation of carbohydrates, while the other is used to achieve the next Calvin Cycle [11].

The carbohydrate profiles of microalgae can vary greatly, but their main monosaccharides are glucose, galactose, mannose and ribose, with others in varying proportions [24]. Although, *Nannochloropsis* profile monosaccharide are predominant glucose and mannose, in addition to galactose, ramosa, ribose and fucosa in smaller proportions [25]. Brown et al., [7] reported that *N. oculata* polysaccharides represented

approximately 88% of total carbohydrate, of which 68.2% is glucose and about 4 to 8% is ramosa, mannose, ribose, xylose, fucose and galactose. Recently, Jia et al., [26] demonstrated the presence of eight monosaccharides in *N. oceanica*: glucose, galactose, mannitol, fucose, rhamnose, mannose, ribose and glucosamine, although the first three are more abundant.

As the polymerization process of any biopolymer polysaccharide generation involves three stages; initiation, elongation and termination of the chain. The principal microalgae polysaccharides are β-1,3-glucans, while the most studied are Chrysolaminarin (branched, β-1,3/1,6 glucans) and Paramylon (linear, β-1,3 glucans) [27]. In marine diatoms (*Skeletonema costatum*), carbohydrates produced through the Calvin Cycle are rapidly converted to glucose-1-phosphate and polymerized into glucan per UDP-glucose pyrophosphorylase and glycosyltransferase [28]. It should be mentioned that *Nannochloropsis* species do not synthesize starch [5]. Moreover, the activity of exo-1,3-β-glucanase (exo-Glu) was detected in several diatoms and upregulation of this activity coincided with βG degradation in *S. costatum* [29]. It has been proposed that exo-Glu and endo-1,3-β-glucanase (endo-Glu), and β-glucosidase (BGL) are the main enzymes involved in βG degradation, as shown in figure 1; in *Eustigamotos* cf. *Polyphem,* single-cell soil [30].

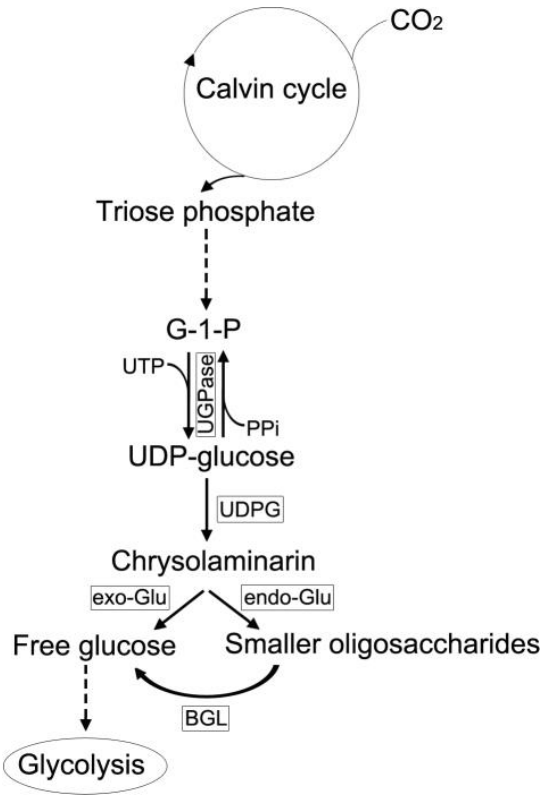

Figure 1. Chrysolaminarin biosynthesis and degradation pathway reconstructed based on the *de novo* assembly and annotation of *E.* cf. *polyphem* transcriptome. Identified enzymes are shown in boxes and include: UGPase, UDP glucose pyrophosphorylase; UDPG, chrysolaminarin synthase; exo-Glu, exo-1,3-b-glucanase; endo Glu, endo-1,3-bglucanase and BGL, b-glucosidases. G-1-P, glucose-1-phosphate; PPi, pyrophosphate (taken from Wan et al., 2012). http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0035142.

1.2. Factors and Strategies to Improve βG Production from *Nannochloropsis*

Culture medium and environmental conditions significantly affect microalgal biomass composition [31]. Therefore, control of some cultivation or environmental conditions seems to be a feasible route for the manipulation of microalgal biomass composition, in order to increase or maximize carbohydrates accumulation [32]. The most frequently reported factors influencing carbohydrate content in microalgal biomass are starvation and/or limitation of nutrients, saline stress, light intensity, temperature and metabolic types [11]. The effects of these factors and reported strategies are described below:

Metabolis Type

There are three main strategies for cultivating microalgae; photoautotrophic, heterotrophic and mixotrophic cultures [33]. Generally, microalgae are cultured through fixation of dissolved inorganic carbon and absorption of light energy; this is a fotoautotrófico culture. In heterotrophic culture, microalgae use organic components as sources of carbon and energy for their growth, without need for light energy. Only some species have this type of metabolism [34]. Moreover, mixotrophic cultures rely on microalgal growth using simultaneously inorganic and organic carbon sources in the presence of light [35]). That is, photoautotrophic and heterotrophic metabolism occur simultaneously.

Perez-Garcia & Bashan [36] indicate that supplementing organic carbon sources to microalgae heterotrophic culture causes an increase in biomass production and lipid/carbohydrate content in cells. Generally, energy storage molecules, such as lipids and carbohydrates accumulate under heterotrophic and mixotrophic conditions. Therefore, the biomass content of these compounds is greater than under photoautotrophic conditions [37]. However, studies on the effect of mixotrophic or heterotrophic cultures on the reserve carbohydrate content in microalgae, particularly βG, are scarce.

Studies with *Nannochloropsis* have demonstrated that it is able to maintain a mixotrophic metabolism in the presence of organic carbon sources such as glucose, glycerol and lactic acid [38–40]. Some of these organic carbon sources are industrial byproducts of biofuel production [41] and fish silage [42]. In mixotrophic cultures with glycerol as a source of organic carbon, *Nannochloropsis* reaches a higher biomass density and lipid production than in photoautotrophic cultures [43].

Temperature

The effect of temperature on microalgae chemical composition is contradictory. Renaud et al., [44] did not observe significant differences in the biochemical composition of tropical microalgae under stress due to temperature variations. However, other research indicates that the effect of temperature is potentially capable of changing the microalgae biochemical composition. For example, in cultures of *Spirulina* sp, an increase in temperature from 25 to 40°C resulted in a carbohydrate increase of 14 to 21% [45]. It is considered that this response is generated per decrease in protein content, while synthesis of carbohydrates is shown to be stimulated [46]. In diatom *Chaetoceros* cf. *wighamii*, carbohydrates were higher when exposed to lower temperatures [47]. Therefore, the effect of temperature on microalgae carbohydrates accumulation seems to be highly dependent on the strains of microalgae used [48]. However, to date there are no studies of this type in *Nannochloropsis*.

Osmotic Stress

Tolerance to salinity variations differs between microalgae. According to this, microalgae can be grouped into; halophiles (require specific salinity for optimal growth) and halotolerant (with response mechanisms to survive in a highly saline environment) [49]. Variations in salinity influence growth and proximal composition of marine microalgae. These respond to osmotic stress accumulating carbohydrates of low molecular weight, which act as osmolytes, therefore an increase in carbohydrate content is a mechanism of protection against damage caused by osmotic stress [50, 51].

Thus, salinity decline is proposed as a unique way to change the biochemical composition of marine microalgae [52], but the role of salinity in carbohydrate metabolism is variable and dependent on the specific nature of the species and of the cultivation conditions.

Yao et al. [52], reported that *Tetraselmis subcordiformis* in nitrogen limitation and a decrease in salinity (20% normal salinity) strengthened biomass and carbohydrate accumulation, regardless of irradiance. The highest carbohydrate content was 58.2% dry weight. Authors argue that reduced salinity combined with nitrogen limitation generate moderate stress that causes an accumulation of carbohydrates. Therefore they propose that the manipulation of salinity can be applied effectively for an improved production of carbohydrates in marine microalgae, for example *Nannochloropsis*.

Lighting Type and Intensity

High light intensity increases polysaccharides production in microalgae cells [53]. In diatoms, βG are synthesized during day and degraded overnight [29, 54]. Moreover, Tredici et al., [55] demonstrated that carbohydrate content in *Spiulina platensis* grown in interperie, was significantly higher on sunny days than on cloudy days. Friedman et al., [56] reported a 0.6 to 3-fold increase in polysaccharides obtained from *Porphyridium* sp. and *Porphyridium aerugineum* cultures, when light intensity increased from 75 to 300 μmol m^{-2} s^{-1}. Similar results were obtained with *Odontella aurita*, where light intensity of 300 μmol m^{-2} s^{-1} produced close to a 2-fold content of Chrysolaminarin compared to 100 μmol m^{-2} s^{-1} [57].

Conversely, a dominant wavelength of light spectrum (blue and red) decreased the content of carbohydrate in *Isochrysis* and *Chaetoceros* [58, 59]. However, *Nannochloropsis* exposure to a monochromatic blue light source resulted in higher biomass productivity than exposure to green or red light, as well as white [43]. We need to know the effect of monochromatic light on βG production in *Nannochloropsis.*

Nutrient Limitation

The nutrient limiting strategy [32] is considered a feasible approach for microalgae production rich in carbohydrate. This technique is practically accessible because it is relatively easy to control the nutrients in the culture medium. According to Markou et al. [11], most studies discuss nutrient limitation as an effective strategy to modify the composition of microalgae, but omit nutrients of interest from the start of microalgal cultures. So, the results of nutrients' omission are decreased growth rates and low biomass production. Therefore, optimization of nutrient concentration is very important [60]. Limited nutrient optimization has a dual scope: support adequate biomass production and act as limiting factor to control biomass composition [11].

Although several nutrients are in the culture medium, the most studied for their effect as limiting are nitrogen and phosphorus. In diatoms, nitrogen depletion results in a significant increase in Chrysolaminarin accumulation, reaching ~ 80% of the total organic carbon of cells [54]. Conversely, when nitrogen is abundant, the Chrysolaminarin is degraded to support protein synthesis [61].

Furthermore, Jia et al., [26] reported total carbohydrate in *N. oceanica* increased from 5.8 to 17.9% of dry biomass during the first 4 days after abrupt nitrogen depletion, and then increased to 19.1% on day 14. In this same work, it was evidenced that 1,3-β-glucans content, increased from 1.63 to 7.87% on day 8, after stress through nitrogen depletion, to finally decay to 6.28% on day 14. The authors concluded that in abundant nitrogen conditions, βG may be the main carbon storage in these cells, which serves as temporary storage of the same and probably competes for carbon sources with triacylglycerol. Conversely, in depleted nitrogen conditions, positive regulation of β-1,3-glucan metabolic pathways together with pyruvate dehydrogenase-mediated acetyl-CoA synthesis and membrane lipid turnover/ degradation contributed to increased triacylglycerol synthesis.

Similarly, the effect of phosphorus starvation on microalgae biomass composition results in simultaneous accumulation of carbohydrates and lipids, in addition to a decrease in protein synthesis [62, 63]. Carbohydrates are significantly promoted in the starvation of phosphorus and their accumulation is more than lipids [64]. However, in some studies, phosphorus starvation was observed to cause only lipid accumulation. For example, in *Nannochloropsis* F&M-M24 cultures, lipids were increased to 50% [65] and *Scenedesmus* LX1 cultures up to 53% [66].

Supply Inorganic Carbon

Inorganic carbon is added to a microalgae culture either in the form of bicarbonate ($NaHCO_3$) or carbon dioxide gas (CO_2). It has been observed that the addition of these compounds promotes both, production of biomass as well as that of lipids and carbohydrates, in addition to improving nitrogen assimilation [67, 68].

Hsueh et al., [69] observed in *N. oculata* an accumulation of total carbohydrates of 29% of dry biomass per supplying 5% CO_2 in aeration, while using 8% CO_2, 37% of total carbohydrates. This suggests that carbohydrate content, as well as the lipid content, is proportional to CO_2 supply.

Production of βG in Two-Stage Process

Some authors suggest that the best option to obtain high-carbohydrate microalgae biomass is the two-stage process; the first stage of the process results in biomass production under optimum conditions of cultivation and in the second stage, microalgae harvests are exposed to stress conditions in order to alter biochemical composition [11]. Therefore, photobioreactors should be efficient systems for this strategy, since biomass can be obtained massively [70] and are suitable for factors control [71, 72] that may be stressful for *Nannochloropsis* as described above, thus causing the production of biomass rich in βG.

1.3. Molecular Structure and Capacity as Immunostimulants of βG

βG have different molecular structures that generally depend on their origin, for example; βG of oats and barley have linear structures with short stretches of β-1,3 and β-1,4 bonds [73], whereas structures of yeasts (*Saccharomyces cerevisiae*) and basidiomycetes (*Schizophyllum commune*), which apparently correspond to the most active form of βG [18, 74, 75], are composed of β-1,3 glucan bonds with β-1,6 branching, which in yeasts are long and short in fungi (β-1,3/1,6 glucans) [76]. Moreover, bacterian βG present a varied group of structures such as β-1,3 glucans, cyclic β-1,3 glucans, β-1,3/1,6 cyclic glucans and β-1,3/1,2 glucans [77].

The βG microalgal reported structures are β-1,3 glucans, β-1,3/1,6 glucans and β-1,6/1,3, see Table 1. β-1,3/1,6 glucans of marine diatoms are known as Chryolaminarin [78–80] and β-1,3 glucans of freshwater *Euglena gracilis* are named Paramylon [81]. It is recognized as important, knowledge about the quantification and characterization of βG in microalgae species. For example: *Chaetoceros debilis* βG present molecular structure β-1,3/1,6 glucans with molecular weight of 4.9 kDa and 30 polymerization degree [80]. *Chaetoceros mulleri*, the molecule has been found and characterized β-1,3/1,6 glucan configuration structure with 19 polymerization degree and 0.005 branching [78]. Moreover, β-1,3/1,6 glucans with molecular weight of 7.75 kDa were reported from *Odontella aurita* [57]. Additionally, also reported was the isolation and characterization of βG from *Isochrysis galvana* with inverse bonds to diatoms molecular structure; β-1,6 /1,3 [82].

However, molecular characterization has not yet been performed for βG from Eustigmatophyta lineage, and no studies are known regarding storage location of its reserve carbohydrates, even though this group includes important microalgae for industrial applications such as *Nannochloropsis* [84].

A variety of βG have the ability to stimulate the immune system. Therefore, pharmacologically they are classified as biological response modifiers [85]. Also, βG are considered nutraceutical compounds, because their consumption has been related to the prevention and/or treatment of diseases [86]; or as additives for management of aquatic animal health [87].

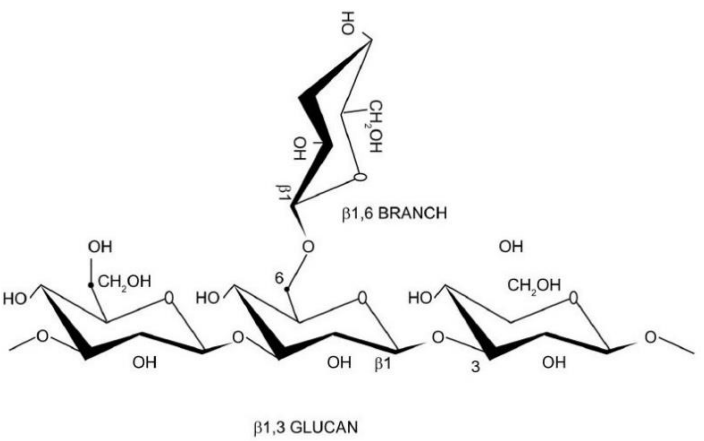

Figure 2. Molecular configuration of β-1,3/1,6 glucans (modified from [83]). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2704234/.

The immunostimulating effect of βG is dependent on several factors related to molecular structure [88]. The most effective molecular weight of clinically useful βG ranges from 100 to 200 kDa, with branching degree between 0.2 and 0.5 [85]. Moreover, in aquaculture, βG with reported immunostimulatory effects are mainly yeast, fungal or microalgae isolates, and have lower molecular weights ranging from 5 kDa of seaweed *Laminaria digitata* [89, 90] to 200 kDa of yeast *Saccharomyces cerevisiae* [91]. The molecular weight interval of principal βG microalgal, Chrysolaminarin, is 4-40 kDa, Table 1.

Table 1. Characteristics and molecular structures of microalgal βG

Microalgae	Structure	MW (kDa)	PD	BD	Reference
Diatom					
Chaetoceros debilis	β-1,3/1,6	4.9	30	-	[80]
Chaetoceros mulleri	β-1,3/1,6		19	0.005	[78]
Odontella aurita	β-1,3/1,6	7.75		-	[92]
Thalassiosira pseudonana	-	-	29	-	[14]
Thalassiosira weissflogii	-	-	5 - 13	-	[79]
Stauroneis amphioxys	-	4	24	-	[93]
Skeletonema costatum	(1,2) y (1,6)	6.2	35	-	[94]
Stephanodiscus meyeri	-	40	-	-	[95]
No diatom					
Euglena gracilis	β-1,3	-	-	-	[96]
Isochrysis galbana	β-1,6/1,3	1.2-5.2	7-29	-	[82]
Chorella pyrenoidosa	β-1,2, y β-1,4/1,6	-	-	-	[97]

MW: molecular weight; PD: polymerization degree; DB: branching degree

Therefore, due to important infectious disease scenarios in aquaculture [98], an alternative called immunostimulation has made an application of compounds to increase or activate immune system of organisms in cultures. Such a strategy has the purpose of boosting the immune system and increasing resistance of the host against infectious diseases [74, 75, 99, 100]. The central axis of these strategies are immunostimulating agents, defined as those natural compounds that modulate the immune system through increasing the resistance of the host to diseases that in a majority of circumstances are caused by pathogens [75]. The result is a change in the number and/or function of cells involved in immune responses. The most proven effect of immunostimulants is to increase functions of phagocytic cells and increase their bactericidal and fungicidal activities [101].

The most promising immunostimulants are β-1,3/1,6 glucans, as they have been extensively studied in different vertebrate and invertebrate species [18]. They have a well-defined chemical structure and their mode of action generates beneficial effects to organisms [15, 102, 103]. Aditionally, their degree of safety has been recognized as generally safe (GRAS) by the US Food and Drug Administration [86].

β-1,3/1,6 glucans, are commonly known as Zymosan when isolated from *S. cerevisiae* and are composed of βG and mannose, and to a lesser extent lipids and proteins [104]. These yeast βG can be found commercially as MacroGard® [105]. But the warning that exists about the problems generated through the impurities of Zymosan should be considered when attempting to describe the molecular mechanisms of immunological responses. When Zymosan is given in animals, it induces a wide range of responses [18, 102, 103], and it is very difficult to identify which component or structure of this compound is inducing what

effect [88]. There are other βG from different sources that present the same structure as Lentinan, Schizophyllan and Chrysolaminaran, and may not present the same problem.

Nevertheless, due to its structure, it has been noted that Zymosan can simultaneously induce two of the major types of PRRs during induction to inflammatory response; C-type lectin receptors (Dectin-1) and Toll-like receptors (TLR2) [106]. It also demonstrated preferential binding of β-1,3/1,6 glucans of fungal origin to same receptors [107] generating responses to mediators of inflammation [108]. Based on the above, it would be possible to obtain similar immunological responses with β-1,3/1,6 microalgal glucans.

Activation of Dectin-1 per its binding to βG increases activation of NF-κB, through synergistic effect of TLR2, and these receptors act together increasing production of cytokines such as IL-12 and TNF-α [108]. Dectin-1 has been reported to be expressed at higher levels in macrophages and neutrophils, but to a lesser extent in dendritic cells and in subpopulation of T cells [109]. Also, it was shown that Dectin-1 in conjunction with Zymosan, triggers production of reactive oxygen species (ROS). For their cooperation with TLRs, βG can promote responses associated with Th1 immunity (cell-mediated immunity: macrophage activation and B-cells to produce opsonizing antibodies) including production of IL-12 [106]. Such responses are critical for defense against many pathogens [75].

Although investigations on the immunostimulatory effect of microalgal βG are scarce, they are considered as potential immunostimulatory agents. For example, β-1,3/1,6 glucan from *C. mulleri* was evaluated as an immunostimulant of atlantic cod larvae (*Gadus morhua*), obtaining an increase in survival and growth of larvae fed with rotifers enriched with these microalgal βG during first feeding, in comparison to larvae fed with rotifers with and without Zymosan. According to the authors, results are due to the low molecular weight and high branching degree of microalgal βG that generate better immune response [12]. Furthermore, in experiments, the biological activity of β-1,6/1,3 from *I. galvana* directly inhibits proliferation of human lymphoma cells of leukemic U937 monocytes, and therefore is predicted to have high potential anti-tumor activity [82]. Moreover, Russo et al., [110] in vitro results indicate that linear βG from *E. gracilis*, obtained per sonication and alkaline treatment can act as safe and effective coadjutant of innate immune system response, regulating pro-inflammatory factors (NO, TNF-α, IL-6 and COX-2) in lymphomonocytes.

Sletmoen & Stokke [88] suggest that primary structure, solution conformation and/or polymer degree may play a determining role in the ability of βG to bind receptor(s) and modulate immunological functions. To date, most reports related to βG effect on fish have proposed these polysaccharides as potent, valuable and promising immunostimulants, however their effects on growth and modulation of the immune system are dependent on the structure, dose, mode, duration or time of administration, ambient temperature, and species under study [18, 111].

1.4. Characterization of Microalgal βG

The first step to achieve the characterization of microalgae βG is proper extraction of a total carbohydrate extract. This first step is relevant so that the choice of the most appropriate extraction method depends on the source and molecular structure of βG expected to be produced [112]. This is because the nature of the extraction procedure has a significant effect on structure and molecular weight of generated βG [113]. For example, differences in

molecular weight estimates of βG may come not only from its source such as *Nannochloropsis*, but also from influence of methods used for extraction and purification. Commonly, the phenomena of aggregation and depolymerization occur during the extraction stage [16]. Therefore, to determine the structural characteristics of molecular βG isolates, extraction procedures need to ensure the integrity of parent molecules, and optimize yield and purity of βG [114]. Currently, sulfuric acid extraction for diatoms and hot water-ethanol for non-diatoms are the most used method to extract microalgal carbohydrates (Table 2). Other methods reported for different sources are alkaline extraction [115], ultrasound assisted extraction [116], microwave assisted extraction [117], enzymes as a trigger for extraction [118] and acid extraction [119]. However, there are some drawbacks with the extraction methods which have to do with demand for long periods of time per process, high costs and little environmental sustainability [112]. Some of the new extraction methods, such as accelerated extraction of solvents [116] and superheated water extraction [120], are useful to mediate with current problems of extraction, along with higher yields.

Hence, post-extraction and before performing rigorous structural analyzes of βG, it is necessary to estimate its purity. That is, to know the proportion of polysaccharide components and presence of impurities. Total and/or reductive carbohydrate assays, as well as assays for other compounds are commonly used to solve this task. The term "total carbohydrates" covers all mono-, oligo- and polysaccharides as compared to non-carbohydrates (proteins, lipids, aromatic compounds, mineral compounds, etc.) which may be present in carbohydrate extracts. In a pure polysaccharide, concentration of reducing sugars (those having a free carbonyl group at reducing ends) represent amount of terminal reducing groups, depending on polymerization and branching degrees. The next analytical task is estimation of bonds and structures, which are typical for non-target polysaccharides, including some glucans [17].

Subsequently, more rigorous structural analyses of purified polysaccharides are required to clarify their structure. For this purpose, a set of spectroscopic, chemical and separation methods is used. Among them, nuclear magnetic resonance (NMR) spectroscopy is recognized as a powerful tool for the structural analysis of glucans in both solution and solid state. Together with chemilolytic methods (methylation analysis, periodate oxidation, partial chemical or enzymatic hydrolysis, etc.), correlation NMR experiments are able to determine the exact structure of polysaccharides tested. Moreover, spectroscopic methods of infrared vibration are sensitive to the anomeric structure of glucans and can be used for purity control. Molecular weight distribution, homogeneity and branching of glucans can be estimated through size exclusion chromatography, laser light scattering and viscometry [17]. Table 2 shows experiments and NMR analysis reported for the characterization of βG of microalgae.

Finally, βG from *Nannocloropsis*, which is an important species for industry and widely used in aquaculture, has not been quantified or characterized. Therefore it is considered of great importance to evaluate the effect of βG from *Nannocloropsis* on immune response modulation of vertebrates and invertebrates. Hence, this chapter’s aim is to quantify the concentration of βG in biomass harvests of three *Nannochloropsis* strains, and describe the qualities and possibilities that project to species of this genus as potential for the production of βG.

Table 2. Methods used for extraction, purification and characterization of βG microalgae

Microalgae	Extraction	Dialysis (kDa)*	Fractionation	Methods RMN	Reference
Chaetoceros mulleri	Sulfuric acid	1	-	hr-MAS ^{13}C ^{1}H HETCOR COSY	[78]
Thalassiosira weissflogii	Sulfuric acid	1	-	^{1}H COSY	[79]
Chaetoceros debilis	Sulfuric acid	1	-	^{1}H ^{13}C-DEPT135 TOCSY ^{1}H-COSY NOESY ^{13}C-HMQC	[80]
Odontella aurita	Sulfuric acid	-	Columna Sephadex G-200 DEAE-52	^{13}C y ^{1}H	[92]
Thalassiosira pseudonana	Hot wáter	3.5	-	^{1}H	[14]
Stephanodiscus meyeri	Sulfuric acid	1	-	^{13}C	[95]
Isochrysis galvana	Hot water-Ethanol	12-14	Spectrum spectra/por 4 Columna Q-Sepharosa Columna Sephadex G-50	^{13}C y ^{1}H (30 y 80 ºC) ^{1}H H^{1}COSY, TOCSY, ROESY ^{1}H-^{13}C HSQC, HSQC-TOCSY, HMBC	[82]
Chorella pyrenoidosa	Hot water-Ethanol	12	Columna Sephadex G-100	HRESIMS	[97]
Durvillaea antartica	Hot water-Ethanol	12-14	Spectrum spectra/por 4	^{1}H	[121]

* Molecular weight cut-off (MWCO).

2. MATERIALS AND METHODS

2.1. Microalgae Strain and Cultures

The present study was carried out in Laboratory Microalgae of Center Research in Food and Development, A.C. Unit Mazatlan. Three *Nannochloropsis* strains were used and are conserved with the keys: NN-X-1, CIB76 and NpUNAM. NN-X-1 strain was obtained from the microalgae collection of Center Scientific Research and Higher Education (CICESE), Ensenada, Baja California Norte, Mexico. CIB76 is characterized as *Nannochloropsis oculata* and belongs to the microalgae collection of Center Biological Research of the Northwest (CIBNOR), Baja California Sur, Mexico. NpUNAM belongs to the microalgae collection of Marine Biotoxins Laboratory, Institute Marine Science and Limnology (ICMyL-UNAM), Mazatlán, Sinaloa, Mexico.

Batch cultures were done in triplicate (n = 9) in 16 liter polycarbonate bottles. The culture medium f/2 [122], prepared with sea water (34 ups), filtered (1 μ) and chlorinated was used. Culture conditions were: 19.2 ± 1.8 °C, 8.6 ± 0.5 pH and continuous flourescent lighting and

aeration [123]. Cell concentration was determined daily with hematocytometer (0.1 mm depth, with improved Neubauer reglilla, Brighline Optik Labor) and composite microscope (Leica model CME) according to Guillard & Sieracki [124]. The cell density was plotted and the logistic model was applied using theCurveExpert Basic 1.4 software (Pindyck & Rubinfeld, 1981) [125]. The cell division rate (μ) of each strain was calculated according to Nieves et al., [126]. The cultures were sampled in stationary phase of growth curve determined per maximum value of $\Sigma\mu$; Considering this cultures phase as a condition of nutrient depletion, mainly nitrogen and phosphorus, and consequently carbohydrates accumulation in cells.

$$\mu = \frac{\ln(C_{t+1}/C_t)}{\ln 2} \quad (1)$$

Where:

C_{t+1}= Cellular concentration at time t+1

C_1= Cellular concentration at time t

2.2. Determination of Dry Weight and Organic and Inorganic Content

Samples of microalgae were collected in triplicate (n = 27) in the stationary stage to determine dry weight, and organic and inorganic content of strains through the method of incineration proposed by Sorokin [127].

2.3. Total Carbohydrates Determination

Total carbohydrates were determined through phenol-sulfuric colorimetric method by Dubois et al. [128], samples from stationary phase in triplicate of each replicate were analysed (n = 27).

2.4. Enzymatic Quantification of Beta Glucans

A Megazyme® enzyme kit developed for adequate measurement of β-1,3/1,6 glucans and α-glucan in fungi and yeast products (CAT # K-YBGL, 12/16; Lot: 161124-3) was utililized. The existence of a unique method for microalgae is unknown. Triplicate samples of lyophilized biomass (pool of replicates) from stationary phase cultures were analyzed. Additionally, quantification in lyophilized biomass of commercial concentrate of non-viable *Nannochloropsis oculata* (strain CCMP 525; Nanno3600®, Lot: 16267; Reed Mariculture Inc.) was performed and as control, yeast beta glucans were quantified with 49% of purity supplied by the kit (Lot: 130905b). Total glucans were determined using controlled acid hydrolysis (H_2SO_4), and released glucose was determined using glucose oxidase/peroxidase reagent (GOPOD). α-glucans were measured after hydrolysis of starch/glycogen to glucose, and from glucoamylase and sucrose to glucose more fructose with invertase. Glucose was

measured with GOPOD reagent. βG were determined through the difference between total glucans and α-glucans [129].

2.5. Volumetric Productivity of Beta Glucans

A literature review per data βG cell concentration ($pg \cdot cel^{-1}$) and volumetric productivity of βG ($mg \cdot l^{-1}$) of various microalgae species were obtained. The same variable for *Nannochloropsis* strains were calculated using following equations:

$$Cellular\ concentration\ \beta G = \frac{\%\ \beta G \cdot DW}{CD} \quad (2)$$

$$Volumetric\ Productivity\ \beta G = \%\ \beta G \cdot DW \cdot CD \cdot V \quad (3)$$

Where,

% βG= βG percentage through enzymatic determination
DW= Dry weight
CD= Cellular density
V= Volume

2.6. Statistical Analysis

In order to compare total carbohydrates obtained in *Nannochloropsis* strains, percentages were transformed using the arcsine function of the square root [130]. Subsequently, the normality and homogeneity of variance for all variables was tested by techniques Levines and Kolmogorov-Smirnov, respectively. Since data was homoscedastic, tests of variance analysis and Tukey's multiple comparisons were performed [131]. Statistical analyses and growth functions were performed with SigamPlot v11 program.

3. RESULTS

3.1. Growth Curve

The highest cell density and cell division rate were registered on different culture days (d) for strains. CIB76 showed the highest cell density (64.5 x 10^6 $cells \cdot ml^{-1}$ d15), followed by NN-X-1 (63.3 x 10^6 $cells \cdot ml^{-1}$, d13) and NpUNAM (63.2 x 10^6 $cells \cdot ml^{-1}$, d22). Values of cell division rate (μ) were 1.05, 1.0 and 0.98 for CIB76, NN-X-1 and NpUNAM, respectively. Therefore, the beginning of the stationary phase, and consequently sampling day, also were registered in different culture days: NN-X-1, d17; CIB76, d19 and NpUNAM, d25 (Figure 3).

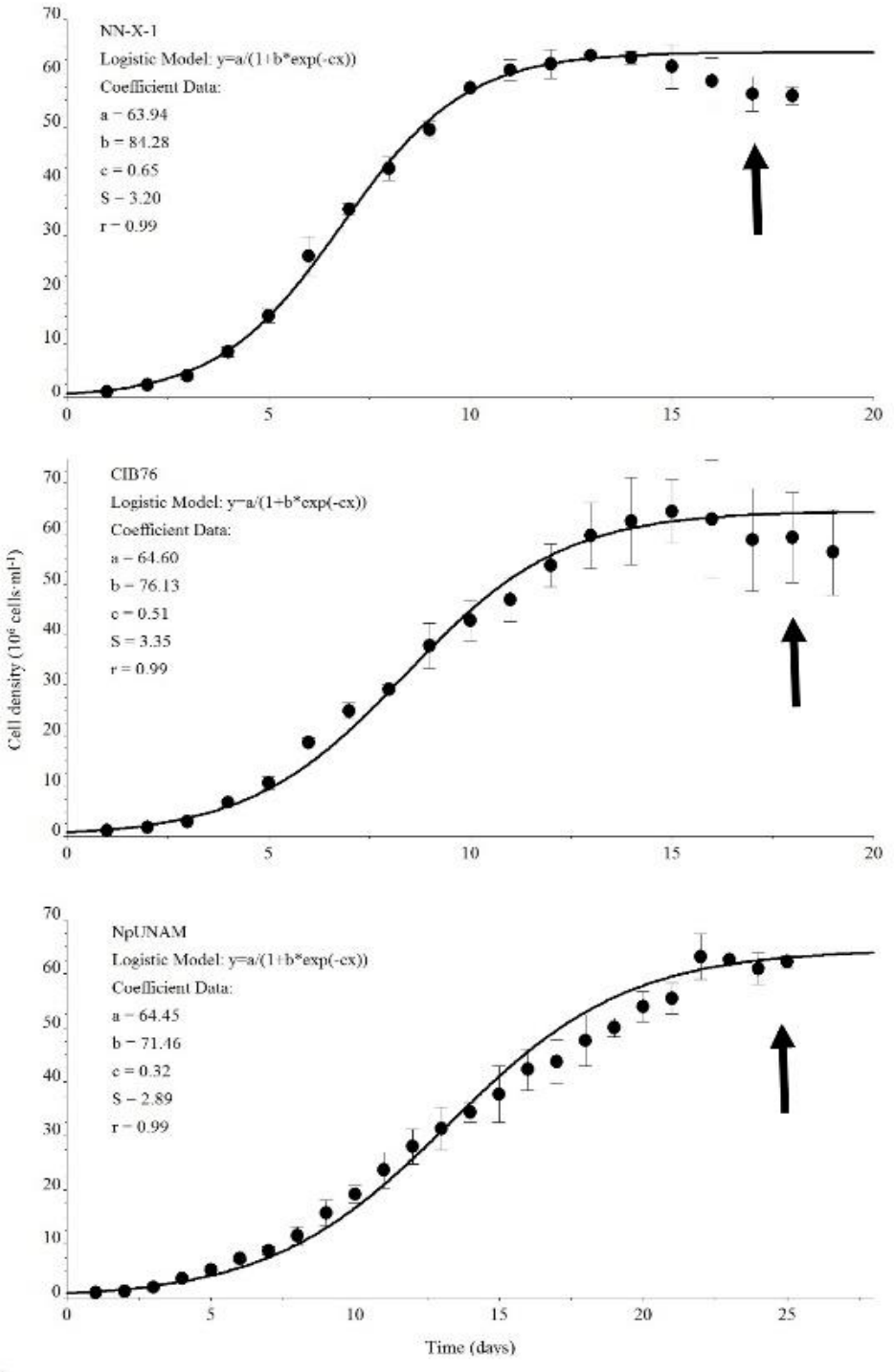

Figure 3. Population growth of *Nannochloropsis* strains. Circles represent the mean values (± SD) of cell density (n = 3), while the line is the approximation determined with a logistic model: a = Maximum value in the curve, b = Maximum load capacity and c = Slope of the curve. The arrow indicates when the culture reached the stationary phase.

3.2. Volumetric Production of Biomass and Carbohydrates Accumulation

Biomass production in total dry weight did not show significant differences ($P < 0.05$) among strains. The highest yield was 0.40 $g \cdot l^{-1}$ obtained with NN-X-1. Cellular organic composition, in terms of individual dry weight, organic and inorganic dry weight and total carbohydrates did not present significant differences ($P < 0.05$) between strains. Percentage of total carbohydrates of NpUNAM 30.9 ± 3.0 was higher than CIB76, 23.5 ± 7.1% and NN-X-1 22.7 ± 4.3%. However, there were no significant differences ($P < 0.05$) (Table 3).

Table 3. Biomass production in cultures of three strains *Nannochloropsis*, no significant differences ($P < 0.05$) (Mean ± s.d., n = 3)

Strains	Total dry weight	Dry weight	Organic dry weight	Inorganic dry weight	Total carbohydrates	Total carbohydrates
	($g \cdot l^{-1}$ ± s.d.)	($pg \cdot cell^{-1}$ ± s.d.)				(% ± s.d.)
NN-X-1	0.40 ± 0.04	6.6 ± 1.1	5.7 ± 0.8	0.9 ± 0.4	1.3 ± 0.2	22.7 ± 4.3
CIB76	0.39 ± 0.03	6.7 ± 0.9	5.7 ± 0.9	0.9 ± 0.5	1.3 ± 0.4	23.5 ± 7.1
NpUNAM	0.38 ± 0.04	6.0 ± 0.3	5.5 ± 0.3	0.5 ± 0.2	1.8 ± 0.2	30.9 ± 3.0

3.3. Cellular and Volumetric Productivity of Beta Glucans

NpUNAM presented the highest percentage of total glucans, 23.4 ± 0.7, while similar percentages of NN-X-1 and CIB76 were 15.8 ± 0.9 and 16.8 ± 0.5, respectively. Besides, α-glucans in all sources account interval 1.1-1.7%. The lowest percentages of βG were registered in Nanno3600® (6.2 ± 0.1%). With respect to the strains, NpUNAM presented βG a higher percentage (21.7 ± 0.7%) than CIB 76 (15.4 ± 0.5%) and NN-X-1 (14.3 ± 0.9%) strains. Yeast βG control presented total glucan approximate values (48.8 ± 1.4%), specified by the kit (~ 49%), Figure 4.

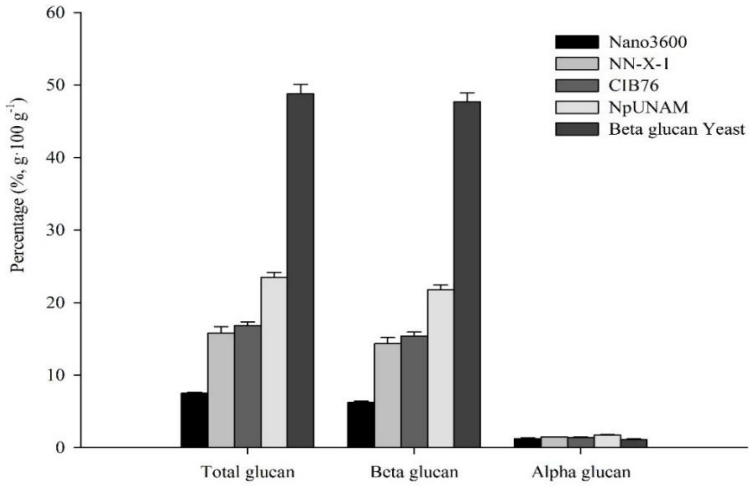

Figure 4. Cellular content of beta glucans in *Nannochloropsis* strains through enzymatic determination (Mean ± s.d., n=3).

Table 4 shows the high content of βG (Chrysolaminarin) in diatom cells reported in literature, up to 411.9 pg·cell^{-1} in *Thalassiosira weissflogii* [79]. Comparatively, *Nannochlopsis* strains presented low concentrations, 1.1, 1.0 and 0.9 pg·cell^{-1} βG in CIB76, NpUNAM and NN-X-1, respectively. NpUNAM and NN-X-1 were significantly different ($P > 0.05$) in concentrations of βG cellular (pg·cell^{-1}), and other comparisons were not significant.

However, volumetric productivity also depends on harvested cell density. Therefore, the diatom with the highest productivity in batch culture is *Chaetoceros mulleri*, with 71.57 mg·l^{-1} [79]. In contrast, *Nannochlopsis* strains showed similar volumetric productivity, NN-X-1: 57.63 mg·l^{-1} and CIB76: 60.50 mg·l^{-1}. However, it should be noted that NpUNAM has a volumetric productivity of 81.55 mg·l^{-1}, which is higher than diatoms reported in batch cultures. Although the volumetric production registered with 306 mg·l^{-1}·d^{-1} was reported in the photobioreactor system with the marine diatome *Odontella aurita* [92]. Regarding volumetric productivity, NpUNAM was significantly different ($P > 0.05$) than other strains, and volumetric productivity of NN-X-1 and CIB76 were not different ($P < 0.05$).

Table 4. Cellular and volumetric productivity of beta glucans in microalgae species, (Mean, n=3)

Microalgae	Beta glucan (pg·cell^{-1})	βG productivity (mg·l^{-1})	Reference
Eustigmatales			
NN-X-1	0.9^{a}	57.63^{a}	Present study
CIB76	1.1^{ab}	60.50^{a}	Present study
NpUNAM	1.0^{b}	81.55^{b}	Present study
Nannochloropsis salina	3.6	64.80	[132]
Diatoms			
Odontella aurita	-	306*	[92]
Thalassiosira weissflogii	411.9	31.72	[79]
Chaetoceros mulleri	18.2	71.57	[79]
Skeletonema costatum	17	-	[54]
Chaetoceros debilis	15.8	17.5	[80]

*Photobioreactor production, mg·l^{-1}·d^{-1}.
Statistical analysis only for evaluated strains, different letters mean significant differences ($P > 0.05$).

4. DISCUSSION

Total carbohydrate content depends on the *Nannochloropsis* strain. NpUNAM presented the highest content of total carbohydrates from stationary phase, 30.9%. Some microalgae may naturally contain more than 50% of their weight as carbohydrates [48]. In particular cases such as non-photosynthetic mutant of *E. gracilis*, Paramylon accumulates up to 90% of dry cell weight with glucose as a carbon source, therefore total carbohydrates are a higher percentage [133]. With respect to *I. galbana*, carbohydrates account for about 13% of dry weight [82].

Regarding *Nannochloropsis*, in some cases it has been reported as one of the species with the lowest percentage of carbohydrates presented, Templeton et al. [25] reported a value less than 10%. Nevertheless, Hsueh et al. [69], observed in *N. oculata* an accumulation of total

carbohydrates of 29% of dry biomass, per supplying 5% CO_2 in aeration, while using 8% of CO_2 obtained 37% of total carbohydrates. Moreover, Jia et al. [26] reported that total carbohydrate content in *N. oceanica* increased from 5.8% to 17.9% of its dry biomass during the first 4 days due to abrupt nitrogen depletion in culture medium and then increased to 19.1% on day 14. This indicates that total carbohydrates *Nannochloropsis* biomass can vary from 10% to 40% of its dry weight, depending on growing conditions and time of harvest, but it can still be up to 60% when NN-X-1 grown under conditions of nutrient limitation (Piña-Valdez PhD, unpublished data). Obviously, *Nannochloropsis* has high potential as a carbohydrate source for a wide industrial use.

The βG represented 21.7, 15.4, 14.3 and 6.2% dry biomass of NpUNAM, CIB76, NN-X-1 and Nanno 3600®, respectively. In diatoms, it has been reported that Chrysolaminarin has a representation of up to 64.86% of dry weight [57]. It should be noted that with Nanno 3600® 6.2% of βG was registered and CIB76 presented 15.4% of βG even though both sources are considered to be *Nannochloropsis oculata*. This may indicate that other monosaccharides, not glucose, are represented in concentration commercial biomass. This is evidenced in the percentage of total carbohydrates for each source; Nanno 3600®, 62.5%; NN-X-1, 69.5%; CIB76, 71.5% and NpUNAM, 75.8%. Moreover, Mohammady et al. [132], reported 3.6 $pg \cdot cell^{-1}$ Bg in *N. salina* under conditions of stress per diesel fuel pollution. However, the determination was of total carbohydrates and referred to them as βG, but even so, total carbohydrates per cell were higher than those reported in present study.

This is the first study on enzymatic determination of βG content in microalgae cells, especially *Nannochloropsis* (K-YBGL 12/16; Megazyme®). This method is relatively easy to use and its principle allows for reliable beta glucans quantification in sources not yet studied.

However, the most important variable for determining the potential of microalgae as a source of βG is volumetric productivity. The registered 306 $mg \cdot l^{-1} \cdot d^{-1}$ was obtained with *O. aurita* in a photobioreactor system under nitrogen limitation which registered production of 6.36 $g \cdot l^{-1}$ of biomass [57]. Another important production of βG was achieved with *Chaetoceros mulleri* for batch culture, 71.57 $mg \cdot l^{-1}$ [79]. In the present study, *Nannochlopsis* strains showed similar volumetric productivity, NN-X-1: 57.63 $mg \cdot l^{-1}$ and CIB76: 60.50 $mg \cdot l^{-1}$. But it should be noted that NpUNAM has a volumetric productivity of 81.55 $mg \cdot l^{-1}$, which is higher than diatoms reported in batch cultures. However, considering that production of *Nannochloropsis* dry biomass (strain 211/78) in flat plate photobioreactor systems has been reported from 4.7 $g \cdot l^{-1}$ [134], then it is possible to speculate a high yield of βG from production massive biomass of *Nannochloropsis* strains, for example NpUNAM, in photobioreactor systems.

Moreover, the molecular weight of microalgae βG could be directly relateds to the cell size of microalgae. For example, diatom *O. aurita* has the highest MW of 7.75 kDa [57] and a greater cell size of 10-95 microns (μm). Conversely, *C. debilis* with lower MW 4.9 kDa was registered [80] and less cell size of 10-40 μm. Likewise, from *I. galbana* MW of βG was reported 1.2-5.2 kDa [82] and cell size of 4-8 μm. Therefore, it could be expected that for cellular size of *Nannochloropsis* (1-2 μm), the molecular weight of its βG is smaller than diatoms and *I. galvana* (Cell size from [4]). This may be determinant in its molecular characteristics such as low polymerization degree and high branching degree.

In summary, the advantages of *Nannochloropsis* as a potential source of βG are as follows: I) Fresh water is not required to culture most of its species [135]. II) Biomass can be obtained on industrial scale from photobioreactors [134]. III) It is possible to modify the

biochemical composition of biomass to increasing fraction of carbohydrates [32]. IV) High volumetric productivity of βG in batch cultures (81.55 $mg \cdot l^{-1}$). V) Microalgal βG can be obtained for simple extraction techniques [79] because they are not bound for covalent bonds to other structural components of cells, facilitating purification of these polysaccharides from biomass [135]. VI) Possibly present molecular structure similar to their homologues of levaduduras and diatoms [13, 80].

CONCLUSION

Nannochloropsis strains (NN-X-1, CIB76 & NpUNAM) present 23% to 31% dry weight in the form of carbohydrates, of which 14% to 21% are βG, from stationary phase in batch cultures. Individual content of βG is ~ 1 $pg \cdot cell^{-1}$, with a maximum volumetric productivity of 81.55 $mg \cdot l^{-1}$. *Nannochloropsis* (particularly NpUNAM), has great potential for βG industrialized production and due to its possible molecular characteristics, it is estimated that there are potential applications as immunostimulants. Future research is necessary to characterize βG isolated to *Nannochloropsis*, optimize its production and assess its capacity as an immunostimulant.

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Chapter 56

PULSED ELECTRIC FIELD (PEF) ASSISTED PROTEIN EXTRACTION FROM *NANNOCHLOROPSIS*

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ABSTRACT

Extraction of cytoplasmic soluble proteins is one of the challenges for successful development of microalgae biotechnological potential. The consequence of the delivery of properly selected Pulsed Electric Fields is the release of cytoplasmic soluble proteins.

Keywords: electroporation, nannochloropsis, batch, flow process, pulsed electric field

1. INTRODUCTION

Nannochloropsis is a robust industrial alga, which can extensively grow in outdoor ponds and photo bioreactors for aquaculture (Sukenik A et al., 2009). Photosynthetic performance of outdoor *Nannochloropsis* mass cultures has been evaluated under a wide range of environmental conditions. *Nannochloropsis* is a rich source of high-quality protein (Volkman JK et al., 1993).

Extraction of cytoplasmic soluble proteins is one of the challenges for successful development of microalgae biotechnological potential. Several approaches are under investigation. Among those PEF (Pulsed electric field) is proposed as one of the most promising (Günerken E et al., 2015; Vanthoor-Koopmans M et al., 2013). Pulsed electric fields (PEFs), that are routinely used to perturb the cell membrane of microorganisms (electropermeabilization), are directly applied on an aqueous cell suspension. This process triggers a permeabilization of the cell membrane and a perturbation of the cell envelope,

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which are associated with an enhanced transport of natively non-permeant molecules. Previous studies showed that PEFs were effective for the plasma membrane electropermeabilization of walled eukaryotes but the cell wall prevented the transport of macromolecules (Azencott et al., 2008; Ganeva et al., 1995; Ganeva et al., 2014). Nevertheless, after optimization of the treatment, PEF is a routine method for gene transfer valid for bacteria, plant cell and yeasts. More recently, gene electrotransfer was performed on microalgae, including *Nannochloropsis* (Kilian O, et al., 2011; Li et al., 2014), allowing gene editing (Wang et al., 2016).

The consequence of the delivery of properly selected PEF can be the release of cytoplasmic content including proteins. This was validated on yeast, with a flow process system, for its development at the industrial level (Ganeva et al 2003). The technology was then developed to be efficient on *Nannochloropsis*.

Different protocols are available where a train of successive electric pulses is delivered on the microalgae aqueous suspension. Pulses can be low field long duration (ms) (LFLD) (Coustets et al, 2013), high field short duration (μs) (HFSD) or high voltage electrical discharge (HVED) (Grimi et al, 2014). Those protocols will apply different physical and chemical stresses on the cell envelope. The cell suspension buffer (during PEF) is water, which is poorly conductive in order to limit the Joule heating effect. Pulses can be applied on the microalgae suspension either on a batch or on a flow process system.

2. BASIC TECHNOLOGICAL DESCRIPTION OF ELECTROPULSATION

Pulsed electric fields (PEF) are obtained by delivering a controlled voltage between a set of two electrodes during a short duration of time.

As the electrodes are flat and parallel in the electroextraction process, the field is

$$E = V/d \tag{1}$$

where d is the gap between the electrodes.

The voltage is either constant during the pulse duration (T) (square wave used in the LFLP approach) or exponentially decaying (capacitor discharge system used in the HFSP process). The decay is characterized by a time constant τ_C that depends on the charge storage capacitor (C) (where the high voltage is stored) and on the load R (the electrical resistance due to the cell suspension between the electrodes).

$$\tau_C = RC \tag{2}$$

As the cell suspension electrical characteristics would change during the voltage delivery (i.e., R is not constant due to the leakage of cytoplasmic ionic content), there is a need to monitor the voltage profile during the pulse delivery with a digitized oscilloscope (Figure 2).

In all applications, a train of repetitive pulses is delivered on the suspension. Each single pulse in the train can be similar and the delay between each of them (or the frequency) controls the effect. The pulse in a train can be either monopolar or bipolar. In the latter, two

delays should be described as the train may be the application of successive pairs of bipolar pulses (with a short delay) with a longer delay between each pair.

The energy consumption W that is delivered to the sample during the train of N pulses is

i) for square wave pulses

$$W = VINT \tag{3}$$

ii) and for capacitive discharge systems

$$W = CV^2N/2 \tag{4}$$

where C (=200 nF) is the capacitance of the capacitor for the HFSP and HVED models.

This electrical energy is mostly converted in heat. Indeed the power supply associated to the pulse generator has to be suited for such an energy storage. Interestingly, the Joule heating effect depends on the conductivity of the microalgae suspension only in the case of square wave pulses, as the energy is related to the current I, i.e., a function of the conductivity through the Ohm's law. This explains the need to work with low conductivity buffers (0.2 mS/cm).

3. A BIOPHYSICAL DESCRIPTION

3.1. An Electric Field Has Physical Effects

Physicochemical effects of electrical pulses are complex as contributions are of physical and electrochemical nature (Rems and Miklavcic, 2016).

3.1.1. Electrochemistry at the Electrodes (pH, Corrosion)

Delivering a voltage between two electrodes on a conductive liquid is associated to many electrochemical reactions at the metal-solution interface. Large currents are delivered across the electrode-liquid interface in a PEF treatment chamber. These currents would result in large-scale electrochemical reactions along the electrode surface (Morren et al., 2003).

Electrode reactions induce changes to the chemical structure of the liquids and produce toxic chemicals release such as reactive oxygen species (ROS). Electropulsed solutions where chlorine is present are toxic even a long period after pulse delivery. PEF associated electrochemistry in the solution is complex. A cascade of reactions is induced with short term and long term biological consequences. Changes of pH are observed in the bulk suspension (Turjanski et al., 2011). In non-buffered conditions (as used for microalgae electroextraction), pH-shifts of more than 4 units are predicted and observed as a result of a treatment time shorter than ms at electric field strength of 10 kV/cm, used for HFSP approaches. Interestingly, the use of bipolar pulses with a short inter-pulse delay prevents the interfacial electrochemical reactions and the potentially damaging pH shifts. Bipolar pulses were used in the LFLP process.

3.1.2. Electrophoresis in the Suspension

Microorganisms have a surface negative charge resulting in electrophoretic drift when submitted to an electric field. The microalgae are pushed toward one of the electrode surfaces. The formation of a film adjacent to the treatment electrode (fouling) causes local electric field distortion and can result in arcing. The bipolar approach prevents fouling.

3.1.3. Electrophoresis in the Cell Membrane

Membrane components, which are not linked covalently to the cytoskeleton or the extracellular matrix, are freely mobile in the cell membrane (glycolipids, proteins). As their polar groups in the external solution are highly charged, which is the case of glycosylated by-products, a force is present when the external field is applied. This results in an electrophoretically driven lateral movement. An accumulation of the affected molecules towards one pole of the cell is observed (Poo 1981). Charged domains are formed as a result and non-covalently bounded components of the cell wall are affected.

3.1.4. Stretching of the Cell

In the presence of electric fields, vesicles are deformed, because of an electric stress brought by the external electric field on the membrane (considered as a dielectric flexible sheet such as a lipid bilayer), that is given by the Maxwell stress tensor. The deformation results from a gradient of electrical conductivities between the external buffer and the internal content of the vesicle. This gradient is high under PEF conditions, where a buffer of low conductivity reduces the Joule heating effect and energy costs. Stretching of the pulsed cells under electric Maxwell stress results in vesicle elongation. The cell membrane is connected to the cytoskeleton and/or to the cell wall. Its mechanical properties are such that deformations under a mechanical stress (as induced by the electric field) are dependent on the rate of the stress and of course on its duration (Evans et al., 2003). Irreversible deformations may appear. When a small stress is present for a sufficiently long duration, irreversible strains become large and a dramatic elongation affects the cell assembly.

3.1.5. Induction of Acoustic Shock Waves

PEF mediates non-electrical factors, such as pressure transients, that bring external mechanical stress on the cell membrane. Pressure transients can result in inertial cavitation shock waves as observed optically (Roth et al., 2015). Such disruptive effects are present under the LFLP conditions as observed by the fragmentation of lipid vesicles (Wasungu et al., 2014). Pressure transients affected the wall organization in ultrasound treatment of microalgae.

3.2. The External Field Induces Membrane Potential Difference Modulation

An external electric field modulates the transmembrane potential (TMP) of a cell that can be considered as a spherical capacitor [Rems and Miklavcic 2016]. The TMP induced by the electric field after a (capacitive) charging time, $\Delta\Psi_i$ is a complex function $g(\lambda)$ of specific conductivities of the membrane (λ_m), pulsing buffer (λ_o), cytoplasm (λ_i), of membrane thickness, cell size (r) and packing. Thus, when assuming that a cell is a sphere,

$$\Delta\Psi_i = f\, g(\lambda)\, r\, E \cos(\theta)(1-\exp(-t/\tau_m)) \quad (5)$$

in which θ designates the angle between the direction of the normal to the membrane at a considered point on the cell surface and the field direction, E the field intensity, r the radius of the cell and f, a shape factor (a cell being a spheroid, 1.5 for a sphere). $\Delta\Psi_i$ is a linear function of the cell size. Higher fields must be applied on small cells to get the same $\Delta\Psi_i$ as induced on the large cells. Induced TMP is the highest at the positions of the cell facing the electrodes. The last term in Eq. 5 is telling that the TMP steady value is obtained only after a charging period τ_m. τ_m depends on the cell size r and on the external buffer conductivity. The associated loading time strongly depends on the external conductivity λ_o.

$$\tau_m = rC_{membr}\,(\lambda_i + 2\lambda_o)\,(2\lambda_i\lambda_o) \quad (6)$$

Charging is fast with small microorganisms such as Nannochloropsis. A decrease in the charging time of TMP occurs with an increase of the external solution conductivity. This charging time was calculated to be long (50 μs) in a very low conductivity buffer (3 μS/cm) and affected the steady value of $\Delta\Psi_i$ with short pulses. With more classical buffers (0.2 mS/cm and 1 mS/cm), τ_m were as short as 1 μs, meaning that the steady state TMP should be reached only during LFLP pulse (Blanckaert et al., 2015).

3.3. Electropermeabilization

When the resulting transmembrane potential difference $\Delta\Psi$ (i.e., the total between the resting value of cell membrane $\Delta\Psi_o$ and the electro induced value $\Delta\Psi_i$) reaches locally more than 250 mV ($\Delta\Psi_{iperm}$), that part of the membrane becomes permeable for small charged molecules.

The field strength controls permeabilization. Field intensities larger than a critical value ($E_{p,r}$) must be applied to the cell suspension. $E_{p,r}$ is such that:

$$\Delta\Psi_{iperm} = f\, g(\lambda)\, r\, E_{p,r} \quad (7)$$

Permeabilization is therefore a local process on the cell surface. The permeabilized surface on a spherical cell, A_{perm}, is given by:

$$A_{perm} = A_{tot}\,(1- E_{p,r}/E) \quad (8)$$

where A_{tot} is the cell surface and E is the applied field intensity. Increasing the field strength will increase the part of the cell surface, where an electropermeabilized state is present.

Post pulse leakage results from a concentration driven diffusion transport described by using the Fick equation on the cell electropermeabilized part. A permeability coefficient for the molecule s, Ps, across the electropermeabilized part is induced. The permeabilized part of the cell surface is a linear function of the reciprocal of the field intensity.

Under low-conductivity conditions, the electric stretching force contributes significantly to the increase in "electroleaks" across the plasma membrane, i.e., in Ps [Sukhorukov et al., 1998).

Experimental results, obtained either by monitoring conductance changes or by observation of the leakage of metabolites, show that the pulse duration strongly controls the density of the local alterations. Delivery of a train of successive pulses leads to an increase in the leakage with an increase of their number.

4. MATERIALS

The marine microalgae *N. salina* used under the LFLP conditions was grown in an on-site closed photo bioreactor system using 3-W white LEDs. Growth was checked by measuring the density of algae under an inverted microscope with a Malassez slide. A 100 mL volume of *N. salina* suspension in water was centrifuged at 1400 ×g during 10 min at room temperature. Supernatant was removed and the cells were resuspended in 100 mL of distilled water. The final conductivity was adjusted to limit the Joule heating effect (final conductivity = 100 μS/cm). The cells had approximately a spherical shape, and the mean diameter was found to be about 2 μm (Figure 6).

The suspensions of *Nannochloropsis sp*. microalgae used for the HFSP and HVED trials were produced in two steps. In a first step, a large biomass was produced under non-stressful conditions and, to maximize the accumulation of lipids, the culture was then processed under conditions of nitrogen starvation. *Nannochloropsis sp.* was obtained as a frozen algae paste (12–15% solid content). The biomass was thawed at ambient temperature and then harvested by centrifugation and washed 3 times with deionized water to obtain a 1% (w/w) untreated (U) suspension with an initial electrical low conductivity of 0.020 ± 0.002 mS/cm.

It was determined that the microalgae suspension contained 20% of proteins (w/w dry weight biomass).

4.1. Analytical Methods

4.1.1. Analysis of Ionic Components

The ionic components extraction is estimated by the measurements of the electrical conductivity σ of the supernatant before and after the electrical treatment of the sample.

4.1.2. Analysis of Proteins

Cells were pulsed and, after an overnight incubation at room temperature, centrifuged to form a pellet. The concentration of proteins, C_p (mg of bovine serum albumin equivalent/g of dry matter), in the supernatant was determined by a Bradford assay (Bradford 1976).

With the LFLP method, results are given in protein amount, while with the two other methods, the degree of extraction of proteins Z_p is estimated as:

$$Z_p = (P - P_i)/(P_{max} - P_i) \quad (9)$$

where P is the protein content after treatment, P_i is the initial value of P before treatment, and P_{max} corresponds to the protein content in samples containing the largest amount of disintegrated cells.

4.2. Physical Techniques of Cell Disruption

4.2.1. LFLP (Low Field Long Pulse)

The PEF delivery system was on a flow system suited for the treatment of large volumes (Figure 1). Pulse generators (DEEX-Bio, Betatech, France) and flow through applicators (CNRS) were designed to apply trains of bipolar pulses on the flow. In the pulsing chamber, the distance between the parallel plate electrodes was 3 mm.

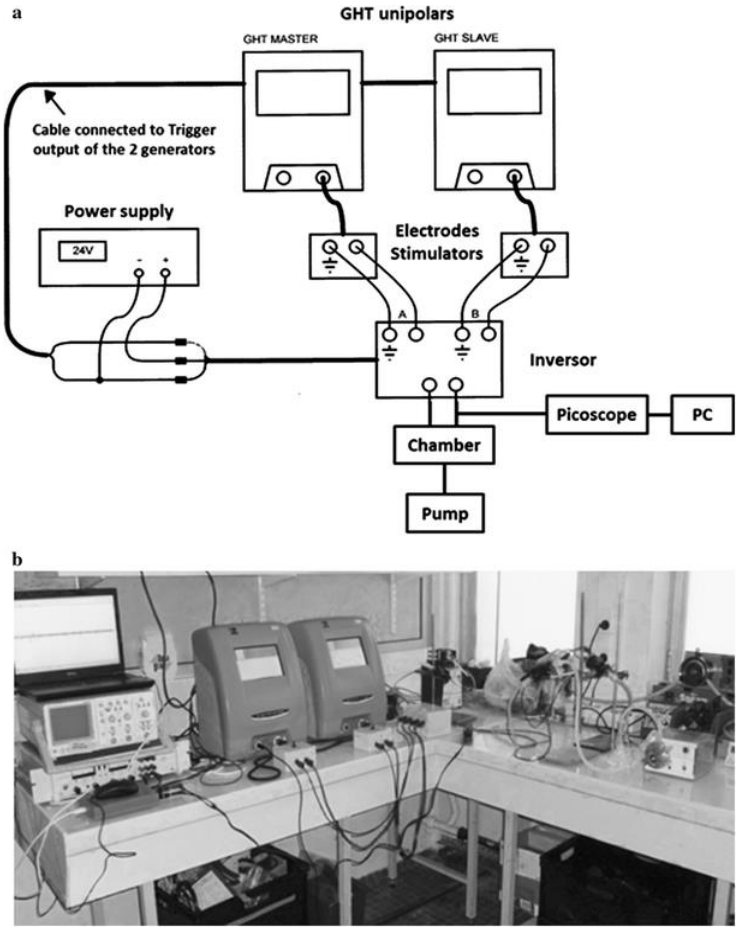

Figure 1. Scheme and picture (b) of the electroextraction system. (a) Scheme representing the electrical device, in which two Betatech S20 pulse generators are successively delivering similar pulses to the flow applicator with a controlled delay in between. A relay reverses the polarity of the second pulse in order to obtain bipolar symmetric pulses within the flow applicator. The bipolar pulse train is delivered at a frequency higher than 16 Hz (from *J Membr Biol*. 2013 Oct; 246(10):751-60 by courtesy of Springer).

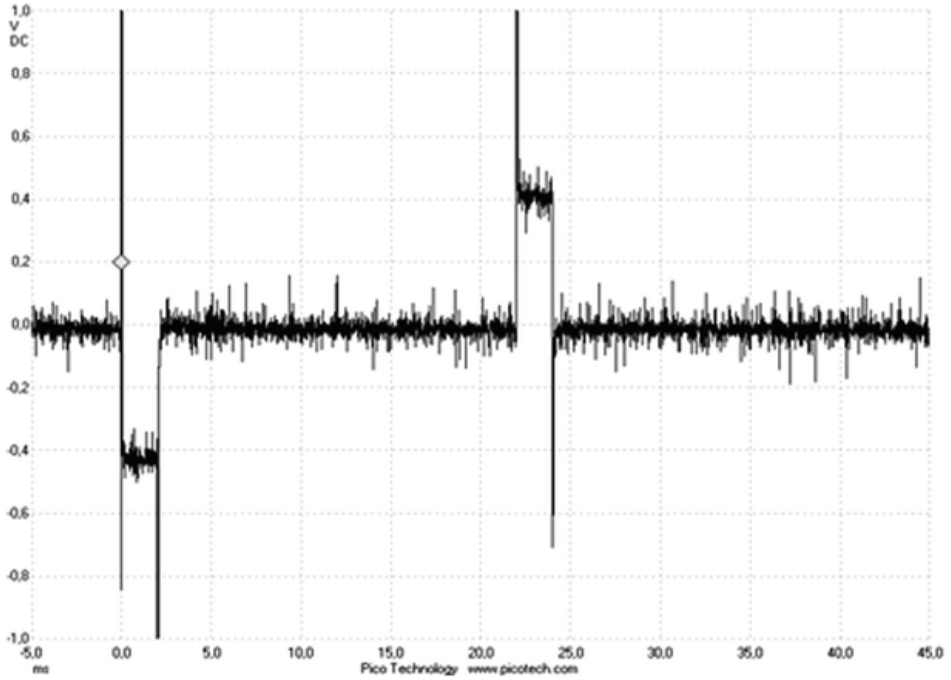

Figure 2. Current profile. A current probe monitored on line the delivery of the electric pulse train. Symmetrical square waved profiles were observed (from *J Membr Biol*. 2013 Oct;246(10):751-60 by courtesy of Springer).

Washed suspensions of microalgae were flow processed through the pulsing chamber, according to the parameters (E = 6 kV/cm, duration of pulses = 2 ms, bipolar pulses delayed by 16 ms, flow rate = 150 µl/s) (Figure 2). Those were selected by taking into account the size of the microalgae, which was close to the one of the *E. coli* bacteria (Figure 4 and 5a). This electric treatment was effective for protein extraction (Coustets *et al.*, 2013).

The number of bipolar pulses delivered on average to each cell was 15 (30 pulses as a total). After the electric treatment, the pulsed suspension was collected and diluted five times in water or in a phosphate buffer (PB/DTT = PB 105 mM, 0.3 M glycerol, 1 mM DTT, pH 7). DTT (dithiothreitol) was shown to enhance the extraction from yeasts when present in the post PEF incubation medium (Ganeva et al., 2003). Samples were incubated overnight at room temperature. Supernatants were collected after centrifuging for 5 min at 2500×g.

4.2.2. HFSP (High Field Short Pulse)

HFSP PEF treatment was carried out in a cylindrical batch chamber between two parallel plate electrodes (Figure 3a). The distance between the electrodes was fixed at 2 cm and the corresponding electric field strength E was 20 kV/cm. PEF treatment comprised application of N successive pulses (N = 1 to 400). The voltage between the electrodes decayed along the discharge, $V(t) = V_0 \exp(t/t_p)$ with an effective decay time (t_p) of 10.0 ± 0.1 µs. A delay of 2 s was present between each pulse. This system resulted in the delivery of pulses according to the following parameters: 20 kV/cm, pulse application duration 1–4 ms, 13.3–53.1 kJ/kg.

4.2.3. HVED (High Voltage Electrical Discharge)

HVED treatment was done in the laboratory batch chamber with needle-plate geometry of electrodes (Figure 3b). The distance between the tip of the central stainless steel needle (Φ10 mm) and the grounded dish plate (Φ 25 mm) electrodes was 10 mm. HVED treatment consists in the application of repetitive pulses (N = 1 to 400) with an initial voltage peak amplitude of V_0 = 40 kV, delivered at a frequency of 0.5 Hz, giving a treatment time ranging between 2 and 800 s. The temporal profile of each single pulse was complex with damped oscillations, at a period of 0.5 ± 0.1 µs superimposed on the exponential decay of voltage V exp (t/t_p) with an effective decay time (t_p) of 10.0 ± 0.1 µs. The parameters of the pulses were 40 kV/cm, pulse application duration 1–4 ms (N= 100 to 400), 13.3–53.1 kJ/kg. A polarity inversion of the field was repetitively present in HVED, due to the damped oscillations. A pulsed streamer discharge in water is associated to the HVED (V_0 = 40 kV, 1–4 ms, 13.3–53.1 kJ/kg, duration 200-800 s). It is usually accompanied with localized high electric fields (heterogeneous spatial distribution), production of short ultraviolet light, strong shockwaves, cavitations, liquid turbulences, ozone, hydroxide radicals (ROS) production, etc. These secondary phenomena to the electric field effect induce cell structure damages and facilitate externalization of intracellular compounds, but may also induce protein inactivation.

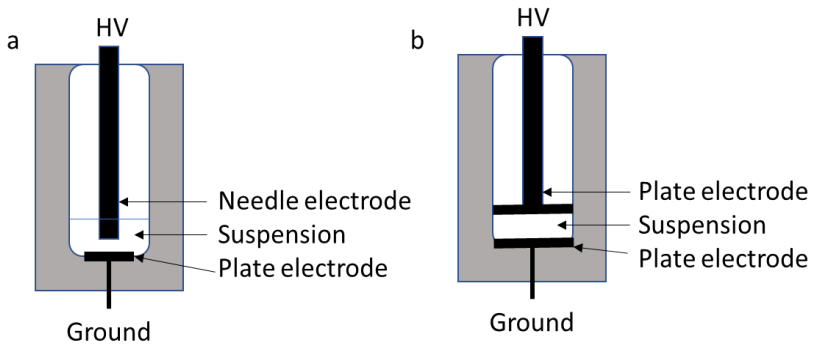

Figure 3. The schemes of the experimental pulsing chambers used for HVED treatment (a), PEF treatment (b) (Redrawn from *Food Bioprocess Technol* (2013) 6:576).

5. RESULTS

5.1. LFLP

5.1.1. Microalgae Electropermeabilization

The current delivered by the pulse generator at a given applied voltage was a direct assay of the conductivity of the solution present in the pulsing chamber (Figure 2). This current was much higher than the value measured before the pulse delivery, just after washing the cultures (100 µS/cm). This high value was further checked by measuring the conductivity of the recovered pulsed samples. A 2.5 to 3 times increase was observed for a pulsed suspension of $5x10^7$ cells /mL. This conductivity increase was larger when the microorganism density was brought from $5×10^7$ to 10^8 cells/ml. This observation was indicative of the release of the ions

present in the cytoplasm due to the plasma membrane electropermeabilization. A 3 kV/cm applied field did not trigger such ionic leakages.

This ion leakage resulted in a sharp temperature increase due to the Joule heating effect. Temperatures up to 50°C were transiently observed but cooling was also observed in the downstream tubing. The temperature in the collector was close to the room temperature.

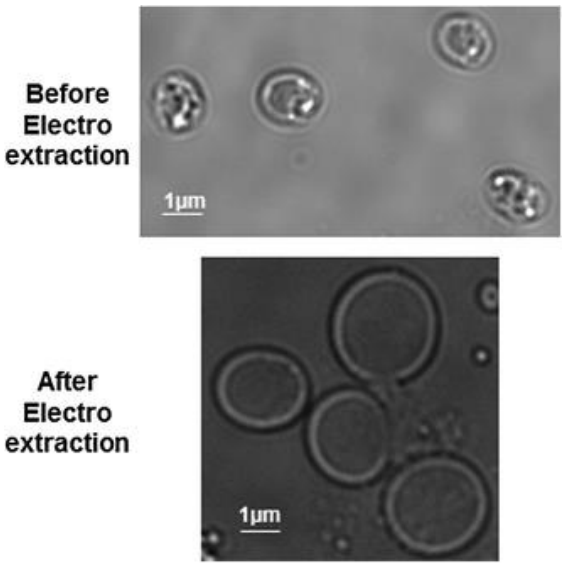

Figure 4. Phase-contrast microscopic observation of the effect of the electric pulse train. *Nannochloropsis* was observed before and after the electro treatment (from *J Membr Biol*. 2013 Oct; 246(10):751-60 by courtesy of Springer).

Phase-contrast microscopic observations of the samples, before and after electric treatment, showed a loss of contrast after pulse delivery and a swelling of the cells (Figure 4). Both results indicated that the cytoplasmic content of the pulsed sample was strongly affected, compared to the control, due to the membrane electropermeabilization.

5.1.2. Protein Extraction from N. salina

A spontaneous and rather low protein leakage was observed, after the overnight incubation, from the negative control incubated in water without any treatment applied to the suspension. This export of cytoplasmic soluble proteins overnight was also observed in yeast experiments (unpublished results). This phenomenon may be the result of an endogenous secretion of eukaryotic cells and/or passive diffusion through the plasma membrane and the cell wall of cytosolic proteins during the incubation period. The electroextraction method, with water as a post-pulse incubation medium, improved the recovery of soluble proteins by 400% after overnight incubation compared to the negative control (Figure 5b). No increase, above the low leakage observed on the negative control, was obtained when cells were treated with a field of 3 kV/cm.

Those results confirmed that the train of electric pulses permeabilized the plasma membranes of *N. salina* (as shown already by the conductance assay), but as demonstrated on yeasts previously, it induced a structural reorganization of the wall. Cytoplasmic proteins

could leak out indicating that the wall was permeable. These proteins were easily recovered, as no debris were present in the supernatant.

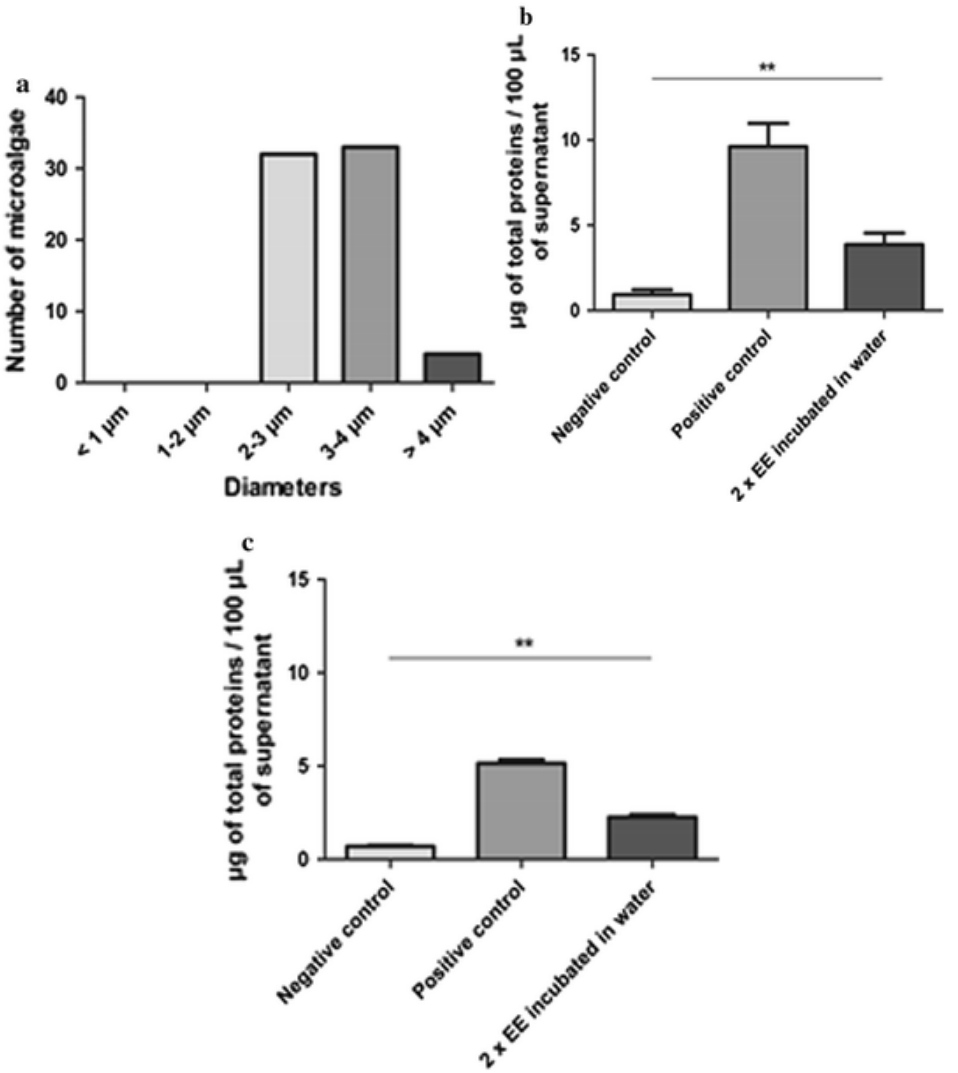

Figure 5. Total protein assay of *Nannochloropsis* electroextraction. (a) Size histogram of the population. (b) The concentration of microalgae suspension in those assays was 1.10^8 cells/ml. Unpulsed suspensions of microalgae, respectively incubated in water or methanol and vortexed, provide negative and positive controls. The remaining suspension in water was processed by a 6kV/cm electroextraction and then incubated in water. The incubation period of all samples and controls was 18 h at room temperature in the dark. Concentrations of total proteins were determined by a classical Bradford assay, using BSA as a standard (n = 6). (c) Same as in (b), but those assays were conducted at a concentration of 5.10^7 cells/ml (n = 2). Statistical analyses for b and c were made by Prism software using standard error means and a t test (from *J Membr Biol*. 2013 Oct;246(10):751-60 by courtesy of Springer).

The possibility for treatment of more concentrated suspensions (from 5.10^7 up to 10^8 cells/ml) was checked. Levels of extraction were two times higher with the higher density suspension in comparison to the lower density (Figure 5c). However, the conductivity of the

pulsed suspension increased with the higher microorganism load. A higher current was delivered, resulting in an increase in the Joule heating effect. A deformation of the electrical pulse associated voltage and current was observed as the conductivity is temperature dependent.

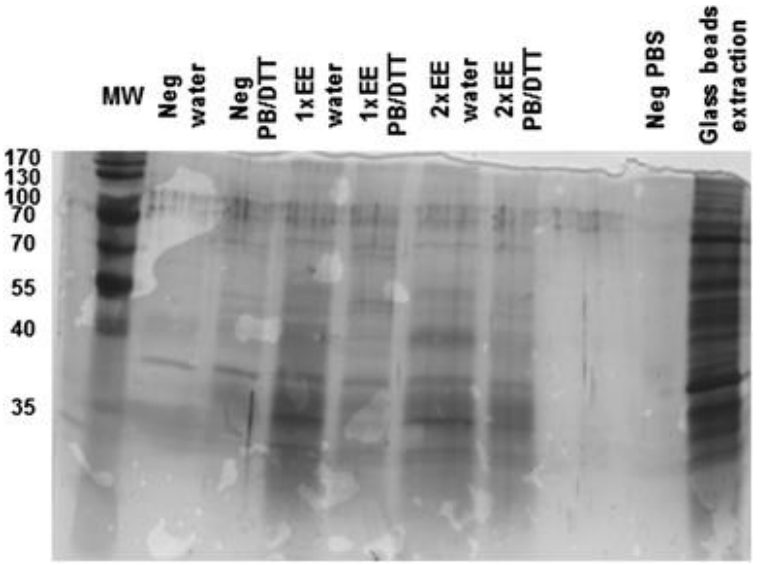

Figure 6. Electrophoretic analysis of proteins in cell extract and supernatant of control, electrically treated and mechanically extracted cells. Protein samples in SDS-PAGE sample buffer containing 2-mercaptoethanol were boiled for 3 min at 90°C prior to electrophoresis on 12% acrylamide gel. Gels were stained for protein by silver nitrate (from *J Membr Biol*. 2013 Oct;246(10):751-60 by courtesy of Springer).

The proteins from supernatants of control, electrically treated and mechanically extracted cells were analyzed by SDS-PAGE under reducing conditions (Figure 6). Bands were detected between 35 and 170 kDa. The bands present in negative controls appeared on pulsed samples with a higher intensity. Several new proteins appeared after electroextraction in comparison to the negative controls. Most of the proteins recovered by the glass bead extraction, where the cell wall was completely disrupted, were also present after electroextraction.

5.2. HFSP and HVED Treatments

5.2.1. Microalgae Electropermeabilization

Disrupted algae suspension characteristics were measured between successive applications of the HVED or PEF pulses.

Application of HFSP PEF brought only an insignificant increase of buffer low conductivity up to 0.07 mS/cm. The application of the HVED technique resulted in a more powerful cell disintegration and in a larger increase of conductivity, up to 0.3 mS/cm. This release of cytoplasmic ionic compounds was the direct effect of the cell membrane electropermeabilization.

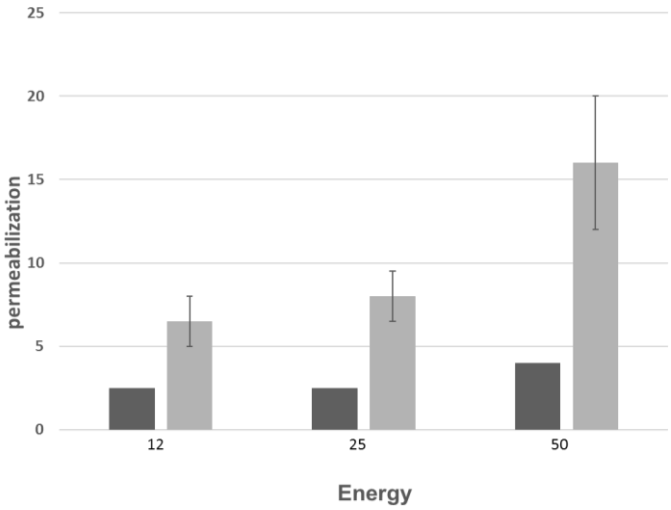

Figure 7. Microalgae permeabilization. It was assayed by the Ratio of electrical conductivities of the supernatant after and before treatment σ/σ_i versus the specific power consumption W expressed in kJ/kg. Results are shown in black after PEF treatment and in grey after the HVED treatment (adapted from Bioresource Technology 153 (2014) 254–259).

This increase in conductivity, along the field discharge, resulted in a Joule heating effect. The initial temperature before HFSP PEF or HVED treatments was 22°C and the temperature elevation after electrical treatment never exceeded 30°C.

5.2.2. Microscopic Observations

Under the microscope, the cells were observed to be free and almost not aggregated in untreated (U) and HFSP PEF treated suspensions. This was not the case with the HVED treatment, where the microalgae treated suspensions were noticeably agglomerated and formed large clusters. Those results indicated that large damages were induced by the treatment, changing the hydrophobicity of the wall. The HVED-produced shockwaves, cavitations and liquid turbulences can facilitate aggregation and provide micronization, a set of physical stresses destructive for the cell wall. The treatment affected sedimentation of microalgae because of cell debris formation. Thus, sedimentation was acting on large aggregates and small cell debris.

5.2.3. Protein Extraction

The treatment was done sequentially, meaning that the protein content in the supernatant was first checked with control cells (U), then after a HFSP treatment (PEF) and eventually after addition of a HVED treatment (HVED). Analysis of the supernatant was operated just after the electrical treatment with no further incubation.

The total quantity of extracted water-soluble proteins in the control was Z_p = 0.7% (w/w DW biomass) while the microalgae proteins content was 20.0% ± 0.8% (w/w DW biomass). The effect of HFSP treatment was associated with Z_p = 5.2% and should be considered as rather significant when compared with the low supplementary contributions of HVED (1.15%) (Figure 8).

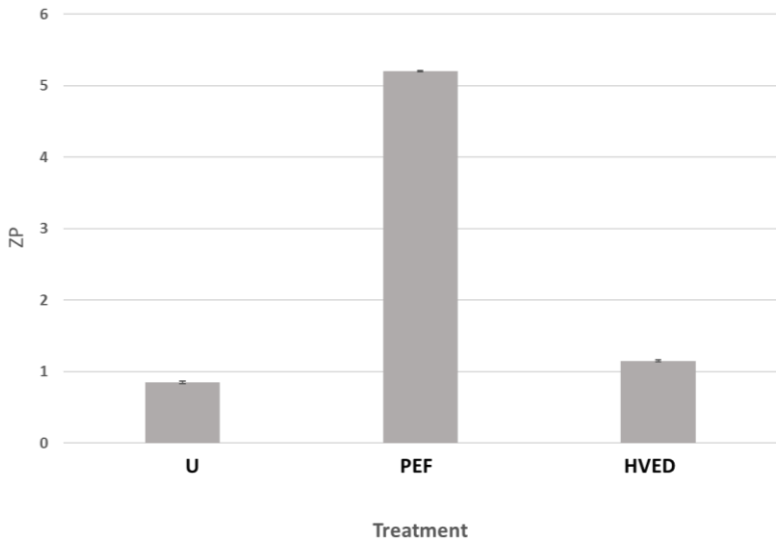

Figure 8. Degree of extraction of water-soluble proteins Z_p (%) (Eq. (9)) for 1% microalgae suspensions: untreated (U), -treated by PEF (20 kV/cm, 4 ms), and HVED (40 kV/cm, 4 ms), redrawn from *Bioresource Technology* 153 (2014) 254–259.

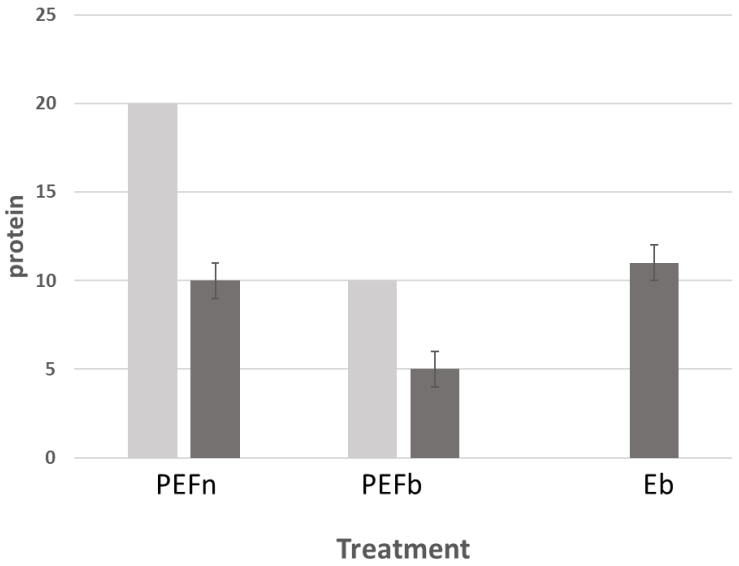

Figure 9. Concentration of proteins (mg/g), after basic aqueous extraction coupled to PEF treatment. The data (light grey) are presented for extracts obtained using PEF_n (neutral pH), PEF_b (basic pH), and E_b extraction procedures. The effects of post PEF aqueous extraction are shown in dark grey. The pulse time duration was t_{PEF} = 4 ms (N=400), and t_{Eb} = 10,800 s for PEF and E_b extraction procedures, respectively (Adapted from *Algal Research* 8 (2015) 128–134).

A direct aqueous extraction of algae proteins from *Nannochloropsis* biomass was obtained at pH 11 and a temperature of 333 °K (Gerde et al., 2013). The relevance of this pH dependent spontaneous extraction was investigated on the PEF induced extraction.

HFSP PEF were delivered (N=400) on a suspension either at a close to neutral pH (8.5) (PEF_n) or under basic conditions (pH 11) (PEF_b). The neutral condition was more effective

for protein extraction (Figure 9). A supplementary basic extraction ($+E_b$) was done under the optimized conditions described for the reported basic extraction (E_b) method (pH=11, T=323°K, incubation during 10,800 s). This additive treatment brought an improvement to the protein extraction, regardless of the PEF treatment pH, but with a slightly larger effect after PEF_n. The most important conclusion was that the PEF pretreatment improved the yield of protein extraction. The concentration of proteins in the supernatant was increased 3 times when the pH 11 extraction was operated on a suspension, which was already pretreated by PEF at pH 8.5 (Figure 9).

Those observations clearly showed the improvement in protein extraction brought by a PEF pretreatment application in a normal medium (pH = 8.5) followed by a basic medium supplementary extraction (pH = 11) (Figure 9). The potential of PEF pre-treatment was established as a preliminary step of pH-assisted aqueous extraction of algae components from *Nannochloropsis* suspensions.

CONCLUSION

The electrical methods provide soluble protein extraction from microalgae. The results were very interesting with the LFLP approach in which the PAGE results provided the evidence of the lack of damage on the extracted proteins. Previous results on yeasts showed that the protein activity was preserved (Ganeva et al., 2003).

The use of low conductivities buffers is problematic, when using short (μs) pulses, as the associated capacitive loading times are long. This means that the induced TMP does not reach its steady state value. Only a limited effect is present to induce permeabilization. This may be one reason why the extraction is more effective with the LFLP than with the HFSP processes. The cumulated time of PEF application is much longer with the LFLP (60 ms) than with the two other methods (less than 4 ms). The effect on the wall is associated with the plasma membrane permeabilization that may affect the covalent interactions between the two assemblies. However, a mechanical effect is associated with the LFLP procedure. Stretching of the cells brings an electromechanical stress on the wall and can induce rupture of molecular interactions. The DTT reduction of the disulfide bonds during the post pulse overnight incubation further increased the level of extraction. Another difference is that the LFLP technology is delivered on fresh cultures, in which the membrane organization was not pre-affected.

What is still missing to appreciate the advantages of the methodology is basic investigations of the biophysical processes that are responsible for membrane and wall alterations of the treated microorganisms. Cells are more than a vesicle with a dielectric shell. One should consider the different effects associated with the train of electric pulses and their control by the electric parameters (field strength, pulse duration, delay between pulses). Another level of complexity is the organization of the cell envelope and its complex response, not only to the physical (electromechanical) constraints, but also to metabolic stresses, which are associated with high-level membrane electropermeabilization.

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Chapter 57

RECENT ADVANCES IN THE UTILIZATION OF *NANNOCHLOROPSIS* BIOMASS FOR COMMODITY CHEMICALS, FEEDS, HIGH VALUE PRODUCTS, BIOFUELS, COSMETICS, FERTILIZERS, AND MATERIALS PRODUCTION

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ABSTRACT

Nannochloropsis is a genus of marine microalgae including six known species (*N. gaditana, N. granulata, N. limnetica, N. oceanica, N. oculata, N. salina*), which are considered as promising microalgal cell factories for lipids production and bioactive compounds. Currently, *Nannochloropsis* is commercially cultured to produce biomass for various industrial applications. Due to its high nutrition profile, the marine microalgal is mainly used as an energy-rich food source for aquaculture such as fish larvae and rotifers. Nevertheless, it has become more widely recognized as potential lipids source for biodiesel production, while generating biomass residuals that have been utilized for bioH_2 and lactic acid production. Ingredients (mainly polysaccharides, vitamin B12, Vitamin C) extracted from *N. oculata* biomass were also used for cosmetics formulation. Moreover, *N. oculata* biomass was recently explored as a high-value organic slow-release fertilizer to enhance carotenoid and sugars content in tomatoes. More recently, lipid-extracted *N.*

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salina residual and whole biomass of *Nannochloropsis* sp. were utilized as fillers for biocomposites fabrication with polymers. Industrial large-scale cultivation of *Nannochloropsis* is expanding, meanwhile biorefinery is evolving to valorize all biomass constituents (e.g., proteins, lipids, carbohydrates, pigments) for products development in relevant industries. This chapter presents recent advances in the utilization of *Nannochloropsis* biomass for commodity chemicals, feeds, high value products, biofuels, cosmetics, fertilizers, and materials production. Challenges and future perspectives in application of *Nannochloropsis* biomass for biorefinery development in microalgae-based industry are also highlighted.

Keywords: *Nannochloropsis*, high value products, commodity chemicals, feeds, biofuels, cosmetics, biofertilizers, materials, biorefinery

1. INTRODUCTION

The greatest challenges that society faces this century are climate, energy, and food security. BioEnergy with Carbon Capture and Storage (BECCS) has been proposed as a primary measure for mitigation of CO_2 emission while simultaneously producing renewable energy on global scale [1]. However, since BECCS based on utilization of terrestrial biomass, the method has many negative environmental impacts for land, nutrient, and water use as well as biodiversity and food production. Contrastingly, industrial-scale cultivation of marine microalgae for algal biomass production could provide a more environmental favorable approach for satisfying the climate goals that were agreed and singed at the 2015 Paris Climate Conference. Marine microalgae is an attractive alternative to traditional crops-based biomass and have been considered as the third generation feedstock for bioenergy production [2]. They offer substantial advantages over terrestrial plants, due to higher photoconversion efficiency, possibility to be grown in brackish, saline or wastewater and cultivated on non-arable land, with low seasonal variation of production mode and biomass productivity [3]. Furthermore, microalgae accumulate a broad range of potential compositions, mainly polysaccharides, proteins, and fatty acids (lipids), which are much more suitable for processing in a biorefinery [4]. Particularly, microalgae biomass also contains many minor components including antioxidants, vitamins, and pigments such as astaxanthin, lutein, etc, that can be extracted and purified as high value ingredients for pharmaceutical [5, 6] and cosmeceutical industries [7].

Nannochloropsis is a typical marine microalgal strain that is potential for industrial cultivation. Many studies have been carried out to investigate pilot culture of *Nannochloropsis* in outdoor open race way pond [8], flat panel photobioreactors [9], photobioreactors [10], and annular reactor [11]. *Nannochloropsis* was also used for pilot-test to grow in anaerobic digestion of municipal wastewater (as nutrient source) with dilution rate of 15-30% in seawater in both tubular and raceway reactors [12]. The marine microalgal has high productivity, high protein content and lipid composition, making it a promising source of nutrition [13]. Due to its high accumulation of polyunsaturated fatty acids (PUFAs) [14], it has recognized as a potential candidate for nutraceuticals (e.g., EPA) and raised growing interest for the investigation of biodiesel production [15-17]. It is also known that *Nannochloropsis* could accumulate variety of pigments including chlorophyll *a*, zeaxanthin, canthaxanthin and astaxanthin, thus it is considered as a source of valuable pigments [18].

Biomass production is a preliminary step for commercialization of algae-based industrial products. Most commercial facilities of *Nannochloropsis* focus on one single product such as whole biomass for aquaculture or animal feed, or EPA for nutraceutical industry. However, since *Nannochloropsis* biomass simultaneously contains lipids, carbohydrates, proteins, pigments, therefore utilization of all these components to develop specific products will maximize exploitation of the biomass and allocate all constituents in different markets. In order to implement the task, biorefinery research is prerequisite. Biorefinery of microalgal biomass is the next step for the commercialization of products from algae. The biorefinery process strategy depends on the product portfolio, and may result in different market scenarios for biofuels, chemicals, food/feed, food additives, and cosmetics/healthcare. The present chapter provides updated research activities on the utilization of *Nannochloropsis* biomass for the development and production of commodity chemicals, feeds, high value products, biofuels, cosmetics, fertilizers, and materials. Different biorefinery scenarios based on four primary products production from *Nannochloropsis* are also highlighted.

2. COMMODITY CHEMICALS PRODUCTION FROM *NANNOCHLOROPSIS*

2.1. Lipids Production via Solvent Extraction

Lipids extraction from algal biomass is a crucial step before subsequent conversion to biofuels or lipids-based bioproducts. Because lipids body is embedded in internal compartment of microalgal cells and protected by highly rigid cell walls. Therefore, cell disruption is necessary to make lipids droplets to be accessible to extracting solvents. Moreover, extraction of lipids from wet algal biomass is considered as more costly effective than that from dry biomass because dewatering step can be omitted. The use of solvents to recover crude lipids from disrupted algal biomass is necessary. Searching for an efficient solvent is also important. Solvent used to extract lipids should provide a reasonable partition coefficient, forcing the solute to migrate to the solvent phase [19]. Lipids should have high partition coefficient in the selected solvent. Principally, hydrophobic lipids (e.g., neutral lipids such as TAG) is preferably partition into the nonpolar solvent phase (chloroform, hexane), whereas polar lipids are not so easy to dissolve in nonpolar solvents due to their bindings with biomass matrices. Therefore, co-solvents are traditionally applied to break the linkages between the polar lipids and biomass, leading to increase the solubility of the polar lipids. Recent study conducted by Angles et al. [20] pointed out that the cell disruption appeared to be the main controlling step if low water soluble solvents were used for *Nannochloropsis* sp. Furthermore, it was found that extraction of lipids from suspension of disrupted microalgae was more efficient than extraction from dried biomass (same solvent, same energy, and time) and water liberated with the disrupted biomass improved selectively for saturated fatty acids (SFA) recovery. Lipids extraction yield achieved 50% when disruption rate of 84% by high pressure disruption cell was obtained in 10 min, while the SFA content was enriched up to 72% of the total extracted lipids. Two solvents including methyl tert-butyl ether (MTBE) and cyclo pentyl methyl ether (CPME) were screened out from 11 experimental solvents (cyclohexane, heptane, chloroform, toluene, methyl isobutyl ketone, ethyl acetate, dimethyl carbonate, R-limonene, 2-methyltetrahydrofuran, methyl ter-butyl ether, and cyclo pentyl

methyl ether) showed to have the highest lipids recovery, while ease to be recyclable and safety. Chloroform ($CHCl_3$) is the most encountered in lipid extraction from dry algal biomass for standard analytical methods. However, it was rarely found to apply for microalgae oil recovery from wet form. In a study carried out by Chatsungnoen and Chisti [21], implementing optimization of oil extraction from *N. salina* biomass paste (72% water) using chloroform, methanol and water as co-solvent. The optimal volumetric ratio of chloroform/methanol/water was 5.7:3:1, which was then applied for single-step extraction experiments to optimize the extraction time, temperature and the volume of the solvent mixture required for oil recovery from 1 g dry biomass (equivalent to the wet paste). The optimal extraction conditions were 25ºC, 2 h using 33 mL of solvent mixture. The developed protocol resulted in saving by 48% of solvent and 78% of extraction time when compared to the conventional Bligh & Dyer process [21].

2.2. Biocrude Production via Thermal Process

Algal biocrude from *Nannochloropsis* biomass can be produced via two typical thermal processes which are pyrolysis and hydrothermal liquefaction (HTL). Adamczyk and Sajdak performed pyrolytic experiments using *N. gaditana* biomass at 400-600ºC under N_2 (50 mL/h) in a 1 kg fixed bed reactor [22]. Char, bio-oil, and gas were main products (of the pyrolysis process), which were characterized by a gas chromatography (GC) with mass detection. The bio-oil produced under 600ºC achieved 38-40% efficiency with the highest heating value of 12.6 MJ/kg, while the highest composition of bio-oil including alkanes and alkenes were determined under 500ºC. In the gas phase, methane content was the largest composition with greater 50%. Biochar contained up to 70% ash that can be used as a fertilizer. When catalyst (HZM-5) was used at 1/1 loading over dry weight of algal biomass, the pyrolysis process of *Nannochloropsis* sp. resulted in a 20.7% bio-oil at 400ºC, which was significantly lower than 31.1% that obtained from pyrolysis without catalyst [23]. Bio-oil products produced via pyrolysis are a complex consisting of numerous chemical compounds having various functional groups and a wide range of molecular weights that may cause un-wanted characteristics. Therefore, the pyrolytic bio-oil needs to undergo further upgrading or treatment in order to enhance its properties for the targeted use. Nam et al. [24] employed atmospheric fraction distillation and vacuum distillation for physical upgrading *N. ocutala*-derived bio-oil (pyrolyzed at 500ºC in a batch reactor in 30 min [25]) for alternative transport fuels. Properties of each distillates were analyzed for energy and mass yields, elemental, and chemical composition, total acid number (TAN) to compare with characteristics of petroleum-based fuels. It was noted that the similar tendency of physical and chemical characteristics of each fraction from both distillation methods was observed. The middle fraction had heating value of 41.2 MJ/kg and water content of less than 1.25%, but the TAN as high as 17-25 mg KOH/g (higher than 0.12 mg KOH/g measured for the light fraction). The lowest viscosity of 0.61 cSt was obtained for the light fraction from fractional distillation to be equivalent to that of gasoline fuel, while the largest chemical compositions in the light fraction were olefins (26-31%), aromatics (14-17%), and paraffins (43-49%) and alcohols (4-5%) were predominant in the middles fraction. Nitriles were evaluated to account for 25-42% in the heavy fraction. To meet the petroleum transportation fuel substitute, the physically upgraded pyrolytic bio-oil was chemically upgraded using Pd/C catalyst [26]. The light, middle fraction

and raw bio-oil was catalytically treated under temperature of 130-250ºC and pressure of 4.1-8.3 MPa using response surface methodology (RSM). Results revealed that the physical distillation prior to catalytic upgrading led to a better quality of upgraded bio-oil when compared to the direct bio-oil upgrades. Moreover, both the oxygen and hydrogen contents of light and middle fraction upgrades were enhanced, while the upgraded raw bio-oil showed a limited improvement. The higher heating value (HHV) and TAN for the middle fraction was respectively improved to 42.9 MJ/kg and 1.09 mg/KOH due to most of the ketones in upgrades were removed under severe conditions. Paraffin and aromatic chemical groups were remarkably generated via hydrogenation and hydrodeoxygenation of olefin groups. These results indicated that sequentially physical distillation followed by catalytic upgrading effectively enhanced the quality of biofuel that can be petroleum fuels substitutes or additives.

Hydrothermal liquefaction (HTL) can be alternatively applied to convert algal biomass paste to biocrude, but not necessary under absent of oxygen. Recent HTL for *Nannochloropsis* sp. was systematically investigated by Vanldez et al. [27] at a range of temperatures of 250-400ºC, times of 10-90 min, water densities of 0.3-0.5 g/mL, and biomass loading of 5-35%. Biocrude of HTL for algal biomass commonly includes light and heavy fractions that are associated with gaseous, aqueous, and solid by-product fractions. It was reported that the yields of light and heavy biocrude depend on reaction time and temperature, while the total yield depends on temperature only. Among biocrude-related products, carbon proportion in aqueous phase and the light biocrude accounted for 51 and 75 wt%, respectively, regardless of reaction time and temperature. Moreover, approximate 67% of N in the raw algal biomass was distributed to the aqueous phase with 84% partition here, whereas up to 85% of the P was distributed to the aqueous phase in the form of free phosphate. This interesting data provides a fundamental evidence to recover N and P from aqueous phase for nutrient recycling. Based on these quantitative results, a reaction network for *Nannochloropsis* sp. liquefaction was developed, that comprises the pathways for consumption and formation of each product fraction [28]. Based upon the reaction network, quantitative kinetic model for HTL of *Nannochloropsis* sp. was also developed [29] to describe influence of time and temperature on the gravimetric yield of gas, solid, biocrude, and aqueous-phase products from isothermal HTL of 15 wt% slurry.

2.3. Sugars Production via Enzymatic and Acidic Hydrolysis

Microalgae can produce carbohydrates as major products via photosynthesis and the carbon fixation metabolism. The carbohydrates are accumulated as starch in plastids or cellulose in algal cell wall with total content up to 16.4-70% dry cell weight (DCW) depended on cultivation conditions [30, 31]. The reducing saccharides generated from algal carbohydrates mainly consists of glucose, xylose, mannose, galactose, rhamnose, which can be utilized by bacteria or yeast for liquids (i.e., ethanol, butanol, etc.) or gas fuels (i.e., hydrogen, etc.) production via fermentation [31]. Microgalgal sugars can be produced via enzymatic hydrolysis [32, 33] or chemical pretreatment. Due to high cost of enzyme, thermochemical treatment of algal biomass for sugars production are currently implemented with the aid of chemical acid catalysts (i.e., H_2SO_4, etc.) [34, 35]. The primary research has focused on development of algal lipids-based biofuels (i.e., biodiesel, jet-fuels), while the leftover was used for biogas, electricity, animal feed, fertilizer, or materials production. But

the biomass residual after lipid extraction is approximately estimated as a haft of total algal biomass weight, which generally contains carbohydrates that can be derived for further producing other liquid fuels such as ethanol. Therefore, Mirsiaghi and Reardon [36] made an effort to develop enzymatic hydrolysis and acid-aided degradation process of lipid-extracted algal biomass of *N. salina* (LEAB) for sugar recovery. For enzymatic hydrolysis, an optimal enzyme mixture of Accellerase 1500, 0.05–0.25 mL/g biomass; Accellerase XC, 0.0125–0.125 mL/g biomass; Accellerase XY, 0.005–0.05 mL/g biomass; and pectinase, 0.04–0.153 mL/g biomass was made to use for biodegradation of the LEAB under 50°C for 5 h with 5% (w/v) biomass loading, giving the yield of sugar release and maximum sugar release rate of 77.9 mg sugars/g LEAB and 87.0 mg sugar/g LEAB/h, respectively. For the acid hydrolysis, the highest yield of released sugar (243.2 mg/g LEAB) was achieved via one-step sulfuric acid process with 10% acid concentration (w/w) at 90°C for 5 h (biomass loading of 5%, w/v), while using 10% HCl catalyst gave maximum sugars release yield of 164.6 mg sugars/g biomass under the same conditions applied for H_2SO_4. It was also reported that yeast can be grown on the sugars-rich hydrolysate after enzymatic and acid degradation without addition of other nutrients. The study demonstrated that development of value-added chemicals (i.e., sugars, ethanol, etc.) from LEAB via development of biorefinery can improve economic feasibility of microalgae-based biofuels production [36].

2.4. Lactic Acid Production via Fermentation

Dry biomass of *N. salina* contains a total lipid and carbohydrate content of 39.8% and 18.2%, respectively, was subjected to pretreatment with sulfuric acid (5%, w/v) at 120°C for 1 h to disrupt the algal cells and degrade polysaccharides to monosugars. The hydrolysate was employed for lipid extraction with hexane (40 °C and 200 rpm) to obtain lipids, while recovering lipids-free hydrolyzed mixture containing reducing sugars (3-25 g/L, mainly glucose and xylose) [37]. The resulted mixture was neutralized to remove acid and used as a carbon source for anaerobic fermentation with *L. pentosus* at 30°C under shaking rate of 150 rpm for 48 h, giving lactic acid (LA) concentration of 10.1 g/L and LA yield of of 92.8%. The sugar and lipid yields after the acid hydrolysis of the algal biomass were measured as 64.3% and 85.6%, respectively, presenting a high potential for simultaneous lipids extraction and sequential sugar recovery and microbial LA production from algal biomass via acid pretreatment [37]. However, the hydrolysate has to be deionized for maintaining LA yield of over 90% at a sugar concentration of over 11.2 g/L. Moreover, it was estimated that, the volumetric productivity of LA of 0.45 g/L/h is low and needs to be improved for cost effective production of LA at the industrial scale [37].

2.5. Protein Isolation from Defatted *Nannochloropsis* sp. Biomass

Generally, after lipid extraction, algal residue contains a countable amount of proteins that can be isolated as a co-product for specific uses. This can improve the economic feasibility and sustainability of the feedstock. Temperature and pH were optimized for maximizing protein recovery from *Nannochloropsis* biomass [38]. Maximum quantity of protein was solubilized at 60°C and pH 11 and recovered at pH 3.2. The isolated protein

fractions contained 56.9% and 40.5% protein when using isopropanol defatted and non-defatted biomass as starting materials, obtaining protein yields of 16 and 30%, respectively. This indicated that protein extraction yield was decreased when isopropanol pretreatment was applied. Although the isolation process produced a protein-rich product that may have unique functional properties due to the high degree of glycosylation, the overall recovery of the protein is relatively low, thus it should be further improved [38].

3. AQUACULTURE AND ANIMAL FEEDS PRODUCTION FROM *NANNOCHLOROPSIS*

3.1. Aquaculture Feed Production from *Nannochloropsis*

The global market for aquaculture products such as carp, molluscs, crustaceans, salmon, trout and other fish was estimated at 156.27 billion US$ in 2015 (~ 71,190 $\times 10^3$ tons) and is expected to reach 209.42 billion US$ in 2021, growing rate at a CAGR of 5.0% between 2016 and 2021 [39]. Several microalgal species such as *Chlorella, Tetraselmis, Isochrysis, Pavlova, Phaeodactylum, Chaetoceros, Nannochloropsis, Skeletonema, Thalassiosira* [40] and *Schizochytrium* sp. [41] are considered as outstanding sources of proteins, carbohydrates, lipids, and vitamins and have been utilized widely as food and/or feed additives for aquaculture larvae for over 40 years [42, 43]. Moreover, microalgal cultures are "green-water" maker for larvae tanks. Thus, microalgae are importantly functioned in aquaculture hatchery in terms of the nutritional values from natural food chain as well as maintenance of water quality in the hatchery environment [44]. *Nannochloropsis* is well known as a cell factory to produce pigments such as chlorophyll *a*, zeaxanthin, canthaxathin, and astaxanthin [18], vitamins [45], protein [46] and polyunsaturated fatty acids, mainly EPA (C20:5n3) [47-50]. It is commonly grew in fish hatcheries as a primary food for zooplankton production, such as rotifers and copeponds, which is then feed for rearing larvae of diverse species of mollusk, crustaceans, and fish [51]. Particularly, the broadly nutritional profile of *Nannochloropsis* is comprehensively summarized in Table 1 to 4, representing a potential live and formulated feed source for aquaculture.

Table 1. Nutritional profile of *Nannochloropsis*

Strain	Chlorophyll *a* (%DCW)	Protein (%DCW)	Carbohydrate (%DCW)	Lipid (%DCW)	EPA (%DCW)	Ref.
N. oculata	0.89	35	7.8	18	ND	[63]
Nannochloropsis sp.	0.5-1.4	17.2-41.0	8.1-11.3	22.9-39.4	1.6-3.8	[64]
N. granulata[a]	ND	48.76-50.41	14.91-16.59	25.62-28.55	ND	[65]

[a]Data was reproduced from [65].
ND: Not determined.
DCW: Dry cell weight.
Ref.: Reference.

Table 2. Amino acid composition of protein from *Nannochloropsis*

Amino acids	Strain				
	N. oculata[a] [63]	*Nannochloropsis* sp.[a] [64]	*N. granulata*[b] [65]	*N. oculata*[a] [66]	*Nannochloropsis* sp.[b] [56]
Essential					
Arginine	7.3	5.1-5.5	25.4-26.3	6.2-6.7	2.68
Histidine	2.1	1.0-1.7	7.5-7.8	2.6-3.0	0.86
Isoleucine	4.8	4.4-5.1	17.4-18.0	4.7-5.1	2.11
Leucine	7.8	7.6-8.4	32.4-33.1	7.3-8.1	4.17
Lysine	6.1	5.8-6.4	21.2-24.1	6.3-7.5	2.91
Methionine	1.6	2.0-2.3	8.7-9.0	2.6-3.2	0.67
Phenylalanine	6.2	4.3-4.9	19.1-19.3	5.6-6.3	2.30
Proline	9.3	16.9-23.1	28.1-29.9	5.6-9.4	2.24
Threonine	5.5	4.0-4.8	16.5-18.1	4.5-5.3	2.11
Tryptophan	1.6	ND	0.3-0.4	2.0-3.8	ND
Valine	6.5	5.9-6.9	21.5-22.7	5.6-5.9	2.76
Non-essential					
Alanine	7.4	5.8-6.8	23.0-24.9	5.8-6.7	3.57
Aspartate	7.6	7.2-8.0	35.5-38.4	6.8-8.7	4.0
Cystine	0.41	0.8-1.2	2.7-3.0	0.77-1.35	ND
Glutamate	10.1	7.0-9.8	40.4-43.2	9.7-10.9	5.80
Glycine	5.5	5.1-5.4	18.3-19.7	5.2-5.8	2.71
Hydroxy-proline	0.05	ND	0.1	ND	ND
Ornithine	0.35	ND	1.4-1.5	0.30-0.53	ND
Serine	5.0	3.1-3.7	14.8-16.1	5.1-5.5	1.86
Tyrosine	4.2	3.5-4.1	14.1-14.6	5.0-5.7	1.66
γ-aminobutyric acid	0.78	ND	ND	ND	ND

[a]Weight percentage of total amino acids.
[b]Unit is expressed as mg/g DCW.
ND: Not detected.
DCW: Dry cell weight

Table 3. Sugar composition of carbohydrates from *Nannochloropsis*

Sugar	Strain			
	N. oculata[a] [63]	*Nannochloropsis* sp.[b] [67]	*N. oceanica*[a] [68]	*N. oceanica* CCMP 1779[c] [69]
Arabinose	ND	ND	ND	0.28
Fucose	4.4	0.4	ND	1.59
Galactose	3.8	1.4-1.8	21	1.54
Glucose	68.2	3.8-4.2	27	81.68
Mannose	6.1	0.2-2.8	5	3.31
Rhamnose	8.3	0.4-0.6	13	1.68
Ribose	4.6	0.5-0.6	14	0.00
Xylose	4.4	0.2	20	0.61

[a]Unit is expressed as weight percentage of total weight of carbohydrates.
[b]Unit is expressed percentage of dry cell weight (DCW).
[c]Unit is express as µg/mg of alcohol-insoluble residue biomass.
ND: Not detected.

Table 4. Fatty acids composition of lipids from *Nannochloropsis* (expressed as weight percentage of total fatty acids)

Fatty acids	Strain			
	Nannochloropsis sp.[a] [64]	*Nannochloropsis* sp.[b] [64]	*N. gaditana*[c] [54]	*N. limnetica*[c] [54]
C14:0	3.4-3.8	4.8-7.7	10.1	8.1
C16:0	18.2-36.4	38.1-48.4	27.5	27.2
C16:1	17.6-25.8	24.6-28.5	22.5	25
C18:0	ND	1.8-2.7	ND	ND
C18:1n9	3.3-3.7	5.0-11.3	3.75	4.0
C18:2n6	5.1-7.6	0.9-1.4	2.5	2.45
C20:4n6	6.1-1.2	1.8-3.3	6.0	6.5
C20:5n3	7.8-4.6	5.0-10.7	23.0	23.5

[a]Data obtained for *Nannochloropsis* sp. grew under irradiance level of 35 and 290 µmol E/m^2/s, respectively.
[b]Data obtained for *Nannochloropsis* sp. grew at dilution rate of 0.2-0.92 (1/day)
[c]Data obtained in semi-continuous cultures maintained with daily renewable rate of 40% of the volume of the cultures
ND: Not detected.

For the live feed, *Nannochloropsis* sp. was cultured in 1200 L plant made of ten 50 cm annular reactors with combined illumination to produce 270 g dry biomass annually. The concentrated algal suspension (50 g/L) was successfully used by fish hatcheries as a live feed for rotifers and rearing seabream larvae with the green-water techniques [11]. Biomass of *Nannochloropsis* sp. rich in EPA was produced in a flat plate (10 cm light-path), vertical reactor made of 10 mm glass plates glues together to form 500 to 1000 L reaching the optimal cell density of 6×10^8 cells/mL (12 g DCW/m^2/day and 650 mg EPA/m^2/day) for aquaculture [52]. To produce sufficient the algal biomass for production of rotifer quantity required by seabream hatchery producing 8×10^6 fingerlings annually, production of *Nannochloropsis* sp. was scaled up in 2000 L reactor to harvest 175 kg dry biomass per year [52]. Semicontinuous cultivation of *N. gaditana* for live feed production for the rotifer *Brachionus plicatilis* enrichment was investigated [53]. It was figured out that selection of the appropriate culture conditions is crucial for the production of microalgae having high biomass and nutritive profile for rotifer enrichment, because only microalgal cultures subjected to high growth rates and nutrient availability are suitable to improve the biochemical composition of rotifers, as indicated by very high positive correlations between the contents of EPA and ω-3 fatty acids in *N. gaditana* and *B. plicatilis* in the short time of exposure periods 24 h [53]. Marine *Nannochloropsis* is commonly cultured due to their high growth rate and proportion of polysaturated fatty acids (particularly EPA). However, freshwater species such as *N. limnetica* also owns a potential for rotifer nutrition. Recent study described that *N. limnetica* grew well in the range of 15-27°C and reached maximum biomass productivity of 0.64 g/L/day at 22°C, while its fatty acid profiles was found to be similar to that of well-known marine species *N. gaditana* [54]. Interestingly, growth and egg-ratio of the rotifer *B. plicatilis* cultured with *N. limnetica* in laboratorial scale experiments were two-fold higher than that when cultured with the same concentrations of *N. gaditana*, while fatty acids profile in the algae-rotifers mixture was similarly maintained [54]. Moreover, hatchery-scale experiments within five days indicated that *B. plicatilis* fed with baker's yeast supplemented with either on-site produced fresh microalgal cultures or with concentrated algae achieved similar growth rate with both *N. limnetica* and *N. gaditana*, but obtained higher dry weight and slightly better

egg ratio with the freshwater species [54]. This implies that *N. limnetica* can be a potential substitute of the freshwater *Chlorella* and marine *N. gaditana* in live feed production. Another study used *N. limnetica* for fatty acids production carried out by Krienitz et al. [47] pointed out that with a high content of phosphate (40 mg/L K_2HPO_4) in the culture medium under non-aerated suspension culture, *N. limnetica* can accumulate 22.19 mg/g DCW linoleic acid, 10.52 mg/g DCW arachidonic acid (ARA), and 55.56 mg/g DCW eicosapentaenoic acid (EPA), which were ten-fold higher than in the other picoplankton *Choricystis minor* and *Pseudodictyosphaerium jurisii*, and higher than in the nanoplanktonic green algae *Chlorella vulgaris*, *Monoraphidium neglectum* and *Scenedesmus obtusiusculus* indicating a high-quality food resource in fresh food chains [47]. Culturing large volumes of single microalgal for rotifer cultivation is heavy burden on hatcheries. Therefore, effect of the combination feeding of both high density of *N. oculata* (N) and condensed freshwater *Chlorella* (FC) on the fatty acid composition of L-type rotifer *B. plicatilis* (R) in a continuous culture system was performed [55]. It was pointed out that, the combination N+FC-feeding positively affected rotifer density when the culture was conducted at 24ºC and 25–27 psu (salinity) in a 2000 mL bottle with 50% of water exchanged daily. Moreover, the fatty acid profile (e.g., ∑n-6/∑n-3 ratio, EPA and DPA content, and polar/non-polar lipids ratio) of the N+FC-R and FC-R grew in a continuous culture system was affected by both N and FC [55]. The artificial detritus (detritus) originated from macroalgae *Ulva pertusa*, *Undaria pinnatifida*, and *Chondrus ocellatus* were used as individual substitute diets or combination with *Nannochloropsis* for investigation of population growth and nutritional content (amino acid and fatty acid compositions) of *B. plicatilis* [56]. In terms of population growth, maximum population density, and population growth rate, macroalgal detritus are not as good as *Nannochloropsis*, as they contain lower nutritional profile than *Nannochloropsis*. Interestingly, when the macroalgae-based detritus mixed with *Nannochloropsis*, the *B. plicatilis*'s populations maintained high levels and achieved higher maximum population densities as well as population growth rates [56]. Regarding to nutritional value of *B. plicatilis* fed with *U. pertusa* and *Nannochloropsis*, particularly, the amino acids, polyunsaturated fatty acids (PUFA), and docosahehaenoic acid (DHA), were recorded to be higher than those of other diets. Notably, DHA/EPA ratio in rotifers feed with *U. pertusa* and *Nannochloropsis* was reached 6.9 times higher than that fed with *Nannochloropsis* only. This indicates that *U. pertusa* can be partly substituted in the feed for enrichment of *B. plicatilis*, potentially reducing requirement of large volume of microalgae culture (e.g., *Nannochloropsis*, *Chlorella*, etc.) [56]. Concentrated algal paste of *Nannochloropsis* used for the production of *B. plicatilis* was studied by Pfeiffer and Ludwig [57]. Experimentally, rotifers were cultured in four translucent and low-density polyethylene tanks with a culture volume of 45 L of artificial seawater. The feed solution was a mixture of commercially available *Nannochloropsis* algal paste and artificial seawater. During a 21 days culture period, the rotifer population in each of four tanks was maintained between 500 and 1,500 rotifers/mL. A volume of 15 L was harvested when the rotifer count was 1,500 rotifers/mL or more. The results indicated that the simple and inexpensive use of a concentrated algal paste as a food source is suitable for the production of rotifers [57]. Very recently, fish growth hormone (GH) production in *N. ocutata* was studied by Chen et al. [58]. In the study, plasmid phr-YPGHs containing fish GH cDNA driven by a heat shock protein 79A promoter and a RUBISCO SSU 2 promoter was introduced into the protoplast of *N. ocutata* by electroporation. It was reported that four transgenic clones were screened and treated with heat shock for GH

production, which was determined as 0.27-0.42 µg/mL from 50 mL culture medium. The transgenic *N. oculata* was incubated with artemia for 6 h and fed to redtilapia larvae. Result revealed that the growth rate of larvae fed with artemia incubated with transgenic microalgae was greater than that of larvae feed with artemia inculated with nontransgenic microalgae with respective to weight gain (316% vs 104%) and body length (217% vs 146%). Moreover, the transgenic microalgae were existed stably at least until the 50th generation. Therefore, engineering *N. oculata* can enable production of functional GHs for aquaculture [58].

For the form of solid feed, commercial aquaculture products produced from *Nannochloropsis* are presently available in the forms of chilled, frozen, condensed or freeze dried, which have been shown to support good rotifer growth [59-61].

As early mentioned, aquaculture industry is rapidly growing, therefore it needs stable supply of feed source of both quantity and quality for its future growth, particularly the DHA and EPA-rich feeds which have been long originated from fish oils. According to a recent report, approximate 70% fish oil is still utilized for current aquafeeds production [62]. However, the global supply of fish oils is limited, while other emerging ω-3 markets are growing and competing with the traditional resource. DHA and EPA from microalgae are considered as a promising alternative which possess a highly potential to fulfill the global demand. However, current microalgae production technology is not yet to be economically feasible. A recent techno-economic analysis (TEA) of microalgae production implemented based on biological and technical parameters published in the literature under various scenarios indicated that, biological productivity, geographical locations and production technology are important factors which must need to be optimized to lower production cost [62]. Specifically, cost of EPA and DHA production was estimated to be the lowest for flat panel photobioreactors in locations with near sky conditions. Moreover, sensitivity analysis revealed that optimizing photosynthetic efficiency and doubling of the EPA and DHA yield could reduce the cost to 11.9 USD/kg total (EPA and DHA), which is a great potential for aquaculture feed formulation [62]. This is the targeting goals of coming researches on microalgae-based aquaculture feed production.

3.2. Animal Feed Production from *Nannochloropsis*

Microalgae have received much attention as a source of biomolecules and whole biomass for feed formulation. The application of microalgae biomass in poultry nutrition [70] and livestock [71] has been comprehensively reviewed. They are not only formulated into poultry diets as a DHA/EPA-rich source, but they can also be used as a source of protein, microelements, vitamin and antioxidants, and pigmentation agents for skin and egg yolks. The incorporation of microalgae biomass into poultry feed can have beneficial effects on meat and egg quality, i.e., increasing ω-3 fatty acids and carotenoids, as well as enhancing performance indices and immune function of the poultry [70]. The use of whole algal biomass in animal feeds has been studied for over 50 years [72]. Initially, algae were cultured in ponds followed by sequential harvesting, drying and formulating/using as a protein source for animal diets. Recently, lipid-extracted microalgae biomass generated from biofuels production has been utilized to study as feed supplement for various animal diets, mainly originated from *Spirulina*, *Chlorella* [70], but also other lipids-rich strains such as diatom

Staurosira sp. [73], *Schizochytrium* [74], and astaxanthin-rich algal meal *Haematococcus pluvalis* [75].

The early study on utilization of *Nannochloropsis* as a supplement feed for rats was dated back to 1992, when chemical composition, nutritional and toxilogical evaluation of *Nannochloropsis* sp. was determined [76]. Data revealed that, the algal biomass produced in an outdoor photobioreactor (diameter = 4 cm, volume = 64 L) enriched with seawater medium, consisted of 40 wt% protein, 25.5 wt% lipids, 5 wt% crude fiber, 11.5% non-nitrogen extract, and 10% ash. The main fatty acids found in total lipids of the algal biomass were palmitic (23 wt%), palmitoleic (24.5 wt%), and eicosapentaenoic (23 wt%), while the minerals content presented an absence of bioconcentration of toxic elements. The whole nutritive value (fiber, protein, lipids, minerals and energy composition) of *N. oculata* has been examined to evaluate its potential as a source of animal feed like steam-flaked maize and soybean oil meal [77]. Results after evaluating the feeds and *N. oculata* showed variations in the protein content as well as amino acid contents, however such variations can be adjusted during formulation of feeds for animal. This study confirmed the promising potential of *N. oculata* as an ingredient in animal feeds [77]. Moreover, safely assessment of *N. oculata* carried out on male and female Sprague-Dawley rats administered a 0 or 10 mL *N. oculata*/kg rat (10^8 viable cells/mL) suspension by oral gavage once daily for 14 consecutive days indicated no mortalities occurred and no signs of toxicity were observed during the study, confirming that the algal strain used in the production of omega-3 oil is not pathogenic when administered orally to rats [78].

Bruneel et al. [79] reported an increased content of DHA in egg yolks of hens fed a diet containing *N. gaditana* and suggested that this algae may be used as an alternative to current sources of ω-3 for the production of DHA-enriched eggs. Lemahieu et al. [80] compared the efficacy of four different algae species (*Phaeodactylum tricornutum*, *Nannochloropsis oculata*, *Isochrysis galbana* and *Chlorella fusca*) on the enrichment of egg yolks in ω-3. They reported that the highest enrichment with ω-3 as well as increased yolk color was achieved with supplementation using *Phaeodactylum* or *Isochrysis*, and these two microalgae could be used as an alternative to current sources for the enrichment of eggs. A study carried out to evaluate three microalgae (*Nannochloropsis oceanica*, *Phaeodactylum tricornutum* and *Isochrysis galbana*) as potential nutrient sources in diets for monogastric animals by Skrede et al. [81] reported that there was a significant linear reduction in crude protein (CP) digestibility with increasing dietary inclusion of all algae products at 60, 120 and 240 g/kg as is, replacing fish meal (*N. oceanica* and *P. tricornutum* had similar crude protein (CP) content (47.7 and 49.0% of DM, respectively), amino acid composition and lipid content (8.4 and 7.4%, respectively), whereas *I. galbana* contained 20.1% CP and 16.2% lipids). The apparent CP digestibility determined by linear regression for *N. oceanica*, *P. tricornutum* and *I. galbana* was 35.5, 79.9 and 18.8%, respectively. The individual amino acid digestibility showed acceptable values for *P. tricornutum*, but low and highly variable values for *N. oceanica* and *I. galbana*. Although the algae contributed a minor proportion of dietary lipids, lipid digestibility declined with increasing inclusion of all algae and especially with the highest level of *N. oceanica*. It was concluded from the mink study that among the investigated algae, *P. tricornutum* was the preferable source of digestible nutrients [81].

4. HIGH VALUE PRODUCTS PRODUCTION FROM *NANNOCHLOROPSIS*

4.1. Production of Eicosapentaenoic Acid (EPA) from *Nannochloropsis*

Nannochloropsis sp. is well known to accumulate high content of eicosapentaenoic acid (EPA) (around 2-5% DCW) [82, 83], therefore large scale autotrophic outdoor cultivation of *Nannochloropsis* sp. in tubular photobioreactors [84-86] and flat panel photobioreactors [87] for EPA production has been widely investigated. Several techniques have been developed and applied for extraction of EPA including ultrasound-assisted extraction [88], hydrothermal-acid treatment [89], solvent extraction [90], and pressurized fluid extraction [91].

The using of ultrasound is to disrupt cell wall of the microalgal to make lipids body and fatty acids to be readily accessible to extracting solvent. It was reported that recovery of EPA was significantly influenced by extraction temperature and time, whereas hexane to biomass ratio (v/w) was not statistically important variable for EPA yield [88]. The optimal conditions established for extraction temperature, reaction time and solvent to biomass ratio were 27.6^{o}C, 34 min, and 21:3 (mL/mg), respectively, at which the EPA yield and content in total fatty acids reached 62.8% and 16.25%, respectively [88]. Supercritical carbon dioxide (SC-CO_2) is regarded as safer and more environmental friendly than hexane-based extraction because the process uses CO_2 as a solvent, short extraction time and low temperature, and giving high quality final product. Andrich et al. [92] applied SC-CO_2 for bioactive lipids extraction from *Nannochloropsis* sp. at temperature and pressure of 40-55°C and 10-77 MPa, respectively, resulted in 39.3-44% DCW total lipids containing 29.4-33 wt% EPA in 6 h extraction and CO_2 flow rate of 10 kg/h. Pressurized fluid extraction (PFE, trades as "Accelerated Solvent Extraction," ASE®) was considered as an efficient solvent extraction methodology because it employs elevated temperatures and high pressure to accelerate penetration of solvent into biological matrix, while dissolving interested compounds. This is particularly beneficial when microalgae having highly-rigid cell walls are utilized as extraction substrates. ASE is a promising alternative to supercritical CO_2 extraction (SC-CO_2). Pieber et al. [91] used ASE for EPA extraction from N. oculata using alternative solvents including hexane, hexane/propan-2-ol (2:1, v/v) and ethanol (96 vol.%). Results revealed that the highest extraction yield of total lipids, total fatty acids, and EPA obtained from ASE with ethanolic solvent were 36%, 16.7%, and 3.7%, respectively. This indicated that EPA production from N. oculata and extraction with ethanol via ASE is economically beneficial because ethanol is generally recognized as safe (GRAS) solvent, which can be completely removed without toxic effect on EPA nutrition ingredient. Furthermore, ethanol liberated with residual biomass after the extraction can be also completely removed without effect on protein quality of the leftover, which can be utilized for other applications (animal feed, aquaculture) with respect to biorefinery [91]. Recently, hydrothermal acid pretreatment of microalgae biomass for lipids extraction was intensively studied. Acids such as sulfuric acid, hydrochloric acid, and nitric acid are highly active and efficient in disruption of microalgal cells and hydrolysis of algal cells wall containing cellulose and make lipids and fatty acids in algal biomass to be accessible to extracting solvents. The optimal conditions for hydrothermal acid treatment of wet *N. salina* and EPA extraction were identified using statistical technique (response surface methodology, RSM) [89]. It was reported that

maximum EPA yield of 43.93 mg/g DCW was obtained with 1.27% of sulfuric acid, 113.34ºC of temperature and 36.71 min of reaction time, when using hexane (96 vol.%) for 1 h extraction. The study pointed out that the acid treatment can be indeed a noteworthy alternative for EPA extraction [89].

4.2. Production of Pigments from *Nannochloropsis*

Pigment composition was reported to vary among six strains of *Nannochloropsis*. Ketocarotenoids astaxanthin and canthaxanthin was accumulated in higher concentration in *N. salina* and *N. gaditana* than in other strains [18]. Zeaxanthin content in *N. gaditana* could increase up to 100% when culture salinity experimented in the range of 15-100 practical units. In culture with cell density of 109 cells/mL, pigment production reached: 350 mg/L for chlorophyll *a*, 50 mg/L for violaxanthin, 5 mg/L for canthanxanthin, and 3 mg/L for astaxanthin. Total canthaxanthin and astaxanthin in *N. gaditana* can reach 0.7% dry cell weight [18]. Moreover, the highest accumulation of beta-carotene was observed in *N. salina* among six microalgae including *Phaeodactylum* sp. (Bacillariophyceae), *Nannochloropsis* sp. (Eustigmatophyceae), *Chlorella* sp., *Dunaniella* sp., and *Desmodesmus* sp. (Chlorophyta) when they were grown on municipal wastewater [93]. The availability of diverse pigments including chlorophyll *a*, zeaxanthin, canthaxanthin and astaxanthin, each with high production levels indicating that *Nannochloropsis* is a source of valuable pigments.

5. BIOFUELS PRODUCTION FROM *NANNOCHLOROPSIS*

5.1. Biodiesel Production from *Nannochloropsis*

Since *Nannochloropsis* contains high lipids content, the biomass has been widely used as an interested feedstock for biodiesel production study [94, 95]. Conventionally, lipids of *Nannochloropsis* can be primarily extracted before subsequent conversion to biodiesel with alcohols using chemical homogeneous and heterogeneous catalysts, biocatalyst, and supercritical conditions. However, due to its high cost, direct conversion of oil-rich *Nannochloropsis* biomass with alcohols to biodiesel were investigated extensively. A recent application of *Nannochloropsis* biomass for biodiesel production is summarized in Table 5.

5.2. Biodiesel Production from *Nannochloropsis* via Chemical Heterogeneous Catalysis

Typically, Umdu et al. [96] described fatty acid methyl esters (FAME, biodiesel) synthesis from *N. oculata*-extracted lipid over solid base catalysts (CaO/Al_2O_3 and MgO/Al_2O_3) at 50ºC. It was found that single CaO and MgO metal oxide powder did not show any activity, while CaO/Al_2O_3 binary synthesized powder (80 wt% CaO loading) was the highest active catalyst, which resulted in 97.5% biodiesel yield when methanol to oil molar ratio applied of 30:1 combined with catalyst loading of 2 wt% (based on oil weight),

Table 5. Conversion of oil-rich *Nannochloropsis* biomass to biodiesel

Strain	Feedstock	Catalyst/Method	Conditions	Biodiesel yield (%)	Ref.
N. oculata	Lipid	Transesterification of algal extracted lipid over solid base catalyst (80% CaO/Al_2O_3)	MeOH/lipid molar ratio: 30:1 Catalyst loading: 2 wt% oil Temp.: 50ºC Stirring rate: 1100 rpm Reaction time: 4 h	97.5%	[96]
N. gaditana	Lipid	Transesterification of algal-extracted lipid over solid Beta (h-Beta) zeolite as heterogeneous catalysts	MeOH/lipid molar ratio: 100:1 Catalyst loading: 2 wt% (oil + MeOH) Temp.: 115ºC Stirring rate: 1100 rpm Reaction time: 4 h	25%	[97]
N. salina	Lipid	Selective esterification over zeolite as a catalyst (HZSM-5)	MeOH/FFA: 30:1 Catalyst loading: 10 wt% Temp: 85ºC Stirring rate: 150 rpm Reaction time: 3 h	65%	[98]
N. gaditana (wet, 86% water)	Saponifiable lipids (SLs)	H_2SO_4	Dry crude lipids (71.0 wt% SLs): 100 mg Hexane: 1.4 mL/g SL MeOH: 12-20 mL/g SL H_2SO_4: 0.22 g/g SL Temp.: 60-80ºC Time: 2-4 h	95-97	[100]
Nannochloropsis sp. (wet paste, 75% water)	Extracted lipids	H_2SO_4/KOH	Two-step catalytic conversion *1st step: Pre-esterification* Algal oil: 5 g MeOH: 30 wt% H_2SO_4: 1 wt% Temp.: 65ºC Time: 2 h *2nd step: Transesterification* Pre-esterified oil: 5 g (acid value of 2 mg KOH/g) MeOH/oil molar ratio: 12:1 KOH: 2 wt% Temp.: 65ºC Time: 30 min	70.4	[101]
N. salina (wet paste, 76% water)	Whole biomass	H_2SO_4	Biomass: 200 mg MeOH: 2 mL (or EtOH, 1 mL) Temp.: 105ºC H_2SO_4: 2.5-5% Time: 30 min	99.7 (for MeOH) 94.6 (for EtOH)	[103]
			Biomass: 100 g MeOH: 1 L (or EtOH, 500 mL) Temp.: 105ºC H_2SO_4: 2.5-5% (v/v in MeOH or EtOH) Time: 30 min	100.4 (for MeOH) 101.0 (for EtOH)	[103]
N. gaditana (wet, 73.6% water and 31.1% total lipids)	Extracted lipids (saponifiable lipids, SL)	Novozym 435	SL/MeOH ratio: 11:1 *t*-butanol: 10 mL/g SL Catalyst: 0.225 g/g SL Temp.: 40ºC Time: 48 h	94.7	[106]
N. gaditana (wet, 73.6% water and 31.1% total lipids)	Extracted lipids (saponifiable lipids, SL)	*Rhizopus oryzae* intracellular lipase	SL/MeOH ratio: 11:1 *t*-butanol: 10 mL/g SL Catalyst: 0.7 g/g SL Temp.: 35ºC Time: 72 h	83	[107]

MeOH, Methanol; EtOH, Ethanol; FFA, Free fatty acids; Temp., Temperature; SL, Saponifiable lipids; Ref., Reference.

stirring rate of 1100 rpm and reaction time of 4 h (Table 5). Lipids extracted from *N. gaditana* with methanol and stirring under reflux was employed for biodiesel production using synthesized hierarchical zeolites (ZSM-5, h-ZSM-5, Beta, and h-Beta) as heterogeneous catalysts [97]. It was reported that hierarchical h-Beta zeolite catalyst showed the highest activity in the transesterification of *N. gaditana*'s oil with methanol, which gave biodiesel as high as 25% under the reaction conditions of methanol to oil molar ratio of 100:1, catalyst loading of 2 wt% (oil + methanol), temperature of 115°C, stirring rate of 1100 rpm, and reaction time of 4 h (Table 5). Lipids extracted from *N. salina* refined with 1% (v/v) phosphoric acid (chlorophyll removal), followed by refluxed with KOH 1M and acidified with sulfuric acid to obtain free fatty acids (FFA), which was then utilized as a substrate for selective esterification with methanol over heterogeneous HZSM-5 catalyst for simultaneous biodiesel production and polyunsaturated fatty acids (PUFA) enrichment [98]. Results indicated that the zeolite catalyst preferentially converted shorter-chain fatty acids (SCFA) into fatty acid methyl esters (FAME) achieving 65% conversion and enriched high-value PUFA 78% in the unreacted free fatty acids (FFA) stream ([98], Table 5).

Microwave and ultrasound are alternatively physical means to assist cell rapture during direct biodiesel conversion from *Nannochloropsis* biomass [99]. It was reported that microwave oven method appeared to be the most simple and efficient technique among two applied processes for single-stage direct transesterification of *Nannochloropsis* with chloroform/methanol (2/1, v/v) catalyzed by base SrO solid catalyst (30 wt% DCW) at 50-60°C. Biodiesel yield obtained by microwave oven and ultrasound reached 40 wt% and 20 wt% based on dry biomass, respectively [99].

5.3. Biodiesel Production from *Nannochloropsis* via Chemical Homogeneous Catalysis

Saponifiable lipids (SLs) (with SL purity of 71% and low polyunsaturated fatty acids of 16.9 wt%) were derived from *N. gaditana* biomass (86% water) using homogenization at 1700 bar and low temperature of 20-22°C [100]. The crude SLs was then used for biodiesel conversion via H_2SO_4-catalyzed transesterification with methanol at 60-80°C during 2-4 h reaction. Data indicated that FAME conversion reached 95-97% using methanol/SL ratio of 12-20 mL/g and H_2SO_4/SL ratio of 0.22 g/g ([100], Table 5). Wet paste of *Nannochloropsis* sp. (75% water) was employed for lipids extraction using high pressure extractor under extractive conditions of 1.5 MPa, 120°C, and 50 min to obtain crude oils containing acid value of 6.5 mg KOH/g [101]. The crude oil was used for biodiesel production via two-step sequential catalytic process, which consisted of H_2SO_4-catalyzed-preesterification followed by KOH-catalyzed transesterification with methanol. The optimal conditions for biodiesel yield of 70.4% at the same reaction temperate of 65°C were 30% methanol, 1% H_2SO_4, and 2 h reaction (for pre-esterification) and methanol to oil molar ratio of 12:1, KOH loading of 2%, and reaction time of 30 min (for the transesterification) ([101], Table 5). Recently, Hita Peña et al. [102] developed a five-steps process producing biodiesel from saponifiable lipids (SLs) extracted from wet *Nannochloropsis gaditana* biomass. Firstly, wet biomass containing fatty acids was transformed to potassium fatty acid salts using KOH/ethanol (96%) at 60°C for 1 h. Secondly, water was supplied to the fatty acid salt to make a solution with 30% (w/w) water, which was then mixed with hexane (1:1, v/v) at 20°C for extraction of unsaponifiable

lipids (hexane phase). Thirdly, ethanol phase containing fatty acids was acidified to pH = 5 with HCl 37 wt% and subsequently supplied with hexane at volume ratio of 1:1 (v/v) (hexane/ethanol-water) for fatty acid extraction (hexane phase). Fourthly, the extracted fatty acids were converted to fatty acid methyl esters (FAME) via methylated esterification catalyzed by 1.8 wt% H_2SO_4 (based on FA weight) at 60°C, 200 rpm, 2 h with molar ratio of methanol to FA of 10:1, resulted in biodiesel yield and purity of 82 wt% and 74.8 wt%, respectively. The crude biodiesel was further purified with bentonite (bentonite/crude biodiesel ratio 2:1 w/w) at 40°C for 24 h to achieve FAME purity of 96.5% [102].

Direct conversion of algal biomass to biodiesel is a promising process as it can lower total cost of biodiesel production. Kim et al. [103] used H_2SO_4 as homogeneous catalyst for simultaneous disruption of *Nannochloropsis salina* cell (wet paste) and catalysis of transesterification of the algal lipids with alcohols (methanol and ethanol) to fatty acid methyl (and ethyl) esters [103]. It was described that biodiesel yield reached over 90% at 105°C with 2.5-5% H_2SO_4 (v/v in alcohol) in 30 min reaction for 200 mg biomass used. Moreover, the pre-determined reaction conditions were found to work well with biomass scale up to 100 g, which resulted in over 100% conversion of biodiesel presenting highly potential for large scale application ([103], Table 5). To maximize value of biodiesel production, Im et al. [104] recently developed and reported a protocol for concurrent production of biodiesel and chemicals (ethyl levulinate (EL), ethyl formate (EF), diethyl ether (DEE)) via wet in *situ* transesterification of *N. gaditana* with ethanol in chloroform and catalyzed by H_2SO_4. It was evaluated that EL, EF, and DEE were significantly generated up to 23.1%, 10.3%, and 52.1%, respectively, of the fatty acid ethyl esters (FAEE) with the FAEE yield reached over 90% at 125°C [104]. The formation of EL, EF, and DEE is attributed to the hydrolysis of algal cell wall (contains cellulose) by acid, leading to generation of reducing sugars and lactic acid (LA) and formic acid (FA) in presence of ethanol. In turn, LA and FA become precursors for esterification reaction to generate EL and EF in excess ethanol [104]. This is a promising ideal for newly establishment of biorefinery of microalgae biomass for biofuels and chemical production. Using homogeneous acid catalysts (e.g., H_2SO_4, HCl, etc.) in conversion of lipids-rich algal biomass with alcohols can directly produce fatty acid alkyl esters via simultaneous transesterification and esterification. In contrast, catalysis of algal lipids with alcohols by alkaline (e.g., KOH, NaOH, etc.) generates soap because algal lipids contain certain amount of free fatty acids. Therefore, in order to obtain biodiesel, the soap should be precipitated by a common inorganic salt to form a precipitation which is then can be separated by filtration for further reaction to produce biodiesel when acid catalysts are employed. A recent study carried out by Binnal and Babu [105] reported that, direct saponification of wet paste of *N. oculata* (80 wt % moisture) harvested at the end of the exponential phase was subjected to direct saponification in a low-cost microwave reactor using ethanolic KOH as a alkaline catalyst to convert the algal lipids to soap. The soap floated as an upper layer was precipitated and separated from other unsaponifiable matters by saturated KCl and subjected to simultaneous acidulation and esterification with methanol catalyzed by H_2SO_4 to form biodiesel of high purity (97.04% FAME in final biodiesel). Optimum parameters of esterification of fatty acids in microwave reactor were: microwave power of 450 W, molar ratio of methanol to fatty acids of 80:1, concentration of sulphuric acid of 2.5 wt %, reaction time of 11 min and reaction temperature of 60°C, under which the final biodiesel yield of 97.11% and reaction rate constant of esterification of 0.452 min^{-1}

were achieved. Moreover, Cetane number and oxidation stability index of biodiesel were evaluated as 59.349 and 3.298 h, respectively [105].

5.4. Biodiesel Production from *Nannochloropsis* via Enzymatic Catalysis

Biocatalyst (enzyme) owns a promising capability to catalyze esterification and transesterification of algal lipids (containing free fatty acids) with alcohols to biodiesel because of its high activity, low reaction temperature, and greener than chemical catalysts. The main disadvantage of the biocatalysis is costly due to high cost of biocatalyst production. However, application of biocatalyst for biodiesel production from microalgae lipids have been widely documented in the literature. Navarro Lopez et al. [106] recently used wet paste of *N. gaditana* for saponifiable lipids (SLs) extraction and subsequently conversion to biodiesel by lipase-catalyzed transesterification using a commercial enzyme Novozym 435. Results revealed that lipids extracted by ethanol (96%)-hexane had 31% SLs, which was then increased up to 95% after degumming with cold acetone at 4°C. The transesterification of the SLs were performed at 40°C using 0.225 g biocatalyst/g SL in 10 mL *t*-butanol/g SLs as solvent, and 11:1 methanol to SL molar ratio as acyl acceptor for three step addition of alcohol, to result in 94.7% biodiesel yield (Table 5). This best conversion was reported to decrease to 9.8% after three cycles of reusability of the biocatalyst [106]. On the other hand, the optimal transesterification conditions for the SLs conversion to biodiesel with the highest yield of 83% when using *Rhizopus oryzae* intracellular lipase as biocatalyst were methanol to SLs molar ratio of 11:1, biocatalyst loading of 70 wt% SLs, 10 mL *t*-butanol/g SLs, temperature of 35°C, and reaction time of 72 hr [107]. Although biodiesel yield achieved by *Rhizopus oryzae* lipase was lower than that with Novozym 435, the FAME conversion was remained as high as 71% of initial conversion after three cycles of reusability, indicating that *Rhizopus oryzae* lipase had higher durability than Novozym 435 [106].

5.5. Biodiesel Production from *Nannochloropsis* via Supercritical CO_2

Supercritical CO_2 was employed to catalyze transesterification of lipids-rich *N. oculata* biomass with methanol and extract FAME at low temperature (37 and 55°C) and pressure (5.9 and 7.6 MPa) [108]. The presence of FAME peaks in methanolic extracts indicated that extraction and transesterification is achievable in a single step process with SC-CO_2 using methanol as acyl acceptor. In term of energy consumption, it seems that, this data was superior to results of previous study that was reported by Patil et al. [109] conducting reactions at the harsh conditions (high temperature (255°C) and pressure (1200 psi)) on a wet *Nannochloropsis* sp. paste (90% water), however, further investigation should be carried out (e.g., measuring biodiesel yield, biodiesel productivity and biodiesel purity) in order to fully evaluate the efficiency of two conversion processes. Although FAME can be simultaneously converted and extracted to a certain level under low supercritical conditions, the quality FAME product may not be satisfy. This is because FAME utilized for purification to reach fuel grade biodiesel requires purity of over 95 wt% which is usually obtained under a high temperature and pressure supercritical technology to achieve a high FAME yield.

5.6. BioH_2 Production from *Nannochloropsis*

Hydrogen is a green fuel (zero-emission fuel) because combustion of H_2 generates H_2O only and releases energy, which is completely unharmful for the environment. Production of hydrogen via fermentation of sugars derived from biomass (i.e., lignocelluloses, algae, etc.) is considered as an alternative route over the conventional hydrogen-producing processes such as water electrolysis and steam reforming because of its high sustainability [110]. Microalgae biomass is a promising feedstock for bioH_2 production due to fast growing, high carbohydrate content, and diverse distribution [31]. The main biochemical compositions of *Nannochloropsis* species used for biological fermentation are carbohydrates (20-38%) and proteins (10-43%), in which carbohydrates are assimilated as carbon sources, while proteins are utilized as nutrition for anaerobic bacteria [111, 112]. However, high molecular weight polymers such as carbohydrates and proteins are not ready to be utilized by the hydrogen-producing bacteria. It requires a pretreatment step to degrade these polymers to reducing saccharides and amino acids, which are easily metabolized by the bacteria for hydrogen production. In a recent study, Xia et al. [113] reported a microwave-assisted pretreatment of acidic *N. oceanica* biomass for sugars and amino acids generation before utilization for bioH_2 production via three-stage process of dark fermentation (at 30°C), photofermentation (at 30°C), and methanogenesis (at 35°C) with *Clostridium butyricum*, *Rhodopseudomonas palustris*, and *Methanosarcina* and *Methanothrix*, respectively. The optimal conditions of the microwave-assisted pretreatment obtained for sugars production were temperature of 140°C, reaction time of 15 min, 1% (v/v) H_2SO_4 of catalyst loading, and 50 g/L biomass loading, which achieved a sugars and amino acids concentration of 4.8 g/L and 0.87 g/L, respectively, that was much higher than that in raw algal biomass [113]. The thermodynamic analysis for dark fermentation between amino acids and reducing sugars from *N. oceanica* hydrolysate revealed that the total utilization efficiencies of amino acids and reducing sugars were equivalent to 95%. However, the consumption time of the reducing sugars is two times faster than that of amino acids. In three-stage process on utilization of sugars- and amino acids-rich *N. oceanica* hydrolysate, the maximum hydrogen and methane yields were estimated as 183.9 ml/g-total volatile solids (TVS) and 161.3 ml/g-TVS, respectively. Furthermore, the total energy yield of hydrogen and methane produced from *N. oceanica* via the developed method (three-stage) was respectively 1.7 and 1.3 times higher than those via two-stage (dark fermentation and methanogenesis) and single-stage (methanogenesis) methods [113]. More recently, biomass of *Nannochloropsis* sp. strain was firstly used for lipids and pigments extraction via supercritical CO_2, subsequently followed by the utilization of the leftover as carbohydrates-rich feedstock for bioH_2 production via dark fermentation [114]. It was reported that under the supercritical conditions of 40°C, 300 bar, and CO_2 flow rate of 0.62 g/min, 33 g lipids can be recovered from 100 g dry biomass. Moreover, supplement of 20 wt% ethanol with CO_2 as co-solvent resulted in lipids recovery of 45 g/100 g dry biomass and recovered 70% pigments. The residual biomass obtained after the supercritical extraction was employed for dark fermentation with *Enterobacter aerogenes* resulting in a hydrogen production yield of 60.6 mL/g dry biomass [114]. This experiment was used as a biorefinery template for energy and CO_2 emission and economic analysis toward evaluation of the potential of microalgae as energy sources for biofuel production. The energy consumption, economic assessment, as well as CO_2 emission from whole refining process including *Nannochloropsis* sp. cultivation, harvesting, dewatering, milling (pretreatment), extraction

(using soxhlet extraction (SE) and supercritical fluid extraction (SFE)), and fermentation of residual for bioH_2 production were simultaneously evaluated. It was reported that the oil production pathway by SE showed the lowest value of energy consumption and CO_2 emissions, which were estimated as 177-245 MJ/MJ(prod) and 13-15 kg CO_2/MJ(prod), respectively. Although the SFE consumed and emitted c.a. 20% more than the SE pathway, production of oil via SFE proved to be more economically viable, with a cost of 365 €/kg oil produced, while simultaneously recovering of high-value pigments. The bioH_2 as co-product may be advantageous in terms of product yield or profit [115].

6. COSMECEUTICALS PRODUCTION FROM *NANNOCHLOROPSIS*

Cosmeceutical originated from cosmetics and pharmaceutical is the term that was coined by the dermatologist Albert Kligman since 1980 [116, 117]. Cosmeceuticals are cosmetics products having biologically active ingredients, which own medical or drug-like benefits by treating and preventing imperfections of the skin. Microalgae are a source of natural products including proteins, vitamins, polysaccharides, and minerals, which are potential applications for cosmeceuticals [118]. Metabolites produced from cyanobacterium under extreme conditions in hyper-arid habitats was reported to have high water retention capacity and potentially to be used in cosmetic products as moisturizers [119]. The cyanobacterium also produce UV screening compounds such as mycosporine-like amino acids and scytonemin, which are good candidates as alternatives to current synthetic UV filters [119]. Benefits of microalgae in skin care are: (i) microalgae are a good source of ω-3 fatty acids, which help reduce the appearance of fine lines and wrinkles, prevent dryness and fight skin problems like eczema and acne, (ii) algae regulate the production of sebum that is necessary for the skin to be moisturized and thereby prevent dehydration of the skin, (iii) algae offer antioxidant properties that avoid free radical damage which causes of skin aging. Moreover, algae help to fight free radicals and also help aid in the production of collagen and elastin, which are essential to having firmer skin, (iv) algae are loaded with essential minerals and vitamins that offer regenerative, protective and intense rejuvenating properties to prevent the aging process while also repairing skin damage.

Spirulina and *Chlorella* are the main species that are well established in skin care industry [120]. Recently, it was reported that tyrosinase activities were inhibited by the extract of several microalgae [121]. For the development of *Nannochloropsis*-based cosmetics, antityrosinase zeaxanthin from *N. oculata* has been recently examined [122-124]. In commercial market, many *N. oculata*-based skin care products have been launched. Typically, new products made from ingredients (polysaccharides, amino acids and vitamins, particularly vitamin C (an effective antioxidant) and vitamin B_{12}) of *N. oculata* with excellent skin-tightening properties (short and long-term effects) (Pepha®-Tight) has been marketed by Pentapharm (Basel, Swizerland). NuFountain LLC (Colorado, USA) recently developed and launched C20 Vitamin C Serum for skin care which used purified *N. oculata* extract containing Vitamin C and B_{12} as main ingredients. Algoid Technologies (Florida, USA) also developed and marketed a “Morning Rose Facial Elixir” for skin care made from 100% naturally-derived ingredients of live *N. oculata, I. galbana* (T-iso) and *T. chuii* plankton. AlgaeEnergy (Madrid, Spain) has developed diverse research and developments programs for

Spirulina, *C. vulgaris*, *T. suecica*, *I. galbana*, and *N. gaditana* strains in order to increase the value of cosmetic formulae. Their products will be launched in coming years. Solazyme (San Francisco, USA), an algae-based fuel startup company recently unveiled Algenist, a luxurious anti-aging cream skincare line that used compounds-originated microalgae as a main ingredient. The products are marketed to help visibly restore the skin resiliency and elasticity, delivering a firmer, tighter, more toned appearance. It also visibly strengthens the most fragile skin texture and softens facial contours, minimizing the appearance of fine lines and deep wrinkles to unveil skin's youthful radiance.

7. SOLID BIOFERTILIZERS PRODUCTION FROM *NANNOCHLOROPSIS*

The use of chemical fertilizers is well known to have adverse effect on soil, plant, and the environment. To mitigate this issue, the interesting in organic farming has attracted considerable attention recently. Microalgae can accumulate high content of proteins, lipids, vitamins, and minerals under favorable conditions. Particularly, microalgae can be used as a biological agents to utilize nitrogen, phosphorous, and organic carbon source in wastewater for wastewater bioremediation purpose via photosynthesis using solar energy, while simultaneously producing biomass and generating oxygen, which is beneficial for the environment. Microalgae biomass can be directly introduced into soil inoculation to increase crop productivity, offering an alternative to vicious cycle of chemical fertilizers, soil deterioration, and dependency on imports. Moreover, algae fertilizers can also boost the profitability of wastewater treatment industry by generating additional income from fertilizer's sale. Although numerous studies have reported positive responses on using algal fertilizers for crop plants [125-129], the current development of algal fertilizers has not been well-established. The first study using blue-green microalgal *Tolypothrix tenuis* was observed to increase in rice yield in Japan [130]. Later, the increase in germination of rice seeds compared to control samples was also recorded when the rice seeds pretreated with cyanobacteria *Phormidium foveolrum* [131], which were silimar to observation of Tripathi et al. [132] and Saadatnia and Riahi [133]. The combination of *Aulosira fertilissima* (2.25 g) + *Spirulina platensis* (2.25 g) + diamonium phosphate (DAP) (0.5 g) was found to increase number of tomato fruits and productivity up to 522% and 977%, respectively [134]. Extract, culture medium, and whole biomass of microalgal strain *Acutodesmus dimorphus* was recorded to notably increase germination rate of seeds and improve growth and productivity of of Roma tomato [127]. More recently, *Spirulina plantensis* was used to treat aquacutlure wastewater containing nitrogen (mainly amonia and nitrate) and produce biomass, which was utilized as a organic fertilizer for study of seed germination and growth of several crop plants including *Arugula* (*Eruca sativa*), *Bayman Red Bayam Red* (*Ameranthus gangeticus*) và *Pak Choy* (*Brassica rapa* ssp. *Chinensis*) [128]. Data revealed that the pretreatment with the algal suspenstion culture remarkably increased germination rate of seeds of Chinese Cabbage (*B. rapa* ssp. *Chinensis*) and Kai Lan (*Brassica oleracea alboglabra*). Furthermore, the supplement of solid biomass with standard soild improved growth rate of Arugula (*Eruca sativa*), Bayman Red Bayam Red (*Ameranthus gangeticus*) and Pak Choy (*Brassica rapa* ssp. *Chinensis*). Interestingly, a recent study reported that extract and culture medium of several microalgae contain phytohormones (e.g., gibberellins, auxin, and cytokinin), which play an

important roles in plant developement [135]. Algal turf scrubbers (ATS) biomass produced via growing in N- and P-rich anaerobically digested dairy manure has been successfully employed as an organic slow-release fertilizer [136, 137]. The dried biomass was amended with potting mix as N- and P-slow release fertilizers to grow cucumber and corn deedlings, which presented equivalent growth rate (seedling dry weight and nutrient content) with comparable levels of chemical fertilizer-supplemented potting mixes. This suggested that dried ATS grew in wastewater can be a substitute for commericial fertilizers as a potting substrate for crop production [137]. The using of *Nannochloropsis* for wastewater treatment and CO_2 sequestration from flue gas to produce biomass for biofertilizers application was recently carried out by Coppens et al. [125]. Microalgal bacterial flocs (MaB-flocs) grew in a raceway pond treating aquaculture wastewater and *N. ocutala* phototrophically cultivated on a landfill site using CO_2 from flue gas were harvested and used to study their slow-release fertilizer potential for tomato cultivation in comparison with conventional inorganic and organic horticulture fertilizer systems [125]. It was reported that there was no significant difference in growth rate of the tomato inoculated in microalga and commercial organic fertilizers. Interestingly, although lower tomato yield was achieved, the microalgal fertilizer enhanced the fruit quality as it increased sugar and carotenoid content of the tomato fruit. However, further study is needed to determine the optimal fertilizer mixture that produce high quality of fruits with satisfactory yields. In term of economic point of view, optimization of the amendment ratio of conventional fertilizers with algal biomass produced from N- and P-rich and cheap medium (wastewaters) could make microalgae-based fertilizers to be economically feasible [125].

8. MATERIALS PRODUCTION FROM *NANNOCHLOROPSIS*

Nannochloropsis is a potential candidate for simultaneous CO_2 sequestration and lipid production [138, 139]. Therefore, it is tremendously promising to use *Nannochloropsis* to convert CO_2 from industrial flue gas as a cheap feedstock for biomaterial production, while mitigating CO_2 release, which is mainly responsible for the global warming cause [140]. However, one CO_2 is captured and transformed to macromolecules (lipids, carbohydrates, proteins) in the microalgal biomass, the sustainable utilization of the biomass should be taken into account. This is because, algae biomass can be degraded and re-transformed to CO_2 and CH_4, which are then released back to the atmosphere, if it is not captured permanently [141]. Conversion of algae into useful polymeric materials can be easily accomplished by a simply extrusion technology. A determined proportion of algae biomass i.e., 20% can be formulated with matrices originated from non-biodegradable polymers such as polyolefin, which is highly resistant to abiotic or biotic degradation [142]. Interestingly, one algae biomass is incorporated into polymeric matrices, it is long stored in a form of material and will not be fastly decomposed to release CO_2. Shi et al. [142] recently blended *Nannochloropsis* biomass with starch to form a formulated filler for composite fabrication with commercial polypropylene (Polypropylene Pro-fax® SV954 (PP)), glycerin, mono-di-glycerin as matrix, plasticizer, and surfactant, respectively. Although polymeric material fabricated by 80% PP and 20% algal/starch (70/30, w/w) exhibited lower mechanical strength than the neat PP

([142] and Table 6), these properties are acceptable for a number of industrial applications such as packaging, personal care products, agriculture film, container, building materials, etc.

Nannochloropsis offers excellent nutrient composition with a substantial amount of PUFAs, a broad spectrum of vitamins (especially B_{12} and E), and contains all essential amino acids and polysaccharides, hence, using whole *Nannochloropsis* for composite production, which is considered as a low value material, may not be applicable. However, lipids-rich *Nannochloropsis* can be converted to biodiesel via in situ transesterification, while generating residual microalgae biomass (RMB) as a byproduct of biodiesel production for biocomposites formulation instead of whole biomass [143]. Torres et al. [143] reported that RMB of *Nannochloropsis* can be formulated with biodegradable polymer poly(butylene adipate-co-terephthalate) (PBAT) at proportion of 10, 20, and 30% with supplement of glycerol and urea as plasticizers. The best extruded formulation was a compound of 70% RMB, 30% of glycerol, 10 phr of water, and 7.5 phr of urea before employed for biocomposite fabrication with 80% PBAT, which resulted in improvement of mechanical properties of PBAT/RMB (80/20) (Table 6). The synthesized composite can be used for mulching film in agricultural industry without threatening the environment because it is biodegradable. The study provides an alternative way to produce biodiesel from *Nannochloropsis*, while simultaneously generating co-product (biocomposite). More recently, we developed a solution casting protocol for fabrication of biocomposite from lipid-extracted *Nannochloropsis salina* biomass (LEA) with poly(vinyl alcohol) (PVA) as a matrix [144]. Our result revealed that incorporation of LEA into PVA resulted in enhancement of thermal properties, but reduction in mechanical properties of PVA/LEA when compared to the neat PVA. Interestingly, addition of poly(diallyldimethylammonium chloride) (PD) as a plasticizer significantly improved mechanical properties of PVA/LEA/PD compared to PVA/LEA composites [144]. PVA was compounded with 20% LEA and 12% PD to synthesize the PVA68LEA20PD12 biocomposite (Table 6). In contrast to neat PVA, this biocomposite recorded the similar mechanical property but the enhanced thermal property. This type of biocomposite film can be applied in commercial industries as specialty materials, for instance, 3D printing material. This helps to improve the economic feasibility of microalgae-based biofuel production. Practically, the residual biomass after lipid extraction still contains high element content of carbon (41.82%), hydrogen (6.25%), nitrogen (5.63%), sulfur (3.94%) [144] which can be gasified to produce syngas for liquid fuels or methanol synthesis [145]. The leftover after gasification process is mineral ash which is rich in inorganic compounds such as SiO_2, SO_3, P_2O_5, CaO, etc. [146]. The ash is a potential filler for composite fabrication with PVA [146]. P**rior to** using it as a filler for composite fabrication with poly(vinyl alcohol), raw ash (RASH) was activated with NaOH and surface modified with (3-aminopropyl)triethoxysilane**.** Surface modification of activated ash (PASH) significantly improved interfacial interaction between surface-modified ash (GASH) and polymer matrix (Table 6). Higher ultimate tensile strength of PVA/GASH composites was recorded, compared with PVA/RASH and PVA/PASH. Young's modulus of biocomposites appeared to increase proportionally to loading of the fillers. Our preliminary results revealed that microalgae-derived ash can be potentially integrated to downstream processing of microalgal biorefinery, while reducing the total operating cost of microalgal-based facility via production of value-added byproduct ashes.

Table 6. Biocomposite fabrication from *Nannochloropsis*-based biomass

Strain	Biomass type	Matrix	Plasticizer/ surfactant	Fabrication method	Optimal formulation	Mechanical properties	Potential application	Ref.
Nannochloropsis	Whole biomass/ Glucosol® 800 polymer (70/30)	Polypropylene Pro-fax® SV954 (PP)	Glycerol/ mono-di-glyceride (22/2)	Co-extrusion	PP/algae-starch (80/20)	TS = 17 MPa TM = 1030 MPa EB = 9.36% BS = 16 MPa	Packaging Personal care products Agriculture film Container Building materials	[142]
N. gaditana	Resisual microalgae biomass (RMB)	Poly(butylene adipate-co-terephthalate) (PBAT)		Extrusion	PBAT/RMB (80/20)	TS = 14 MPa TM = 90 MPa EB = 370%	Packaging Agriculture film Container	[143]
N. gaditana	Resisual microalgae biomass (RMB)	Poly(butylene adipate-co-terephthalate) (PBAT)	Glycerol Urea H_2O	Extrusion	PBAT/RMB-p (72/28/10 phr water/7.5 phr urea)	TS = 9 MPa TM = 57 MPa EB = 500%	Packaging Agriculture film Container	[143]
N. salina	Lipid-extracted algal biomass (LEA)	Poly(vinyl alcohol) (PVA)	Poly(diallyldimethylammonium chloride) (PD)	Solution casting	PVA/LEA/PD (68/20/12)	TS = 19.5 MPa TM = 1430 MPa EB = 502%	Packaging Agriculture film Container 3D printing materials	[144]
N. salina	Mineral ash	Poly(vinyl alcohol) (PVA)	3-minopropyl triethoxysilane (APTES)	Solution casting	PVA/PASH (95/5)	TS = 34.6 MPa TM = 0.94 MPa EB = 180%	Packaging Agriculture film Container 3D printing materials	[146]
					PVA/GASH (95/5)	TS = 40.7 MPa TM = 0.98 MPa EB = 240%		
					PVA/PASH (90/10)	TS = 37.9 MPa TM = 1.43 MPa EB = 187%		

TS: Tensile strength; TM: Tensile modulus; EB: Elongation at break; BS: Break stress; NA: Not available; phr: Part per hundred; LEA, Lipid-extracted algal biomass; PVA, Poly(vinyl alcohol); PD, Poly(diallyldimethylammonium chloride; Ref., Reference. Number in parentheses are weight percentage of each material component formulated into biocomposite materials).

9. CHALLENGES AND FUTURE PERSPECTIVES OF *NANNOCHLOROPSIS* FOR BIOREFINERY

Nannochloropsis presents a unique potential with respect to the biorefinery. The strain comprises of cellular compounds which can promisingly be valorized into multiple products, positioning small or large end markets. In order to valorize all constituents of the *Nannochloropsis* biomass, it is necessary to develop economically feasible production process. Since the composition and amount of residual biomass can vary significantly depending on cell stoichiometry and cultivation techniques, therefore, it requires appropriate biorefinery side-process strategies to be adapted to production of the primary product. Based upon biochemical processes which were presented in the above sections, production scenarios for biorefinery from *Nannochloropsis* can be developed based on four differently typical products which have different market volumes, including high- and medium- value products (e.g., pigments, EPA, neutraceuticals), whole biomass (e.g., aquaculture and animal feeds) and biofuels (e.g., biodiesel, bio-jet fuels). The biorefinery of *Nannochloropsis* can be principally presented in Table 7.

Scenario 1: Simultaneous production of high-value products and utilization of residual biomass (RB). The main ideal of this scenario is to use RB from production of high-value products. One high-value products (pigments such as astaxanthin with commercial price of over 1000 US$/kg) are extracted from *Nannochloropsis* biomass, the RB containing carbohydrates and protein can be used to produce ethanol or biogas (via fermentation), aquaculture and animal feeds, fertilizers, biocomposite materials, and biocrudes (via hydrothermal process). Integrating production of products from RB will increase additionally economic benefits for the high-value products-based biorefinery that may facilitate to reduce the price of the high value products. Nitrogen and phosphorous released into liquid phase via extraction and hydrothermal treatment can be recycled as nutrients for *Nannochloropsis* cultivation.

Scenario 2: Combined utilization of medium-value bulk products from *Nannochloropsis*. This biorefinery focuses on production of commodity chemicals with large market volume and price ranging from few US$/kg to 100 US$/kg that are important segment of entire products portfolio of microalgae-based industry. Bulk chemicals produced from *Nannochloropsis* could be based on three main components including lipids, carbohydrates, and proteins. This biorefinery (e.g., lipids extraction) usually generates large amount of RB, therefore study on utilization of RB (fertilizers, materials, etc.) is very important for economic optimization of the process. Nitrogen and phosphorous released into liquid phase via extraction, hydrothermal treatment can be recycled as nutrients for *Nannochloropsis* cultivation.

Scenario 3: Utilization of whole cell for food and feed: Raw *Nannochloropsis* biomass (live culture or wet paste) having high nutritional value is directly utilized as an energy-rich food source for aquaculture. The raw biomass can also be further dewatered via a spray drying method to obtain algal powder, which is then directly used or formulated into tablets as a food additive for human and animals without releasing RB.

Scenario 4: Production of lipids-based biofuels (biodiesel, jet-fuels) coupling with utilization of RB: This biorefinery mainly converts *Nannochloropsis*-based lipids to biodiesel (fatty acid alkyl esters) or bio-jet fuels (hydrocarbons), while generating large amount of RB

that contain carbohydrates and protein. The RB can be further processed to produce ethanol, bioH_2, lactic acid (via biochemical processes), materials, syngas and biocrudes (via thermal chemical process such as pyrolysis). Nitrogen and phosphorous released into liquid phase via extraction and hydrothermal treatment can be recycled as nutrients for *Nannochloropsis* cultivation.

Table 7. Production of commodity chemicals, feeds, high value products, biofuels, cosmetics, fertilizers, and materials from *Nannochloropsis* via different biorefinery scenarios

Biorefinery scenario	Biorefinery 1	Biorefinery 2	Biorefinery 3	Biorefinery 4
Primary product	High value products	Medium value commodity products	Whole cells	Lipids-based biofuels
Stoichiometry	Minor part of biomass	Major constituents of biomass (lipids, carbohydrates, protein)	Total biomass	Lipids
Residual biomass utilization	Small amount of RB due to production conditions Ethanol, biogas Biocrude Aquaculture and animal feed Biofertilizers Materials N and P recycling	Large amount of RB Biocrude Aquaculture and animal feed Biofertilizers Materials N and P recycling	No residual biomass	Large amount of RB Biocrude BioH_2 Biogas Lactic acid Aquaculture and animal feed Biofertilizers Materials N and P recycling
Market position	Growing market	Open markets	High potential	Huge market

RB, Residual biomass.

Despite the promising feedstock for industrial application, the total quantity of microalgal biomass in general and *Nannochloropsis* in particular produced in current industrial process is relatively low and production processes are usually not economically viable [147]. Furthermore, although many chemical/biochemical processes have been developed to convert *Nannochloropsis* biomass to specific products, no *Nannochloropsis*-based biorefinery has not been established. We are still facing immature technologies for production and technologies not specifically designed for algae biorefinery [148]. Accordingly, this field is continuously evolving and reductions in costs can be expected in the coming years. The advancement in strain improvement (via genetic engineering) and cultivation is extremely important since enhancements in productivity, quality, and composition of the biomass are tightly relevant to both up- and down-stream processing, and finally affecting market price. Furthermore, the development of efficient and novel biochemical, chemical and thermal chemical processes to valorize all constituents of *Nannochloropsis* biomass to different products having different markets will facilitate complete commercialization of *Nannochloropsis*-based biorefinery.

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Chapter 58

GENETIC IMPROVEMENT OF MICROALGAE *NANNOCHLOROPSIS* SPECIES

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ABSTRACT

Microaglae of the *Nannochloropsis* genus are essential phytoplankton able to survive in both fresh- and seawater. Using a low-cost prokaryotic microbial culture system can yield high biomass of these microalgae that contain high polyunsaturated fatty acid and high antioxidant pigments. These advantages endow *Nannochloropsis* species with high commercial potential as photoautotrophic green cell factories for application in aquaculture, biofuel and nutraceutical industries. Recently, nuclear genome sequencing of *N. oceanica* and *N. gaditana*, as well as mitochondrial and plastid genomes' sequencing of *N. gaditana*, were performed. The results lay the groundwork for analyzing metabolic pathways and cellular processes in addition to performing genetic manipulation. Meanwhile, many genetic improvement approaches have come to the forefront, including genetic transformation platforms, non-antibiotic selection markers, targeted gene silencing, insertion and mutagenesis methods. With such biotechnological advancements, transgenic *Nannochloropsis* varieties can be generated with the phenotypic traits required to serve as biofuel producers, protein and unsaturated fatty acid bioreactors, and food supplements. As a result of their asexual reproduction, *Nannochloropsis* species have little gene flow, being considered as environmental safety. Therefore, we suggest that transgenic *Nannochloropsis* microalgae possess a broad spectrum of applications in marine and plant biotechnology and that they represent a source of raw material for a wide range of industries, as detailed in this review. We also present a perspective outlining the future uses of this genus in the context of genetic improvement for new biotechnological applications.

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Keywords: *Nannochloropsis*, genetic modification, gene transfer system, selective marker, reporter, recombinant foreign protein, reverse genetic approach

INTRODUCTION

Belonging to the Eustigmatophyceae class, *Nannochloropsis* species are photoautotrophic eukaryotic unicellular nonflagellate microalgae that include *N. gaditana*, *N. granulata*, *N. limnetica*, *N. oceanica*, *N. oculata* and *N. salina*. All of these species are spherical or ovoid in shape with diameter ranging from 2 to 4 μm and almost no difference in morphological characteristics. *N. gaditana*, *N. granulata*, *N. oceanica*, *N. oculata*, and *N. salina* are distributed primarily in the marine environment, but it has been reported that *N. limnetica* was found in freshwater and brackish habitats (Krienitz et al. 2000; Fawley and Fawley 2007).

Nannochloropsis spp. are emerging models of oil-producing photoautotrophic microalgae owing to high biomass and tolerance of extreme environmental conditions (Wei et al. 2013). This genus is capable of synthesizing large quantities of lipids, mainly in the form of triacylglycerols (TAGs) and marine derived n-3 long-chain polyunsaturated fatty acids (LCPUFA) (e.g., eicosapentaenoic acid (EPA) (20:5 n-3) and docosahexaenoic acid (DHA) (Ma et al. 2016). They produce and accumulate lipid contents up to 28.7% (Ash-Free Dry Weight (AFDW)), or even exceed 50% under nitrogen deprivation (Gouveia and Oliveira 2009). With their high TAGs and EPA contents, *Nannochloropsis* spp. are considered ideal organisms for use in aquaculture as natural feed for the cultivation of fish, Mollusca, and shrimp. They may also be applied industrially for biofuel production. These species can also produce a high concentration of pigments with antioxidant properties, such as astaxanthin, zeaxanthin and canthaxanthin. For example, *N. gaditana* can synthesize canthaxanthin and astaxanthin in 0.7% dry weight and zeaxanthin in 0.6% dry weight (Lubian et al. 2000). Thus, *Nannochloropsis* spp. can serve as an excellent source of high-value nutraceutical products.

As green factories, *Nannochloropsis* spp. can produce valuable lipids, pigments and even heterologous proteins for biotechnological applications. Important to this study, genetic manipulation of *Nannochloropsis* spp. can produce varieties with phenotypic traits useful in the biosciences. More specifically, the nuclear genome sequencing of six *Nannochloropsis* strains have been investigated and annotated partially (Pan et al. 2011; Liang et al. 2012; Radakovits et al. 2012; Vieler et al. 2012; Corteggiani Carpinelli et al. 2014; Wei et al. 2013) as well as chloroplast genome sequencing of *Nannochloropsis* spp. were performed (Radakovits et al. 2012; Wei et al. 2013; Starkenburg et al. 2014; Corteggiani Carpinelli et al. 2014) Moreover, transcriptome, proteome and lipidome analyses of *Nannochloropsis* spp. have also been reported (Vieler et al. 2012; Corteggiani Carpinelli et al. 2014; Zheng et al. 2014; Dong et al. 2013; Hu et al. 2014; Li et al. 2014). The results lay the groundwork for analyzing metabolic pathways and cellular processes, as well as gene expression, in addition to performing genetic manipulation. Mutagenesis of *Nannochloropsis* spp. has been performed with the aim of manipulating endogenous genes beneficial for developing lipid biosynthesis pathways and improving productivity of pigments, biomass and lipids (Schneider et al. 1995; Chaturvedi et al. 2004; Anandarajah et al. 2012; Ma et al. 2013; Perin et al. 2015; Beacham et al. 2015; Wang et al. 2016). Moreover, we can employ genetic engineering to express exogenous and endogenous functional proteins for lab research and

industrial applications. Genetic modification approaches for nuclear transformation of *Nannochloropsis* spp. include electroporation, particle bombardment and *Agrobacterium*-mediated gene transfer, allowing the introduction of exogenous DNA into host nuclear genome to display any desirable genetic traits (Chen et al. 2008; Kilian et al. 2011; Kang et al. 2015a, 2015b; Cha et al. 2011). These genetically modified (GM) *Nannochloropsis* species show faithful expression of foreign proteins and enhancement of biomass and lipid productivity (Kaye et al. 2015; Iwai et al. 2015; Kang et al. 2015b; Li et al. 2016; Beacham and Ali 2016).

Similarly, with the development of a workable reporter gene applied to species of this genus, researchers could realize cellular localization and quantify the expression level of target genes in cells. Screening putative transformants after gene transfer on *Nannochloropsis* spp. is tedious, but the choice of proper reporter gene, or selective markers, would be a key factor in improving the screening efficiency and future application of transgenic cells. In fact, a new direction in GM *Nannochloropsis* transgenic strains has emerged and is compliant with all applicable rules and regulations (Li and Tsai 2009; Cha et al. 2011; Kang et al. 2015a; Moog et al. 2015; Shih et al. 2015). For example, the environmentally friendly, non-antibiotic-based selection maker purple chromoprotein (CP) has been isolated from the sea anemone *Stichodacyla haddoni* (sh) (Chiang et al. 2014). With a favorable food safety profile, shCP and its derivatives represent a consumable protein of marine food chain origin (Shih et al. 2015). More recently, gene knockdown, knockout and knockin techniques have been achieved for *Nannochloropsis* species. This has implications for the development of new approaches to functional gene analysis (Kilian et al. 2011; Wang et al. 2016; Wei et al. 2017). In this article, we will introduce current genetic engineering and manipulation techniques, including many genetic improvement approaches, such as genetic transformation platforms, non-antibiotic selection markers, targeted gene silencing and mutagenesis methods, all forging a path toward the genetic improvement of *Nannochloropsis* species.

GENETIC MODIFICATION APPROACHES IN *NANNOCHLOROPSIS* SPECIES

Thus far, we highlighted four approaches that have been overwhelmingly used to generate GM *Nannochloropsis* varieties, including particle bombardment, electroporation, *Agrobacterium tumefaciens*-mediated genetic transformation and mutagenesis (Table 1). The first two approaches physically deliver the foreign DNA into the host genome, while *Agrobacterium* is known for its ability to transfer the exogenous gene between itself and the host genome after infection, and mutagenesis utilizes methods such as UV induction and mutagen manipulation. Originally designed to generate higher plants, these genetic modification platforms have been gradually modified for use in transgenic microalgae.

Table 1. Current genetic modification tools for *Nannochloropsis* species

Transgenic method	Method		Host	Reference
Particle bombardment	Low pressure delivery with microcarrier gold particle		*N. salina* CCMP1776	Kang et al. 2015a
Electroporation	Cell wall removal (protoplast)[a]		*N. oculata*	Chen et al. 2008
	Intact cell wall (direct)[b]		*Nannochloropsis* W2J3B	Kilian et al. 2011
			N. gaditana CCMP526	Radakovits et al. 2012
			N. oceanica CCMP1779	Vieler et al. 2012
A. tumefaciens-mediated	via T-DNA vector		*Nannochloropsis* species UMT-M3	Cha et al. 2011
			N. salina CCAP 849/3	Beacham and Ali 2016
Mutagenesis	Physical way	UV	*Nannochloropsis* species BR2	Sharma and Schenk 2015
			N. salina CCAP 849/3	Beacham et al. 2015
		Heavy-ion	*N. oceanica* IMET1	Ma et al. 2013
		Gamma ray	*Nannochloropsis* species	Schneider et al. 1995
	Chemical way	EMS	*N. oculata* ST-6	Chaturvedi and Fujita 2006
			Nannochloropsis species	Schneider et al. 1995
			N. salina CCAP 849/3	Beacham et al. 2015
			N. gaditana CCAP 849/5	Perin et al. 2015
		MNU	*N. oculata*	Chaturvedi et al. 2004
		NTG	*N. oceanica*	Wang et al. 2016

[a] protoplast: protoplast-based electroporation; [b] direct: direct electroporation.

Particle Bombardment

Particle bombardment, commonly known as the "gene gun", is said to be an effective and highly reproducible transformation method for establishing GM algae strains (Qin et al. 2012). This method mainly uses heavy metal coated with plasmid DNA which is then shot by air pressure, allowing DNA fragments to penetrate the target cell wall and integrate into nuclear chromosome, or organelles like chloroplasts, to generate transgenic microalgal varieties.

However, the "gene gun" strategy requires costly specialized equipment and has a relatively low rate of transformant recovery. Still, it can deliver multiple copies of exogenous DNA into the host genome, thereby increasing transformation efficiency (Potvin and Zhang 2010), suggesting that it is an effective way to establish nuclear or chloroplast transformation in a variety of microalgal species. For example, the sfCherry fluorescent protein was introduced in *N. salina* CCMP 1776, allowing the fluorescent signal to be microscopically detected within cells (Kang et al. 2015a) and, hence, serving as a new genetic manipulation platform for selecting transformant cells. Kang and coworkers (2015b) also showed that overexpression of a basic helix-loop-helix (bHLH) transcription factor (NsbHLH2) could increase biomass productivity and production of fatty acid methyl esters (FAME).

Electroporation

Electroporation is simple and effective, and it is commonly used for nuclear transformation in *Nannochloropsis*. An intense electrical field is applied to ensure that the cell wall and lipid bilayers are disrupted so that exogenous DNA fragments can penetrate the genome of transformants. However, its efficiency is dependent on field strength, pulse length, medium composition, temperature, membrane characteristics and DNA concentration (Potvin and Zhang 2010). This strategy involves either removal of the cell wall to allow protoplast to ingest foreign materials (Chen et al. 2008) or genetic transformation of intact call walls by direct electroporation, as in *Nannochloropsis* cells (Kilian et al. 2011).

Electroporation for nuclear transformation was first reported by Chen et al. (2008), who were successful in transferring exogenous DNA fragments containing fish growth hormone cDNA into the protoplast of *N. oculata* cells. They demonstrated that the growth of fish larvae fed with the transformed *N. oculata* could be enhanced. Two subsequently published articles reported the establishment of transgenic *N. oculata* strains by following this protoplast-based electroporation approach (Li and Tsai 2009; Shih et al. 2015). As noted above, Kilian et al. (2011) used genetic transformation of intact call walls to establish a transgenic *Nannochloropsis* species W2J3B. The transformants were generated by direct electroporation with a DNA construct which knocked out two genes involved in nitrogen metabolism by homologous recombination. This electroporation protocol is now commonly used for genetic transformation of *Nannochloropsis*.

Agrobacterium tumefaciens-Mediated Genetic Transformation

A. tumefaciens is a Gram-negative soil bacterium. It is a widely used genetic modification tool for establishing transformed plants. *A. tumefaciens* harbors a Ti (tumor-inducing) plasmid which contains a DNA fragment, namely T-DNA, which can be transferred and randomly integrated into the chromosomal genome of host plant cells. Hence, the T-DNA region can be exchanged with exogenous DNA and transformed into the target cells to generate GM plants. Recently, this transformation approach has been successfully applied in microalgae, such as *Porphyra yezoensis* (Cheney et al. 2001), *Chlamydomonas reinhardtii* (Kumar et al. 2004), *Haematococcus pluvialis* (Kathiresan et al. 2009) and *Dunaliella bardawil* (Anila et al. 2011). Investigators claimed that stable transgenic *C. reinhardtii* and *H. pluvialis* strains could be obtained and that the transferred gene could be stably maintained for 18 months and 2.5 years, respectively.

The first successful case of applying *Agrobacterium*-mediated gene transfer for nuclear transformation in *Nannochloropsis* spp. was reported by Cha et al. (2011), who applied GUS assay to evaluate the transformation efficiency among acetosyringone, cinnamic acid, vanillin and coumarin, suggesting that, instead of acetosyringone, the other three phenolic compounds could become potential alternatives to *Agrobacterium vir* gene induction to achieve high *Agrobacterium*- mediated transformation efficiency for *Nannochloropsis* species UMT-M3. Interestingly, *A. tumefaciens*-mediated transformation was applied to transfer the *Saccharomyces cerevisiae* type 2 diacylglycerol acyltransferase *DGA1* gene into *N. salina* CCAP 849/3 (Beacham and Ali 2016). They found improved lipid content because an additional copy of the exogenous *DGA1* gene was introduced into *N. salina*. On the other

hand, they observed that transformants displayed transient expression because of gene silencing.

Mutagenesis

Genetic transformation can also be achieved through mutagenesis, as demonstrated by manipulating and altering the metabolite productivity and composition or increasing the photosynthesis efficiency and biomass productivity in the genus *Nannochloropsis*. The strategy can be implemented though either classical physical or chemical means. For example, ultraviolet (UV) radiation is a simple and commonly used physical method to acquire mutant *Nannochloropsis* strains (Sharma and Schenk 2015; Beacham et al. 2015). Gamma ray and heavy ion radiation were also applied to generate a mutant *Nannochloropsis* strain owing to its wide mutation spectrum and higher mutation frequency compared with the UV method (Schneider et al. 1995; Ma et al. 2013). Three alkylating mutagens have also been used to develop *Nannochloropsis* mutant strains, including ethyl methane sulfonate (EMS), N-methyl-N-nitrosourea (MNU) and 1-methyl-3-nitro-1-nitrosoguanidine (NTG). These mutagens are alkylating agents capable of causing a high nucleotide substitution or transition frequency. Therefore, they are commonly used to induce mutation in the genus *Nannochloropsis*.

Mutants of *N. salina* CCAP849/3 and other *Nannochloropsis* species were reported to display distinct phenotypes, such as improvement of total cellular lipid contents, EPA, PUFA and FAMEs, using UV-induced mutagenesis (Sharma and Schenk 2015; Beacham et al. 2015). In addition, mutation for the change of lipid composition induced by gamma ray in *Nannochloropsis* species was reported by Schneider et al. (1995). Meanwhile, Ma et al. (2013) reported that the mutant of *N. oceanica* IMET1 obtained from heavy ion irradiation mutagenesis had higher biomass accumulation, growth rate and lipid productivity compared with wild type. EMS is a commonly used mutagen to induce overproduction of metabolites, such as xanthophyll, TAGs and EPA in the genus *Nannochloropsis* (Perin et al. 2014). In contrast, only Chaturvedi et al. (2004) and Wang et al. (2016) have reported that the mutagens MNU and NTG can be used to establish mutant *Nannochloropsis* strains to enhance lipid productivity.

SELECTION MARKERS APPLIED TO THE TRANSFORMATION

Recent studies have confirmed the use of antibiotic- and herbicide- resistant genes, or viral proteins, as selective markers for screening putative clones with the aim of generating transgenic *Nannochloropsis* strains. Reporter protein systems were also established for *in vivo* protein localization studies (Moog et al. 2015) and served as a substitute for normal antibiotics and herbicide selective markers (Shih et al. 2015). Several studies have also reported genetic modifications for either endogenous or exogenous gene overexpression, resulting in the improvement of lipid and protein production. Meanwhile, different *Nannochloropsis* mutants displayed distinctive biological traits or enhanced metabolites compared with wild type.

Selective Marker of Antibiotic-Resistant and Reporter Genes

The development of GM strains requires a proper and suitable selective marker system for identifying and isolating the putative transformed cells, and two major systems have been developed to select transformed *Nannochloropsis* cells (Table 2). One involves antibiotic- and herbicide- resistant genes, such as *sh ble* (Kilian et al. 2011), *HygR* (Kilian et al. 2011), *Bsr* (Kilian et al. 2011) and *aph7* (Vieler et al. 2012). These markers allow direct selection of transformed cells by conferring genes resistant to Zeocin or Hygromycin B. Another involves reporter genes, such as the red fluorescent protein gene (DsRed) (Li and Tsai 2009), sfCherry fluorescent protein gene (Kang et al. 2015a) and shCP gene (Shih et al. 2015). These markers do not exert direct selective pressure on transformed cells. Instead, they display distinctive coloration or fluorescent signal that can be identified by visual detection or fluorescent microscopy observation.

Antibiotic and herbicide selective markers are commonly used to isolate transformed microalgal cells. For example, Kilian et al. (2011) applied *sh ble*, *Bsr* and *HygR* antibiotic selective markers for nuclear transformation of *Nannochloropsis* species W2J3B after direct electroporation following with homologous recombination, conferring resistance to Zeocin or Hygromycin B. The transformation efficiency was 2.5×10^{-6} per μg of plasmid DNA (Umaima et al. 2016). The *aph7* selective marker gene was also introduced into *N. oceanica* CCMP1779 by direct electroporation, allowing the transformed cells to resist Hygromycin B and resulting in a transformation efficiency of $1.25 \times 10^{-6} \pm 0.6 \times 10^{-6}$ per μg of plasmid DNA (Vieler et al. 2012). However, GMOs that originate from antibiotic gene selection raise public concerns over environmental and food safety. Therefore, the search for safe alternative selection markers is necessary. Attempts have been made to develop various environmentally safe reporter genes as selective markers. Although these reporters do not exert direct selective pressure on transformed cells, they do allow the selection of putative transformants based on the distinctive characteristic of phenotypes they present. More importantly, since these selective genes encode naturally consumable proteins, they may be considered environmentally safe for the selection of transformed cells. For example, DsRed originated from coral was the first fluorescent reporter to be employed as a selective marker for screening transgenic *N. oculata* strains using protoplast-based electroporation (Li and Tsai 2009). The red fluorescent signal could be detectable in transformants under fluorescence microscopy. After culturing colonies on agar plates, it does take some two months for the red fluorescence to appear, but when it does, it is easy to distinguish between transformants and non-transformants. However, the endogenous fluorescent signals from chlorophyll and pigments in the host microalga *N. oculata* could occasionally interfere with the identification of red fluorescent signal (Shih et al. 2015). To address this problem, Kang et al. (2015a) introduced the sfCherry reporter into *N. salina* CCMP1776 using particle bombardment. They demonstrated that the sfCherry reporter, which was derived from mCherry through directed mutagenesis with better folding and higher intensity of fluorescence signal (Shaner et al. 2004; Nguyen et al. 2013), could be an effective alternative to DsRed by the absence of interference between endogenous host fluorescent signals and those of the reporter. Still, reporters like sfCherry require expensive fluorescent instrumentation for screening of transformed cells that express fluorescent signals. To overcome this drawback, Shih et al. (2015) developed to use novel selection marker, CP. This marker is easily detected by the naked eye, and, more importantly, it meets food and environmental safety standards as well.

Gene coding for CP was cloned from the sea anemone *S. haddoni* (sh) in Tsai's lab. When the shCP marker was introduced into *N. oculata* NIES-2146 by protoplast-based electroporation, the transformed cells displayed a distinctive dark brown coloration, making it easy to distinguish transformants from non-transformants by direct visual detection without employing complicated fluorescence equipment. Since shCP is not a toxic protein, we noticed that no selective pressure was present on the agar plate, allowing both transformed and non-transformed microalgae to grow. Thus, screening positive transformants from among the many hundreds of cells grown on agar is, indeed, time-consuming. However, despite this disadvantage, shCP is a protein originated from sea anemone, which belongs one of member in marine food chain, and considered as an edible natural resources. Therefore, it complies with both environmental and food safety requirements, making this reporter protein simple and ecologically safe for selecting transgenic *Nannochloropsis* cells.

GUS and GFP Reporter Genes Applied to *Nannochloropsis* Species

The protein produced by reporter genes is easy to observe, detect and quantify. Hence, reporter genes are commonly used to evaluate the promoter activity of target gene in microalgal cells. Meanwhile, the GUS gene encoding β-glucuronidase and *gfp* cDNA encoding green fluorescent protein (GFP) are widely used as a reporter gene to assess transformation efficiency, monitor gene expression, search for protein localization, and trace the dynamic change of target gene within *Nannochloropsis* cells (Table 2).

GUS reporter gene has been applied in several different microalgae strains, such as dinoflagellates, including *Amphidinium* species, *Symbiodinium microadriaticum* (Michael and Miller 1998); the diatom *Thalassiosira weissflogii* (Falciatore et al. 1999) and *Chlorella* species (Wang et al. 2007). Cha et al. (2011) first reported the use of a GUS reporter gene to assess the influence of transformation efficiency of four phenolic compounds on *A. tumefaciens*-mediated genetic transformation in *Nannochloropsis* species UMT-M3. They demonstrated that cinnamic acid, vanillin and coumarin could produce a higher percentage of GUS-positive cells than those of acetosyringone. Subsequently, GUS assay was also used to evaluate the transformation efficiency between PCR fragment based-transformation and plasmid based-transformation systems among six *Nannochloropsis* species. As a result, they suggest that the transformation efficiency of PCR fragment based-method is higher than that of plasmid based-method (Li et al. 2014). Apart from GUS, GFP has also been used to trace target protein in *Nannochloropsis* and monitor gene expression, as well as trace protein localization, in microalgae, e.g., the diatoms *Phaeodactylum tricornutum* (Zaslavskaia et al. 2000) and *C. reinhardtii* (Kumar et al. 2004) and the red alga *Cyanidioschyzon merolae* (Watanabe et al. 2011). Moog et al. (2015) was the first the use the GFP reporter to perform an *in vivo* localization study in *N. oceanica* CCMP1779 after direct electroporation. They demonstrated that the terminal sequences could drive the location of proteins within the cellular compartments, such as plastid transport through endoplasmic reticulum and mitochondria of transformed cells.

Table 2. Current selective markers and reporters used in the transgenesis of *Nannochloropsis* species

Category	Transgenic method	Gene or protein	Selection strategy	Purpose	Host	Reference
Antibiotics	Direct electroporation	sh ble	Zeocin resistance	Screening	*Nannochloropsis* species W2J3B	Kilian et al. 2011
	Direct electroporation	HygR	Hygromycin B resistance	Screening		
	Direct electroporation	Bsr	Blasticidin S resistance	Screening		
	Direct electroporation	aph7	Hygromycin B resistance	Screening	*N. oceanica* CCMP1779	Vieler et al. 2012
Reporter	Protoplast electroporation	DsRed	Emit red fluorescent	Screening	*N. oculata*	Li and Tsai 2009
	Protoplast electroporation	shCP	Display dark brown coloration	Screening	*N. oculata* NIES-2146	Shih et al. 2015
	Particle bombardment	sfCherry	Emit sfCherry fluorescent	Screening	*N. salina* CCMP1776	Kang et al. 2015a
	A. tumefaciens mediated	GUS	Display blue coloration	Statistical analysis	*Nannochloropsis* species UMT-M3	Cha et al. 2011
	Direct electroporation	GFP	Emit green fluorescent	Localization study	*N. oceanica* CCMP1779	Moog et al. 2015

EXPRESSION OF FOREIGN PROTEINS APPLIED FOR AQUACULTURE AND LIPID BIOSYNTHESIS

Since *Nannochloropsis* species have high biomass productivity and high commercial potential for producing valuable lipids, pigments and proteins, they are commonly used in industrial fields, such as aquaculture, as well as biofuel, bioreactor and nutraceutical industries. GM approaches to *Nannochloropsis* spp. are effective and well developed (Table 3), but the production of functional and valuable recombinant proteins obtained from different distinctive phenotypes can be improved.

From this perspective, *N. oculata* is frequently used as live feed for larvae of fish and shellfish in the aquaculture industry (Duerr et al. 1998). Chen et al. (2008) generated a transgenic line of *N. oculata* to produce functional yellowfin porgy growth hormone (YPGH) from marine fish *Acanthopagrus latus*. This transgenic *N. oculata*, which harbors a YPGH cDNA using protoplast-based electroporation, was first fed *Artemia*, a zooplankton. Then, these microalgae-containing *Artemia* was fed to red tilapia larvae. As a result, the growth of red tilapia larvae was greatly increased. Interestingly, Li and Tsai (2008) also demonstrated that the transgenic *N. oculata* harbors an antimicrobial peptide, bovine lactoferricin (LFB), using protoplast-based electroporation. After challenge by the pathogen *Vibrio parahaemolyticus*, the average survival rate of fish larvae orally fed with transgenic microalgae was increased to 85 ± 7.1% compared with 5 ± 7.1% of control group orally fed with non-transgenic microalgae, demonstrating the enhanced bacterial resistance of fish larvae fed with LFB-containing transgenic microalgae.

Moreover, several studies reported the overexpression of specific genes to increase lipid or biomass productivity. Genome, proteome and lipidome analyses of *Nannochloropsis* species are well established and, hence, the relevant knowledge of metabolic pathways in *Nannochloropsis* spp. For example, the overexpression of endogenous *N. oceanica* microsomal-likeΔ12 desaturase (*NoD12*), which is capable of regulating the primary step of LC-PUFA biosynthesis by transforming oleic acid (18:1 n-9) into linoleic acid (18:2 n-6) in *N. oceanica* CCMP1779, was achieved using direct electroporation (Kaye et al. 2015). In addition, the overexpression of homologous *NoD12* resulted in altering total lipid composition, which not only enhanced ratio of phosphatidylcholine by 18:2 in the triacylglycerol (TAG) under nitrogen starvation conditions but also converted part of linoleic acid (18:2 n-6) to arachidonic acid (20:4 n-6) without showing negative effect on the growth of cells. Furthermore, Iwai et al. (2015) isolated type-2 diacylglycerol acyl-CoA acyltransferase (CrDGTT4) from *C. reinhardtii* which is driven by sulfoquinovosyl-diacylglycerol synthase 2 (SQD2) promoter. CrDGTT4 is able to induce the accumulation of TAGs under phosphorus starvation condition. They introduced *CrDGTT4* gene into *Nannochloropsis* species NIES-2145, resulting in the improvement of TAG accumulation and oleic acid content (18:1 n-9) and altering the lipid composition of the host to become more similar to *C. reinhardtii* under phosphorus starvation condition. Meanwhile, Li et al. (2016) reported the overexpression of type 2 diacylglycerol acyltransferase (DGAT2), which serves as a rate-limiting enzyme in TAG biosynthesis, in *N. oceanica* CCAP 849/10. The results showed that overexpression of *DAGT2* gene increased saturated fatty acids and PUFA, but

Table 3. Exogenous and endogenous genes expressed in transgenic *Nannochloropsis* species

Category	Gene/Protein	Source	Transgenic method	Outcome	Host	Reference
Exogenous	YPGH	*A. latus*	Protoplast electroporation	Improve growth rate of fish larvae	*N. oculata*	Chen et al. 2008
	LFB	Bovine	Protoplast electroporation	Against aquaculture pathogens	*N. oculata*	Li and Tsai 2009
	CrDGTT4	*C. reinhardtii*	Direct electroporation	Improve TAG accumulation and change in lipid content	*Nannochloropsis* species NIES-2145	Iwai et al. 2015
	DAG1	*S. cerevisiae*	*A. tumefaciens* mediated	Improve FAME content	*N. salina* CCAP 849/3	Beacham and Ali 2016
Endogenous	NoD12	*N. oceanica*	Direct electroporation	Increase lipid productivity and alter lipid composition	*N. oceanica* CCMP1779	Kaye et al. 2015
	bHLH	*N. salina*	Particle bombardment	Improve biomass and FAME productivity	*N. salina* CCMP1776	Kang et al. 2015b
	DGAT2	*N. oceanica*	Direct electroporation	Improve TAG biosynthesis	*N. oceanica* CCAP 849/10	Li et al. 2016

decreased the content of monounsaturated fatty acids. Additionally, neutral lipid content was increased by 69%, and TAG biosynthesis was also accelerated. *N. salina* can accumulate a high amount of palmitic acid (C16:0) and oleic acid (C18:1 n-9), according to Van Wagenen et al. (2012). Consequently, this microalgal strain has become a suitable recipient for transforming the *S. cerevisiae DGA1* gene to utilize acetyl-CoA substrate, such as palityl-CoA (C16:0) and oleoyl-CoA (C18:1). The transgenic *N. salina* CCAP 849/3 harboring the *DGA1* gene was generated through the *A. tumefaciens*-mediated genetic transformation approach (Beacham and Ali 2016). As a result, this transformed variety showed improved Fatty Acid Methyl Ester (FAME) content by 38%, although it caused the silencing of the transgenic gene. Recently, a basic helix-loop-helix (bHLH) transcription factor (TF), which functions as a regulator of growth, development and stress response, was overexpressed in *N. salina* CCMP1776 using particle bombardment (Kang et al. 2015b). When two bHLH TFs, named as *NsbHLH1* and *NsbHLH2*, were individually isolated and overexpressed in *N. salina*, *NsbHLH2*- overexpressing transformants displayed enhanced biomass productivity by 36% under normal condition and FAME productivity by 33% under nitrogen starvation.

REVERSE GENETIC APPROACHES OF SILENCING, KNOCKOUT AND KNOCKDOWN IN *NANNOCHLOROPSIS* SPECIES

Reverse genetic approaches, or gene editing tools, such as homologous recombination (Sodeinde and Kindle 1993), RNA-mediated silencing (Rohr et al. 2004) and CRISPR/Cas9 (Shin et al. 2016) have been established and applied in a few species of microalgae (Wei et al. 2017). Unlike forward genetic engineering, reverse genetic approaches are commonly applied in studying specific gene functions or modifying the gene of interest to develop a desirable phenotype through knockout or silencing of target gene in the study organisms (Hlavova et al. 2015). Since *Nannochloropsis* species are emerging as an excellent microalgal model organism, these reverse genetic tools are now available for extensive genetic investigation and profiling. Three major studies have reported on the application of reverse genetics to *Nannochloropsis* spp. (Kilian et al. 2011; Wang et al. 2016; Wei et al. 2017) (Table 4).

Table 4. Reverse genetic tools used for *Nannochloropsis* species

Tool	Transgenic method	Target gene	Host	Reference
Homologous recombination	Direct electroporation	nitrate or nitrite reductase	*Nannochloropsis* species W2J3B	Kilian et al. 2011
CRISPR/Cas9 system	Direct electroporation	nitrate reductase	*N. oceanica* IMET1	Wang et al. 2016
RNA interference	Direct electroporation	carbonic anhydrase and bicarbonate transporter	*N. oceanica* IMET1 and CCMP1779	Wei et al. 2017

Homologous Recombination

Nannochloropsis spp. are potential feedstock candidates for biofuel and biochemical production, but our knowledge of the genes and enzymes involved in biosynthesis is lacking (Weeks 2011). To remedy this, homologous recombination is a common tool used to perform site-specific target gene knockout and replacement, enabling the integration of exogenous DNA into a specific region of the target genome and expression of the gene of interest. Kilian et al. (2011) demonstrated gene replacement of *Nannochloropsis* species W2J3B by homologous recombination after direct electroporation. The target endogenous nitrate or nitrite reductase gene was knocked out, and the desired transgene was inserted, resulting in a transgenic strain unable to rescue on nitrate- and nitrate/nitrite-containing medium. This work successfully opened the door for the functional analysis of genes, thus improving the outlook for future studies of genes involved in metabolic processes.

CRISPR/Cas9 System

Clustered, Regularly Interspaced, Short Palindromic Repeats-associated Endonuclease 9 (CRISPR/Cas9) has been used as a reverse genetic tool for functional analysis study in microalgae, such as *C. reinhardtii*, *Thalassiosira pseudonana* and *P. tricornutum* (Baek et al. 2016; Shin et al. 2016; Hopes et al. 2016; Nymark et al. 2016). The CRISPR/Cas9 system contains a specific guide RNA (gRNA) and a nonspecific CRISPR-associated endonuclease protein (Cas9), allowing sequence-specific cleavage of DNA double-strand breaks (DSB) in the target DNA loci region through base pairing of corresponding gRNA. As the result, it generates a knockout or replacement of the target gene in recipient cells or organisms (Brasila et al. 2017). More recently, heterologous genome editing to knock out the target gene through the CRISPR/Cas9 system was successfully achieved in *N. oceanica* IMET1 via direct electroporation (Wang et al. 2016). Five base-pairs were precisely knocked out, causing a frameshift of endogenous nitrate reductase by CRISPR/Cas9. As a result, transgenic *N. oceanica* failed to grow on nitrate-containing medium. This work demonstrated that the CRISPR/Cas9 system could potentially serve as an efficient strategy for genomic sequence editing in *Nannochloropsis* genomics and metabolomics.

RNA Interference (RNAi)-Mediated Gene Silencing

Application of RNAi-mediated gene silencing has been reported in a wide range of microalgae, e.g., *C. reinhardtii*, *T. pseudonana*, *Dunaliella salina*, *P. yezoensis*, *P. tricornutum* and *Penium margaritaceum* (Rohr et al. 2004; Armbrust et al. 2004; Jia et al. 2009; De Riso et al. 2009; Liang et al. 2010; Sorensen et al. 2014). RNAi-mediated gene silencing is also a commonly used reverse genetic tool to silence target genes in different microalgae. However, some species of algae with small nuclear genomes have lost the RNAi machinery (Cerutti et al. 2011). Generally, RNAi is a post-transcriptional process which is triggered by double-stranded RNA (dsRNA) for targeted gene silencing, resulting in the degradation of specific target transcripts. Most recently, RNAi-mediated gene silencing was used to knock down carbonic anhydrase (CA) and bicarbonate transporter (BCT) in strains of

N. oceanica IMET1 and CCMP 1779 (Wei et al. 2017). CA and BCT play a key role in the inorganic carbon concentration mechanism (CCM) which represents biological adaptation to low carbon dioxide concentrations in the environment (Moroney and Ynalvez, 2007). Wei et al. (2017) reported that the transcription level of CA in the RNAi-knockdown strain was inhibited by 62-83% and that the photosynthetic oxygen evolution (POE) rate was increased by 68-100% under pH 6.0 but decrease by 39-45% under pH 9.0 compared with wild-type (WT), revealing the crucial correlation between CA and CCM. This work demonstrated that RNAi-mediated gene silencing is a potentially effective tool for knocking down target genes in *Nannochloropsis* for functional gene studies.

BIOSYNTHESIS MUTATION IN *NANNOCHLOROPSIS*

Random mutagenesis is still another genetic manipulation tool commonly used to alter the biosynthesis pathway in *Nannochloropsis* species since this genus is recognized as a potential feedstock of lipid and nutrients. A variety of desirable mutant *Nannochloropsis* strains were obtained through random mutagenesis, resulting in the enhancement of its metabolite productivity, biomass productivity and photosynthetic efficiency. The development of a selective marker for mutant strains was also reported (Table 5).

Table 5. Current mutagenesis applied on *Nannochloropsis* species

Method	Approach	Host	Phenotype	Reference
Physical	Heavy-ion	*N. oceanica* IMET1	Improved lipid productivity and photosynthesis efficiency	Ma et al., 2013
	UV	*Nannochloropsis* species BR2	Two-fold increase in EPA content	Sharma and Schenk 2015
Chemical	EMS	*N. oculata* ST-6	Obtained antibiotics resistance mutants with improved EPA and some long-chain fatty acid	Chaturvedi and Fujita 2006
		Nannochloropsis species	Four-fold increase of total fatty acid content and palmitoleic acid and decrease in EPA	Doan and Obbard 2012
		N. oculata	Improved two-fold violaxanthin, three-fold zeaxanthin and 1.8-fold xanthophyll content and decrease in lutein content	Lee et al., 2006
		N. gaditana CCAP 849/5	Increased in biomass productivity and decreased chlorophyll	Perin et al. 2015
		Nannochloropsis species	Improved biomass productivity, chlorophyll and lipid content	Anandarajah et al. 2012
	MNU	*N. oculata*	Improved total fatty acid, TAG, PUFA and EPA	Chaturvedi et al. 2004
	NTG	*N. oceanica*	Improved lipid productivity and decrease in PUAF and TAG	Wang et al. 2016
Physical and Chemical	Gamma ray and EMS	*Nannochloropsis* species	Devoid EPA, reduction in TAG and slower growth rate	Schneider et al. 1995
	UV and EMS	*N. salina* CCAP 849/3	Three-fold increase in lipid accumulation and decrease in growth rate and overall lipid productivity	Beacham et al. 2015

Mutation to Enhance Lipid Productivity and Change Lipid Content

Nannochloropsis species are widely known for their distinctive characteristics of rapid growth and high lipid productivity. Several studies have reported on improving lipid metabolism through mutagenesis, successfully establishing a variety of mutant *Nannochloropsis* strains with different enhancement in specific lipid contents, lipid accumulation and change in lipid composition. Several studies successfully used chemical mutagens, such as EMS, MNU and NTG, to trigger mutation for changing lipid profiles in *Nannochloropsis*. The enhancement of eicosapentaenoic acid (EPA) content in *N. oculata* ST-6 using EMS demonstrated that cerulenin- and erythromycin-resistant mutated strains increased EPA content by 29 and 12%, respectively (Chaturvedi and Fujita, 2006). Meanwhile, increase in the content of fatty acids (C14:0), (C16:0), (C16:1), (C18:0) and (C20:4 n-6) was also obtained. Moreover, Doan and Obbard (2012) reported a mutant of *Nannochloropsis* species obtained from EMS treatment that increased by four-fold the total fatty acid content with a 30% increase in palmitoleic acid (16:1) and a 45% decrease of EPA compared to wild-type strain, suggesting that it is possible to obtain mutants with enhanced fatty acid. In addition, Beacham et al. (2015) demonstrated a double-mutated *N. salina* CCAP849/3. This strain was first treated with EMS and displayed a change in PUFA content, which improved total FAME up to 156% compared with wild type. Then, the EMS-mutant strains were further subjected to UV radiation, obtaining double mutant strains with a three-fold higher lipid accumulation than wild type. However, the growth rate of these mutants was reduced, which, in turn, caused an overall decrease in lipid productivity. Meanwhile, two mutants of *N. oculata* obtained from MNU treatment can resist quizalofop, an inhibitor of acetyl-CoA carboxylase (ACCase). Thus, they were able to change the fatty acid metabolism. As a result, total fatty acid content, PUFA, TAG, linoleic acid (18:2), arachidonic acid (20:4 n-6) and EPA (20:5 n-3) were improved (Chaturvedi et al. 2004). Recently, Wang et al. (2016) reported three mutants of *N. oceanica* through EMS and NTG mutagenesis, resulting in the improvement of lipid productivity. As a result, three mutants displayed increases of 33.54, 30.93 and 21.44% in lipid productivity, and lipid content was increased by 17.4, 23.7 and 29.4%, respectively. One of mutants was able to accumulate lipid as high as 31.34% of dry biomass and increased in (C16) to (C18) lipid content by 12.56%, while it was absent in some proportions of TAG composition, resulting in the decrease of PUFA content than that of wild type. This study also reported that NTG showed more mutation efficiency for establishing strains with high lipid content.

Physical mutagenesis was used to develop mutants of *Nannochloropsis* as well. Schneider et al. (1995) applied EMS and gamma irradiation to obtain a mutant of *Nannochloropsis* species which was completely devoid of EPA (20:5 n-3) but increased in the relative lipid content of (20:4 n-6) and TAG. Alternation of several lipid composition and membrane lipid levels in mutants was also observed. For example, mutants displayed the reduction in the percentage of (20:4 n-6) in monogalactosyldiacylglycerol, digalactosyldiacylglycerol and phosphatidylglycerol following by the improvement of the percentage of (20:4 n-6) in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, compared with wild type level of (20:5 n-3). The negative influence on mutant was slower growth rate, fewer thylakoid stacks per chloroplast and a four-fold-decrease of membrane lipid level. Heavy-ion irradiation mutagenesis was applied to improve lipid productivity and TAG content in *N. oceanica* IMET1 (Ma et al. 2013). The biomass

accumulation, lipid productivity and growth rate in this mutant were increased by 19, 6 and 28%, respectively. Meanwhile, the analysis of lipid composition has revealed that TAG was increased by 14% and polar lipid content was decreased by 15% compared with those of wild type. However, the improvement in photosynthetic efficiency in this mutant was also revealed. Enhanced lipid content in mutant *Nannochloropsis* species BR2 could also be obtained by UV radiation, which displayed a two-fold-increase of EPA content within 24 h that accounted to be approximately 30% of total fatty acids and constituted up to 12% of total dry weight at higher dose of UV treatment (1000 mJ/cm^2) (Sharma and Schenk 2015).

Mutation to Improve Pigment Productivity

Antenna pigments are another valuable commercial product which can be extracted from *Nannochloropsis* species. Accordingly, the improvement of pigment production in the mutant of *N. oculata* strain was achieved through EMS treatment (Lee et al. 2006). Two mutants exhibited abnormal xanthophyll biosynthesis, and thus increased violaxanthin content by two-fold, zeaxanthin content by two to three-fold and xanthophyll pool size by 1.8-fold under low light conditions, followed by a decrease in lutein content under low light conditions, while comparing with wild type with no negative influence on growth and cellular chemical composition. Although the investigators claimed high light condition may cause negative influence on the reduction of violaxanthin, a potential method for producing zeaxanthin can be applied in microalgal industries.

Mutation to Increase Photosynthesis Efficiency and Biomass Productivity

Since photosynthesis efficiency is highly associated with lipid and biomass productivity, improving photosynthesis should help microalgae to convert light energy to biomass and lipids. Therefore, mutation of the photosynthesis apparatus in *Nannochloropsis* strains was attempted based on this concept. Perin et al. (2015) did obtain improved photosynthetic activity in the mutant *N. gaditana* CCAP 849/5 using EMS. Although several mutants displayed reduction in cellular chlorophyll contents, some mutants exhibited improved photosynthesis productivity, and thus increased biomass productivity. In addition to improvement in biomass, two *Nannochloropsis* mutants produced chlorophyll a and lipids, resulting in increased biomass productivity by 25% under high light and by 21.3% under low light in one mutant strain. Chlorophyll a content in this mutant was also improved by 16.9% under high light. Meanwhile, lipid productivity was increased by 11.9 and 21.7% in both mutants, respectively. Yet, no significant change was observed in lipid composition (Anandarajah et al. 2012).

FUTURE PERSPECTIVES

The genus *Nannochloropsis* is an important feedstock which provides a variety of natural compounds for biofuels, aquaculture and healthy foodstuffs. In response to technological

advances over the last decade, genomic sequencing, transcriptomics, lipidomics, proteomics, metabolomics, and forward and reverse genetic approaches have all been developed. Knowledge of this genus has increased, and a platform for its biological and genetic enhancement is well established. Although molecular biotechnological techniques have been applied to develop GM strains for generating commercial products, establishment of an effective and feasible platform for genetic manipulation in *Nannochloropsis* is still needed, especially with respect to transformation efficiency, expression of desired products, and exhibition of stable phenotypes.

The Expression Level of Foreign Protein in Chloroplast

Foreign genes expressed in cellular organelles within microalgae have been demonstrated, but the expression level has varied. Nuclear transformation is a commonly used method to obtain transformed microalgal strains. However, this technique has some limitations. One key disadvantage of nuclear transformation involves the expression level of foreign gene in transgenic microalgae, which is relatively low owing to positional effect, RNA silencing, compact chromatin structure, and unconventional epigenetic effects (Potvin and Zhang 2010).

In contrast, the level of foreign protein expression in chloroplast transformation is high as a result of viable protein folding, limited proteolytic pathways, and protection from degradation of foreign protein in chloroplast envelope (Potvin and Zhang 2010). The number of chloroplasts in a cell is abundant compared to one nucleus. In addition, chloroplasts are important components in algal cells by their involvement in several metabolite pathways, allowing alternative genetic manipulation options for analyzing or improving their endogenous gene functions (Umaima et al. 2016). The genomics of *Nannochloropsis* chloroplasts has been investigated, thus allowing a better understanding of gene expression in the chloroplast machinery. Consequently, chloroplast genetic engineering should be considered as a potential microalgal cell factory for producing highly valuable compounds or studying its metabolite pathways.

Promoter Used in Gene Construct for Transformation

Promoters control and drive the expression of foreign protein. As such, they can regulate the yield of desirable products. Selection of an effective promoter for expressing foreign gene at high level depends on the target organisms, cellular compartments and protein types which enable the correct recognition of elements associated with transcriptional machinery in the expression system. Thus, several exogenous and endogenous sources of promoters have been applied to the transformation of *Nannochloropsis*, resulting in variable expression efficiency and stability of foreign proteins.

Three types of promoters are available. First, the CaMV 35S promoter from Cauliflower mosaic virus (CaMV) is a widely used promoter, even though lacking the genetic information of endogenous promoter in microalgae strains (Qin et al. 2012). Cha et al. (2011) demonstrated the application of CaMV 35S promoter in *Nannochloropsis* species UMT-M3 and successfully drove the expression of GUS gene. Second, heat shock protein 70A

(HSP70A), a chimeric promoter, was combined with small subunit of RUBISCO (RbcS2) inducible promoter isolated from the model microalgal organism *C. reinhardtii*. Foreign proteins, such as yellowfin porgy growth hormone, antimicrobial peptide bovine lactoferricin and sea anemone (*Stichodacyla haddoni*) purple chromoprotein, were driven to conditionally express in *N. oculata* by this hybrid promoter (Chen et al. 2008; Li and Tsai 2009; Shih et al. 2015). In addition, SQD2-stress-inducible promoter from *C. reinhardtii* was also used to drive type-2 diacylglycerol acyl-CoA acyltransferase under phosphorus starvation in *Nannochloropsis* species NIES-2145. Third, *Nannochloropsis* is a source of native promoters, such as violaxanthin/chlorophyll *a*-binding protein (VCP) 2, lipid droplet surface protein (LDSP), β-tubulin (TUB), HSP70A and ubiquitin extension protein (UEP). Unlinked VCP2 bidirectional promoters coupled with VCP1 3' untranslated region from each gene were established to drive antibiotic *sh ble* (Zeocin- resistant) gene in *Nannochloropsis* species W2J3B (Kilian et al. 2011). This bidirectional promoter was also used to drive the gfp gene in *N. oceanica* CCMP1779. In addition, Radakovits et al. (2012) selected TUB, HSP70A and UEP native promoters to drive *sh ble* gene in *N. gaditana* CCMP526. The UEP promoter was also used to drive the bHLH transcription factor and sfCherry in *N. salina* CCMP1776. Additionally, Vieler et al. (2012) developed LDSP endogenous promoter from *N. oceanica* CCMP1779 to drive the *aphVII* gene (Hygromycin resistance).

Codon Usage

Although several studies reported the expression of foreign proteins in transgenic *Nannochloropsis* strains, the expression efficiency of exogenous genes from other organisms was hindered by the variation of codon usage pattern in the transformed cells, resulting in poor translation rate or incorrect expression of certain amino acids within the coding region, thus either losing functionality of target protein products or reducing overall protein productivity. These phenomena were observed while developing several transgenic microalgae strains (Qin et al. 2012). Hence, the optimization of codon usage of transferred genes can improve the expression level of foreign protein and reduce the gene silencing of nuclear or chloroplast transformation in host cells. In particular, because the codon preference in chloroplast genome is unique, the exogenous gene derived from animals should be optimized. Recently, *Nannochloropsis* genome research has provided data for codon optimization which is a relatively effective way to increase protein stability and expression efficiency and avoid undesirable gene silencing. In addition, several software programs have been developed to estimate Codon Adaptation Index (CAI), which serves as a quantitative tool to predict foreign gene expression based on codon usage within different organisms (Shih et al. 2015), thereby allowing feasible optimization of codon usage of nuclear genes and chloroplast in *Nannochloropsis* strains.

Further Improvement of Selective Marker System

Two types of selective marker systems have been successfully applied in developing transgenic *Nannochloropsis* strains. Antibiotics-related selective markers can confer selective pressure directly on transformed cells, which is considered a simple, but effective, method for

isolating the desired transgenic clones. However, the application of such marker genes in generating GMO has raised public concern over environmental and food safety issues. Consequently, these markers with antibiotics-resistant genes have become an obstacle for transgenic strains developed for foodstuffs. As a result, reporters, such as sfCherry, endow the host cells with fluorescent signal and thus serve as a selective marker, even while not exerting selective pressure or requiring fluorescent microscopy to screen transgenic *Nannochloropsis* strains (Kang et al. 2015a). The shCP isolated from marine invertebrate and its derivatives can circumvent this disadvantage, enabling the transformed cells to display dark brown coloration which can be easily observed and screened by the naked eye, although transformation efficiency is low (Umaima et al. 2016) owing to the absence of selection pressure.

Nevertheless, it is possible to solve some problems associated with the disadvantages noted above. For example, marker excision methods, such as direct-repeat-mediated excision via homologous recombination (Fischer et al. 1996), co-transformation (Rochaix 1997) and site-specific recombination (Landy 1989; Dale and Ow 1991; Onouchi et al. 1995), have all been reported to produce marker-free transgenic algae and other plants. This strategy provides a feasible way to eliminate antibiotics markers within the host genome, allowing the development of transgenic strains via antibiotic selective markers without violating any laws and regulations. However, no study has reported on the application of marker excision methods for transgenic *Nannochloropsis* strains.

Nonetheless, several methods are available to improve the transformation efficiency of shCP for establishing transgenic *Nannochloropsis* strains. For instance, transformation protocol, promoter and coding gene sequence can be further optimized, thereby increasing transformation efficiency and paving a way for yet another selective marker option for generating transgenic *Nannochloropsis* strains.

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Chapter 59

NANNOCHLOROPSIS OCULATA AND INTEGRATED BIOREFINERY BASED ON PALM OIL MILLING

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ABSTRACT

The challenges in addressing sustainability goals include minimizing the environmental impact, energy consumption and wastes, and maximizing socio-economic benefits especially for the poor segments of the society, apart from the industrial players. Biorefining is a sustainable conversion of whole crop, green or lignocellulosic feedstocks via acid/enzymatic, chemical, gasification or pyrolysis processes into useful products for energy, fuels, pharmaceuticals or chemicals. Oil palm (*Elaeis guineensis Jacq*), a monoecious plant, is an eminent source of oil for food and biodiesel, and its agro-wastes are equally important for biomaterials. The palm oil industry has contributed significantly in meeting the demand and supply of the oil and fat production in the world market, and serving the agro-industry communities. In the oil palm biorefinery set-up, microalgae can be a unique component, not only as the source of biochemicals and bioproducts, but also as a part of integrated scheme of waste treatment and bioenergy co-generation, with the extraction of important commodities such as bio-oil, celluloses, lignins or biomaterials. In this review, the general characteristics, its potential and the cultivation of *Nannochloropsis oculata* are described using an integrated biorefinery approach based on palm oil milling as a model system. Issues addressed include the bioengineering aspects for the conversion of light into biomass, co-location strategy to take advantages of the industrial wastes, energy, water and nutrients, productivities of the different reactor systems and configuration, utilization of palm oil mill effluent (POME) as an alternative source of nutrients, biomaterials co-utilization, bioenergy co-generation and downstream processing aspects for extraction, purification and conversion into value-added products for pharmaceuticals and functional foods.

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Keywords: *Nannochloropsis oculata*, oil palm, biofuels, bioenergy, biomaterials, nutrient recycling, lipid, palm oil mill effluent

1. INTRODUCTION

The major thrust for the production of fuels and chemicals from renewable sources stems mainly from environmental considerations, and the concerns with regards to the depletion of fuel reserves and the instability of fossil fuel supplies and prices (du Preez, 2016). Physical, biochemical or chemical routes to the conversion of any raw materials to the end products may be more economical when implemented within the context of an integrated biorefinery set-up. In bioprocesses, enzymes, microorganisms, mammalian or plant cells, wild or recombinant, may convert the feedstocks into antibiotics, biofuels, protein, and sugar hydrolysates, enzymes, vaccines, biopesticides, beverage, organic acids, and others. The bioprocess may be at its simplest and advantageous when put next to the routes involving chemical synthesis for bioproducts (Damaso et al. 2014). Industrial biorefineries producing multiple products can take advantage of the differences in biomass components and intermediates and maximizing the value derived from the biomass feedstock. A biorefinery therefore may produce one or several low volume, but high-value chemical products and a low-value, but high-volume liquid transportation fuel, whilst generating electricity and process heat for its own use or for sale. These 'green and clean' biorefineries are the basis of bio-molecular transformations that contribute to the production of biochemicals, biofuels and biomaterials (Doelle & DaSilva 2009; Abdullah et al. 2015a, b, 2016a, b, c).

Microalgae, consisting of small eukaryotic algae and prokaryotic cyanobacteria, have the advantages of converting the solar energy and CO_2 into bioproducts. Algae perform oxygenic photosynthesis, and like higher plants with a large variety of species, live in a wide range of environmental conditions. Capable of capturing carbon from many sources, some species actually hold higher potential as the oil-producer than oil seed crops, and may be processed into a wide range of products including biodiesel, green diesel, gasoline replacements, bioethanol, methane, fertilizer, bio-oil, animal feed, or biopharmaceuticals (Christenson & Sims 2011; Abdullah et al. 2016a; 2017). Algae can be cultured in open ponds, in photobioreactors (PBRs) or in hybrid modes. Heterotrophic algal culture may give high oil productivities but is prone to contamination especially in open ponds, and the cost of organic carbon source can be astronomical. Phototrophic cultivation is attractive for large scale investment, but the operation costs for mixotrophic and photoheterotrophic are high because of the need for a special PBR to avoid contamination risk and satisfy the light requirements (Chen et al. 2011). Furthermore, all stages involved in the microalgal biodiesel production chain may require high energy which adds up to the high production costs as compared to the conventional fuels.

Despite the algal technology for biofuels production being greatly advanced over the past decade, the commercial endeavour suffers from the constraints of yield, productivity and price (du Preez, 2016). The cost of algae-based bio-crude has been reduced from $240 to $7.50 per gallon, but to meet the long term goal of $3/gasoline gallon equivalent, a combination of improvements in all key technologies together with productivity, conversion, and processing, must be made (Picardo et al. 2013; Yang et al. 2016). In this regard, the

integrated biorefinery concept within the palm oil milling set-up is an ideal solution as the organic residues from the oil palm industries can be used as the carbon source and for the value-added conversion of biomaterials, and the co-cultivation with microalgal biomass is attractive for renewable generation of biofuels and bioproducts whilst remediating the environment. This represent a new route to the production of high-value biocompounds with much lower impact on the environment and less interference in the food supply than the conventional oil seed crops. The whole processes from upstream to downstream, and the production of high value, low volume and high volume, low value products can be optimized (González-Delgado & Kafarov 2011; Abdullah et al. 2016a; 2017). Starch rich microalgae are extensively studied (Choi et al. 2010), and species such as *Nannochloropsis*, *Chlorella*, *Dunaliella*, *Chlamydomonas*, *Scenedesmus* and *Spirulina* are some of the most abundant organisms on earth, existing in salt or fresh water (Ruiz et al. 2013). *Nannochloropsis* sp. is one of the few species accepted as fuel and food and is therefore of broad interest as a highly productive commodity for biorefinery application.

This chapter covers the analysis of the productivities of the most recent culture systems, co-location of the algae biomass production process to core industry, the use of POME as alternative sources of nutrients, and the challenges in the development of a biorefinery system based on *N. oculata*. The sustainability of the process while minimizing the energy consumption, the socio-economic benefits and the importance of large-scale integrated microalgae-based biorefinery for the production of biofuel and bioproducts including biofertilizers and aquafeed, using marine water and nutrients from POME, are also highlighted.

2. FACTORS AFFECTING *NANNOCHLOROPSIS OCULATA* CULTIVATION

2.1. Effects of pH, Salinity and Light Intensity

N. oculata are marine eustigmatophyceae, possesing a high biomass productivity which may reach up to 3 $gL^{-1}day^{-1}$ and up to 30–70% lipid by dry weight, which is suitable for biodiesel production (Rodolfi et al., 2009; Chen et al., 2011). Factors such as pH, salinity, light intensity and duration, availability of macro and micronutrients and reactor configuration and mode of operation could all influence the biomass and lipid productivity. Different geographical strains have different preferences towards salinity and optimal salinity may be a function of immediate conditions from which the strain is initially isolated. Species isolated at higher salinities will grow better at higher salinities and may not be as good at lower salinities (Cucchiari et al. 2008). Light is the main source of photosynthesis and light intensity influences cell photo-acclimatization for microalgal growth and lipid synthesis, where strong illumination may induce their accumulation (Takagi & Yoshida, 2006). The quantity of photon energy absorbed by each cell is further affected by factors such as cell density, photoperiod, length of optical path, thickness of layers, photon flux density and rate of agitation. The responses of microalgal strains to different light intensities are indication of the light antenna accessibility on cell surface and cell responds by modulating the composition of its photosynthetic apparatus, a response called acclimation (Simionato et al. 2011). Larger quantity of light energy may induce more metabolic flux channelled into lipid

accumulation on a unit biomass basis. The high lipid content at high light exposure is measured as a total global radiation (TGR) where light and photoperiod may actually influence the cultivation temperature. Light and temperature therefore play a synergistic role in algal lipid synthesis and accumulation (Weldy & Huesemann, 2007).

In comparing the performance of two green microalgae (*N. oculata* and *Tetraselmis suecica*) with two brown microalgae (*Isochrysis galbana* and *Pavlova lutheri*) in Conway media in 250ml flask cultivation over two weeks period, all isolated from marine environment that thrive at high salinity, *N. oculata* shows superior performance in terms of biomass and lipid productivity. Only *P. lutheri* shows high lipid level comparable to *N. oculata*. The highest cell density of 75.5×10^6 cells mL^{-1} (maximum biomass of 0.80 gL^{-1}) achieved at 35-40 ppt salinity by *N. oculata*, is almost 1.5-5 fold higher than the other 3 strains. Generally, the shortest doubling time is observed in pH 7–9 media, while the longest at pH 5-6 and 10. *N. oculata* and *P. lutheri* attain the highest lipid contents of 31.3-36.5% at pH 8 and 35 ppt salinity, while the lipid contents for *T. suecica* and *I. galbana* are lower, around 23.4-26.3% (Shah, 2014). The transition from low to high photoperiod and light conditions enhance both cell growth and lipid accumulation by 2-fold (Shah, 2014). This is in agreement with a study on *N. salina* which has the lipid enhanced from 10 to 70% when the light intensity is increased from 150 µE m^{-2} s^{-1} to 350 µE m^{-2} s^{-1} (Sforza, et al. 2012), and *Ochromonas danica* where the cell growth and lipid content increase with increasing light intensity (Verma et al. 2010). Increased microalgal lipid productions to 26-36% at temperatures 25-30°C for several microalgal species have been reported. However, reduced lipid level to 15-20% at extremes of low (15°C) and high temperature (30°C) has been observed in *I. galbana and Nanochloropsis* species (Sayegh & Montagnes, 2011). Low temperatures reduce enzyme activity in glycolysis and the Krebs cycle and consequently the metabolism of carbon sources.

2.2. Effects of Macro and Micronutrients

The effects of nitrate, phosphate and iron investigated at 120–150 gL^{-1} KNO_3, 12–15 g L^{-1} Na_2HPO_4 and 4–5 g L^{-1} $FeCl_3$ show that *N. oculata* maintain the highest cell density (72.3×10^6 cells mL^{-1}) and maximum biomass (0.81 g L^{-1}), followed by *T. suecica* with 46.5×10^6 cells mL^{-1} density and 0.70 g L^{-1} biomass. The maximum specific growth rates, μ_{max}, are 0.16–18 d^{-1}, with the doubling time, t_d, of 3.78–4.62 days. Both *I. galbana* and *P. lutheri* only achieve 17×10^6 cells mL^{-1} though almost comparable dry weight at 0.7 g L^{-1}. Although cell growth appears to require excesses of nitrate, phosphate and iron, lipid production is only induced under conditions of nutrients limitation with the highest lipid content achieved by *P. lutheri* at 34.2-37.1% (in 40 g L^{-1} KNO_3, 2 g L^{-1} $FeCl_3$ and 4 g L^{-1} Na_2HPO_4) and *N. oculata* at 33.1-35.3% (in 10 g L^{-1} KNO_3, 4 g L^{-1} Na_2HPO_4 and 2 g L^{-1} $FeCl_3$). *I. galbana* and *T. suecica* only attain around 23.8-26.8% lipid. Lipid production is therefore highly affected by nitrate and phosphate concentration where excesses could inhibit lipid production while iron shows little effect (Shah, 2014). The total lipid contents of 35.9, 29.8, and 32.1% respectively, have been reported for *N. oculata* SHOU-S14, *T. subcordiformis* SHOU-S05, and *P. viridis* SHOU-S16 cultured in different amounts of nitrogen level (Bondioli et al. 2012). The increase of lipid contents in *N. oculata* from 7.9% to 15.31% and *C. vulgaris* from 5.9% to 16.41% are achieved at reduced nitrogen as low as 75% (Harwood & Guschina, 2009).

Table 1. Establishment of kinetics between 5 L PBR and 300 L open tank cultures at optimized conditions as predicted by Response Surface Methodology (Shah, 2014)

Media Conditions		*N. oculata* [a]				*T. suecica* [b]				*I. galbana* [c]				*P. lutheri* [d]			
		X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)	X'_{max} ($gL^{-1}d^{-1}$)	μ_{max} (d^{-1})	t_d (day)	Lipid (%)
pH and Salinity	PBR	0.176	0.24	2.92	38.6 ± 1.54	0.173	0.22	3.15	30.3 ± 4.38	0.143	0.23	2.99	32.2 ± 2.11	0.135	0.21	3.30	40.2 ± 3.31
	Open Tank	0.161	0.23	2.98	35.5 ± 1.81	0.162	0.20	3.53	27.5 ± 2.98	0.131	0.21	3.36	28.6 ± 2.75	0.134	0.19	3.61	36.1 ± 3.65
Photoperiod and Light Intensity	PBR	0.185	0.24	2.92	40.1 ± 2.77	0.172	0.22	3.16	30.8 ± 2.87	0.148	0.23	2.98	32.8 ± 3.44	0.142	0.22	3.11	41.8 ± 0.78
	Open Tank	0.171	0.20	3.40	30.7 ± 2.52	0.166	0.21	3.32	25.6 ± 3.90	0.120	0.20	3.45	24.8 ± 3.13	0.120	0.18	3.82	32.1 ± 2.57
Nitrate, Phosphate and Iron	PBR	0.172	0.21	3.27	42.2 ± 3.78	0.157	0.21	3.25	31.6 ± 4.33	0.134	0.21	3.30	33.5 ± 2.27	0.137	0.19	3.65	41.2 ± 1.92
	Open Tank	0.168	0.19	3.63	36.2 ± 2.47	0.152	0.20	3.48	30.5 ± 1.84	0.126	0.18	3.75	32.4 ± 1.47	0.126	0.17	3.96	38.5 ± 0.76

[a] *N. oculata* : pH 8, Salinity (35 ppt), photoperiod (24 hrs), light intensity (188 µmol photons m^{-2} s^{-1}) KNO3 (10 gL^{-1}), Na_2HPO_4 (6 gL^{-1}) and $FeCl_3$ (2.53 gL^{-1})

[b] *T. suecica* : pH 7.9 Salinity (32 ppt), photoperiod (24 hrs), light intensity (196.5 µmol photons m^{-2} s^{-1}), KNO3 (13.7 gL^{-1}), Na_2HPO_4 (5.6 gL^{-1}) and $FeCl_3$ (2.50 gL^{-1})

[c] *I. galbana* : pH 9, Salinity (39.2 ppt), photoperiod (20.5 hrs), light intensity (188.7 µmol photons m^{-2} s^{-1}), KNO3 (75.4gL^{-1}), Na_2HPO_4 (8.9gL^{-1}) and $FeCl_3$ (2.8gL^{-1})

[d] *P. lutheri* : pH 7.9, Salinity (35.5 ppt), photoperiod (24 hrs), light intensity (198 µmol photons m^{-2} s^{-1}), KNO3 (62.5gL^{-1}), Na_2HPO_4 (3.92gL^{-1}) and $FeCl_3$ (2.63gL^{-1})

Note: For 300 L open tank photoperiod was 12 hrs and light intensity was 165-250 µmol photons $m^{-2}s^{-1}$ (shaded from direct sunlight).

Table 2. Fatty acid profile at optimized pH and salinity (Shah, 2014)

Experimental Conditions		Total fatty acids composition (%)														
		C14:0	C15:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:2	C20:3	C20:4	C20:5	C22:6
N. oculata	PBR	4.60	6.10	38.23	10.96	3.12	5.80	6.10	3.28	2.1	5.96	ND	ND	5.8	4.3	2.34
	Open Tank	3.36	14.73	25.23	5.23	1.67	6.35	7.86	3.13	2.4	3.69	1.08	ND	ND	ND	2.79
T. suecica	PBR	4.16	5.20	22.95	5.98	12.67	5.07	2.49	6.45	0.54	3.70	ND	ND	ND	ND	4.20
	Open Tank	6.02	15.51	8.91	2.75	3.61	5.33	1.44	2.36	ND	3.98	ND	ND	ND	ND	4.87
I. galbana	PBR	3.32	15.68	13.94	2.61	3.15	4.72	11.67	ND	ND	3.50	2.38	ND	8.43	2.23	2.66
	Open Tank	2.72	9.49	11.43	3.67	1.92	2.64	8.35	ND	ND	2.21	1.54	ND	5.43	1.32	2.37
P. lutheri	PBR	2.69	3.52	32.34	22.42	4.04	0.41	12.10	0.34	1.36	3.86	ND	ND	ND	7.14	5.69
	Open Tank	1.54	2.62	28.38	19.37	2.87	ND	8.28	ND	1.25	2.64	ND	ND	ND	5.27	4.84

Nitrogen stress on *N. oculata* (75 gL^{-1}, 37.5 g L^{-1} and 150 g L^{-1}) and *C. vulgaris* (75 g L^{-1}, 150 g L^{-1} and 300 g L^{-1}) with nitrate in the form of $NaNO_3$ have been reported under light intensity of 70 μE m^{-2} s^{-1} at 20ºC. Low nitrogen in the growth medium increases the lipid production, as the higher nitrogen level could extend the growth cycle thus lowering the lipid productivity (Harwood & Guschina, 2009).

2.3. Effects of Reactor Configuration

Reactor configuration may exert different hydrodynamics conditions that could affect algal productivity (Shah et al. 2014a; Abdullah et al. 2015b; 2016a; 2017). Comparing between cultivation in 250mL flask with 1L, 5L and 30L tanks, *N. oculata* and *P.* lutheri show the highest cell densities of 64.6 x 10^6 and 14.5 x 10^6 cells mL^{-1}, respectively, in 250 mL flask, but the densities are lower at 52.6 x 10^6 and 10.7 x 10^6 cells mL^{-1}, respectively, in 30 L tank. Only *N. oculata* and *P. lutheri* show higher lipid content at 32-37%, while the highest μ_{max} of 0.15 d^{-1}, t_d of 4.62 days and lipid content of 36.8 are achieved by *N. oculata* in 250 ml shake-flask (Shah, 2014; Shah et al. 2014a; Abdullah et al. 2015b). Comparing between cultivation in 5L PBR and 300L open tanks (Table 1), the highest cell density and biomass are achieved by *N. oculata* at 78.8 × 10^6 in 5 L PBR and 55.5 × 10^6 cells mL^{-1} in 300 L tank with 0.93 and 0.65 gL^{-1} biomass, respectively. All strains show the highest μ_{max} of 0.21-0.24 d^{-1} (t_d of 2.92-3.30 days) in 5 L PBR as compared to μ_{max} of 0.19-0.23 d^{-1} (t_d of 2.98-3.61 days) in 300 L open tank. The lipid content remains higher in 5 L PBR at 42.2 and 41.2% as compared to 300 L open tank at 36.2 and 38.5% for *N. oculata* and *P. lutheri*, respectively (Shah, 2014). Table 2 shows that pentadecanoic acid C15:0 (6.1%, 14.7%); palmitic acid C16:0 (38.2%, 25.2%); palmitoleic acid C16:1 (11.0%, 5.2%); oleic acid C18:1 (6.1%, 7.9%); and eicosanoic acid C20:0 (6.0%, 3.7%) are the major components of the oil derived from *N. oculata* cultivated in PBR and open tank, respectively. The total saturated fatty acids (TSFA) (63.8%, 55.03%); monounsaturated fatty acids (MUFA) (17.1%, 13.1%); and (polyunsaturated fatty acids) (PUFA) (15.7%, 9.4%) are obtained for *N. oculata*, respectively. The major components of *P. lutheri* are palmitic acid C16:0 (32.3%, 28.4%); palmitoleic acid C16:1 (22.4%, 19.4%); oleic acid C18:1 (12.1%, 8.3%); and docosahexaenoic acid (DHA) C22:6 (5.7%, 4.8%) with the TSFA (46.9%, 38.1%); MUFA (34.5%, 27.6%); and PUFA (14.5%, 11.4%) respectively, for cultivation in PBR and open tank (Shah, 2014; Shah et al. 2014a; Abdullah et al. 2015b).

3. DIFFERENT TYPES OF BIOREFINERY

Algae are like microscopic factories producing all sorts of useful compounds. They are composed of different lipids (7-23%), carbohydrates (5-23%) and proteins (6-52%) (Zhu, 2015). A way to improve the productivity is to obtain a full valorization of each microalgae components with products of the highest possible value (Vanthoor-Koopmans et al. 2013). Integrated biorefinery converts biomass into multiple end products to produce energy in the form of heat or by producing biofuels, molecules for fine chemistry, cosmetics or medical applications, materials such as polymers or plastics and sources of human food and animal

feed. Figure 1 illustrates the generalized outline of a biorefinery (González-Delgado & Kafarov 2011) which is derived from petroleum refineries, producing not only fuels, but also fine chemicals for the chemical industry (Markou & Nerantzis 2013). It combines in a single facility the production of various products by multiple steps, arranged into a cascade chain that use all the raw material components and prevent losses. But care must be taken at each step to not damage one or more of the products (Vanthoor-Koopmans et al. 2013). Moreover, several steps of any type of biorefinery need improvements to lower the global cost of the process. The different types of biorefinery are discussed below.

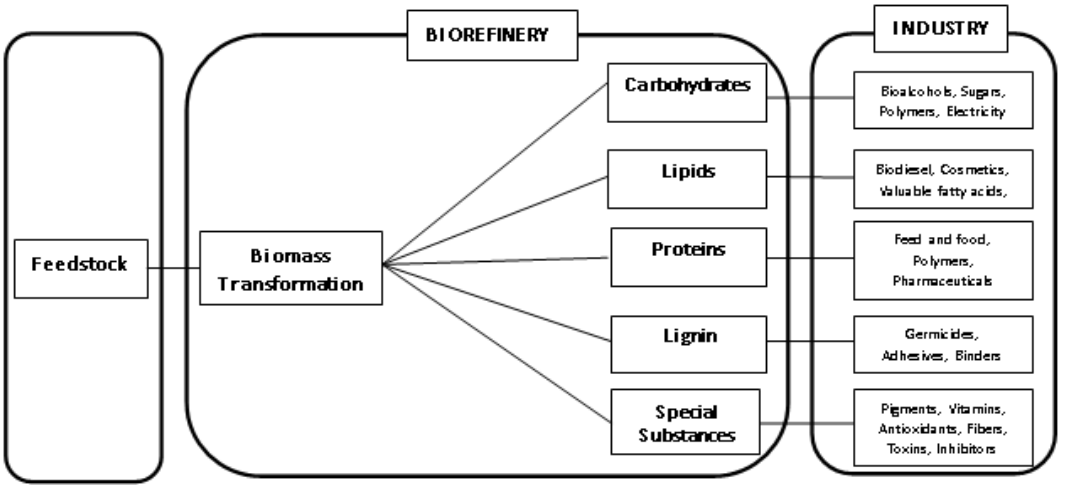

Figure 1. General outline of the biorefinery concept (adapted from González-Delgado & Kafarov 2011).

3.1. Oil Recovery Biorefinery

Current production methods for liquid biofuel production from microalgae produce approximately 60–70% residual biomass that is currently a byproduct. The oil recovery biorefinery uses transesterification process in the treatment of oil and fat, and domestic cooking oils originating from soybean, corn, palm, and canola, to yield fatty acid methyl esters commonly known as biodiesel. The conversion of triacylglycerides (TAGs) extracted from algae biomass to fatty acid methyl esters (FAMEs) is called transesterification reaction, which displaces an alcohol group from an ester by another alcohol (Demirbas 2008). Direct transesterification brings wet microalgae directly into transesterification process, before methanol recovery and biodiesel produced. The indirect method, in contrast, involves cell disruption, solvent extraction, and solvent recovery before transesterification, methanol and biodiesel recovery (Park et al. 2015).

Transesterification can be performed using different heating systems via catalytic or non-catalytic reaction systems. The technology is mature and used as a standard in the conversion of vegetable oils into biodiesel (Hossain et al. 2008) with ethanol and sodium ethanolate serving as the catalyst (Zhou and Boocock 2006). The products are separated by adding ether and salt water to the solution and mixed well. Biodiesel is separated from the ether by a vaporizer under a high vacuum. Base-catalysed transesterification is faster, but would leave

free fatty acids behind and un-transesterified. This saponification can also result in significant losses due to the water solubility of the fatty acid soaps (Laurens et al. 2012). Another route in biodiesel production from algae is acid-catalyzed transesterification (Wahlen et al. 2008). The replacement of soluble bases by liquid-acid catalysts such as H_2SO_4, HCl, or H_2PO_4, is attractive as the acidic catalysts are less sensitive to the presence of water and free acids, which mitigate saponification and emulsification, to enhance product recovery (Ataya et al. 2007). However, these are more applicable to the transesterification of different lipid types and also have lower activity than the conventional alkaline catalysts.

The development of immobilized heterogeneous or homogeneous catalysts or enzymes that are efficient and inexpensive is needed (McNeff et al. 2008) and may hold a key role for economical method of biocatalytic transesterification (Gerken et al. 2013). Acid and base catalysts could be classified as Bronsted or Lewis catalysts. Lewis acid catalysts such as $AlCl_3$ or $ZnCl_2$, have been proven as a viable means of converting TAGs into fatty acid methyl esters while the presence of a co-solvent such as tehtrahydrofuran, may be vital to achieve high conversion efficiencies of up to 98% (Soriano et al. 2009). Hetero-polyacids catalyzed transesterification of vegetable oil achieves higher reaction rates than conventional mineral acids due to their higher acid strength (Xu et al. 2008). Microwave heating can improve the kinetics of transesterification and the presence of catalysts further enhance the impact of microwave irradiation (Yuan et al. 2008). In the ultrasonic reactor, ultrasonic waves cause the reaction mixture to produce and collapse bubbles constantly and simultaneously, providing the mixing and heating for transesterification process (Armenta et al. 2007) with reduced reaction time, temperatures, and energy input (Kalva et al. 2008).

Chemical processes through high conversion of TAGs, resulting in their corresponding esters, are energy-intensive, and require the removal of glycerol and alkaline catalysts and the treatment of alkaline wastewater. Biocatalysts such as lipases offer more environmentally attractive option (Svensson and Adlercreutz 2008), but have not been scaled up due to the relatively high price of lipase and the short operational life caused by excessive methanol and co-product glycerol. Solvents are sometimes necessary to enhance the solubility of the TAGs during the extraction process that the enzymes used must be solvent and temperature tolerant to enable cost-effective biofuel production (Fang et al. 2006). Novel enzymes with desired characteristics may be produced by bioprospecting for the enzymes in extreme environments (Guncheva et al. 2008). Another route for a more powerful extraction than the traditional solvent-separation method is the supercritical processing of algal oil or extracts. The supercritical fluids are selective, providing high purity and product concentrations with no organic solvent residues in the extract or spent biomass (Demirbas 2009). They can be used on the whole algae without dewatering or coupled to transesterification reaction scheme to enable a "one-pot" approach to biofuel production (Anitescu et al. 2008). The extraction is efficient at modest operating temperature of less than 50°C, ensuring product stability and quality. Supercritical methanol or ethanol can be both the oil-extraction medium and the reagent for transesterification (Warabi et al. 2004; Hawash et al. 2009). The catalyst-free, supercritical ethanol transesterification, is capable of tolerating water, with conversion yield similar to anhydrous process for the conversion of vegetable oils. Water in the reaction medium however affects process efficiency but the decomposition of fatty acids is the main factor that limits the attainable ester content (Vieitez et al. 2009).

3.2. Biogas Biorefinery

The refinery uses natural consortia of microorganisms in anaerobic digesters, producing methane from decomposing organic material, organic wastes and manures. Combining and integrating alternative energy technologies with algal production, will foster synergistic development, supporting a self-sustaining community system (Shen, 2014, Abdullah & Ahmad, 2016). Three pathways have been defined for the implementation of anaerobic digestion into algal production processes:- Pathway 1 for the direct anaerobic digestion after the harvesting of biomass, or utilized in a wastewater process where the cell wall is degraded by bacterial activity within the digester; Pathway 2 for the anaerobic digestion of biomass after cell wall disruption prior to conversion; and Pathway 3 for the traditional biodiesel practice where lipid is extracted and residual algal biomass is converted to biogas by anaerobic digestion and methane fermentation (Ward et al., 2014). The system using anaerobically digested agricultural waste materials to feed algal cultures exhibits CO_2 abatement and reclamation and the use of other waste streams such as water and heat (e.g., from the digester and co-generation) supports optimum sustainable algal growth (Figure 2) (Shen, 2014). Anaerobic digestion is cost-effective as it eliminates processes such as drying, extraction and fuel conversion which incur high costs of algal biofuels. Although the cost of growing algae is high, methane production is a low value product. It can be utilized to generate *on site* electrical power or thermal heat to offset biomass processing and extraction processes, and allows nutrient recovery (such as nitrogen and phosphorous) from lipid extracted biomass. The methane produced readily separates from water without energy or chemical input, and can be converted to poly-hydroxybutyrate (bioplastics) blends to increase the value of biogas (Myung et al. 2015).

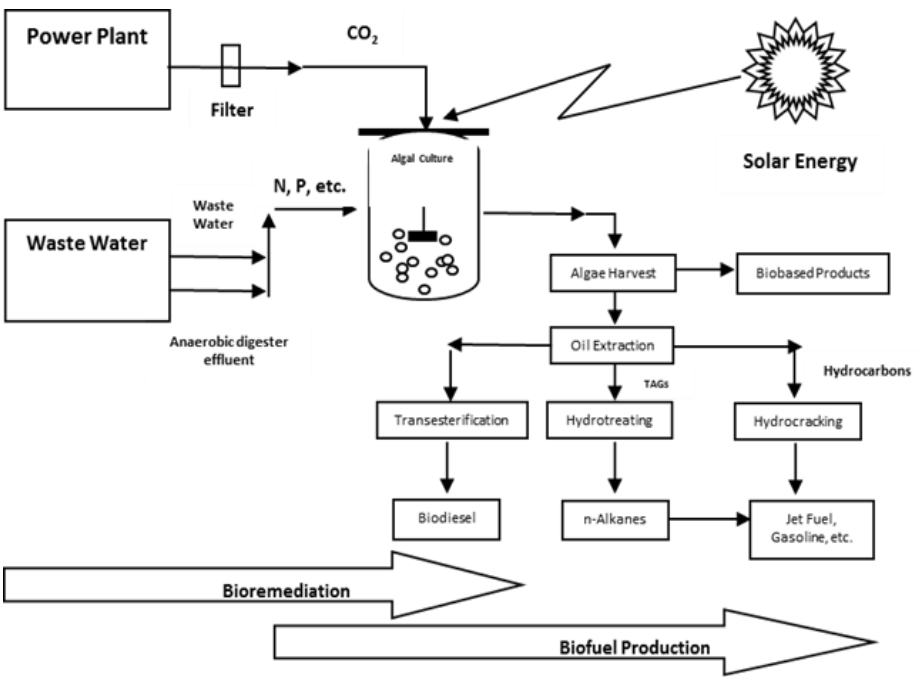

Figure 2. Schematics of an integrated algal culture system for bioremediation and biofuel production (adapted from Shen, 2014).

Scaling up to industrially relevant scales and optimizing the species-specific effects on methane production are two key challenges. Other issues include the low concentrations of digestible, algal biodegradable substrate, recalcitrant-substrate constituents, algal cell wall degradability, algal low carbon to nitrogen ratio, ammonia toxicity, and effects from salinity and associated metal ions (Ward et al. 2014). Co-digestion of other feedstocks with algal biomass may raise the carbon to nitrogen ratio, or proteins may be pre-extracted to use as co-products prior to digestion. A two stage anaerobic digestion with different strains of algae could produce 180.4 $mlg^{-1}d^{-1}$ of biogas with 65% methane concentration (Vergara-Fernández et al. 2008). Fugitive methane emissions must be managed via engineering or process flow as the emissions could be large (Frank et al. 2012). When processes are integrated and operated simultaneously, the benefits to microalgae biofuel production and wastewater treatment derived energy production are enhanced (Ward et al., 2014).

3.3. Sugar Feedstock Biorefinery

High-carbohydrate content from holocellulose-based cell walls and starch-based cytoplasm make algal biomass a suitable feedstock for bioethanol production. During microbial hydrolysis, carbohydrate polymers are broken into simple sugars, followed by fermentation to yield bioethanol (Kumar et al. 2013). Bioethanol production from algal biomass takes place by either the sugar or syngas pathway. Algae are directly fermented to produce bioethanol by the sugar pathway, while via the syngas pathway, hydrocarbons of algal biomass are converted to syngas through gasification followed by fermentation of syngas to produce bioethanol. Similar to cellulosic ethanol, algal bioethanol production requires four major unit operations:- pretreatment, hydrolysis, fermentation, and distillation. Different pretreatment methods have been evaluated to help separate cellulose, hemicellulose, and lignin so that the complex carbohydrate molecules in the algae cell can be broken down by enzyme-catalyzed hydrolysis into simple sugars. The fermentable sugars can be fermented into ethanol by ethanol-producing microorganisms before finally recovered and purified to meet the fuel specifications. Some separated solids can be recovered and utilized as a fuel to provide process heat and electricity at the production facility (Li et al. 2014 a).

Another promising fuel, butanol, has several advantages over bioethanol as it has higher energy content than ethanol and more similar to diesel fuels, in terms of energy content (Tigunova et al., 2013) and it could be blended with gasoline at any percentages. Butanol also has lower vapor pressure, absorbs less moisture and less corrosive as transported fuel. Butanol (C_4H_9OH) is a colorless liquid among the four carbon alcohols, and can be produced by chemical and biological methods. The economy of butanol production by chemical methods is highly dependant on the oil price while in biological methods, the cost of the raw material used is the determining factor. Due to the unstable price of crude oil, biological process is a promising route (Amiri et al., 2015), for a more economical and energy-efficient butanol production from biomass than ethanol, as some bacteria digest not only starch and sugars, but also cellulose (Huesemann et al. 2010). Different processes including batch, fed-batch, and continuous fermentation with and without *in situ* product removal using native and modified strains in the free and immobilized mode, have been reported (Chen et al., 2014; Rathore et al., 2015).

Overproduction of butanol can be achieved by mutagenesis, evolutionary engineering, and genomic studies and transcriptional analysis (Cooksley et al., 2012). For example, *Clostridium* produce acetone, butanol and ethanol (ABE) by anaerobic fermentation of both hexoses (C6) and pentoses (C5) (Van der Wal et al. 2013), but butanol is inhibitory to the fermentation which limits the yield and productivity. The fermentation of *Ulva lactuca* by *Clostridium* strains have produced butanol with a yield of 0.16 g butanol g^{-1} C5 and C6 sugars, but the yield of butanol is lower than that of ethanol produced under comparable conditions (Nikolaison et al. 2012). Using evolutionary dynamics and natural selection, referred to as artificial simulation of bio-evolution which is a repetitive evolutionary training, *C. acetobutylicum* could tolerate upto 4% butanol (Liu et al., 2013). Naturally occurring macroalgae (*Ulva*) has been found as a suitable feedstock for butanol (Potts et al. 2012). *Ulva lactuca* solubilised by hot water treatment, followed by hydrolysis using commercial cellulases to produce sugars, is used as a feedstock for fermentation by *Clostridium* for the production of ABE with a yield of 0.35 g ABE g^{-1} sugar (Van der Wal et al. 2013). However, ABE produced is proposed for the subsequent production of 1, 2 propanediol (propylene glycol) in a seaweed biorefinery to replace fossil fuel derived product rather than as a source of butanol. Macroalgae (e.g., seaweed) has also been examined for bioethanol fermentation due to the presence of sugars such as mannitol and laminarin (Ruiz et al. 2013). However, brown algae (*Saccharina*), produce low yields at 0.12 g g^{-1} extracted soluble solids (Huesemann et al. 2012) where the main fermentation substrates are mannitol and glucose derived from laminarin, and abundant. Brown algae, such as *Laminaria hyperobea*, is composed of organic fraction in the form of alginate and abundant alginates are recalcitrant and do not undergo fermentation (Horn et al. 2000).

3.4. Thermo-Chemical Biorefinery

Hydrothermal liquefaction (HTL) processing technology is a representation of the natural geological processes in the formation of petroleum based fossil fuels. HTL of biomass converts wet algal slurry to a range of liquid fuels in a hot (523 K–647 K) and pressurized (4–22 MPa) water environment at 3–5 mins residence times to disassemble cells into liquid components (Elliott 2016). At lower temperatures (<275°C) and pressure (<2 MPa), hydrochar is produced, and at higher temperatures (>374°C) and pressures (>22.1 MPa), syngas is produced. The main product of liquefaction biocrude, which can be upgraded to the entire distillate range of petroleum derived fuels, typically accounts for 45% wt. of the feedstock on ash free dry weight basis for fast growing, low total lipid algae. The yields are much higher for high total lipid algae such as *Nannochloropsis* sp. cultivated to varying composition to achieve HTL biocrude yield of 33–68 wt% (Leow et al. 2015). The HTL processing of algae requires dewatering, usually accomplished by mechanical means to a slurry of typically 10–20% dry solids. Continuous-flow HTL reactor of microalgae yields between 38–64% (ash free dry weight basis) with an energy recovery of 60–78% (Jazrawi et al. 2013). Algal lipid, protein, and carbohydrate composition influences HTL-conversion efficiency, where higher total-lipid content and lower protein yield higher energy recovery (Li et al. 2014 b). High-protein content may result in higher nitrogen (N) concentration in the biocrude requiring significant denitrification during upgrading to limit nitrogen-oxide emissions (Sudasinghe et al. 2014). Potential production strains of algae have higher N

contents (4–10%) as compared to the lignocellulosic feedstocks (< 1%) (Vardon et al. 2012). HTL application to algae has the potential of nutrient recycling through the precipitation of solids, allowing phosphorous recovery and the transformation of N into ammonium that can be fed back into the algae pond (Elliott et al. 2015). Two step sequential HTL (SEQHTL) has been developed to isolate polysaccharides or other high-value products (Chakraborty et al. 2012) to produce biocrude with low N content (Prapaiwatcharapan et al. 2015), or to collect aqueous phase rich in organic carbon and nutrients to enhance biomass productivity (Selvaratnam et al. 2015). With SEQHT, there may be a trade-off between N removal and overall biocrude yield (Jazrawi et al. 2015) but higher biocrude yield is achieved as compared to HTL (Prapaiwatcharapan et al. 2015). The algal HTL processing to fuels is currently scaled up to pilot plant operations and several small algae processing companies are developing HTL of whole algae (Elliott 2016).

The thermochemical treatment of algae, or other biomass can result in a wide range of products, depending on the reaction parameters. Liquid product yield tends to favor short residence time, fast heating rates, and moderate temperatures (Huber et al. 2006). Pyrolysis is another common chemical decomposition method for a condensed substance by heating in the absence of O_2. Pyrolysis is extremely fast, in comparison to other conversion methods, with reaction times of the order of seconds to minutes. Higher efficiency can be achieved by flash pyrolysis or fast pyrolysis technology, where finely ground feedstock is quickly heated to 350–500°C for less than 2 seconds. For flash pyrolysis, typical biomass feedstocks must be ground into fine particles and dried to <10% moisture. Several commercial plants for fast pyrolysis of biomass have been built in Finland, Canada, and Netherlands to produce fuel oil for heating. Fast pyrolysis bio-oil is also marketed by Ensyn as food flavorings and fuel oil (López Barreiro 2013). The pyrolysis of algae takes place at 400–600°C (López Barreiro 2013). Algae have major advantages over other biomass sources because it exists fundamentally in small units with no fiber tissue and more homogeneous than most biomass or coal. However the major barrier to pyrolysis of algae is moisture content. The cost of algal drying has resulted in the overall process to be energy negative (Jena et al. 2011). Comparison of bio-oil from pyrolysis of algae and biocrude from HTL of algae shows that bio-oil from pyrolysis has lower energy content and also less stable (Jena et al. 2011). Elemental analysis of pyrolitic bio-oil production from the wastewater treatment pond of algal biomass indicates that the bio-oil contain >65 wt.% carbon, 6-9 wt.% N, 8–10.2 wt.% hydrogen and an energy content of 34.4-37 kJ/g, all with higher values at higher temperature (400°C–500°C) except for nitrogen (Mehrabadi et al. 2016). Biocrude produced from HTL and slow pyrolysis of *Spirulina* and *Scenedesmus* are similar in energy density, but the net energy yield of HTL is more favorable (Vardon et al. 2012). Pyrolysis may not be cost-competitive over the short-term unless an inexpensive dewatering or extraction process is developed with the bio-oil demonstrating superior properties to oil produced from other conversion processes.

4. PALM OIL MILL BIOREFINERY

Palm oil industry in Malaysia has generated large amount of biomass and effluent in the form of empty fruit bunches (EFB), palm kernel shells, fibers, fronds, trunks and POME. The

palm oil mill biorefinery involves the utilization of oil palm biomass for the production of biofuels, the conversion of biomass residues into value-added products and the microalgal co-cultivation developed within palm oil milling processes for environmental remediation and subsequent recovery of bio-products (Abdullah et al. 2016a, b, c; Abdullah & Ahmad, 2016). The refined crude palm oil (CPO) methyl ester can be used directly or blended with petroleum diesel as biodiesel but the use of CPO raises the issue of food versus fuel that need to be addressed. EFB contains cellulose, hemicelluloses, lignin, and extractives that can be converted into bio-oil; or into bioethanol. The solid residues such as shells and fibres can be used for steam and electricity generation in the mill, the EFBs re-used as fertilizer and the palm kernel cake composted. POME is considered one of the most polluting agro-industrial effluent due to its high values of COD and BOD concentrations ranging from 50,000 to 90,000 mg L^{-1} (Damayanti et al. 2010). Without effective treatment, considerable environmental problems could occur such as the percolation of POME into the waterways and ecosystems, land and water pollution and destruction of aquatic biota (Singh et al. 2011). POME contains high concentration of organics and inorganics materials, and is normally treated in the ponding system with low investment cost (Abdullah & Ahmad, 2016). Raw EFB has a high potential as a substrate to produce methane. The study at the lab scale on co-substrate addition (EFB and palm kernel) in the anaerobic digestion process of POME in a 500 ml reaction vessel attains maximum specific biogas production rate (0.0574 m^3/kg chemical oxygen demand per day) and methane level (25.6%) at 47.8^oC operating temperature, 50.4 mL POME volume, and 5.7 g EFB addition (Saleh et al. 2012).

4.1. Algal Biomass Co-Utilization

Conventional treatment of municipal wastewater involves primary and secondary biological treatment but it may only remove a fraction of nitrogen and phosphorus (Orpez et al. 2009). Algal treatment offers more efficient means as it could reduce the eutrophication potential with a more environmentally sound approach (Orpez et al. 2009). In biorefinery, microalgal cultivation is the major and critical step and the use of wastewater (municipal, industrial, agricultural) could reduce the cost substantially due to easy access to substrate and abundantly available free nutrients whilst reducing large amount of freshwater consumption (Pittman et al. 2011; Zhang et al. 216; Abdullah & Ahmad, 2016). It allows sharing of waste management and is one of the viable option to obtain biofuels able to compete with petroleum based ones. As sludge disposal of wastewater treatment plants is one of the major challenges of sustainable wastewater engineering, utilizing and producing algal biomass in excess not only produces an oxygenated effluent whilst remediating the pollutants and eliminating nitrogen, phosphorus or metals without organic carbon requirement, but also becomes a source of biomethane and high-value products (Ruiz et al. 2011; Abdullah et al. 2015a; Abdullah & Ahmad 2016). Contamination may be the major challenge though, that resistance must be considered as fundamental when selecting microalgal strains (Zhang et al. 216; Roux et al. 2017). The selected species must be adapted to large scale cultivation, grow quickly and produce high amount of high quality lipids (Rashid et al. 2014).

Table 3. Box-Behnken design and responses of multi-algal species and EFB anaerobic co-cultivation (Ahmad et al. 2016)

Run	Independent Variables				Specific biogas (m^3 kg^{-1} COD d^{-1})	Biomethane (mL CH_4 L^{-1} POME d^{-1})	CO_2 (mL CO_2 L^{-1} POME d^{-1})
	N. oculata (mL mL^{-1} POME)	*Chlorella* sp. (mL mL^{-1} POME)	*T. suecica* (mL mL^{-1} POME)	EFB (g mL^{-1} POME)	Experimental Value	Experimental Value	Experimental Value
Group A							
28	0	0	0	0.12	0.125	3,539.0	3,534.0
6	1	1	0	0.12	0.124	4,651.9	2,265.9
3	1	0	1	0.12	0.101	2,765.2	2,036.6
22	0	1	1	0.12	0.104	3,541.6	1,556.5
Group B							
17	1	0	0	0.06	0.095	3,030.6	1,730.0
10	0	1	0	0.06	0.121	3,165.0	1,883.4
8	0	0	1	0.06	0.099	2,853.6	1,550.6
23	1	1	1	0.06	0.108	3,132.2	1,853.6
9	1	1	1	0.06	0.111	3,072.2	2,272.0
Group C							
27	0	0	0	0	0.104	2,540.0	2,532.0
7	1	1	0	0	0.099	2,579.8	2,301.5
13	1	0	1	0	0.099	2,778.9	1,475.1
19	0	1	1	0	0.099	2,353.0	1,939.8
Group D							
18	2	1	1	0.12	0.097	4,018.9	2,079.5
2	1	2	1	0.12	0.099	2,787.5	2,272.4
26	1	1	2	0.12	0.108	1,064.8	914.38
Group E							
24	2	1	0	0.06	0.124	1,224.8	873.7
14	2	0	1	0.06	0.121	2,123.6	1,643.4
1	0	2	1	0.06	0.104	2,224.8	1,654.3
15	1	2	0	0.06	0.107	2,787.5	1,753.9
5	1	0	2	0.06	0.106	1,229.4	1,153.9
16	0	1	2	0.06	0.089	1,026.7	934.6
Group F							
12	2	2	1	0.06	0.077	952.74	864.8
11	2	1	2	0.06	0.081	943.74	853.8
21	1	2	2	0.06	0.082	952.74	863.8
Group G							
4	2	1	1	0	0.108	3,601.3	1,543.3
20	1	2	1	0	0.096	2,424.0	1,984.9
25	1	1	2	0	0.088	870.5	803.4

Anaerobic co-cultivation of mono-culture *N. oculata* at 2 mL mL^{-1} POME with EFB at 0.12 g mL^{-1} POME, attains the highest specific biogas production rate of 0.113–0.114 m^3 kg^{-1} COD d^{-1} and the methane rate of 4606–5018 mL CH_4 L^{-1} POME d^{-1} (Ahmad et al. 2015). However, it is the co-digestion of mono-culture *Chlorella* sp. with EFB, POME and sludge inocula which achieves the highest biomethane (5276 mL L^{-1} POME d^{-1}) and specific biogas production (0.129 m^3 kg^{-1} COD d^{-1}) rates at 2 mL mL^{-1} POME and EFB of 0.12 g mL^{-1}

POME, after 3 days of cultivation, with high removal efficiencies of COD (95 ~98%), BOD (90 ~ 98%), TOC (80 ~ 86%), and TN (80%) after 7 days treatment (Ahmad et al. 2014). The multi-algal co-cultivation of *N. oculata* and *Chlorella* sp. (each at 1 mL mL^{-1} POME) with EFB (0.12 g mL^{-1} POME) (Table 3) achieves the highest biomethane (4651.9 mL CH_4 L^{-1} POME d^{-1}) and the specific biogas production (0.124 m^3 kg^{-1} COD d^{-1}) rates with CO_2 (2265.9mL CO_2 L^{-1} POME d^{-1}) (Ahmad et al. 2016). Utilizing *N. oculata*, and *Chlorella* sp. achieve the anaerobic removal of COD (95-98%), BOD (90-98%), TOC (80-86%) and TN (80%), far better than without microalgae (Ahmad et al. 2014; 2015).

Table 4. Chemical characteristics of POME at different compositions in sea water before and after inoculation of *N. oculata* and *T. suecica* (Shah et al. 2016)

POME level	pH*	COD	BOD	TOC	TN	Oil and grease
1. Raw						
1%	6.5-7	627	183	33.4	5.43	30.3
5%	6.2	2974	843	153.1	28.6	144.5
10%	5.5	5839	1642	285.5	56.7	285.1
15%	4.7	8947	2448	456.8	80.3	364.5
2. N. oculate						
1%	7	63	21	12	0.5	5.3
5%	7.5	145	32	53	4	9.8
10%	7.8	375	47	87	7	15.6
15%	8.3	558	94	115	13	18.7
3. T. suecica						
1%	7	84	34	19	2	9.3
5%	6.5	196	47	67	7	16.7
10%	7.3	463	62	104	12	22.8
15%	7.2	638	114	132	18	31.7

* All values are in mg L^{-1} except for pH

Different concentrations of filtered and centrifuged POME in sea water (1, 5, 10 and 15%) has been successfully utilized as an alternative medium for algal cell growth and lipid production (Shah et al. 2016). Both *N. oculata* and *T. suecica* show enhanced cell growth and lipid accumulation at 10% POME with maximum specific growth rate of 0.21 d^{-1} and 0.20 d^{-1}, and lipid content of 39.1 and 27.0%, respectively, after 16 days of flask cultivation. In comparison, both *I. galbana* and *P. lutheri* also show enhanced cell growth and lipid accumulation but at 15% POME level and with lower maximum specific growth rate (0.16 d^{-1} and 0.14 d^{-1}) and lipid content (26.3 and 34.5%), respectively, after 16 days of cultivation (Shah et al. 2014). The TSFA (59.24%, 68.74%), MUFA (15.14%, 12.26%); and PUFA (9.07%, 8.88%) are obtained for *N. oculata* and *T. suecica*, respectively at 10% POME. Both *N. oculata* and *T. suecica* are potential candidates for biodiesel production where *N. oculata* contains high palmitic acid (C16:0) at 28.22% and palmitolic (C16:1) at 9.37%, while *T. suecica* contains high palmitic acid (C16:0) at 36.48% and pentadecanoic acid (C15:0) at 9.21%. Table 4 shows that *N. oculata* and *T. suecica* addition also further reduce the BOD, COD, TOC, TN and oil and grease in POME (Shah et al. 2016).

4.2. Cellulose Extraction and the Biocomposite Materials

EFB and the fibers can be moulded into products for the manufacturing of furniture, building, electronics, packaging and automobile industries (Malaysian Palm Oil Council, 2006). Eco-friendly extraction of Purely-Extracted Cellulose (PEC) from EFB has been achieved by ultrasonic (SONO-CHEM) extraction with H_2O_2 at 40 kHz at room temperature, and autoclave (AUTO-CHEM) extraction with a mixture of H_2O_2 and formic acid and further bleaching with H_2O_2 at 80°C. The AUTO-CHEM yields 64% PEC with the α-cellulose content of 93.7% and crystallinity of 70%, while the SONO-CHEM yields 49% PEC with the α-cellulose content of 91.3%, and crystallinity of 68.7% (Nazir et al. 2013; Abdullah et al; 2016c). The 25% PEC loading with polypropylene (PP) composites using injection-moulding technique achieves the tensile strength of 26.7-27.3 Mpa, without any addition of coupling agents (Abdullah et al; 2016c). The PECs are also successfully surface-engineered with carboxylic acids where EDTA treatment achieves 232.9-236.7 mg/g Pb(II) sorption (Nazir, 2013) and 39.3-54.2 mg g^{-1} for the Mn(II), Ni(II) and Cu(II) sorptions (Daneshfozoun et al. 2014). The Pb-loaded modified PEC achieves higher sulphur removal from diesel than without metal-modified sorbents (Nazir, 2013). Agro-based magnetic biosorbents (AMBs) from *Ceiba pentandra* (RKF), EFB and PECs have also been developed, using a novel, simple and cost-effective preparation technique for the removal of heavy metal ions from aqueous solutions. The AMBs achieve 97.7-99.4% Pb(II) removal efficiency with 10.3% higher efficiency than the raw sorbents and the regeneration is successfully performed for 5 adsorption/desorption cycles (Daneshfozoun et al. 2017). A novel composite of PEC-hydroxyapatite (HAp) with graphite has been fabricated into chemically-modified carbon electrode (PEC-HAp-CME) for qualitative and quantitative analysis of Pb(II) ions in aqueous medium, blood serum and POME, employing cyclic voltammetry and square wave anodic stripping voltammetry. The newly developed PEC-HAp-CME possesses favourable electrochemical characteristics for the measurement of Pb(II) at ppb levels (Ajab 2015).

5. BIOPRODUCTS FROM ALGAL FEEDSTOCK

Bioactive compounds are physiologically active substances with functional properties and effects on human health (Herrero et al. 2013). These include carotenoids, phycocyanins, polyphenols, fatty acids, and polyunsaturated compounds which are of special interest for new products development in medical, pharmaceutical, cosmetic, and food industries (Plaza et al. 2010; de Morais et al. 2015; Abdullah et al. 2017). Biocompounds derived from microalgae like proteins, fatty acids, vitamins, and pigments are often sourced directly from primary metabolism, or from secondary metabolism for compounds with antifungal, antiviral, antialgal, antienzymatic, or antibiotic activities. Cyanovirin, oleic acid, linolenic acid, palmitoleic acid, vitamin E, B12, β-carotene, phycocyanin, lutein, and zeaxanthin from microalgae exhibit antimicrobial, antioxidant, and anti-inflammatory activities, with the potential for the reduction and prevention of diseases (Markou 2013; Ibañez et al. 2013). Most are accumulated intracellularly, but in some cases, these are exometabolites or excreted into the medium. Table 5 summarizes the existing and potential high-value products from microalgae (Borowitzka, 2013).

Table 5. Summary of existing and potential high-value products from microalgae (Borowitzka, 2013)

Product	Potential or existing algal source	Some alternate source(s)	Applications
Carotenoids β-carotene	*Dunaliella salina*	*Blakeslea trispora*, synthetic	Pigmenter (food), pro-vitamin A, antioxidant
Astaxanthin	*Haematococcus pluvialis, Chlorella zofingiensis*	*Xanthophyllomy- ces dendrorhous, synthetic*	Pigmenter (aquaculture), antioxidant
Canthaxanthin	*Chlorella* spp., other green algae	*Dietzia natronolimnaea*, synthetic	Pigmenter (aquaculture, poultry and food)
Zeaxanthin	*Chlorella ellipsoidea*; *Dunaliella salina* (mutant)	Paprika (*Capsicu annuum*); *Tagetes erecta*, synthetic	Anti-oxidant, food pigmenter
Lutein	*Scenedesmus* spp., *Muriellopsis* sp., other green algae	*Tagetes* sp., *Blakeslea trispora*	Antioxidant
Phytoene, phytofluene	*Dunaliella*	Tomato (*Solanum lycopersicum*)	Antioxidant, cosmetics
Echinenone	*Botryococcus braunii*, cyanobacteria		Antioxidant
Fucoxanthin	*Phaeodactylum tricornutum*	Brown algae	Antioxidant
Phycobilins (phycocyanin, phycoerythrin, allophycocyanin) Fatty acids	Cyanobacteria, Rhodophyta, Cryptophyta, Glaucophyta		Natural pigment (e.g., cosmetics and food products), fluorescent conjugates, antioxidant, etc.
Arachidonic acid	*Parietochloris incisa*	*Mortirella* spp.	Nutritional supplement
Eicosapentaenoic acid	*Nannochloropsis* spp., *Phaeodactylum tricornutum, Monodus subterraneus*, etc.	Fish oil	Nutritional supplement

The PUFAs, especially *ω*-3 and *ω*-6 such as eicosapentaenoic (EPA), DHA, and arachidonic (AA) acids, have been applied in the treatment of chronic inflammation including rheumatism and skin diseases (Barrow and Shahidi 2007). *Nannochloropsis*, *Isochrysis*, *Phaeodactylum*, *Pavlova*, and *Thalassiosira* are among species reportedly producing *ω*-3 PUFA as an alternative to fish oil in food. *Nannochloropsis* contains appreciable amount of *ω*-3 fatty acids, mostly in the form of all (5Z,8Z,11Z,14Z,17Z)-eicosapenta-5,8,11,14,17-enoic acid (Adarme-Vega et al., 2012). The biosynthesis of fatty acids starts from acetyl-CoA, where the stearoylCoA (C18) is released from the chloroplast and is desaturated to produce the MUFA oleoyl-CoA (C18:1n9). This fatty acid is then attached to a glycerol backbone to form mono-, di-, or triglycerides. The lipid oleoyl group is desaturated again to form the linoleoyl (C18:2n6) group. This is the intermediate for the synthesis of *ω*-3 and *ω*-6 fatty acids. Under N-starved conditions, 21.65% and 19.26% of the EPA are found in the polar and phospholipid fractions, respectively, of *Nannochloropsis* sp (Bondioli et al. 2012; Chua & Schenk, 2017). Culture media with decreased iron and zinc contents has the EPA level reduced in *N. gaditana* but reducing molybdenum, manganese, and cobalt does not affect the EPA productivity (Alboresi et al. 2016). Biotin (Vitamin B7) is another important culture component for EPA production, while thiamine (Vitamin B1) and cyanocobalamin (Vitamin B12) can be reduced without affecting the growth and EPA content (Camacho-Rodríguez et al. 2015).

Natural pigments derived from microalgae have neuroprotective properties, with effectiveness in the treatment of neurodegenerative diseases. Vitamin E from marine algae

has preventive effects on neurodegenerative diseases such as multiple sclerosis as well as on atherosclerosis and heart disease (Pangestuti and Kim 2011). Vitamin E (α-tocopherol) production by *N. oculata* has been reportedly achieved under nitrogen limitation (Durmaz 2007). Extracts of *Chlorella* sp. containing β-carotene and lutein significantly prevent the cognitive disability accompanying Alzheimer's disease in rats. Among the pigments with anti-inflammatory activity, fucoxanthin carotenoid found in diatoms, is capable of stimulating apoptosis in human cancer cells (Zhao et al. 2014). Lutein from *Chlorella* also reduces the incidence of cancer, while carotenoids from *C. ellipsoidea* and *C. vulgaris* could restrain the growth of colon cancer (Schepetkin and Quinn 2006).

6. COST-ESTIMATION ANALYSES

Cost estimates for algal production vary based on the systems modeled as each analysis evaluates new and different combinations of technologies, with the industry continuously evolving and moving forward. Over the past few years, many technologies have been developed and tested by NAABB scientists (Perrine et al. 2012; Elliott et al. 2013). The market, at about $2.5 billion, is expected to grow in the future for *Spirulina*, *Chlorella*, *Dunaliella*, *Nostoc* and *Aphanizomenon*, and many other strains of algae have been screened for their potential use in the food industry (Batista et al. 2013). *Spirulina* and *Chlorella* represent the majority of the microalgal biomass market with an annual production of 3,000 and 4,000 tons, respectively (Masojídek and Prášil 2010). *Nostoc* biomass has been used in the medical field and as a dietary supplement because of its protein, vitamin, and fatty acid content. The medicinal value is evidenced in the treatment of fistula and for some forms of cancer (Temina et al. 2007). *Nostoc* produces several compounds with antimicrobial, antiviral, and anticancer activity and these encourage its cultivation on a large scale, with great economic potential due to its nutritional and pharmaceutical importance (Deng et al. 2008; Abdullah et al. 2017).

The high value protein sources of *Nannochloropsis* would likely drive the economics of a *Nannochloropsis* biorefinery, as up to $100 per litre of EPA-rich oil may be achievable. Considering approximately 30% lipids in *Nannochloropsis*, a minimum of 3.3 kg biomass would be needed to recover 1 L of oil, for an approximately 2.4 kg of defatted, protein-rich biomass by-product. When compared to other protein-rich feeds such as soybean, the maximum price of $300/ton can be considered realistic. At a production cost of $2/kg algal biomass (dry weight), it would cost $6 to produce 1 L of oil, if the remaining biomass can be sold as animal feed (Chua & Schenk, 2017). The "defatted" biomass may still contain significant amount of EPA for use in aquaculture or as animal feed which typically require supplement such as ω-3 fatty acids. The use of whole, unprocessed *Nannochloropsis* as food and/or feed or health supplement addition to food such as bread, pasta and protein bars, is a possible scenario to create highly nutritious functional food to address the general deficiency of ω-3 fatty acids and minerals such as zinc. In this way, the best economically-sustainable scenario can be achieved if both, the extracted EPA-rich oil and the high-value protein extracts can be utilized (Chua & Schenk, 2017).

In large scale algal production facilities, the total production costs may range from $0.15 to $4.00 per kilogram ($0.30 to $7.99 in 2014) of unprocessed biomass. For a privately

funded $20 million program, the algal oil production cost has been estimated at $84/bbl in 2004 ($105.08 in 2014) (Huntley et al. 2007). In terms of technical feasibility for biodiesel production, the estimated cost is $2.95/gallon for open pond, and $3.80/gallon for PBR in 2006 ($3.46 and $4.46 in 2014, respectively) (Chisti, 2007). The co-production of value-added bioproduct from algal glycerol may help to reduce the biodiesel production cost to as low as $2.79/GGE (Gong and You 2014). The cost of $109 and $77 per gal of bio-oil has been estimated for the open pond and PBR systems, respectively ($112.18 and $79.25 in 2014) (Richardson et al. 2014). The biomass production costs in 2016 have been estimated (including dewatering) at 4.95, 4.16, and 5.96 €/kg for open pond, horizontal PBRs, and flat panel PBRs, respectively ($7.12, $5.98, $8.57 in 2014, respectively) (Norsker et al. 2011). The economic feasibility of open pond and PBR production systems have also been compared based on the differences in biomass production, lipid content, culture crashes, harvesting and extraction costs. Five different production scenarios have been proposed for microalgal biofuel costs:- $28/bbl, $0.17/kWh, $332/bbl, $0.72/kWh, and $240/bbl ($30, $0.18, $360, $0.76, and $247 in 2014, respectively) (Lundquist et al. 2010). The costs for open pond is $12.74 per gallon of lipid for open pond and $32.57 for PBRs, respectively ($14.52 and $37.12 in 2014 respectively) (Richardson et al. 2012).

The processing cost of the supercritical technology at $0.26/gal is nearly half the actual conventional transesterification method ($0.51/gal). The immediate priority is to demonstrate that the supercritical process technologies can tolerate the complex compositions found in the raw, unprocessed algae with no negative impact on other small metabolites and can be applied in the processing of algae, either whole or just the oil extract, with similar yields and at efficiencies level that can be scaled up to the commercial production (Anitescu et al. 2008). The most profitable scenario is the product selling price of $17.49 per gallon of algal oil ($18 in 2014) (Davis et al. 2012). It is therefore, theoretically possible that if the other upstream algal-processing costs could be mitigated through the addition of a transesterification conversion process, the overall algal biorefinery could become cost-competitive with fossil fuels.

CONCLUSION

Nannochloropsis sp. has been cultured for biofuel applications due to its high lipid content but with high capital and operating costs. For a more economically and industrially-viable *Nannochloropsis* farming, considerations for high-value products become pertinent as a part of a multi-product biorefinery. *Nannochloropsis* cultivation based on palm oil milling is one of the practical option for the commercial-scale recovery of biomass as a source of renewable biofuels and bioproducts including a high-value food supplement and for animal or aquaculture feed, with co-utilization and conversion of mill residues and the integration of biofixation and waste water treatment to reduce agricultural waste pollutions. The development of a biorefinery setup should consider the biomass productivity of the *Nannochloropsis* strain as the composition of lipids, carbohydrates, proteins, cellulosic material and other high-value compounds will determine the feasibility for extraction and refinement. The market for different products from microalgal cultivation and palm oil milling, and the suitable technological development with regards to the hybrid reactor system

for cultivation and cell harvesting, the hydrothermal liquefaction or supercritical processing for extraction and transesterification reaction, product purification and biomaterials conversion, must all be evaluated. These will determine the economics, sustainability and large-scale applicability of a *Nannochloropsis* biorefinery.

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Chapter 60

TRENDS IN COPEPOD STUDIES

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ABSTRACT

Being present in almost any aquatic system and owing to their ecological role, copepods have been the focus of a large number of studies from taxonomy to global patterns. However, despite the wealth of information available today, our knowledge of their distribution, biology and ecology is still incomplete. Apparently, the more we learn about them, the more we are spurred on to advancing our research on these tiny crustaceans to fully grasp their significance and role. Through the contributions collected, this volume aims at providing new insight in copepod studies and a new foundation for future studies.

Keywords: copepods, distribution, biology, ecology

1. INTRODUCTION

The "insects of the sea", the most abundant metazoans on Earth: over the years, copepods have been labelled in several different ways owing to their diversity of life forms and exceptional colonising ability (Huys and Boxshall, 1991; Hardy, 1970; Wiebe *et al.*, 1992). As reviewed by Schminke (2007), copepods are as successful as insects in terms of both absolute success and – equating swimming to flying - relative success thanks to several features including (but not limited to) phylogenetic age, speciosity and size.

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Since Milne Edwards (1840), copepods have been established as a separate class. Huys and Boxshall (1991) identified ten orders, which were subsequently reduced to nine by Boxshall and Halsey (2004) upon the discovery of the Fratiidae family (Ho *et al.*, 1998). Among them, Calanoida are the most effective colonizers of the pelagic environment (Bradford-Grieve, 2002), comprising more than 2,600 species in the marine (Razouls *et al.*, 2005-2017) and approximately 550 species in freshwater (Boxshall and Defaye, 2008) environments.

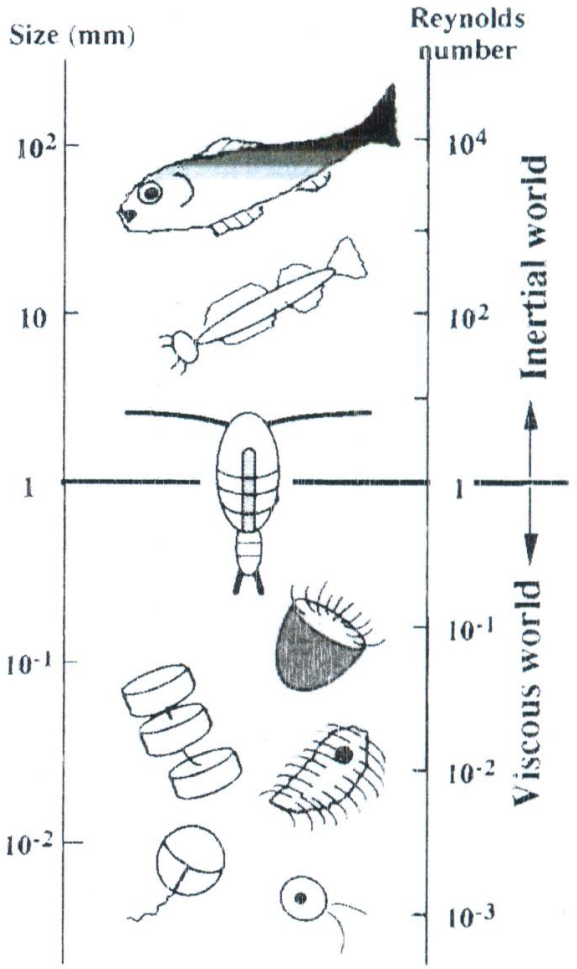

Figure 1. Ecological role of copepods, linking lower and higher trophic levels, respectively characterised by low (<1) and high (>1) Reynolds numbers (Reproduced from Naganuma, 1996, with permission).

Free-living copepods have populated any available habitat, from the pelagic to the subterranean ones (Huys and Boxshall, 1991), through several independent colonisations (Boxshall and Jaume, 2000; Bradford-Grieve, 2002). Copepods are present in any aquatic environment, from deep-ocean trenches to mountain lakes over a vertical range of approximately 20 km (Huys and Boxshall, 1991), and occupy a wide temperature gamut, from polar waters to hydrothermal vents (Walter and Boxshall, 2017).

Copepods represent up to 90-97% of the total biomass of marine zooplankton (Bradford-Grieve *et al.*, 1999), also being abundant in freshwaters (Boxshall and Defaye, 2008). Copepods are primary actors in the functioning and shaping of aquatic ecosystems. They are the linchpin of food webs, preying upon phytoplankton (Marshall, 1973; Turner, 2004) while at the same time representing staple food for higher trophic level organisms, such as chaetognaths (Feigenbaum and Maris, 1984) and fish larvae (Poulet and Williams, 1991). Owing to this, copepods link the viscous and inertial realms, characterised by low and high Reynolds numbers respectively (Naganuma, 1996) (Figure 1). In addition, copepods support the vertical fluxes of carbon through the release of fecal pellets (Fowler and Knauer, 1986), as well as the availability of ammonia to sustain recycled production (Verity, 1985).

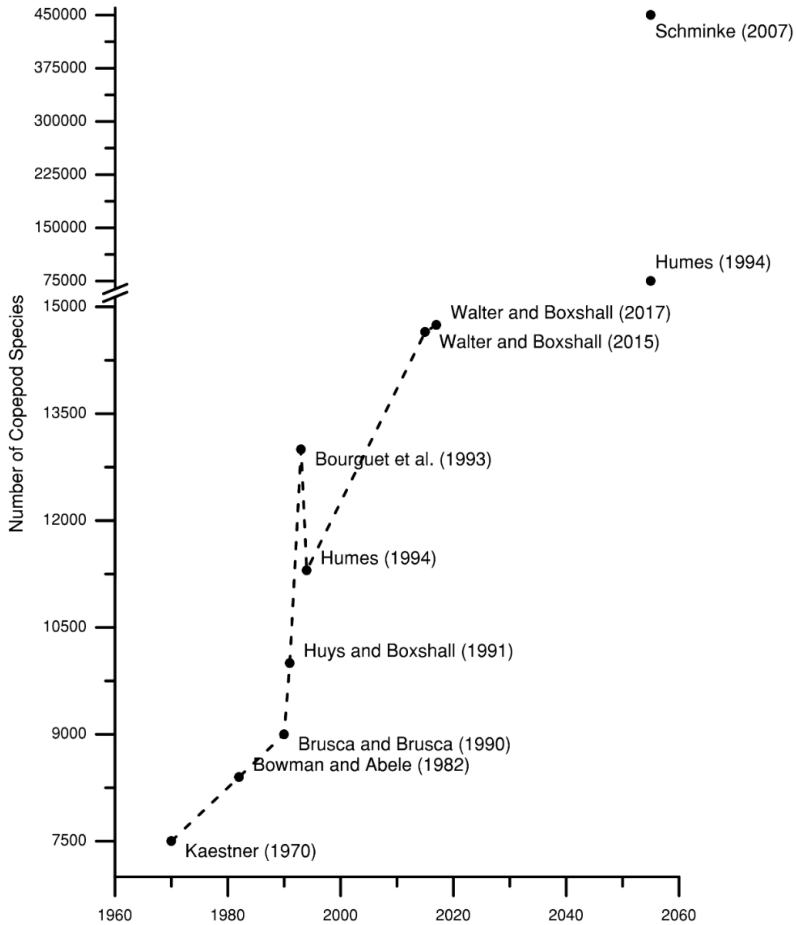

Figure 2. Evolution of the number of copepod species. The future projections by Humes (1994) and Schminke (2007) refer to estimates in the total number of species. It is to note the different line ticking before and after the break along the ordinate axis.

The importance of copepods, however, is not just restricted to this. For example, copepods can be used as beacons of climate change (Beaugrand *et al.*, 2002; Richardson, 2008) and as indicators of the effects of ocean acidification on marine biota (Vehmaa *et al.*, 2013). Nearly half of described species live in association with other organisms (Humes,

1994), host pathogens or have parasitic habits (Bron *et al.*, 2011). These crustaceans can also be extremely useful as model animals for ecotoxicological studies and environmental genomics (Raisuddin *et al.*, 2007; Kulkarni *et al.*, 2013), and a great number of species are well documented as invasive (Zenetos *et al.*, 2012; Sabia *et al.*, 2015). Copepods can also be used as control agents of disease vectors (Kalimuthu *et al.*, 2017). Moreover, despite their small size copepods display peculiar swimming behaviour which mediate their interactions with other organisms and with the surrounding environment (Sabia *et al.*, 2014).

Despite all this, our knowledge on the distribution, biology and ecology of copepods is still incomplete. For example, despite the numerous morphological and phylogenomic records available to date, the total number of copepod species is still indefinite. Over the last fifty years, this figure has risen from approximately 7,500 (Kaestner, 1970) to 14,747 (Walter and Boxshall, 2017) (Figure 2), also thanks to the adoption of molecular techniques particularly useful in the identification of cryptic species (Bucklin *et al.*, 2010; Blanco-Bercial *et al.*, 2014). However, considering that Humes (1994) guessed a grand total of 75,374, subsequently raised by Schminke (2007) to the staggering figure of 450,000 (based on the hypothetical Harpacticoida species counts by Seifried, 2004), much more needs yet to be done.

2. TRENDS IN COPEPOD STUDIES – SUMMARY OF CONTRIBUTIONS

When I was proposed to act as editor of a volume on copepods, I asked myself "Do we really need another book on them?"- a question that many readers may equally pose. After initial indecision, I realised that the answer was simply "Yes". As indicated in the previous section, current copepod studies are exploring multiple lines of research at a fast pace. The intended purpose of the present volume is to provide an up-to-date snapshot of some hot topics in the study of the distribution, biology and ecology of these ubiquitous crustaceans. The following chapters focus on a wide range of processes and scales, from global distribution to molecular investigations, witnessing the interest of the scientific community at different levels of investigation.

Wootton *et al.* (this volume) discuss the functioning, policy issues, strengths and limitations of the survey carried out through the Continuous Plankton Recorded (CPR) since its inception in the 1930s. The authors review the copepod taxa collected by the CPR all over the world, including their biological traits and geographical areas of occurrence of each species. The authors then provide examples of applications of CPR data, and discuss about future perspectives and applications of this unique plankton sampler.

Fernández de Puelles *et al.* (this volume) provide a synthesis of the global distribution of copepods from the tropical and subtropical sectors of the Atlantic, Indian and Pacific Oceans (35°N-40°S) over 15 biogeographical provinces. Samples were collected during the Malaspina Circumnavigation Expedition, from the epipelagic to the bathypelagic. The results highlight horizontal and vertical patterns, and point out great similarities in the assemblage composition among the three oceans, depth playing a pivotal role in their distribution.

The chapter by Zagami *et al.* (this volume) is centred on the invasive cyclopoid *Oithona davisae*. The authors first review the biogeographic distribution, habitat characteristics, ecological traits and dispersal mechanisms of this species. Then, they report on the

introduction and establishment of *O. davisae* in two coastal lakes in the Central Mediterranean Sea, where this cyclopoid has become a dominant species in the zooplankton community. Through a one-year study, the sex ratio and the seasonal variation in abundance for adults, copepodites and nauplii are studied and compared with the literature.

Another invasive copepod, the calanoid *Acartia tonsa*, is the focus of the contributions by Villate *et al.* (this volume), Delpy and Pagano (this volume) and Marques *et al.* (this volume). Villate *et al.* (this volume) report on the impact of *A. tonsa* on the Acartidaee assemblage from the estuaries of Bilbao and Urdaibai along the Basque coast (Spain) over a multiannual period (1998-2005). Their results show that both areas were colonised almost contemporaneously since 2003, with a preference for the innermost salinity habitat of both estuaries, but with distribution characteristics dependent upon the specific features of each estuary. The arrival of *A. tonsa* determined a niche shrinking in the native *Acartia clausi* in both sites, and a seaward shift in *Acartia bifilosa* in the estuary of Urdaibai, while the impact on less abundant Acartiidae species was less easily discernible.

Delpy and Pagano (this volume) compare the relationship between *A. tonsa* and *A. clausi* in the Berre Lagoon (southeast France) before and after a rehabilitation period aimed at reducing salinity fluctuations. Following its introduction in the 1980s, *A. tonsa* dominated the zooplankton community restricting *A. clausi* to the neighbouring coastal area. Upon stabilisation of the salinity fluctuations following the rehabilitation of the lagoon, however, *A. tonsa* decreased its abundance, and the two species started to coexist in the Berre Lagoon with a clear seasonal succession. These signs, in the authors' opinion, might be considered an encouraging starting step in the recovery of the lagoon.

Marques *et al.* (this volume) investigate the distribution of *A. tonsa* and *A. clausi* in the Mondego Estuary (Portugal) over a decade (2003-2013). *A. tonsa* was first observed in 1994, efficiently adapting to the newly invaded site. A spatial segregation between the two species was demonstrated, with *A. tonsa* restricted to the inner part of the estuary and *A. clausi* confined seaward. These results confirm *A. tonsa* as an opportunist species, taking advantage of periods of temperature increase and reduction in freshwater flow recorded in the area.

In their contribution, Svetlichny *et al.* (this volume) provide a detailed review of the behavioural and physiological adaptations in key copepod species from the Black Sea. In particular, the authors focus on specific aspects such as salinity tolerance, effect of temperature on physiology and behaviour, and resistance to oxygen limitations. The examples discussed demonstrate the dependence of copepod distribution and vital rates on environmental parameters, which should be always taken into consideration to fully appreciate the ecological role of these tiny crustaceans.

Lenz and Hartline (this volume) present a comprehensive description of the biology of myelin in calanoid copepods. Using examples from different species, they open with a description of the structure and function of myelin, linking its occurrence to the evolution of copepod families. Subsequently, the authors report on the role of myelin in terms of reaction times and localisation of stimuli, and finish by focusing on the niche separation between myelinate and amyelinate species.

The chapter by Gemmell and Buskey (this volume) concentrates upon the escape strategies used by copepods as a response to the predation threat posed by different foes. The processes of predator detection and the mechanism of escape generation are discussed using examples from the available literature, together with a differentiation between the strategies

used by visual and non-visual predators. The role of environmental features (water motion, temperature and viscosity) are also taken into account.

Langhoff *et al.* (this volume) push the boundary of current knowledge on copepod mating, focusing on the detection by male *Temora longicornis* of the ratio of chemical compounds in the pheromone trails released by the female. Through the design of an olfactory apparatus and the implementation of a Simulated Annealing algorithm for the selection of synaptic weights, the authors demonstrate the capability of ratio detection in copepods, opening to further research on this topic.

The closing chapter by Amato and Carotenuto (this volume) reviews the latest "omics" advances in copepod studies. The authors describe the methodologies presently in use, and provide a review of the most recent advances in molecular studies in planktonic copepods, focusing on gene expression approach, metabolomics and proteomics. The chapter closes with some future perspectives on this rapidly evolving field of research.

The contributions collected in this volume point out the latest developments and case studies on a number of research issues, and will promote discussion and stimulate advances in each field of investigation. As editor, I am confident that readers will appreciate the contents of each chapter and will find in them inspiring suggestions for their research, or even just for their curiosity.

ACKNOWLEDGMENTS

I wish to thank all the authors for having accepted the invitation to contribute to this volume. It was a pleasure and an honour to interact with them all, sharing comments and building together the book as you can read it. I also wish to thank all the colleagues and friends with whom, over these years, I discussed about the different aspects of copepod life – listing them all would take many pages! Insightful comments by Maria Grazia Mazzocchi and Geoff A. Boxshall improved an earlier version of this chapter. I also owe a vote of thanks to Carra Feagaiga at Nova Science Publishers, Inc. for supportive feedback during every stage of the publication process. Thanks are also due to Ian Stewart at Inter-Research Science Center for reprint permission of Figure 1.

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Chapter 61

USING THE CONTINUOUS PLANKTON RECORDER TO STUDY THE DISTRIBUTION AND ECOLOGY OF MARINE PELAGIC COPEPODS

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ABSTRACT

The Continuous Plankton Recorder (CPR) Survey, operated by the Sir Alister Hardy Foundation for Ocean Science (SAHFOS), is the longest running, most geographically extensive marine survey in the world. Since its inception in 1931, the Survey has monitored near surface planktonic communities, including pelagic copepods, providing essential baseline information on the state of the marine environment. Initial observations focused on the North Sea. However, today, in cooperation with sister CPR surveys that have started independent monitoring programmes, the scope extends around the globe, operating across basin scales, providing a truly unique and invaluable dataset for the international community.

CPR data have allowed the description of the geographical distribution of almost 700 planktonic taxa across the North Atlantic, North Pacific and Southern Ocean, monitoring their changes over time. Although both phytoplankton and zooplankton are regularly identified in the Survey, a substantial proportion of the routine analysis is dedicated to the group Copepoda (over 300 taxonomic entities) and forms the focus for this chapter. The CPRs multi-decadal time series allows seasonal cycles and natural variation to be disentangled from changes occurring over a much longer period, such as multiannual oscillations and long-term trends of key copepod species. CPR data have provided evidence for northward distributional shifts of indigenous copepod species, range expansions of non-native species and the presence of pathogenic bacteria on copepods' exoskeletons.

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In this chapter we summarise the main findings on pelagic copepods based on CPR data collected all over the world, highlighting the key policy issues that they have contributed to over time. The strengths and limitations of CPR observations will also be discussed.

Keywords: *Calanus*, regime-shift, policy, phenology, biogeography, CPR, long-term monitoring

1. INTRODUCTION

In the 1920's, Sir Alister Hardy invented a plankton sampling device designed to demonstrate the patchiness (fluctuations in type and quantity) of plankton that he had observed whilst trying to study young herring in the North Sea. Engineered to collect a continuous line of observations, the sampler was first deployed on the Discovery Antarctic expedition (1925-1927) and was given the name Continuous Plankton Recorder (CPR). In subsequent years the sampler underwent a re-design, tailored towards being towed behind commercial vessels and, apart from some relatively minor modifications, is the CPR we see in operation around the globe today. Although regular deployment of the CPR in the North Sea began in the 1930's, consistent data using present-day methodology is available for the period after the Second World War (*i.e.*, post 1948) and represents the longest running and most geographically expansive marine survey in the world.

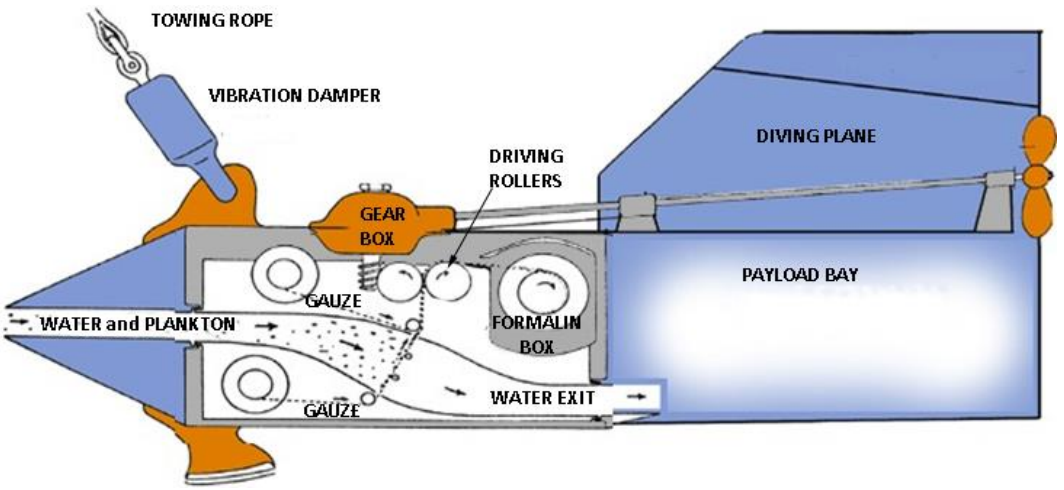

Figure 1. Schematic longitudinal section of a CPR internal mechanism and external body. Water enters through the front aperture and is filtered by a moving band of silk gauze; a second layer of gauze, moving in synchrony, covers the filtering mesh thereby trapping the captured plankton forming a plankton sandwich. As the tow progresses, driving rollers wind the sample into a storage tank containing buffered formaldehyde (fixing the plankton) allowing for the replenishment of fresh gauze and a new plankton sample to be collected.

Over the last 85 years the Survey has evolved into a unique marine monitoring programme, providing the community with its best long-term measure of the state of oceanic plankton (Richardson *et al.*, 2006). Since 1991, the CPR Survey and resulting dataset have

been managed by the Sir Alister Hardy Foundation for Ocean Science (SAHFOS, http://www.sahfos.ac.uk), in Plymouth, UK. Today, in cooperation with sister Surveys that have started independent monitoring programs (at present comprised of 12 regional Surveys) the CPR's scope extends around the globe, operating across basin scales, providing a truly unique and invaluable dataset for the international community. Known as the Global Alliance of Continuous Plankton Recorder Surveys (GACS) (http://www.globalcpr.org), this cooperative union of CPR surveys share their data and expertise with the marine community worldwide.

Truly global studies that use a consistent methodology are rare for planktonic organisms, including copepods, and even more so for open ocean regions. The CPR Survey and GACS community aim to address this but gaps in knowledge still exist, in particular for tropical waters. A recent study by Fernández de Puelles *et al*. (this volume) tackles this issue and impressively describes the tropical to subtropical biogeography of copepods in all three major oceans, from the epipelagic to bathypelagic zones.

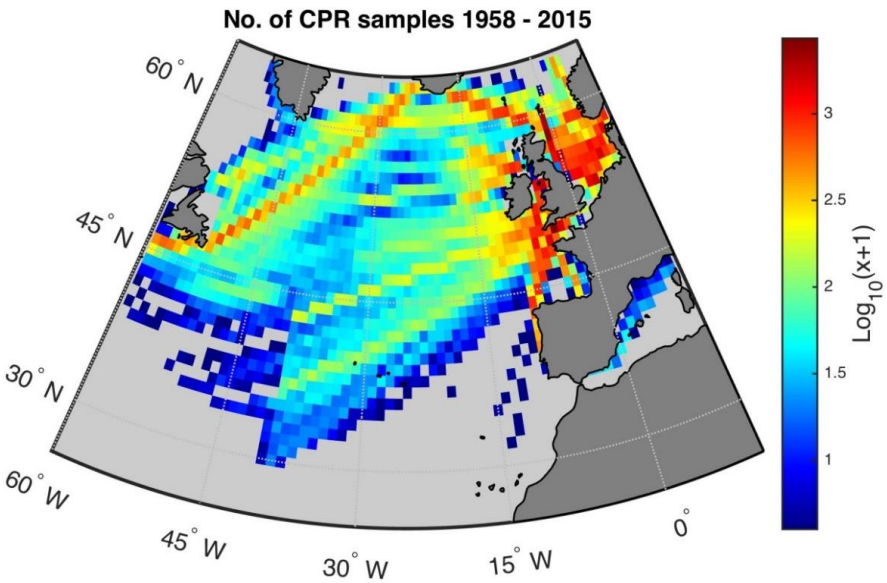

Figure 2. Map of the number of CPR samples collected between 1958 and 2015 in the North Atlantic, gridded onto a 1 by 1 degree grid. Each sample represents 10 nautical miles (18.5 km) of tow.

A Continuous Plankton Recorder (CPR) is a robust semi-automated sampling device that monitors the near-surface plankton communities over hundreds of nautical miles. Towed at approximately 7-9 m depth (Hays and Warner, 1993) behind a Ship of Opportunity (SoOP) the CPR typically collects epipelagic organisms, including a wide range of copepod taxa. Using a simple and reliable mechanism the CPR collects planktonic organisms using a 270 µm silk mesh, which continuously advances as the CPR is towed through the water, typically at a rate of 10 cm per 10 nautical mile sample (Figure 1). Unlike many other sampling devices, the CPR is capable of towing continuously across 500 nautical miles with minimal supervision; the longest single transect towed to date being 3,000 nautical miles, from Canada to Japan. For a detailed description of sampling and analysis methods please refer to Richardson *et al*., (2006). At present the SAHFOS CPR Survey operates across 10,000

nautical miles of ocean each month on 23 routes passing between 20 different countries, with over 6.5 million miles sampled in total over the history of the Survey (see Figure 2). Having undergone minimal modification in design since 1931, the simple yet efficient design of the CPR means that non-scientists can easily deploy the device. Ships' crews require only brief initial training in CPR deployment and operation before becoming fully competent and part of the CPR Survey fleet. As a result, the Survey is able to operate across an extensive geographical range, on a monthly resolution, at significantly reduced cost compared with relying upon specialised research vessels. The collaboration and support of the shipping community is undoubtedly the key to the success and longevity of this unique time-series.

1.1. Why Use a CPR and CPR Methodology?

There are a variety of monitoring programmes and sampling methods used in the study of copepods, each with their own advantages and disadvantages depending upon the research question being asked. Although a detailed debate about the usefulness and limitations of the numerous types of sampling gear in operation is beyond the scope of this chapter, here we briefly highlight some of the reasons why the CPR and the CPR analysis methodology is a useful tool in copepod research (Owens *et al*., 2013).

As previously mentioned, the CPR is capable of sampling over large distances, up to 3,000 nautical miles at present, unlike traditional net sampling which typically sample a single point in space. Depending upon the CPR Survey, each sample represents either 5 or 10 nautical miles of tow, hence the CPR is the ideal tool for large or basin scale studies.

Typical routine analysis of CPR-collected copepods involves identification down to species or genus level for most taxa. Many programmes and analysis methodologies that are able to collect and process such high volume of samples similar to the CPR Survey (such as the digital identification systems ZooSCAN (Grosjean *et al*., 2004) and FlowCam (Le Bourg *et al*., 2015) are able to record 'bulk' indicators such as total zooplankton, total copepods and large *vs* small copepods, and can provide an estimate of bio-volume (and therefore coarse biomass and size spectra). Bulk indicators can provide a valuable quick general picture of processes that take place, but are often limited by their lack of taxonomic resolution. For example, research by Sydeman *et al*., (2010), using CPR data from the North Pacific, showed that seabird species were more closely related to zooplankton taxonomic groups rather than "bulk" measurements of zooplankton abundance or diversity. Many successful studies have been carried out using these automatic identification tools; however, the use of bulk indicators can often mean that subtle differences and relationships between members of the planktonic community are lost. Ultimately the decision on which level of taxonomic resolution is necessary, and hence choice of methodology, depends on what the data generated is to be used for.

The North Atlantic sibling species *Calanus finmarchicus* and *Calanus helgolandicus* are almost morphologically indistinguishable; however, their presence can indicate different water masses as they both have differing thermal niches (Helaouet and Beaugrand, 2007). For this reason they are currently used as copepod 'indicators' (Edwards *et al*., 2008) and are routinely identified to species level in the SAHFOS CPR Survey. Detailed information about plankton community composition at the species level can only be obtained through microscope-based or molecular analysis. Such detailed information, as provided by the CPR

Survey, is essential in providing an accurate insight into changes in biodiversity, food web dynamics and wider issues such as climate change.

A CPR is a compact, robust and reliable piece of sampling equipment. With an average tow success rate of >90%, a CPR works using simple mechanical engineering. Unlike other devices, such as multiple plankton samplers or the Longhurst-Hardy Plankton Sampler, a CPR is free from electrical firing mechanisms which can commonly cause sampling failure. Many other types of sampling gear need to be deployed and monitored by several skilled operators and are only really suitable for use on specifically designed research vessels. In contrast, a CPR can be deployed by non-scientific staff on non-specialised SoOPs (*e.g.*, passenger ferries and container ships), requiring minimal supervision whilst sampling is underway. The body of a CPR also offers a payload area where other sampling equipment can be attached (*e.g.,* temperature, chlorophyll and depth sensors). With oceanographic research vessels costs often prohibitive, it is hard to find a more cost efficient and reliable zooplankton sampler than a CPR.

However, as with any piece of equipment and monitoring programme, there are limitations and the CPR is no exception. Volume of water filtered per sample (3 m^3) is relatively small and sampling depth (typically 7 m) is restricted to the upper water column. Despite this apparent shallow depth, it has been speculated that the actual community sampled may be deeper than originally thought. As the CPR is towed behind a vessel at speed, it is probable that with a large ship draft, possessed by ships used in the Survey, enough turbulence will be caused to mix the water down to do approximately 25m, dependant on ship size, thus allowing the CPR to collect plankton from a wider depth range.

Common to many methods of sampling gear, a consistent CPR tow depth is achieved by deploying a set amount of tow-wire. Since the beginning of the CPR Survey, ship speed has undoubtedly increased over time and has raised questions as to its effect on sampling depth. Data gathered from depth sensors attached to CPRs have shown that tow depth is independent of ship speed (Batten *et al*., 2003).

Due to the nature of sample collection and the subsequent analysis process, some planktonic components are less well represented in the CPR dataset than others. For example, the CPR methodology could underestimate components of the plankton, *e.g*., large plankton like euphausiids, delicate gelatinous plankton, and plankton smaller than the 270 μm mesh (a standard WP2 has a mesh size of 200 μm). In terms of copepods, the CPR Survey likely undersamples the smaller species of *Oithona* and *Oncaea* and some juveniles of larger species. The CPR method of sample collection and analysis is indeed somewhat different to other commonly used techniques, yet comparisons made with other types of sampling gear have demonstrated the CPR's ability to measure changes in seasonal cycles in accordance with other samplers (Richardson *et al*., 2004). In addition, when compared with another sampler (Norpac net) using the same 270 μm mesh, both abundance and species composition have been found to be comparable (Hunt and Hosie, 2003). However, direct comparisons of absolute abundances inevitably can vary within and between sampling devices, including the CPR. Regarding the CPR, this has been well documented and users of CPR data are advised of its limitations and help from experienced SAHFOS researchers is always offered in data interpretation (Owens *et al*., 2013).

Unlike many other sampling initiatives, including some long-term programmes, the strength of the Survey lies in its consistency in sampling technique and analysis methodology for over half a century. There have been very little changes in design and analysis

methodology since 1958, and metadata on changes has been kept. Whilst the Survey might underestimate some taxa, it has operated in a consistent manner over the time period, meaning that long term trends in these taxa are still valid. In addition, as new scientific questions arise, plankton taxa can be speciated or grouped according to need, whilst keeping the core dataset intact.

1.2. Copepod Taxa Recorded by the Survey

Of the 700 taxonomic entities (phytoplankton and zooplankton) recorded by the SAHFOS CPR Survey, 306 belong to the subclass Copepoda, many of which are identified to species level (Table 1). To the end of 2015, this dataset amounts to over a quarter of a million samples analysed for plankton taxa. This equates to over 175 million plankton abundance records; just under a third of these records are related to Copepoda. SAHFOS CPR data can be obtained for *bona fide* research (see https://www.sahfos.ac.uk/data/data-request-form/), and are subject to a data licence agreement. Presence only data for all planktonic taxa analysed by SAHFOS can be accessed via the Ocean Biogeographic Information System (OBIS) portal (http://www.iobis.org/) (Copepoda - http://www.iobis.org/explore/#/taxon/616102).

Table 1 details the copepod taxa recorded in the SAHFOS CPR Survey and their biological traits. For several taxa of specific interest, sex and copepodite stage are also recorded by the Survey, but for simplicity this information has been omitted from the table. For standard North Atlantic analysis it is general practice to include copepodites and adults in the identification level chosen for that taxon, but only where an accurate identification is possible. For example, a record of *Centropages hamatus* would include males, females and most likely all copepodite stages collected, as identification to species level even in juveniles is relatively simple. However, a record of *Calanus helgolandicus* would only include adult males, females and stage V copepodites, because it is generally thought of as impossible to separate *C. helgolandicus* from its sibling species at younger stages using traditional light microscopy. Each taxon in the CPR database will have with it associated metadata such as this, and should be consulted before any interpretations are made.

Biological traits are used to study functional diversity which provides a link between organisms and their ecosystems. With a wide range of trait descriptors used to characterise the role of a species in terms of ecological functioning, here we list the most common: morphological (body size, measured as total length); trophic position (feeding method, defined as herbivore, omnivore, predator or parasite); physical environment (habitat, defined as neritic, oceanic or cosmopolitan) and distribution (sea areas). It must be noted that many of the feeding method traits have been inferred, as the majority of species have not been specifically studied. In this case, traits have been inferred from similar taxa, *i.e.*, species within a genus, or in some cases not explicitly detailed in the reference but alluded to (Johns and Wootton, 2013). Each taxon in the table is also matched with a corresponding Aphia ID, a unique numerical identifier given to each taxon as listed in the World Register of Marine Species (http://www.marinespecies.org/).

Table 1. Copepod taxa identified in the SAHFOS CPR Survey and their biological traits. Ant, Antarctic; IO, Indian Ocean; Med, Mediterranean; NA, North Atlantic; SA South Atlantic; P Pacific

Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Acartia amboinensis	346049	1.3-1.5	herbivore	neritic	IO, P
Acartia danae	346026	0.7-1.3	herbivore	cosmopolitan	IO, Med, NA, NS, P
Acartia longiremis	346037	0.7-1.2	herbivore	neritic	Med, NA, SA,P, North Sea
Acartia negligens	346030	0.8-2.1	herbivore	cosmopolitan	IO, Med, NA,SA, P
Acartia spp.	104108	0.1-2.7	herbivore	cosmopolitan	Arctic, IO, Med, NA, North Sea, SA, P,
Acartia tonsa	345943	0.8-1.5	herbivore	neritic	IO, Med, NA, , P, North Sea
Acartia tumida	346025	1.8-2.1	herbivore	neritic	Arctic, P
Acrocalanus spp.	104192	0.7-1.8	herbivore	cosmopolitan	IO, Med, NA,SA, P
Aetideopsis armatus	356886	2.8-4.5	predator	cosmopolitan	Arctic, IO, Med, NA, SA, P, North Sea
Aetideus acutus	104273	1.2-2.0	omnivore	cosmopolitan	IO, Med, NA,SA, P
Aetideus armatus	104275	1.3-2.3	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Aetideus giesbrechti	104276	1.1-2.2	omnivore	cosmopolitan	IO, Med, NA, SA
Aetideus spp.	104112	1.3-3.0	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea,
Alteutha spp.	115427	0.5-1.3	unknown	neritic	Ant, Arctic, IO, Med, NA, SA, North Sea, P
Amallothrix spp.	104221	1.3-5.4	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P
Anomalocera patersoni	104722	2.5-4.1	predator	neritic	IO, Med, NA, North Sea
Augaptilus spp.	104137	2.0-6.5	predator	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P
Bradyidius armatus	146735	1.5-2.7	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Bradyidius spp.	104115	0.9-4.9	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Calanoides acutus	342434	3.5-5.7	herbivore	cosmopolitan	Ant
Calanoides carinatus	104462	1.6-4.0	herbivore	oceanic	Med, IO, NA, P
Calanopia americana	104723	1.2-2.1	predator	neritic	IO, Med, NA, SA
Calanopia elliptica	104724	1.4-2.1	predator	neritic	IO, Med, NA, SA, P
Calanopia minor	220919	1.1-1.4	predator	neritic	IO, Med, SA, P
Calanopia spp.	104207	1.0-2.6	predator	cosmopolitan	IO, Med, NA, SA, P
Calanus finmarchicus	104464	2.4-5.0	herbivore	cosmopolitan, neritic, oceanic	Arctic, NA, North Sea
Calanus glacialis	104465	3.1-6.0	herbivore	oceanic	Arctic, NA
Calanus helgolandicus	104466	1.9-3.5	herbivore	cosmopolitan	NA, Med, North Sea
Calanus hyperboreus	104467	5.3-10.0	herbivore	oceanic	Arctic, NA
Calanus marshallae	196770	2.9-4.5	herbivore	neritic	Arctic, P
Calanus pacificus	196771	2.2-5.3	herbivore	oceanic	P
Calanus propinquus	342435	4.8-6.0	omnivore	oceanic	Ant, SA
Calanus simillimus	342436	2.5-4.0	herbivore	oceanic	Ant, SA
Calanus spp.	104152	1.9-10 .0	herbivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea

Table 1. (Continued)

Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Calocalanus spp.	104193	0.5-1.5	omnivore	cosmopolitan	IO, Med, NA, SA, P, North Sea
Candacia armata	104474	1.7-3.2	predator	cosmopolitan	Med, NA, North Sea
Candacia bipinnata	104475	1.9-3.2	predator	cosmopolitan	Med, IO, NA, SA, P
Candacia columbiae	349800	3.1-5.0	predator	cosmopolitan	IO, P
Candacia curta	104476	1.5-2.9	predator	cosmopolitan	IO, Med, NA, SA, P
Candacia ethiopica	104478	2.0-3.0	predator	cosmopolitan	IO, Med, NA, SA, P
Candacia giesbrechti	104479	1.7-2.3	predator	neritic	Med
Candacia longimana	104481	2.4-3.9	predator	cosmopolitan	IO, Med, NA, SA, P
Candacia norvegica	104482	2.3-3.6	predator	cosmopolitan	IO, Med, NA, SA, P, North Sea
Candacia pachydactyla	104483	1.5-3.4	predator	cosmopolitan	IO, Med, NA, SA, P
Candacia spp.	104157	1.2-5.1	predator	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Candacia tenuimana	104485	2.4-3.4	predator	cosmopolitan	IO, Med, NA, SA, P
Candacia varicans	104486	1.9-2.7	predator	cosmopolitan	IO, Med, NA, SA, P
Canthocalanus pauper	220834	1.0-2.0	omnivore	cosmopolitan	IO, Med, P
Centropages abdominalis	196774	1.2-2.1	omnivore	neritic	Arctic, P
Centropages brachiatus	104490	1.3-3.0	omnivore	neritic	IO, Med, NA, SA, P, Ant
Centropages bradyi	104491	1.3-2.5	omnivore	cosmopolitan	IO, Med, NA, SA, P
Centropages calaninus	104492	1.7-2.2	omnivore	cosmopolitan	Med, IO, SA, P
Centropages chierchiae	104494	1.5-2.3	omnivore	cosmopolitan	IO, Med, NA, SA
Centropages furcatus	104495	1.4-1.9	omnivore	cosmopolitan	IO, Med, NA, SA, P
Centropages gracilis	220903	1.7-2.2	omnivore	cosmopolitan	IO, Med, NA, SA, P
Centropages hamatus	104496	0.9-1.4	omnivore	neritic	Med, NA, SA, P, North Sea
Centropages spp.	104159	0.2-3.0	omnivore	cosmopolitan	IO, Med, NA, SA, P, Ant, Arctic, North Sea
Centropages typicus	104499	0.8-2.0	omnivore	cosmopolitan	Med, NA, SA, North Sea
Centropages violaceus	104500	1.8-2.2	omnivore	cosmopolitan	IO, Med, NA, SA, P
Clausocalanus spp.	104161	0.5-2.0	omnivore	cosmopolitan	IO, Med, NA, SA, P, North Sea, Ant
Clytemnestrinae	587514	0.4-1.6	unknown	cosmopolitan	IO, Med, NA, SA, P, North Sea
Copilia spp.	128721	1.4-9.0	unknown	cosmopolitan	IO, Med, NA, SA, P
Corycaeus speciosus	128800	0.8-2.3	predator	cosmopolitan	IO, Med, NA, SA, P
Ctenocalanus spp.	104162	0.8-1.7	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P
Ctenocalanus vanus	104510	0.8-2.0	omnivore	cosmopolitan	IO, Med, NA, SA, P
Cyclopinoides longicornis	357973	0.5-1.1	unknown	neritic	P, NA, Med
Diaixis hibernica	104521	0.7-1.2	unknown	neritic	Arctic, Med, NA, SA, North Sea
Diaixis pygmaea	104522	0.7-1.0	unknown	neritic	Med, NA, SA
Drepanopus forcipatus	342452	1.1-1.9	omnivore	neritic	Ant, SA, P
Epilabidocera amphitrites	254330	2.3-4.0	predator	neritic	Arctic, P
Epilabidocera spp.	254329	2.3-4.0	predator	neritic	Arctic, P

Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Eucalanus bungii	254427	4.8-8.0	omnivore	neritic	Arctic, P
Eucalanus hyalinus	345781	3.2-8.3	omnivore	cosmopolitan	Arctic, IO, Med, NA, SA, P, North Sea
Eucalanidae	104085	2.8-8.5	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Euchaeta acuta	104550	3.2-4.8	predator	cosmopolitan	IO, Med, NA, SA, P, North Sea
Euchaeta marina	104552	2.3-3.9	predator	cosmopolitan	IO, Med, NA, SA, P, North Sea
Euchaeta media	104553	3.1-4.8	predator	cosmopolitan	IO, Med, NA, SA, P
Euchaeta paraconcinna	196780	2.3-3.2	predator	cosmopolitan	NA, SA
Euchaeta pubera	104554	2.9-4.4	predator	cosmopolitan	IO, Med, NA, SA, P
Euchaeta spinosa	104555	5.2-7.2	predator	cosmopolitan	IO, Med, NA, SA, P
Euchaetidae	104086	2.1-11.6	predator	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea
Euchirella amoena	104296	2.7-4.0	omnivore	cosmopolitan	IO, Med, NA, SA, P
Euchirella bella	104297	3.1-4.9	omnivore	cosmopolitan	IO, NA, SA, P
Euchirella curticauda	104299	2.5-5.6	omnivore	cosmopolitan	IO, NA, SA, P, North Sea
Euchirella maxima	104300	6.1-8.7	omnivore	neritic	IO, Med, NA, SA, P
Euchirella messinensis	104301	2.8-6.2	omnivore	cosmopolitan	IO, Med, NA, SA, North Sea, P
Euchirella pseudopulchra	196792	3.4-4.4	omnivore	neritic	P
Euchirella pulchra	104302	2.9-4.4	omnivore	cosmopolitan	IO, NA, SA, P, North Sea
Euchirella rostrata	104303	2.0-4.1	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Euchirella spp.	104120	2.0-8.7	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Eurytemora pacifica	232028	0.9-1.3	omnivore	neritic	Arctic, P
Eurytemora spp.	104240	0.9-2.3	omnivore	neritic	Arctic, NA, P, North Sea
Euterpina acutifrons	116162	0.4-0.9	herbivore	neritic	IO, Med, NA, SA, North Sea, P
Farranula gracilis	128813	0.7-1.1	predator	cosmopolitan	Ant, IO, Med, NA, SA, P
Farranula spp.	128636	0.6-1.1	predator	cosmopolitan	Ant, IO, Med, NA, SA, P
Gaetanus minor	104312	1.7-2.4	omnivore	cosmopolitan	IO, Med, NA, SA, P
Gaetanus pungens	342623	2.0-3.8	omnivore	cosmopolitan	Ant, IO, NA, P
Gaetanus simplex	358653	2.7-3.6	omnivore	neritic	NA, P
Gaetanus spp.	104121	1.7-9.7	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Gaetanus tenuispinus	237965	1.8-4.0	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Giardella callianassae	128775	0.8-2.5	parasitic	neritic	North Sea
Giardella thompsoni	128776		parasitic	neritic	North Sea
Haloptilus acutifrons	104422	2.1-4.7	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Haloptilus longicornis	104431	1.2-2.6	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Haloptilus spiniceps	104437	2.6-5.5	omnivore	cosmopolitan	IO, Med, NA, SA, P

Table 1. (Continued)

Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Hemicyclops aberdonensis	413311	1.3-1.6	parasitic	neritic	North Sea
Heterorhabdus abyssalis	104576	2.0-3.7	predator	cosmopolitan	IO, Med, NA, SA, P, North Sea
Heterorhabdus austrinus	343728	2.4-4.0	predator	cosmopolitan	Ant, SA, P (south)
Heterorhabdus clausi	358813	2.0-2.7	predator	cosmopolitan	IO, Med, NA, SA, P
Heterorhabdus norvegicus	104579	2.6-4.6	predator	cosmopolitan	Arctic, NA, SA, North Sea
Heterorhabdus oikoumenikis	346540	2.1-3.5	predator	oceanic	IO, NA, P
Heterorhabdus papilliger	104580	1.6-2.7	predator	cosmopolitan	Ant, IO, Med, NA, SA, P
Heterorhabdus spinifer	104582	1.6-2.0	predator	cosmopolitan	IO, Med, NA, SA, P
Heterorhabdus spp.	104178	1.6-4.6	predator	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P
Heterorhabdus tanneri	196782	3.0-4.9	predator	cosmopolitan	P
Heterostylites longicornis	104586	2.3-4.3	predator	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Isias clavipes	104501	1.3-1.7	omnivore	neritic	Med, NA, SA, North Sea
Labidocera acuta	104726	2.3-3.6	predator	neritic	IO, Med, NA, SA, P
Labidocera acutifrons	104727	3.3-4.7	predator	cosmopolitan	Ant, IO, Med, NA, SA, P
Labidocera aestiva	104728	1.8-3.0	predator	neritic	NA, SA, P
Labidocera spp.	104208	1.1-4.7	predator	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Labidocera wollastoni	104736	2.0-2.6	predator	neritic	IO, Med, NA, SA, North Sea
Lophothrix spp.	104225	2.7-7.4	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P
Lubbockia spp.	128672	1.0-3.0	omnivore	oceanic	Ant, IO, Med, NA, SA, P
Lucicutia spp.	104183	1.0-10.3	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P
Macrosetella gracilis	116382	0.9-1.8	herbivore	oceanic	IO, Med, NA, SA, P
Mecynocera clausi	104616	0.8-1.3	omnivore	cosmopolitan	IO, Med, NA, SA, P
Mesocalanus tenuicornis	104468	1.5-3.4	grazer, herbivore	cosmopolitan	IO, Med, NA, SA, P, North Sea
Metridia gerlachei	344689	2.2-4.3	omnivore	cosmopolitan	Ant, IO, SA, P (south)
Metridia longa	104632	1.6-4.5	omnivore	cosmopolitan	Ant, Arctic, NA, SA, North Sea, P
Metridia lucens	104633	1.5-4.0	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea
Metridia okhotensis	196783	2.8-4.5	omnivore	cosmopolitan	P
Metridia pacifica	196784	1.7-3.5	omnivore	cosmopolitan	Arctic, P
Metridia spp.	104190	1.3-10.5	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea
Microcalanus spp.	104164	0.6-1.1	omnivore	cosmopolitan	Ant, Arctic, IO, NA, SA, P, Med, North Sea
Microsetella spp.	115341	0.3-0.8	omnivore	cosmopolitan	Ant, Arctic, IO, NA, SA, P, Med, North Sea
Miracia efferata	116383	1.2-2.2	herbivore	cosmopolitan	IO, Med, NA, SA, P
Monstrilla longiremis	119805	2.0-4.5	parasitic	neritic	Arctic, IO, NA, P, Med

Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Nannocalanus minor	104469	1.2-2.5	grazer, herbivore	cosmopolitan	Ant, IO, NA, SA, P, Med
Neocalanus cristatus	104470	6.7-10.4	grazer, herbivore	oceanic	Arctic, P
Neocalanus flemingeri	353708	3.8-6.3	grazer, herbivore	oceanic	Arctic, P
Neocalanus gracilis	104471	1.6-4.4	grazer, herbivore	cosmopolitan	Med, NA, SA, IO, P, North Sea
Neocalanus plumchrus	196772	4.0-6.3	grazer, herbivore	oceanic	Arctic, P
Neocalanus robustior	104472	2.8-4.7	grazer, herbivore	oceanic	IO, Med, NA, SA, P
Neocalanus spp.	104155	1.6-10.4	grazer, herbivore	cosmopolitan	Ant, Arctic, Med, NA, SA, IO, P
Neocalanus tonsus	344701	3.1-4.6	grazer, herbivore	oceanic	Ant, IO, SA, P
Oculosetella gracilis	116384	0.7-1.4	herbivore	cosmopolitan	IO, Med, NA, SA, P
Oithona spp.	106485	0.3-2.0	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA,SA, P, North Sea
Oncaea spp.	128690	0.2-1.7	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA,SA, P, North Sea
Paracalanus spp.	104196	0.5-1.5	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Paracandacia bispinosa	220915	1.4-2.2	predator	cosmopolitan	IO, Med, NA, SA, P
Paracandacia simplex	220914	1.6-2.3	predator	cosmopolitan	IO, Med, NA, SA, P
Paraeuchaeta antarctica	344974	5.1-10.4	predator	cosmopolitan	Ant, SA, P (south) IO
Paraeuchaeta elongata	196779	4.1-8.4	predator	neritic	P
Paraeuchaeta glacialis	104560	6.2-11.0	predator	cosmopolitan	Arctic, NA, North Sea
Paraeuchaeta gracilis	104561	5.1-7.0	predator	cosmopolitan	IO, NA, SA, P
Paraeuchaeta hebes	104563	2.6-3.7	predator	cosmopolitan	Med, NA, SA, North Sea
Paraeuchaeta norvegica	104566	5.5-11.0	predator	cosmopolitan	Arctic, Med, NA, North Sea
Paraeuchaeta tonsa	359879	5.1-6.7	predator	cosmopolitan	P
Paraheterorhabdus robustus	368129	2.9-5.3	predator	neritic	IO, NA, SA, P
Parapontella brevicornis	104686	1.3-1.6	unknown	neritic	Med, NA east, North Sea
Parathalestris croni	116598	1.7-2.3	unknown	cosmopolitan	Arctic, NA, North Sea
Phaenna spinifera	104698	1.5-3.0	omnivore	cosmopolitan	IO, NA, SA, P, Med
Pleuromamma abdominalis	104637	2.4-4.5	omnivore	cosmopolitan	IO, Med, NA, SA, P, North Sea
Pleuromamma borealis	104638	1.4-2.5	omnivore	cosmopolitan	IO, Med, NA, SA, P, North Sea
Pleuromamma gracilis	224184	1.5-2.6	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA,SA, P, North Sea
Pleuromamma indica	220900	1.7-2.7	omnivore	oceanic	IO, Med, SA, P
Pleuromamma piseki	104640	1.7-2.4	omnivore	cosmopolitan	IO, Med, NA, SA, P
Pleuromamma robusta	104642	2.1-4.7	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, North Sea, P
Pleuromamma spp.	104191	1.2-6.4	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, North Sea, P
Pleuromamma xiphias	104643	3.3-6.4	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P
Pontella mediterranea	104739	2.4-3.3	predator	neritic	Med, NA (east)
Pontellina plumata	104743	1.0-1.9	predator	cosmopolitan	IO, Med, NA, SA, P
Pontellina securifer	196786	3.2-4.6	predator	cosmopolitan	IO, Med, NA, SA, P
Pontellopsis perspicax	360249	1.8-4.3	predator	cosmopolitan	IO, NA, SA, P
Pontellopsis regalis	104745	1.6-4.5	predator	cosmopolitan	IO, Med, NA, SA, P

Table 1. (Continued)

Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Pseudocalanus spp.	104165	0.5-2.4	herbivore	cosmopolitan	Arctic, Med, NA, SA, North Sea, P
Pseudochirella spp.	104126	3.2-9.7	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P
Pseudodiaptomus marinus	157680	0.8-1.8	herbivore	neritic	P, North Sea, Med, IO
Pseudolubbockia dilatata	128895	1.4-3.4	omnivore	cosmopolitan	NA, P
Rhincalanus cornutus	104542	2.4-4.2	omnivore	cosmopolitan	IO, Med, NA, SA, P
Rhincalanus gigas	220837	6.9-10.0	omnivore	oceanic	Ant, SA, IO, P (south)
Rhincalanus nasutus	104543	2.7-6.1	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, North Sea, P
Sapphirina spp.	128722	1.0-8.0	predator	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Scaphocalanus echinatus	104793	1.3-2.6	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P
Scaphocalanus spp.	104228	0.9-5.8	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P
Scolecithricella spp.	104229	0.7-6.0	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea
Scolecithrix bradyi	104820	1.0-1.6	omnivore	cosmopolitan	IO, Med, NA, SA, P
Scolecithrix danae	104821	1.7-2.5	omnivore	cosmopolitan	IO, Med, NA, SA, P
Scolecithrix spp.	104230	1.0-6.0	omnivore	cosmopolitan	IO, Med, NA, SA, P
Scottocalanus persecans	104832	3.9-5.8	omnivore	cosmopolitan	IO, Med, NA, SA, P
Scottocalanus securifrons	104833	3.8-5.3	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P
Siphonostomatoida	10052	<1.0->22.0	parasitic	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea
Spinocalanus spp.	104236	0.8-5.1	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea
Subeucalanus crassus	104544	2.4-4.6	omnivore	cosmopolitan	IO, Med, NA, SA, P, North Sea
Subeucalanus longiceps	345366	3.9-5.1	omnivore	cosmopolitan	Ant, IO, SA, P (south)
Subeucalanus monachus	104545	1.8-2.8	omnivore	cosmopolitan	IO, Med, NA, SA, P
Subeucalanus mucronatus	104546	2.5-3.5	omnivore	cosmopolitan	IO, Med, NA, P
Subeucalanus pileatus	104547	1.7-2.5	omnivore	cosmopolitan	IO, Med, NA, SA, P
Temora discaudata	220898	1.6-1.8	omnivore	cosmopolitan	IO, Med, P
Temora longicornis	104878	1.0-1.5	omnivore	cosmopolitan	Arctic, Med, NA, SA, North Sea
Temora stylifera	104879	1.4-1.9	omnivore	cosmopolitan	IO, NA, SA, Med, P
Temora turbinata	104880	1.3-1.6	omnivore	neritic	NA, SA, Med, IO, P
Tortanus discaudatus	157684	1.4-3.1	predator	neritic	Arctic, NA west, P
Undeuchaeta intermedia	196795	3.6-4.5	omnivore	oceanic	IO, P
Undeuchaeta major	104342	3.0-6.5	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Undeuchaeta plumosa	104343	2.8-4.7	omnivore	cosmopolitan	IO, Med, NA, SA, P, North Sea
Undeuchaeta spp.	104128	2.8-6.7	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Undinula vulgaris	367334	1.8-3.3	omnivore	cosmopolitan	IO, Med, NA, SA, P
Urocorycaeus spp.	128639	1.2-3.1	predator	cosmopolitan	Ant, IO, Med, NA, SA, P
Xanthocalanus spp.	104204	1.2-8.9	predator	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea

2. WHAT CAN CPR DATA BE USED FOR?

2.1. Mapping Copepod Biogeography

With such a large spatial coverage, data from the CPR Survey is ideally placed to generate biogeographical maps of copepod presence and abundance. Distribution maps are able to be generated for any of the taxa listed in Table 1 and maps of the most common of these taxa in the North Atlantic can be found in Barnard *et al*., (2004).

In addition to sample position, each copepod taxon in the CPR database has metadata associated with it, such as: date taxa first counted, date of collection, abundance, time of collection (day/night). Together, these parameters mean a comprehensive picture of copepod distribution and abundance can be mapped over varying time periods, from short-scale seasonal cycles to long-term decadal or inter-decadal variability, at genus or species level.

Figure 3 shows the average abundances of large (>2 mm) and small (<2 mm) copepods, based on mean adult total length, across the North Atlantic from CPR samples. Although copepods are found throughout the entire region, it can be seen that there is a clear geographical difference in abundance between these two groups, with small copepods dominating in coastal regions. The small cyclopoid copepod *Oithona* is thought to be one of the most abundant and ubiquitous metazoans in the ocean (Gallienne and Robins, 2001), and it is perhaps not surprising that smaller copepods are found widely across the Atlantic. In contrast, the highest abundances of larger copepods seem to be restricted to subarctic waters, notably the Grand Banks and Labrador Sea regions which are known to be amongst the most productive waters in the world.

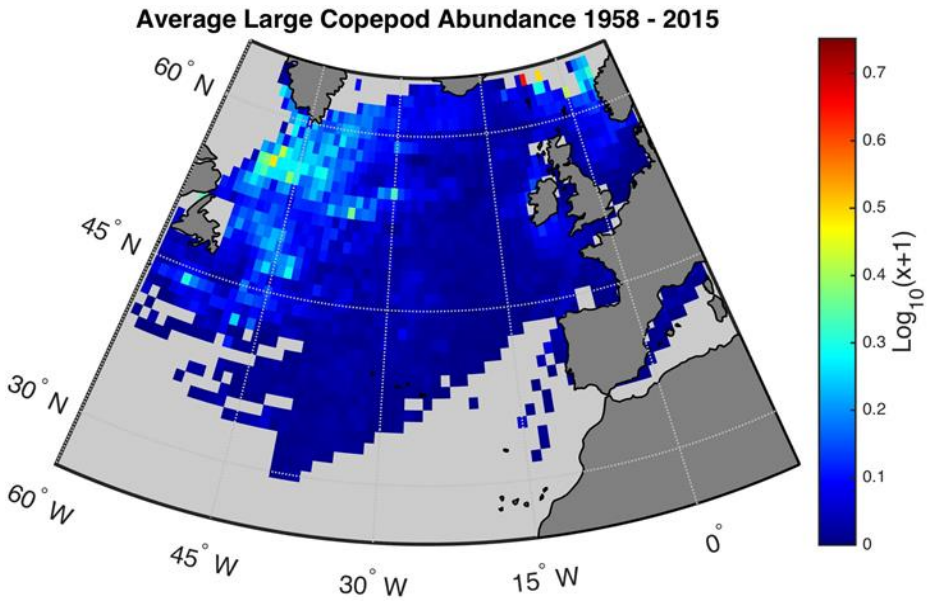

Figure 3. (Continued).

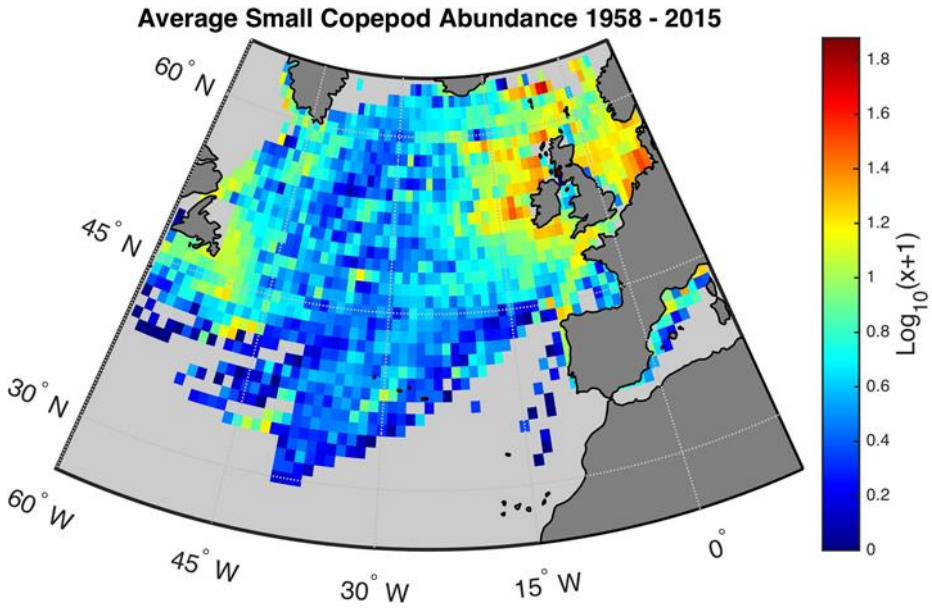

Figure 3. Map of the average monthly abundance of 'large' (>2 mm) (a) and 'small' (<2 mm) (b) copepods, counted in CPR samples between 1958 and 2015 for the North Atlantic, gridded onto a 1 by 1 degree grid.

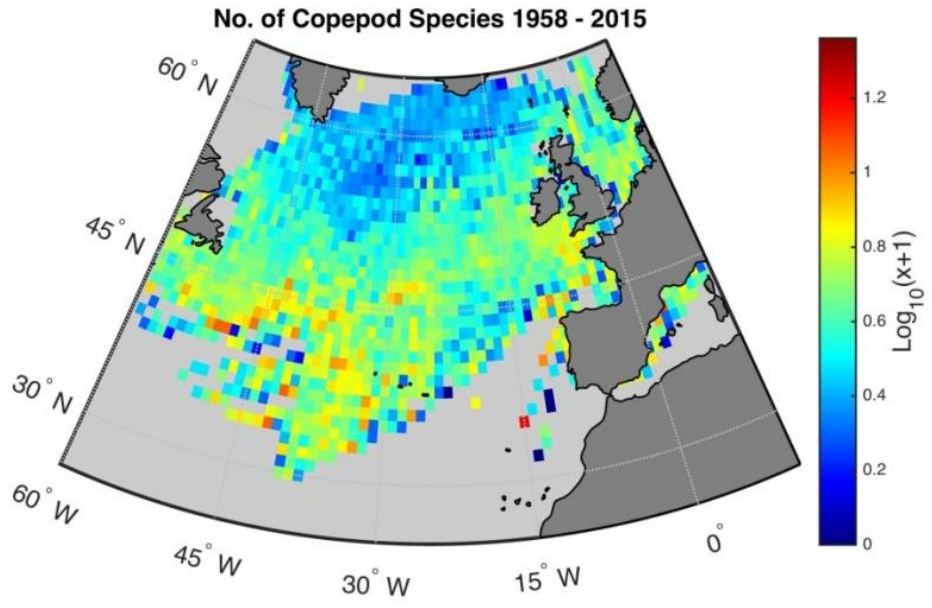

Figure 4. Map of copepod species richness recorded from CPR samples between 1958 and 2015 in the North Atlantic, gridded onto a 1 by 1 degree grid.

It is interesting to note that copepod abundance does not necessarily relate to species richness. Figure 4 shows the number of different copepod species recorded for a given area of ocean. Comparing this with Figure 3 demonstrates that the most speciose regions, such as the Sargasso Sea, coincide with lower copepod abundances. With temperature known to be a

crucial environmental variable affecting marine species richness (Tittensor *et al*., 2010), the map displays the classical theory of species number increasing with latitudinal gradient towards low latitudes, and a decrease in species (but an increase in body-size) towards the poles (Rombouts *et al*., 2009). CPR data have shown that rising sea temperatures in the North Sea have been linked with an increase in overall species diversity (Beaugrand *et al*., 2015). With sea temperatures predicted to rise, it will be interesting to see how this map will change over time.

2.2. Disentangling Short-Term and Rhythmic Variations from Long-Term Trends

Long-term (>30 years) marine monitoring datasets are rare (Edwards *et al*., 2010), but are essential in helping disentangle natural variation, *e.g*., seasonal cycles, from changes in the environment, *e.g*., regime shifts. Extensive time-series provide a baseline of data, a form of "yard-stick," against which new measurements can be compared and changes identified. Since its beginning, the CPR Survey has grown and developed with marine management drivers, linking copepod and other plankton abundances with both short-term events (*e.g*., upwelling, diel vertical migration) and long-term events (*e.g*., climate change, fish stock fluctuations) (Edwards *et al*., 2010). For instance, CPR research has shown that the Atlantic Multidecadal Oscillation accounts for the second most important large scale trend in North Atlantic plankton records, and is responsible for habitat switching (abrupt ecosystem/regime shifts) over multi-decadal scales (Edwards *et al*., 2013); without long-term monitoring programmes like the CPR Survey, our understanding of plankton community drivers would be, at best, limited.

2.3. Monitoring Northward Shifts and Range Expansions

Planktonic copepods are excellent indicators of environmental change (Beaugrand *et al*., 2008). They are quick to respond to changes in their environment, *e.g*., temperature, have rapid generation times and are not generally a harvested resource and consequently they are affected less by direct anthropogenic pressures.

Much of the planktonic research carried out in the North Sea has focused on *Calanus finmarchicus*, a large, lipid rich calanoid copepod (Melle *et al*., 2014). This cold-water species forms the main food source for juvenile cod (Thorisson, 1989) and other fish species, and prefers a temperature range of 0-15°C, with peak abundances from 0-9°C (Melle *et al*., 2014). Its sister species, *Calanus helgolandicus*, similar in size, prefers temperatures between 9-20°C, with peak abundances from 13-17°C (Bonnet, 2005). *C. helgolandicus* abundance has two maxima during the year, the first one from May to June and the second one from September to October (Wilson *et al*., 2016). *C. finmarchicus* on the other hand has its annual maximum around May and is abundant throughout the year (Fromentin and Planque, 1996). Since the 1950s, CPR data have shown a large geographical shift in the distribution of these two species (Beaugrand *et al*., 2001), causing trophic mismatches (Edwards and Richardson, 2004). Notably, there has been a swing in the ratio from a *C. finmarchicus* dominated North Sea to a *C. helgolandicus* dominated North Sea (Figure 5). Coupled with a 70% reduction in

total *Calanus* biomass, this has directly exacerbated the decline in the overexploited Atlantic Cod (*Gadus morhua*) stocks since the mid -1980s (Beaugrand *et al*., 2003), as larvae depend on this prey item for survival (Thorisson, 1989). This step-wise transition, termed a regime shift, is linked to the northward movement of the 9-10°C isotherm in the region and has had effects not only on copepod species biogeography, but on phytoplankton, other types of zooplankton and terrestrial taxa too (Reid *et al*., 2016).

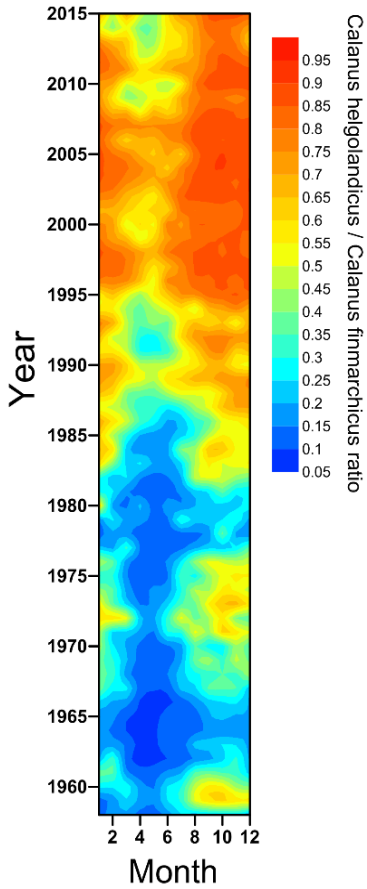

Figure 5. Ratio between the warm-water species (*Calanus helgolandicus*) and the cold-water species (*Calanus finmarchicus*) per month from 1958-2015 in the Central North Sea. Red shades indicate a dominance of the warm-water species and blue the dominance of the cold-water species.

Results from North Atlantic CPR data indicate that the northwards advancement of calanoid copepods is estimated to be 23 Km per year and is closely linked to increasing sea surface temperatures (SSTs) (Beaugrand *et al*., 2009). A similar pattern is also reflected in the North Pacific, with CPR data displaying a close correlation between warm-water copepod species assemblages (examples of taxa incorporated in this grouping include *Mesocalanus tenuicornis*, *Candacia bipinnata* and *Clausocalanus spp.*) and a positive Pacific Decadal Oscillation (PDO). A positive PDO, resulting in warmer SSTs in the northeast Pacific, corresponds with the northward movement of warm water copepod species (Batten and Walne, 2011).

2.4. Observing Phenological Changes

Phenology is defined as the study of cyclic and seasonal natural phenomena, especially in relation to climate, plant and animal life. CPR data have shown that within the copepod assemblage, seasonal timings of peak abundance can have impacts throughout the marine ecosystem (Burthe *et al.*, 2012), and propagate throughout multiple trophic levels, from primary producers to secondary consumers such as fish (Beaugrand *et al.*, 2003) and seabirds (Fauchald *et al.*, 2011; Reiertsen *et al.*, 2014). Due to seasonal variations of the upper-ocean environment, copepods encounter changes that are both intense and prolonged compared to their life spans (Mackas *et al.*, 2012). Changes in zooplankton phenology are often correlated with anomalies of one or more environmental variables, including water temperature (Edwards and Richardson, 2004). Most work on phenology in the zooplankton has focused on functional groups as opposed to individual species (Edwards and Richardson, 2004; Thackeray *et al.*, 2016). Among copepods, the bulk of studies have concentrated on *Calanus*, due to their keystone position in many marine ecosystems (Melle *et al.*, 2014 and references therein).

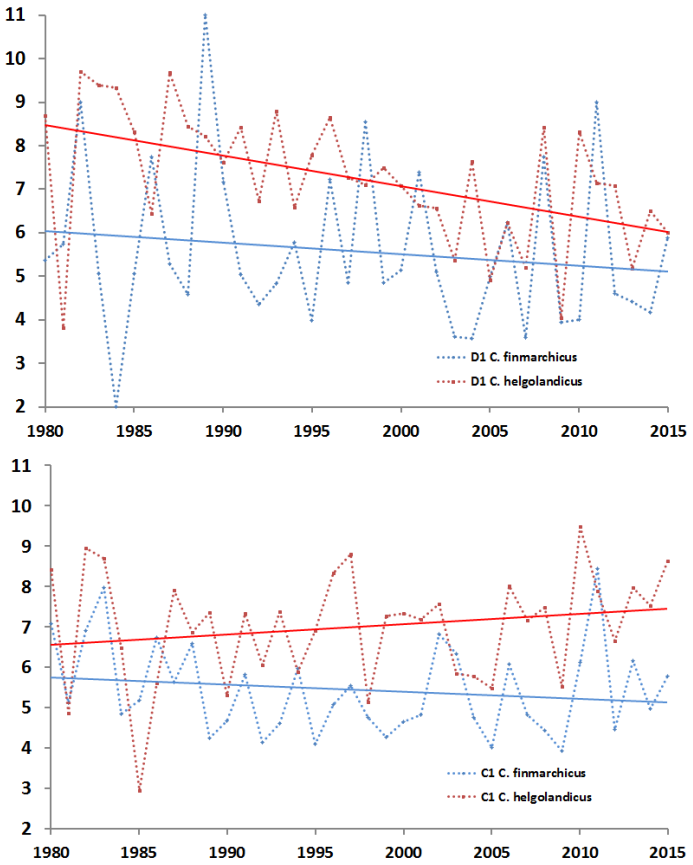

Figure 6. Time of seasonal peak changes in two species of *Calanus* (*Calanus finmarchicus* (blue) and *Calanus helgolandicus* (red)) in the North Sea (D1: Southern North Sea; C1: Central North Sea) from CPR data (Johns, 2017).

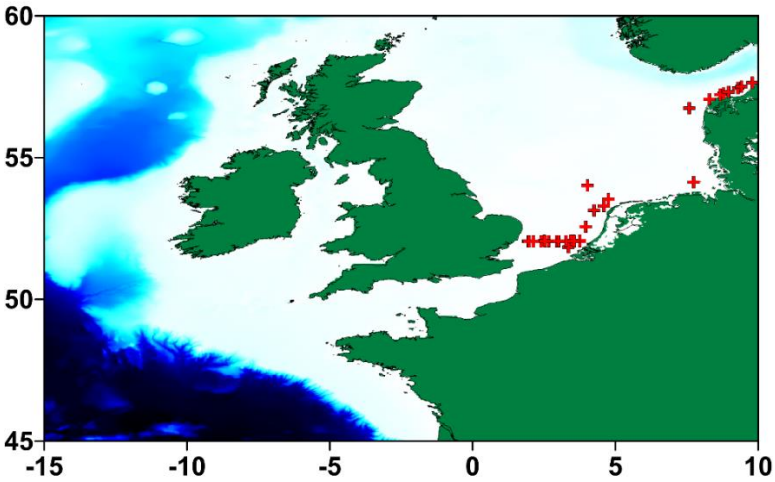

Figure 7. Occurrences of *Pseudodiaptomus marinus* in CPR samples from 2011-2016.

Figure 6 shows the month of seasonal peak for C. *finmarchicus* and C. *helgolandicus*, in two regions of the North Sea (CPR Standard Areas D1, Southern North Sea and C1, Central North Sea). The seasonal peak provides an insight into the phenology of the species in a given year, and the solid lines show long term trends of peak seasonality between 1980 and 2015. It can be seen that although *C. finmarchicus* is appearing slightly earlier in the year nowadays, in both areas *C. helgolandicus* has pronounced differences, moving earlier in the southern area and shifting to a later occurrence in the more northerly region. Both of these species are extremely important to higher trophic levels, such as fish and seabirds, and shifts in their peak periods of abundance can be propagated through the ecosystem – seasonal timing of prey species are often critical for the successful survival of higher trophic levels. This is known as a 'trophic-mismatch,' with seasonal timings becoming asynchronous. The examination of seasonal timings is crucial when looking at such keystone species as *Calanus*, as long-term trends in abundance only inform on part of the impact.

2.5. Detecting Non-Native Species

Research has shown the CPR to be a valuable tool in the discovery and monitoring of non-native species. With an extensive history of regular monthly sampling, the CPR Survey is able to provide a baseline for species diversity. In winter 2011 the Survey collected and identified specimens of the non-native copepod *Pseudodiaptomus marinus* in the southern North Sea (Jha *et al*., 2013). Native to north-western Pacific coastal waters, this species was first recorded in the Atlantic Ocean sector and North Sea in 2010 (Brylinski *et al*., 2012). However, the CPR records highlighted its northwards range extension since its arrival in European waters. Thought to have been introduced to the region via ballast water, *P. marinus* is both eurythermal and euryhaline. Not surprisingly, due to its adaptability, this non-native copepod has established itself throughout coastal regions in North America, the Mediterranean and now the North Sea (Sabia *et al*., 2015). Regularly found in North Sea CPR samples, since 2011, the Survey continues to monitor the spread and persistence of *P. marinus* around European waters (Figure 7).

2.6. CPR Copepod Data and Policy

Since the 1930s, the CPR Survey has co-evolved with marine management drivers, from monitoring North Sea plankton blooms for herring fisheries, to ecosystem-based management today (Edwards *et al*., 2010). The uniqueness of the CPR dataset means SAHFOS holds a distinctive position in the marine scientific policy community. The consistent methodology of the Survey allows exploration of long-term datasets that can be interrogated depending on policy questions. For instance, CPR research has contributed to the most recent Intergovernmental Panel on Climate Change (IPCC) reports (Pörtner *et al*., 2014) and the UN's World Ocean Assessment (Malone *et al*., 2015). Assessments such as these provide a mechanism to transfer scientific information on the state of the marine environment to decision makers, to facilitate evidence-based development of monitoring programmes and policy measures. For example, the European Union's Marine Strategy Framework Directive (MSFD) Directive 2008/56/EC (European Parliament and Council, 2008) seeks to achieve Good Environmental Status (GES) of European seas by 2020. By taking a holistic, ecosystem approach to marine management, this Directive relies on a suite of indicators being developed and monitored, at the member state and OSPAR (Oslo-Paris Convention) level, and monitored towards environmental targets which represent GES. Given the pivotal importance of copepods to the marine web, their inclusion as an indicator group for the monitoring of pelagic habitats is crucial. CPR data have played an integral role in the development of indicators at both national and international levels for multiple components of this Directive and, through the CPR Survey, will continue to contribute to the monitoring of these indicators in an effort to attain GES.

3. FUTURE OF THE CPR SURVEY

3.1. Sample Archive and Molecular Methods

SAHFOS has an extensive sample archive of approximately half a million samples, dating as far back as the late 1950s. Despite samples being preserved in formalin, recently developed molecular techniques have proven to be successful in the genetic analysis of copepod specimens (Kirby *et al*., 2007) as well as other planktonic taxa (Licandro *et al*., 2010). With the development of new techniques, and as interest in how molecular information can inform us about the ecology and physiology of copepods gains momentum (Amato and Carotenuto, this volume), further analyses of these archived samples are possible, thus progressing our understanding of the oceans.

3.2. Copepods and Human Health

Vibrio bacteria are numerous and abundant in the marine environment and are known to be associated with chitinous plankton, such as copepods. Many *Vibrio* species are pathogenic to humans (causing cholera, septicaemia and seafood-related gastroenteritis) as well as causing mass mortality to a range of marine life (Vezzulli *et al*., 2010). *Vibrio* genetic

material has successfully been extracted from archived CPR samples, dating as far back as the 1960s, and has shown that during the last 50 years there has been an increase in the occurrence of *Vibrio* in the North Sea, linked with increased SSTs in the region (Vezzulli *et al.*, 2012). Although there are still many questions regarding the relationship between copepod abundance and *Vibrio*-related outbreaks of disease, the predicted rise in climate change induced SSTs can only warrant further investigation into this subject.

3.3. Instrumentation

To gain a holistic picture of copepod biology and ecology it is important to accurately record many parameters associated within a copepods environment. Since 1994 a range of sensing technologies have been trialled on the CPR, and SAHFOS maintains an archive of these datasets alongside the contemporary presence and abundance data collected. These instruments include miniloggers for temperature and sensors for conductivity, temperature, chlorophyll and depth measurements. Going forward, new techniques are being developed to keep abreast of technological developments. For example, the autonomous Water and Microplankton Sampler (WaMS), which has been developed in collaboration with the Centre for Environment, Fisheries and Aquaculture Science (CEFAS), has already proven its capability to detect bacteria, naked flagellates and other microplankton that would be impossible to be detected with light microscopy alone (Stern *et al.*, 2015). SAHFOS has recently invested in a new FlowCam® Macro for copepods and zooplankton, to be used to advance research capabilities. This newly developed instrument will complement microscopic analysis and SAHFOS' proven world-class taxonomic expertise.

CONCLUSION

Since its inception in 1931 the CPR Survey has grown into the world's most geographically extensive and longest running marine biological survey. Despite sampling and recording a variety of phytoplankton and zooplankton taxa, copepods remain at the core of the Survey in terms of both taxonomic and research expertise. The use of consistent methodology coupled with long-term monitoring has allowed long-term trends to be identified from complex spatial and temporal variations, with data showing range shifts and changes in seasonal timing of copepod species in response to warming waters and climate change.

With an extensive sampling effort over large areas of ocean, CPR data are ideally suited to generate abundance and distribution maps for a wide range of species. Information on over 300 copepod taxonomic entities are routinely collected, yet the majority of research, published both by SAHFOS and the wider scientific community, seems to focus on the sister species *Calanus finmarchicus* and *Calanus helgolandicus*. Important to higher trophic organisms and indicators of different regimes, these two species certainly warrant intense study; however, with regional species diversity on the rise in response to a warming planet, the wealth of other copepod taxa stored in the CPR dataset perhaps also deserve attention and investigation.

As national and international leaders begin to realise the importance of the marine environment, policy and decision makers will be looking for suitable metrics to measure and monitor the health of our oceans. The CPR is the only sampler that covers the extent of the Northwestern European shelf, making it extremely useful for long-term and regional assessments of marine health. At a European level, The CPR Survey has been instrumental in the design of such indicators for the pelagic environment, and has been involved in a number of national and international assessments. With marine protection and policy continuing to progress alongside modelling and statistical techniques, it is likely that the use of such long-term vast spatial time-series will develop and they will become increasingly indispensable.

Criticised by some as antiquated, the CPR method of collection and sample analysis is robust and consistent; however, limitations to this method are acknowledged and advances are being made to expand and modernise the CPR's capabilities in the future. Molecular material has successfully been extracted from copepod specimens collected over 50 years ago and research carried out on a range of subjects from human health to population genetics. Hosting the largest known plankton archive in the world, and with the emergence of new techniques and technologies, the CPR Survey holds in its hands a unique and globally significant asset waiting to be explored.

ACKNOWLEDGMENTS

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Chapter 62

GLOBAL DISTRIBUTION OF TROPICAL AND SUBTROPICAL COPEPODS

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ABSTRACT

Here we show the main distribution characteristics of marine copepods across the subtropical–tropical latitudes and to bathypelagic depths in the Atlantic, Indian and Pacific Oceans (35ºN–40ºS). The copepod samples were collected from December 2010 to June 2011 during the Malaspina Circumnavigation Expedition. Epipelagic (0–200 m), mesopelagic (200–1,000 m) and bathypelagic strata (1,000–3,000 m depth) were sampled using an opening and closing Hydro-Bios Multinet at the following depths: 0–200, 200–500, 500–1,000, 1,000–2,000 and 2,000–3,000 m. As expected, copepods were the most abundant contributors to the zooplankton community (84%), with more than 290 taxonomic categories identified. Other marine organisms observed were chaetognaths (5%), siphonophores (3%), ostracods (2%) and euphausiids (1%). The general distribution patterns observed included low abundances, irregular spatial distributions across the three oceans, and a large decrease of abundance as the depth of the water increased. The lowest abundance was found in the southern and western regions of the Pacific Ocean, while the highest abundances were found close to the upwelling systems of the northeastern Pacific Ocean, off the Cape Vert Islands and in the Benguela current. Large differences were not observed among oceans where depth played the most important role in the structure of the copepod communities.

Keywords: copepods, subtropical–tropical, vertical distribution, global distribution, Malaspina expedition

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1. INTRODUCTION

Marine copepods play a key role in the transfer of matter and energy from the richer upper layers to the deep sea (Gartner, 1997). They are the main components of the zooplankton biomass, they are abundant throughout the whole water column and they are the most abundant group in the marine pelagic ecosystem (Siokou-Frangou *et al*., 2010). They contribute to the ecosystem by transporting organic matter to the deep ocean and egesting faecal pellets during their diel vertical migration (Longhurst, 1995; Hernández-León and Ikeda, 2005). Due to their abundance, biomass, diversity and fast response to environmental fluctuations, they are also good indicators of climate change (Beaugrand *et al*., 2002; Hays *et al.*, 2001). Moreover copepods are one of the most important links to higher trophic levels and a relevant component of the biological pump and the functioning of pelagic food webs (Richardson, 2008). The knowledge of their distribution and structure is useful for the management of marine ecosystems and the assessment of their status and health. Overall distribution and biology in the worldwide oceans are relatively well documented (Razouls *et al*., 2005-2017; Wootton *et al.*, this volume). However, a global-scale baseline assessment of copepod biodiversity and distribution, including long-term monitoring and retrospective analysis, is lacking. Gathering such information will provide a contemporary benchmark that will allow changes in the ocean to be followed over time.

The deep sea is the largest habitat on Earth and also the one that is least known. About 88% of the ocean environment is deeper than 1 km, and 76% of the environment is between a depth of 3 and 6 km (Hering, 2002). The exploration of these zones has been slow because of the inherent difficulties of sampling at great depths. These difficulties are gradually being overcome, which will likely result in an increase in the discovery and identification of new species. Below a depth of 1,500 m, the low abundance of most species requires large volumes of water to be filtered, and sampling takes place over many hours using large systems deployed from research vessels to collect a substantial number of individuals (Bucklin *et al.*, 2010). In addition, zooplankton communities in subtropical–tropical regions are poorly studied, particularly those of the southern hemisphere. They are widely unexplored in comparison to coastal areas, and most studies have been carried out in neritic waters of northern ocean areas (Richardson, 2008). The data on the abundance and distribution of zooplankton is sparse, and it corresponds to different ocean expeditions, mainly in the Atlantic (Gaard *et al*., 2008; Schnack-Schiel *et al*., 2010; Vereshchaka *et al*., 2017). An expedition never covers more than one ocean, and different expeditions use different methodologies, which makes it difficult to compare information about how organisms are distributed and relevant spatial scales. The opportunity to explore three different oceans and to use the same methodologies from surface to the deep ocean in all sampling sites would allow for the generation of a global description of the zooplankton community structure, which is of importance for future studies.

Accordingly, the main goal of this chapter was to give a global view of the copepod distribution down to bathypelagic depths in the subtropical–tropical latitudes in the Atlantic, Indian and the Pacific Oceans. A better understanding of global patterns will provide a baseline for observing future changes.

2. MATERIALS AND METHODS

During the Malaspina Circumnavigation Expedition carried out between December 2010 and July 2011 in the Atlantic, Indian and Pacific Oceans (35ºN–40ºS), samples of zooplankton were collected from 15 biogeographical provinces (Longhurst, 1995), including some poorly studied regions of the Indian and south-western Pacific Oceans (Figure 1). Samples were collected from the surface layer down to a depth of 3,000 m using an opening–closing Multinet that was equipped with a 300 µm mesh. Sample collection took place in five strata at depths of 0–200, 200–500, 500–1,000, 1,000–2,000 and 2,000–3,000 depth m, and samples were always collected during the day (10:00 to 13:00 local time). All samples were preserved on board in 5% buffered formaldehyde. Later, in the laboratory, a total of 38 stations and 198 samples were analysed under a Nikon SMZU stereomicroscope. The main zooplankton groups found were standardized to the number of individuals per m^3 (ind. m^{-3}). Copepods were identified at least to the level of genus and, when possible, to the level of species, using published literature from around the world (Bradford-Grieve, 1994; Bradford-Grieve *et al.*, 1999; Conway *et al.*, 2003; Razouls *et al.*, 2005–2017). For the analysis of copepods and main group linkages, the Primer-6 software package was used (Clarke and Warwick, 2005). A similarity matrix was calculated from the copepod abundance using Log (x+1) and, after averaging main stations by oceans and strata for dendrograms, a cluster analysis was generated based on the Bray–Curtis similarity measure. Due to the particular distribution Benguela station was not included in Figure 5b.

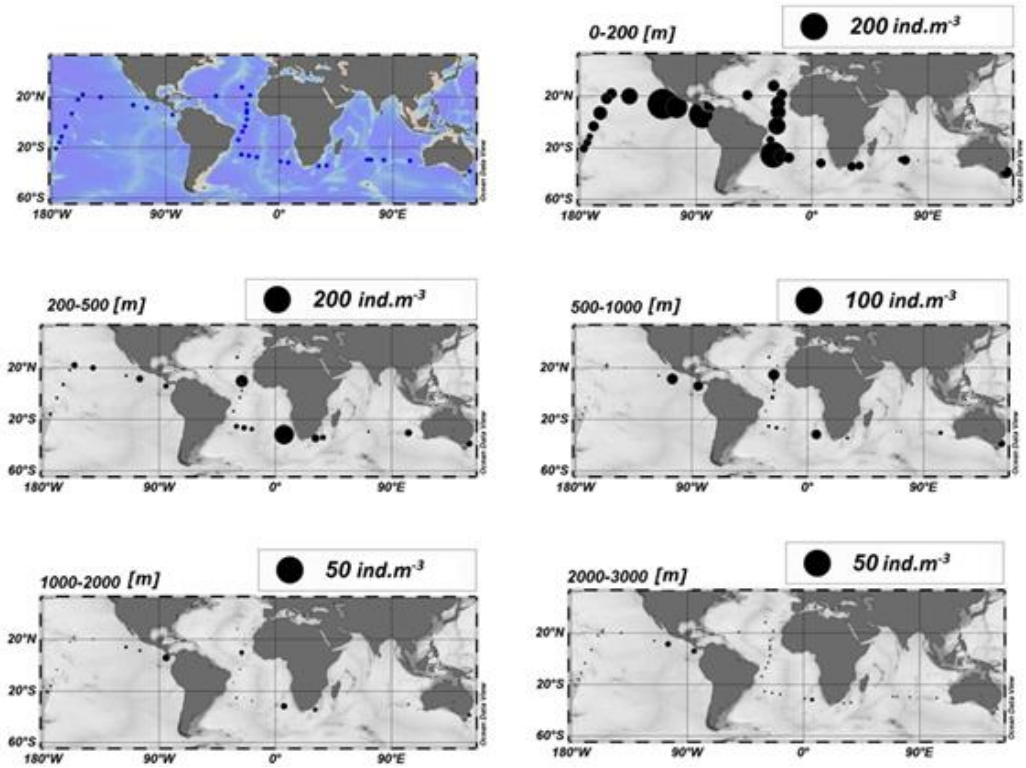


Figure 1. Map of stations and copepod abundance (ind. m^{-3}) for the five layers sampled at each station (circle) during the Malaspina expedition.

3. RESULTS

3.1. Abundance and Distribution

A low abundance of zooplankton was generally found across the entire study area (< 50 ind. m^{-3}), with the highest values corresponding to the upper 200 m of water in the eastern side of the Atlantic and Pacific Oceans (Figure 1). The spatial variability of the copepod abundance was observed among sampling stations, which covered oligotrophic areas with normal trophic conditions and rich areas and upwelling systems (for example, the northeastern Pacific [NEP], the Costa Rica Dome [CRD], off of the Cape Vert Islands [CV] and the Benguela current [BC]).

A drastic decrease of zooplankton abundance was observed as the depth of the water increased. In the epipelagic strata, only a few areas showed values greater than 200 ind. m^{-3}, for example, in the Brazilian waters, NEP and CRD. Large abundances in the mesopelagic strata off of CV and BC were also identified (> 80 ind. m^{-3}). In the bathypelagic strata, very low abundances were generally observed (< 2 ind. m^{-3}), with the exception of slightly higher abundances in NEP waters, CRD and BC (<5 ind. m^{-3}). When samples from the stations of each ocean and strata were averaged (Figure 2), the epipelagic waters of the Pacific Ocean exhibited a higher percentage of copepods (79%) compared to the Atlantic (67%) or the Indian Oceans (43%). In the mesopelagic strata (200–1,000 m depth), the Atlantic and the Indian Ocean copepods were more abundant than in the Pacific. It was interesting to observe that the upper mesopelagic of the Indian Ocean (200–500 m depth) showed a higher percentage of copepods (31%) than the Atlantic (21%) or the Pacific Oceans (8%). Below a depth of 1,000 m, a very low abundance was found in all three oceans.

Seventeen different zooplankton groups were found, but only seven groups had an abundance higher than 1%. Copepods were the dominant group, and they were always found in an abundance higher than 82% (87% in the Indian Ocean). The main characteristic observed in each strata and ocean was the increase in the contribution of copepods at deep strata (Figure 3). However, in the upper mesopelagic (200–500 m depth), the percentage of copepods was the lowest (75%) because other groups such as chaetognaths, ostracods, siphonophores, pteropods and euphausiids were found in higher densities.

Accordingly, the vertical abundance of copepods in the three oceans defined the vertical pattern of total zooplankton, with a decrease from the epipelagic to bathypelagic strata (Figure 4A). The largest decrease was observed in the Pacific Ocean from the upper (42%) to the mesopelagic strata (19%), while in the Indian Ocean the contribution of copepods was higher in the bathypelagic (59%) than in the epipelagic strata (23%). In the Atlantic, abundance of copepods in the epipelagic and mesopelagic copepod strata was similar (35% and 33%, respectively), with a larger decrease down to the bathypelagic depths (19%).

3.2. Copepod Community Structure

In this study, 290 taxonomic categories of copepods were observed, and the highest number of species were found in the epipelagic strata, as expected (Figure 4B). In the mesopelagic layer, the number of species decreased sharply, particularly in the Atlantic Ocean; an increase in species was observed at a depth of 500–1,000 m, similar to the number

found in the epipelagic layer. The sampling from the Indian Ocean exhibited a similar pattern to the Atlantic Ocean. In the Pacific Ocean, a higher number of copepod species was found in the upper bathypelagic layer at a depth of 1,000 to 2,000 m. The vertical profiles of the species number were not linear; an increase was observed at mid-depths and also at deeper water depths in the Pacific Ocean than in the Atlantic or in the Indian Oceans.

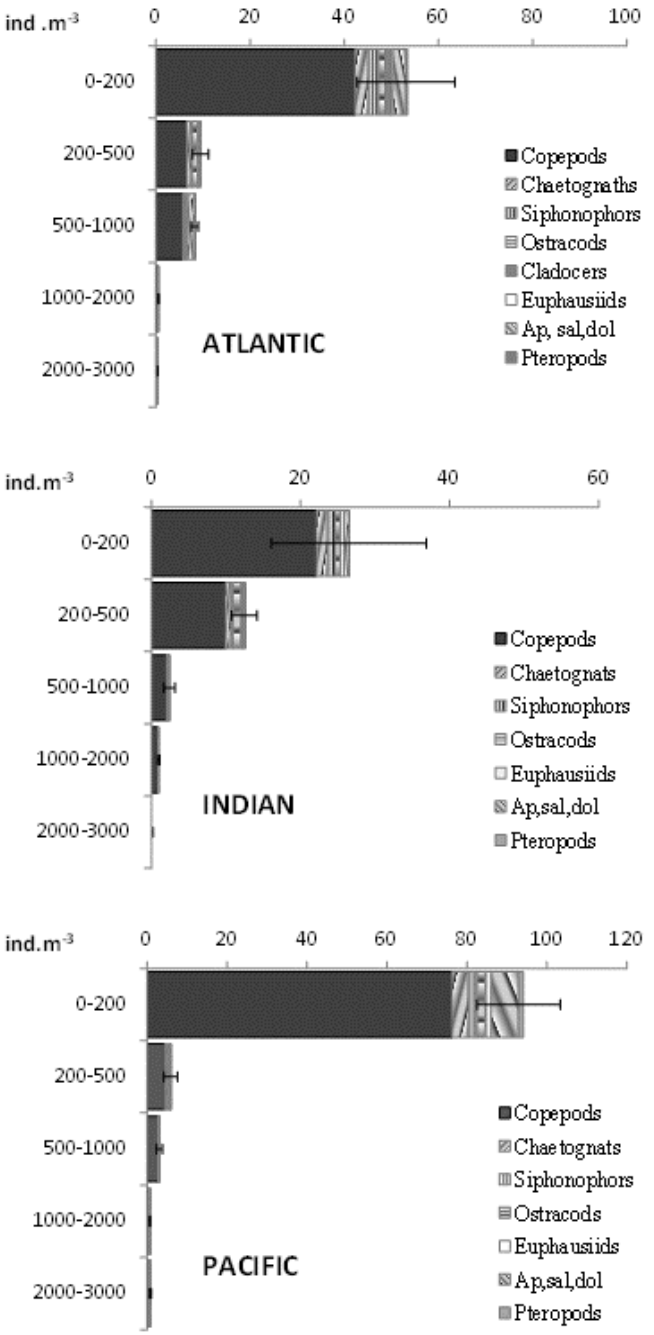


Figure 2. Average zooplankton abundance and their vertical distribution (as ind. m^{-3}) in the Atlantic, Pacific and Indian Oceans (Ap, sal, dol: appendicularians, salps and doliolids).

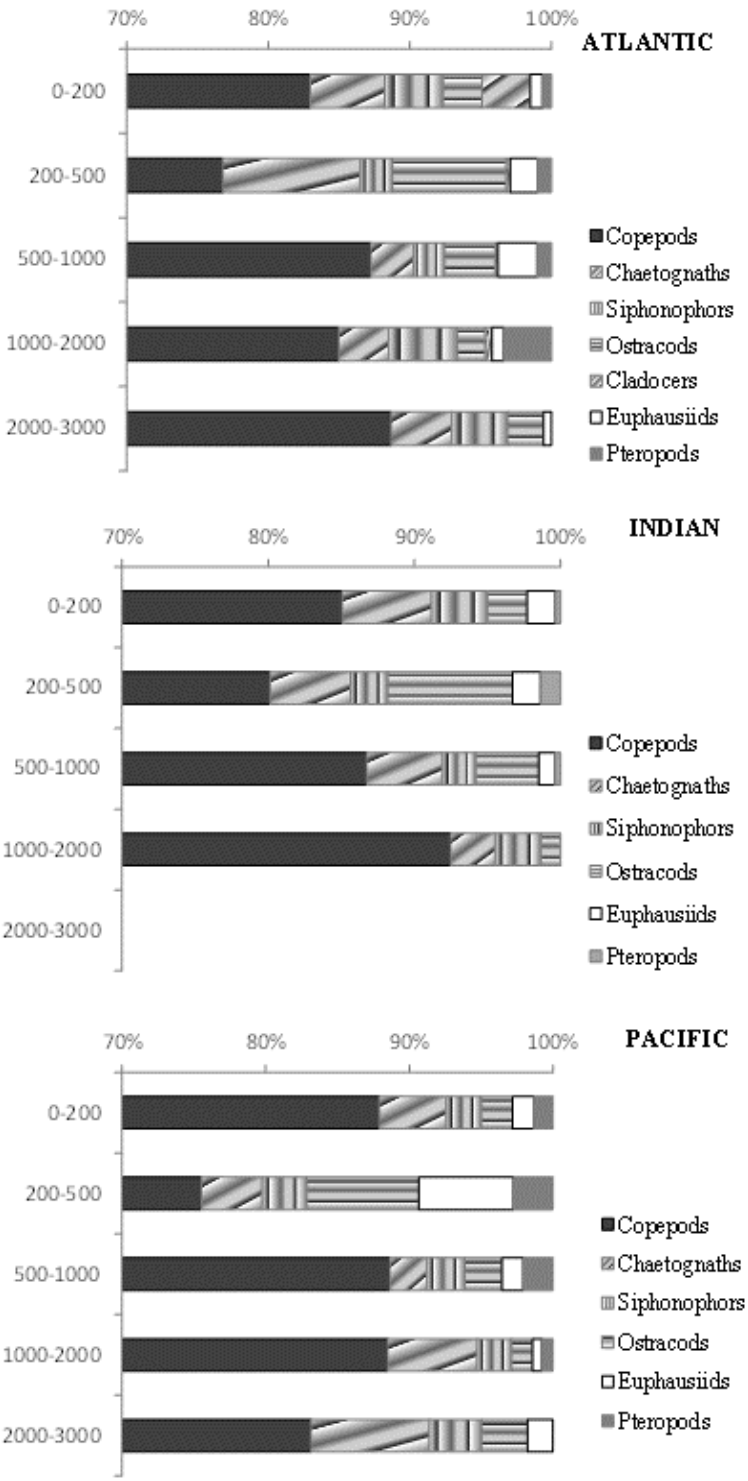


Figure 3. Vertical distribution (%) of the main zooplankton groups in the Atlantic, Pacific and Indian Oceans.

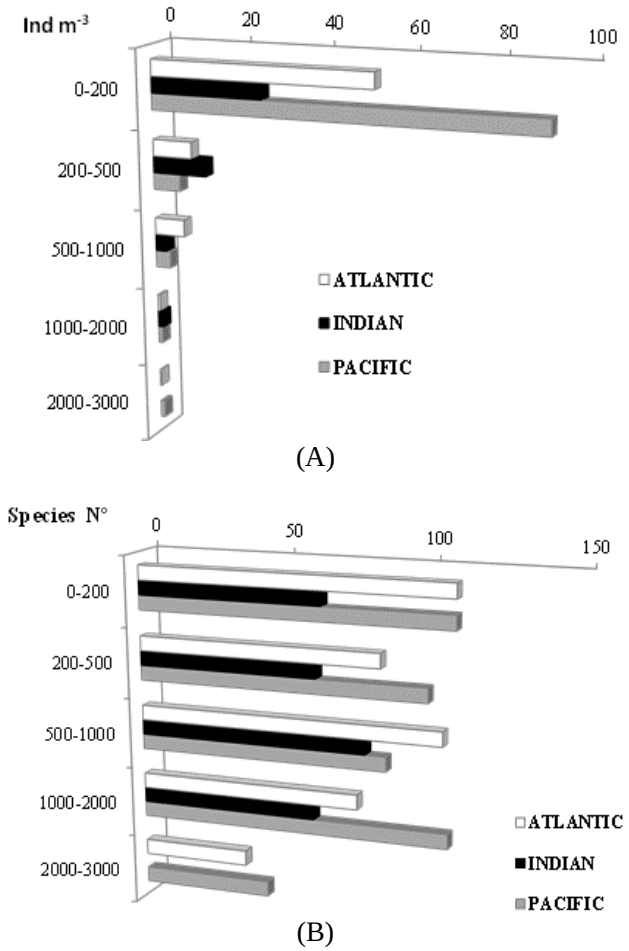


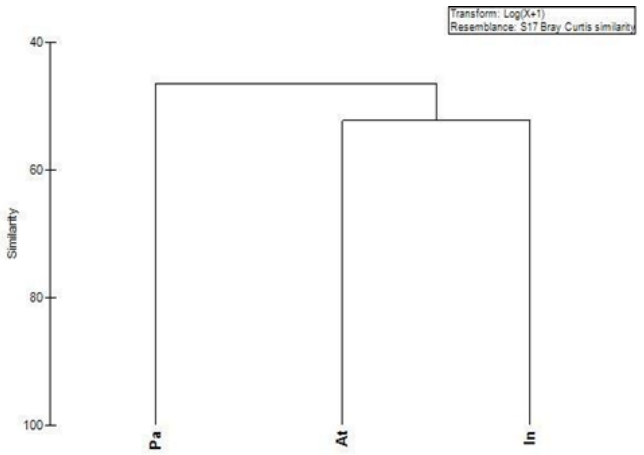
Figure 4. (A) Comparison of average abundances (ind. m^{-3}) and vertical copepod distributions for the Atlantic, Pacific and Indian Oceans. (B) Number of copepod species identified in the Atlantic, Pacific and Indian Oceans.

Although more than 230 species were identified, 55% were consistently rare (less than 1% of abundance). Similar numbers of species were found in the Atlantic and the Pacific Oceans (203 and 206, respectively), and a lower number of species were found in the Indian Ocean (150). Of the calanoids in the epipelagic strata, Clausocalanidae family (16%) had the highest abundance, followed by the Calanidae (14%), Paracalanidae, *Euchaeta* and *Acartia*; all of these accounted around 50% of the total copepods (Table I). Among the non-calanoids, *Oithona* and *Oncaea/Triconia* were dominant (18%). In all three oceans, small cosmopolitan copepods (that is, copepods with a body length ≤ 1 mm) were most common.

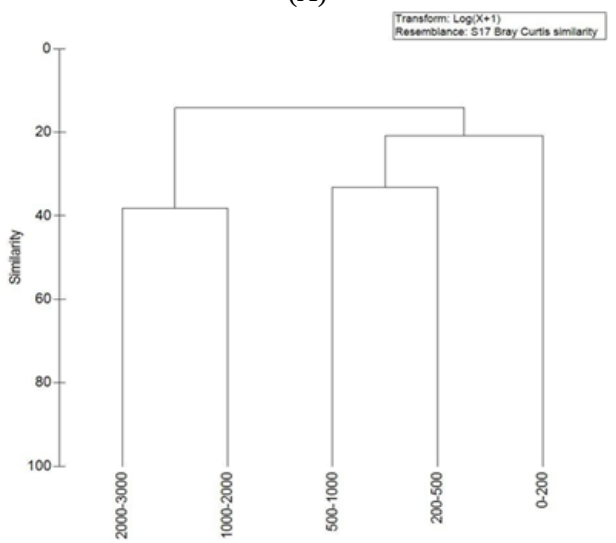
Although highly dispersed throughout the mesopelagic strata, *Pleuromamma* was the dominant copepod taxa, and among the non-calanoids *Oncaea* and *Oithona*. Some *Clausocalanus*, *Lucicutia*, *Pareucalanus* and *Heterorhabdus* were frequently found in the mesopelagic layer. In the bathypelagic layer, although *Oncaea/Triconia* among the non-calanoids were very abundant, some *Paracalanus*, *Metridia*, *Lucicutia* and *Monacilla* were also found. It was noteworthy that at a depth below 1,000 m, a large number of *Neocalanus* juveniles and *Calanoides carinatus* were observed in the Atlantic upwelling system.

Table 1. Main groups of copepods found at the different strata: epipelagic (0-200 m), mesopelagic (200-1,000 m) and bathypelagic (1,000-3,000 m)

Epipelagic	%	Mesopelagic	%	Bathypelagic	%
Clausocalanidae	16	*Pleuromamma*	20	Paracalanidae	15
Calanidae	14	Calanidae	10	*Oncaea/Triconia*	13
Paracalanidae	12	Clausocalanidae	8	Calanidae	12
Oithona	9	*Oncaea*	7	*Metridia*	7
Oncaea/Triconia	9	*Lucicutia*	6	*Oithona*	7
Euchaeta	7	*Oithona*	6	*Lucicutia*	4
Corycaeidae	6	*Pareucalanus*	5	*Pleuromamma*	4
Acartia	5	*Scolecithricella*	4	*Monacilla/Spinocalanus*	4
		Heterorhabdus	4		



(A)



(B)

Figure 5. (A) Clusters of copepod abundances and stations in the Atlantic (At), Indian (In) and Pacific (Pa) (calculated using the Bray–Curtis similarity index after the data (x) had been transformed to Log (x +1)). (B) Cluster showing copepod abundance at different sampled layers (calculated using the Bray–Curtis similarity index after the data had been transformed to Log (x +1)).

A cluster analysis was performed in order to check for similarities between the oceans and the strata by using two way analysis of similarity (ANOSIM; Clarke and Warwick, 2005). Despite the differences in the total abundances between the oceans, the dendograms that resulted from the cluster analysis revealed that at a Bray–Curtis similarity level of 53%, there were more similarities between the Indian and Atlantic Oceans compared to the Pacific Ocean. No significant differences were observed between the three oceans (ANOSIM R: 0.105; significance level of 0.2%) (Figure 5A). However, a significant difference was found between the strata, particularly between the epipelagic layer and other zones (ANOSIM R: 0.508; significance level of 0.1%). The bathypelagic stratum (1,000–3,000 m depth) showed the highest similarity between the upper and the lower strata (40%). The cluster analysis among main stations, copepods and strata (Figure 5B) revealed the presence of three main groups: epipelagic (0–200m), mesopelagic (200–1,000 m) and bathypelagic layers (1,000–3,000 m).

4. DISCUSSION

We analysed the abundance and composition of the zooplankton, and in particular the copepod community, in the water column (0–3,000 m depth) of the subtropical and tropical latitudes. The findings presented in this chapter demonstrate for the first time that three oceans have been sampled using the same methodology. Copepods were always dominant in the three oceans and at all depths (> 70% of total zooplankton abundance), as is usually observed in open ocean areas (Longhurst, 2007; Gaard *et al*., 2008). The dominant copepods observed at each depth were similar to what has been reported by other studies (Bradford-Grieve, 1994; Bradford-Grieve *et al*., 1999; Conway *et al.,* 2003; Dias *et al.,* 2010; Razouls *et al*., 2005–2017). The large decrease in abundance that was observed from the epipelagic to the bathypelagic zones was also found by different authors either in the Atlantic or in the Pacific Oceans (Fernández-Álamo and Farber-Lorda, 2006; Schnack-Schiel *et al.,* 2010; Vereshchaka *et al.*, 2017); this vertical discontinuity is a common pattern. Smaller copepods are usually more numerous at the surface layers, while larger copepods are less abundant at the surface layers and are mainly found in deep waters (Deevey and Brooks, 1977; Webber and Roff, 1995; Brugnano *et al.*, 2012).

The high number of copepods observed here is a common pattern in many zooplankton studies, demonstrating the relevance of this group in transferring energy between different levels of the trophic web. They play a key role in the marine plankton food web by controlling primary production and microzooplankton, and by providing food for animals at higher trophic levels (Chank and Fang, 2004). Knowledge of the vertical distribution of copepods and their variations in space are relevant to understanding the dynamics in pelagic communities and the vertical flux of organic matter through the water column.

An overall finding of this study was the high number of copepod species found; however, the majority of them were found in an abundance of less than 1%, as other authors have reported for the Atlantic Ocean (Piontkovski *et al.,* 2003). The number of species and their abundances tended to decrease with increasing depth, which was another general trend that was observed for the distribution of copepods in the pelagic strata (Longhurst, 1995; Angel, 2003). Nevertheless, the decrease in the number of species was not linear, as a peak was

normally found at mid-water and continued to decrease at deeper layers. This was another trait that has already been mentioned with regard to deep oceans and low latitudes (Bucklin *et al.*, 2010). In the present work, the results from the three oceans did not show significant differences between the compositions of copepods, as 50% of species were common for all three oceans; however, depth was the main factor affecting their distribution. In addition, deeper-dwelling copepods were less likely to be endemic (restricted to a particular region) and more likely to be geographically widespread (Bucklin *et al.*, 2010; Yamaguchi *et al.*, 2015). Moreover, feeding mode varied across all the strata, with filter-feeding herbivorous copepods observed more frequently in the upper layers and omnivores and carnivores more abundant in deeper layers (Benedetti *et al.*, 2015).

Another finding was the widespread distribution of small copepods, such as *Clausocalanus, Paracalanus, Oithona* and *Oncaea*, which is in contrast to other authors who reported limited distribution (Maycas *et al.*, 1999). As expected from their vertical migratory behaviour, large copepods, such as *Pleuromamma, Euchaeta, Pareucalanus, Metridia* and *Lucicutia,* were found in deeper layers (Bradford-Grieve *et al.*, 1999; Razouls *et al.*, 2005–2017). Other copepods, such as *Neocalanus* juveniles with an ontogenetic distribution, were mainly found in upwelling systems and were limited to deep waters and the oxygen-minimum zone (Cavalcanti and Larrazábal, 2004; Fernández-Álamo and Farber-Lorda, 2006; Verheye *et al.*, 2005; Gaard *et al*., 2008; Escribano *et al*., 2009; Teuber *et al.*, 2013).

In summary, we investigated the main characteristics of the copepod community in the subtropical and tropical latitudes (30ºN–40ºS) in the Atlantic, Indian and Pacific Oceans and down to bathypelagic depths. Our results showed irregular spatial abundance from the oligotrophic to the upwelling regions and a large decrease in abundance from the epipelagic to the bathypelagic zones. A large number of copepods were found mainly in the epipelagic layer, but they were also observed at mid-water depths. The overall distribution of copepods in the three oceans was similar; differences between the oceans were mainly due to small differences in genera and species rather than total abundances, and depth was the most relevant factor affecting the copepod distribution. In addition, the oceanic waters were characterized by cosmopolitan small-sized copepods, which dominated the community in the whole water column. In order to understand the role of copepods in the ocean ecosystem, we must expand our knowledge of organisms in deeper water, the patterns of distribution and the diversity of dominant species. This is especially relevant in the deep layers of the subtropical and tropical oceans, which are poorly understood due to the sampling challenges.

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Chapter 63

BIOGEOGRAPHICAL DISTRIBUTION AND ECOLOGY OF THE PLANKTONIC COPEPOD *OITHONA DAVISAE*: RAPID INVASION IN LAKES FARO AND GANZIRRI (CENTRAL MEDITERRANEAN SEA)

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ABSTRACT

The Asiatic copepod species *Oithona davisae* was initially described from the type locality of the Sacramento San Joaquin Estuary, California, USA (Ferrari and Orsi, 1984). Following recent reports of *O. davisae* in the Black Sea, we researched the biogeographical distribution and ecology of this species, based on original data and on a critical review of the literature. *O. davisae* was not recorded for the Mediterranean Sea before 2003, although it is now becoming established in Italian transitional environments. The literature indicates that *O. davisae* is a typical coastal and estuarine copepod species that was originally endemic in the temperate coastal waters of East Asia, and its spread to other regions was due to human-related vectors. *O. davisae* is less than 1 mm in length, and its abundance has often been underestimated because many sampling programmes have used net mesh sizes of 200 μm or larger, which cannot quantify small cyclopoid species. *O. davisae* has most often been recorded in transitional environments that are characterised by high trophic levels and stable waters, which allow its reproduction throughout the year. In Lakes Faro and Ganzirri, its highest abundance occurs in the late spring to early summer, coincident with the highest abundance of small-sized flagellates. The occurrence of dense populations of *O. davisae* in these transitional environments that are used for aquaculture activities confirms its invasive behaviour, with competitive exclusion of the indigenous species *Oithona nana*. After its introduction, *O. davisae* has become the most abundant species of the copepod assemblage in a short time. To date,

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there do not appear to be negative consequences at the community and ecosystem levels. Ship-ballast waters and bivalve transplants appear to be the main means of dispersion of *O. davisae*.

Keywords: copepods; biogeography; ecology; interspecific competition

1. INTRODUCTION

Global human activities have amplified the biogeographical ranges of many invasive species over the last decades. Invasive species are those that are introduced to, and successfully establish themselves in, a region that is outside of their natural range. These have often been considered major threats to ecosystem functions and the maintenance of biodiversity (Carlton, 1989 and 1996; Carlton and Geller, 1993; Ruiz *et al*., 1999; Geller, 1999). The recent increase in studies on such coastal invaders has provided new insights into the human-related vectors, with four main paths of invasion proposed: ship-ballast water, hull fouling, and new connections between water bodies through canals and aquaculture transfers (Ruiz *et al*., 1997; Elston, 1997; Galil, 2009; Zenetos *et al*., 2010). Thus, lagoons, estuaries and coastal marine systems are among the most highly invaded environments for non-indigenous species.

The genus *Oithona* is the most important cyclopoid copepod group in the world oceans in terms of abundance and productivity (Gallienne and Robins, 2001; Turner, 2004). This includes cosmopolitan species, like *Oithona similis* and *Oithona nana*, as well as species with narrower ranges of distribution, such as *Oithona vivida* and *Oithona robusta* (Dahms *et al*., 2015 and references therein). These small-sized oithonids have many ecological roles. They might be an important prey source for the larval stages of some key fishery species (Viñás and Ramirez, 1996), or conversely they might represent energy sinks in metazoan foodwebs (Atkinson and Snÿder, 1997). They directly affect the downward flux of calanoid copepod faecal-pellet material in pelagic zones (González and Smetacek, 1994), contribute to regeneration of nutrients that support primary production (Hiromi, 1995), and facilitate complex trophic interactions between the protozoan and metazoan foodwebs (Nakamura and Turner, 1997). Oithonids differ from better-studied suspension-feeding calanoid copepods in being primarily raptorial predators using hydromechanical signals to detect and capture motile prey (Svensen and Kiørboe, 2000).

Oithona davisae was originally endemic to the temperate coastal waters of East Asia. Over the last forty years, it has been recorded in other regions, which now extend from the Pacific and Atlantic Oceans to northern and southern European waters (Nishida *et al*., 1977; Ferrari and Orsi, 1984; Hirakawa, 1988; Lee *et al*., 2001; Saiz *et al*., 2003; Cordell *et al*., 2009; Lawrence and Cordell, 2010; Temnykh and Nishida, 2012; Cordell *et al*., 2015; Cornils and Wend-Heckmann, 2015; Uriarte *et al*., 2015; Doğan and İşinibilir, 2016). *O. davisae* shows high tolerance to different environments, which allows it to survive and reproduce in many coastal and transitional waters. This ecological plasticity may contribute to its success as an invasive species.

Passive dispersion of *O. davisae* by ship-ballast waters has been widely proposed (Ferrari and Orsi, 1984; Nishida, 1985; Hirakawa, 1988; Cordell *et al*., 2009; Lawrence and Cordell, 2010). Other dispersion vectors have not been considered to have a role in *O. davisae*

dispersion, although aquaculture transfer as a possible means of introduction and spread of invasive non-indigenous copepod species is a well-known possibility (Carlton, 1992a, b; Naylor *et al*., 2001). Aquaculture activities involving the transport of live shellfish, and especially oysters, have been identified as the most important human vector for the introduction of invasive species into coastal marine and estuarine systems (Molnar *et al*., 2008).

The Mediterranean Sea is particularly vulnerable to ship-transported bio-invasions, which have increased greatly in frequency over the last two decades (Galil, 2009; Coll *et al*., 2010; Costello *et al*., 2010). Among the 955 alien species recorded in the Mediterranean Sea, 42 are planktonic copepods (Zenetos *et al*., 2010), three of which have recently been identified in Lakes Faro and Ganzirri, two coastal lakes located in the Central Mediterranean Sea (Sicily, Italy): *Pseudodiaptomus marinus* Sato, 1913; *Acartia tonsa* Dana, 1849; and *O. davisae* Ferrari and Orsi, 1984 (Brugnano, 2006; Zagami and Brugnano, 2013; Pansera *et al*., 2014; Sabia *et al*., 2014; Sabia *et al*., 2015). These above mentioned species have rapidly invaded many coastal and transitional European sites, showing different patterns of coexistence and/or competitive exclusion with indigenous species (Brugnano *et al*., 2011; Altukhov *et al*., 2014; Uriarte *et al*., 2015; Delpy and Pagano, this volume; Marques *et al*., this volume; Villate *et al*., this volume).

In this contribution we first provide a brief revision of the biogeography and ecology of *O. davisae*. We then present results from a year-long sampling programme in Lakes Faro and Ganzirri, showing the seasonal variations in abundance of this species. For the first time we report the introduction of *O. davisae* through aquaculture rather than ballast water, evidenced by the lack of water exchange among the two lakes and the sea. The dynamics observed in these lakes match those recorded in other sites worldwide, provide evidence for the invasiveness of this species and point to further hypothetical alternative vehicles for dispersion. This information broadens our knowledge of the invasive dynamics of this species, and provides new understanding about its biology and ecology.

2. DISTRIBUTION AND ECOLOGY OF *OITHONA DAVISAE*

2.1. Biogeographic Distribution

Oithona davisae is a coastal and estuarine copepod species endemic in the temperate coastal waters of East Asia. *O. davisae* was originally described by Ferrari and Orsi (1984) for Californian estuarine waters, with the type locality of the Sacramento San Joaquin Estuary. It has also been recorded in Chile (Hirakawa, 1988) and in the north-western Mediterranean (Nishida, unpublished observations, cited by Saiz *et al.*, 2003). More recently, *O. davisae* was reported in the Bay of Varna by Mihneva and Stefanova (2013), in the Estuary of Bilbao (eastern Atlantic Ocean, Spain) (Uriarte *et al.*, 2015), Wadden Sea (Cornils and Wend-Heckmann, 2015), Puget Sound (Washington State, USA) (Cordell *et al.*, 2015), Marmara Sea and Golden Horn Estuary (Isinibilir *et al.*, 2016) and Venice Lagoon (northern Adriatic Sea) (Pansera, personal communication) (Figure 1). Data on the literature search on *O. davisae* biogeography are available from Razoul *et al.* (2005-2017) and Walter (2015).

Figure 1. Global distribution of *Oithona davisae* based on the literature data: ● Sacramento San Joaquin Estuary, California (Ferrari and Orsi, 1984); ○ Chile (Hirakawa, 1988); ● Puget Sound, Washington State (Cordell *et al.*, 2015); ■ Eastern Atlantic Ocean, Spain (Uriarte *et al.*, 2015); ▼ North-western Mediterranean (Saiz *et al.*, 2003); ▲ Venice Lagoon, Adriatic Sea (Pansera, personal communication); ◊ Wadden Sea (Cornils and Wend-Heckmann, 2015); ◣ Bay of Varna (Mihneva and Stefanova, 2013); ◗ Black Sea (Temnykh and Nishida, 2012); ◁▷ Korea (Lee *et al.*, 2001; Orui-Sakaguchi *et al.*, 2011); ◆ Japan (Nishida, 1985; Ohtsuka *et al.*, 2008); ► Lakes Faro and Ganzirri, Central Mediterranean (present work).

It is closely related to *Oithona nana*, *Oithona aruensis* and *Oithona brevicornis* (Nishida and Ferrari, 1983; Ferrari and Orsi, 1984). The morphological features that allow *O. davisae* to be distinguished from these other species are: sharply pointed rostrum; very long distal spine on the first inner lobe of the maxillule; and endopod of the maxillule carries only one seta. Owing to this morphological closeness, *O. davisae* has also been misidentified several times, including as *O. brevicornis* f. *minor* (Nishida *et al.*, 1977; Nishida and Ferrari, 1983), *O. aruensis* (Nishida, 1985; Ohtsuka *et al.*, 2008) for the coastal waters of Japan, and as *O. aruensis* in Korea (Lee *et al.*, 2001; Orui-Sakaguchi *et al.*, 2011).

It also appears likely that the *O. brevicornis* specimens that have been reported as recently introduced into the Black Sea (Zagorodnyaya, 2002; Gubanova and Altukhov, 2007; Selifonova, 2009) are also *O. davisae*. This is supported by the specimens collected in an offshore area in the Black Sea off the Crimean Peninsula that were previously identified as *O. brevicornis*, and upon re-examination showed to be *O. davisae* (Temnykh and Nishida, 2012).

The geographic distribution of *O. davisae* shows that this species was originally endemic to the temperate coastal waters of East Asia, in contrast to *O. brevicornis*, which was widely distributed in the subtropical-tropical coastal waters of the Indo-Pacific (Giesbrecht, 1891; Nishida, 1985). The recent record in European waters confirms this non-indigenous species as typical of temperate environments. However, the records of the occurrence of *O. brevicornis* in the Mediterranean Sea (Carazzi and Grandori, 1912; Pesta, 1920; Vaisierre and Seguin 1980; Shuvalov, 1980) should be revised, because these studies were not based on description of the morphological characters that are now known to distinguish *O. brevicornis* from the other related species.

2.2. Habitat Characteristics

O. davisae has mainly been reported in eutrophic land/sea transitional zones, such as coastal lakes, estuaries, lagoons and coastal marine environments, including near-shore waters, inlets and bays. *O. davisae* can tolerate wide salinities from 12 to 35, and temperatures from -1.8 to 29°C (Ferrari and Orsi, 1984; Kimmerer, 2004; Gubanova and Altukohv, 2007; Temnykh and Nishida, 2012; Mihneva and Stefanova, 2013; Cornils and Wend-Heckman, 2015; Doğan and İşinibilir, 2016; Isinibilir *et al*., 2016), although it appears to thrive in relatively warm conditions (20 to 25°C). In the temperate eutrophic inlet of Fukuyama Harbour (Uye and Sano, 1998), in the San Francisco Estuary (Bollens *et al.*, 2011) and inner Estuary of Bilbao (Uriarte *et al*., 2015), *O. davisae* seasonal highest abundances occurred in warmer periods, between late spring and early summer. In Sevastopol Bay (Crimea), the peak abundances of *O. davisae* had high interannual variability, occurring at the end of October (Gubanova and Altukhov, 2007), mid-June and in autumn (Altukhov *et al*., 2014). In the western Black Sea, *O. davisae* abundances peaked during September-October (Mihneva and Stefanova, 2013).

Most of the transitional environments where *O. davisae* has been reported to be abundant, especially in the inner part of estuaries, inlets and semi-enclosed bights, have narrow mouths and shallow channels, limiting water exchange with the sea. This therefore results in little water circulation, eutrophic conditions, and wide salinity variations (Ferrari and Orsi, 1984; Uye and Sano, 1998; Bollens *et al*., 2011; Uriarte *et al*., 2015). These habitat parameters appear to be beneficial for the growth of *O. davisae*. In the Mediterranean Sea, *O. davisae* has only been recorded in some of the many sites that possess these characteristics. One explanation might be that *O. davisae* has often been misidentified as *O. brevicornis* (*e.g*., see Zagami and Brugnano, 2013; Pansera *et al*. 2014), while another might be that the optimal conditions for the success of *O. davisae* invasions are isolated eutrophic environments, resulting in disjunct distributional patterns, and reducing the chances of dissemination of *O. davisae* among coastal transitional environments. In addition, *O. davisae* may have been missed because of the mesh sizes used for copepod sampling. Owing to its small size (< 1 mm body length), *O. davisae* might not be efficiently collected through the WP-2 plankton net (*i.e*., 200 µm mesh size) (UNESCO, 1968; Sameoto *et al*., 2000), which remains the most commonly used net for marine zooplankton sampling. This would lead to underestimation of cyclopoid abundances (Gallienne and Robins, 2001; Pansera *et al*., 2014), but not to their total absence.

2.3. Ecology

With its high egg production rate (11.6 eggs female^{-1} d^{-1} in summer; Uye and Sano, 1995) and invasiveness, *O. davisae* may require only a short time to become the most abundant copepod species in zooplankton communities of many coastal transitional environments (Uye and Sano, 1998; Bollens *et al*., 2011; Altukhov *et al*., 2014; Cornils and Wend-Heckman, 2015; Uriarte *et al*., 2015). O. davis*ae* shows high numerical peaks in both its original and its newly invaded environments, ranging from 5.1×10^3 to 6.0×10^5 ind. m^{-3} (Uye and Sano, 1995). Its highest numbers were recorded for swarms of adults and copepodites attaining 1.34×10^6 ind. m^{-3} for the mud flats of Ariake Bay, Kyushu (Hirota and Tanaka, 1985). The

reasons for such high *O. davisae* abundance might be the low species diversity and the high primary production that characterise these segregated environments, which can result in little inter-specific competition and sufficient or unlimited food supplies.

In several environments like Sevastopol Bay (Altukhov *et al*., 2014), the Marmara Sea and Golden Horn Estuary (Isinibilir *et al*., 2016), and Estuary of Bilbao (Uriarte *et al*., 2016), *O. davisae* has occupied the niche of the indigenous species *O. nana* and it has become the most abundant species of the copepod assemblage. The preferred food of *O. davisae* consists of motile prey, such as flagellates, ciliates and copepod nauplii (Uchima, 1988; Uchima and Hirano, 1986a, b; Saiz *et al*., 2003; Henriksen *et al*., 2007), with either sinking or jumping considered typical foraging movements (Cheng *et al.*, 2014). Also *O. nana* appears to ingest significant levels of non-motile prey, such as diatoms (Lampitt and Gamble, 1982). *O. davisae* might have a significant role in transferring energy from the nanoflagellates and microflagellates and ciliates to the higher trophic levels in eutrophic environments, such as transitional waters (Uye and Sano, 1998).

The dominance of the invasive species *O. davisae* over *O. nana* might be evidence of competitive exclusion of the indigenous species from the zooplankton community. Laboratory experiments and field studies have highlighted the competitive advantage of *O. davisae* over *O. nana*. *O. davisae* is a widely euryhaline species (Svetlichny and Hubareva, 2015; Svetlichny *et al*., this volume), while *O. nana* is a stenohaline species (Kovalev, 1966). In addition, the frequency and speed of routine jumps, and the maximum escape reaction speed in *O. davisae* are significantly higher than for *O. nana* (Isinibilir *et al*., 2016). Comparisons of the biological attributes between these two species show that, at the same temperature, *O. davisae*, has shorter development time than *O. nana*, 17 days (Uye and Sano, 1998) *vs.* 21-24 days (Haq, 1965), higher daily egg-production rate, 11.6 (Uye and Sano, 1995) *vs.* 7.4 eggs female^{-1} day^{-1} (Cepeda *et al*., 2015), and larger clutch size, 28.5 (Uye and Sano, 1995) *vs.* 12.4 eggs female^{-1} (Temperoni *et al*., 2010; Cepeda *et al*., 2015). All of these factors may indicate the higher adaptive potential of *O. davisae* respect to *O. nana*.

2.4. Dispersal

Previous reports on *O. davisae* have shown that it was originally endemic in temperate Japanese coastal waters. Until now, its occurrence in other regions of the world has been explained exclusively by ship-ballast water transport (Ferrari and Orsi, 1984; Nishida, 1985; Hirakawa, 1988; Cordell *et al*., 2009; Kaysan, 2010; Lawrence and Cordell, 2010). Other human-related vectors have not been considered to play a role in *O. davisae* dispersion. However, ballast transport alone does not completely explain the biogeographic distribution pattern of *O. davisae.* Around the world, *O. davisae* has been found mostly in highly anthropogenic environments, and particularly in segregated coastal features, such as bays, estuaries and lagoons, where it can attain high abundances. The optimal conditions for *O. davisae* growth occur in confined eutrophic environments that are characterised by little water circulation, leading to a varied distribution pattern that reduce the chances of the dispersion of this species. Therefore, *O. davisae* occurs in embayments both with and without ballast water discharge.

3. *OITHONA DAVISAE* IN THE CENTRAL MEDITERRANEAN SEA (LAKES FARO AND GANZIRRI, SICILY, ITALY)

3.1. Study Area

Lakes Faro and Ganzirri are formed by two coastal basins in the central area of the Mediterranean Sea (Figure 2). Lake Faro (38°160' N, 15°380' E) is a small coastal basin (0.263 km^2) located in the north-eastern tip of Sicily (Italy), and is connected to the adjacent Lake Ganzirri and to the Strait of Messina by shallow channels. It has a funnel-shaped bottom profile, whereby it has a wide nearshore water area before it reaches its maximum depth of 29 m, in the central part. In this deepest part, Lake Faro is characterised by typical features of a meromictic temperate basin, with its upper oxygenated mixolimnion (up to 15 m in depth) and its lower anoxic and sulphidic monimolimnion (Genovese, 1963; Truper and Genovese, 1968). The nearshore waters of the lake are oxygenated down to the bottom.

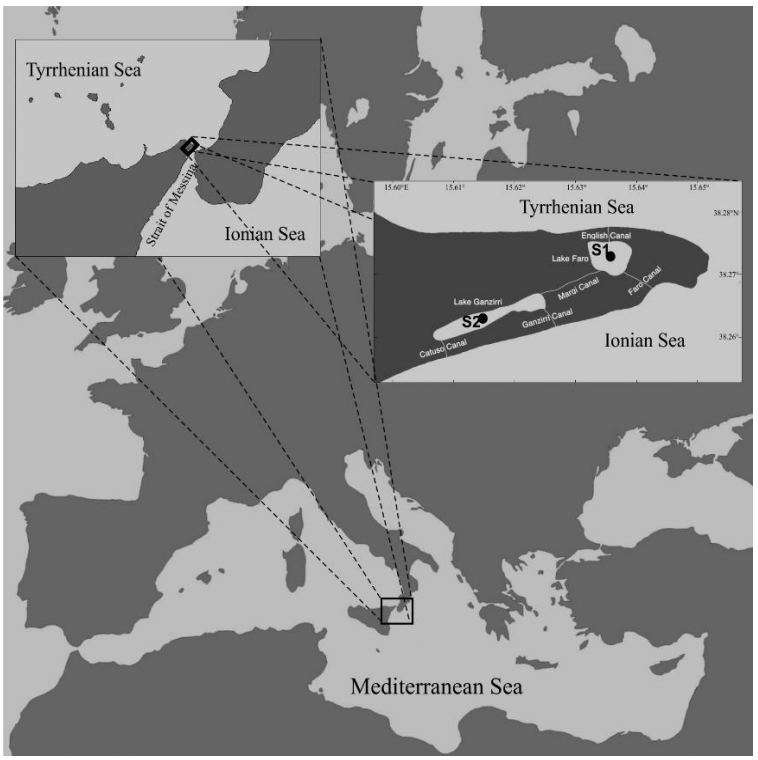

Figure 2. Map of the locations of Lakes Faro and Ganzirri.

Lake Faro shows large seasonal fluctuations in physico-chemical parameters, particularly for temperature (10-28 °C) and salinity (28-37) (Crisafi, 1955; Pansera *et al.*, 2014). Its physical and chemical stratification between the surface water and the water column is significant, particularly in summer when anoxia extends up to the lower mixolimnion zone, resulting in large blooms of photosynthetic sulphur bacteria (Sorokin and Donato, 1975).

Lake Ganzirri (38°150' N, 15°360' E) has an elongated shape with shallow waters, reaching a maximum depth of 5 m thus maintaining oxygenated bottom waters. Through the

year, Lake Ganzirri also has large fluctuations in its physico-chemical parameters, again for temperature and salinity in particular, which range from 10.4 °C to 30.1 °C, and from 21.2 to 28.7, respectively (Crisafi, 1955; Genovese, 1963; Ferrarin *et al*., 2013).

The very long and shallow channel that connects Lake Ganzirri to the sea results in a low marine water turnover rate. Thus, Lake Ganzirri is characterised by typical brackish waters that are supplied more by the surface rainwater than by marine water (Ferrarin *et al*., 2013). For both lakes, the water movements are influenced by the currents of the Strait of Messina. The marine water turnover rate is higher for Lake Faro than Lake Ganzirri, because of its shorter communication channel with the sea, thus Lake Faro has a more marine character than Lake Ganzirri (Genovese, 1963; Ferrarin *et al*., 2013).

In the segregated sectors of both lakes, very little water exchange occurs, leading to ecotoxicological alterations in the zooplankton communities (Minutoli *et al*., 2008). Lake Faro is an important centre for shellfish aquaculture, whereby living bivalves are imported from many Atlantic (Spain, northern France, The Netherlands) and Mediterranean (Venice Lagoon, Thau Lagoon) sites.

The mesozooplankton of Lakes Faro and Ganzirri include a relatively small number of taxonomic groups, of which copepods constitute by far the dominant species (Genovese, 1963; Crisafi *et al*., 1973; Zagami and Guglielmo, 1995). The permanent copepod communities in Lakes Faro and Ganzirri include only a few copepod species, including *Paracartia latisetosa* Krichagin, 1873, *Acartia margalefi* Alcaraz, 1976, and *Oithona nana* Giesbrecht, 1892. For Lake Faro only, there are also temporary coastal copepod species that enter and exit via tides from the Straits of Messina. A very complex current system driven by the flow of waters from the Ionian Sea to the Tyrrhenian Sea, and *vice versa* (Bossolasco and Dagnino, 1959; Mosetti, 1988), takes place in this area. This flow changes every 6 h, and thus has a large influence on the coastal and Lake Faro zooplankton communities (Zagami and Guglielmo, 1995; Zagami *et al*., 1996; Zagami and Brugnano, 2013).

3.2. Materials and Methods

Zooplankton sampling was carried out in Lakes Faro and Ganzirri at approximately monthly intervals from January to December 2014, at a station in the central, deepest area in each lake (Figure 1, S1, S2, respectively). The samples were collected using a net (mouth area, 0.125 m^2; mesh size, 80 μm; Apstein), with a digital flowmeter (Hydro-Bios, Kiel) mounted on the mouth of the net to calculate the filtered water volume. For Lake Faro, each net tow was vertical and taken within the oxygenated layer, from 15 m depth to the surface. For Lake Ganzirri, because of its lesser depth, each net tow was horizontal in the sub-subsurface layer of the water column (*i.e*., 0-1.5 m in depth). A multiparametric conductivity, temperature and depth probe (ISY 6600V2) was used to measure the temperature and salinity at each sampling station. In the laboratory, the zooplankton samples were fixed in 4% neutralised formalin in lake water, and *Oithona davisae* specimens were counted under a stereomicroscope (Wild MDG-17). The body appendages (*i.e*., mandible, maxillule) were removed under a stereomicroscope and mounted on microscope slides for taxonomic identification based on morphological comparisons according to Giesbrecht (1891) and Ferrari and Orsi (1984).

3.3. Results

3.3.1. Seasonal Variations of Environmental Factors

For Lake Faro, the surface water temperature ranged from 12.8°C in January to 29.0°C in August, and a similar range occurred in Lake Ganzirri, 12.5°C to 28.8°C in January and August, respectively (Figure 3). No significant temperature differences were recorded between these two lakes (Student's t-test; $p = 0.89$; n = 24).

For Lake Faro the salinity varied from 26.5 in March to 35.0 in October, while for Lake Ganzirri it varied from 21.3 in April to 28.9 in September. (Figure 4). In general, only salinity showed significant differences between Lakes Faro and Ganzirri (Student's t-test, $p < 0.01$; n = 24).

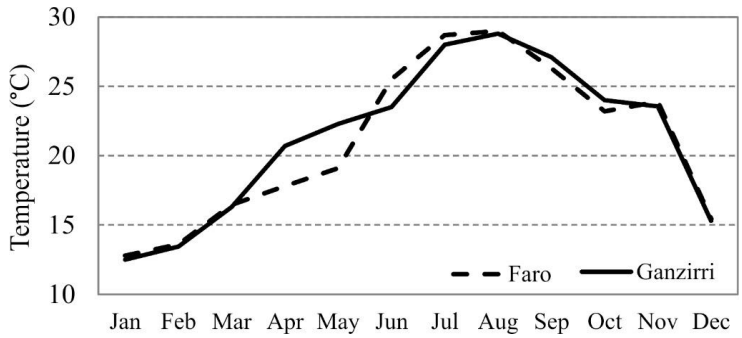

Figure 3. Seasonal variations of surface water temperatures for Lakes Faro and Ganzirri, from January to December 2014.

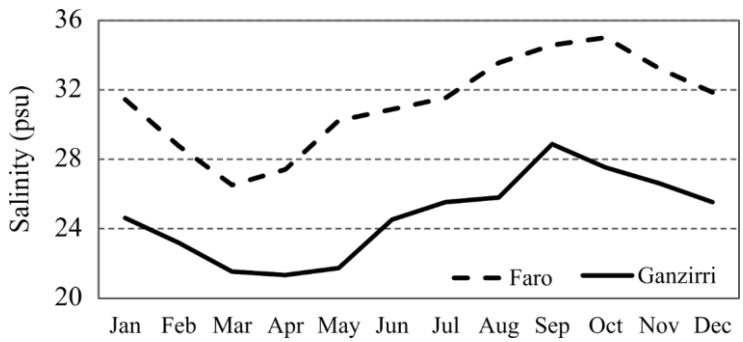

Figure 4. Seasonal variations of surface water salinity for Lakes Faro and Ganzirri, from January to December 2014.

3.3.2. Seasonal Variations in Abundance of Oithona davisae

O. davisae was found in Lakes Faro and Ganzirri during the entire sampling period. Females carrying ovisacs and nauplii were also seen throughout the year, and the two lakes had similar seasonal abundance dynamics. In terms of the mean density of *O. davisae* including adults plus all copepodite stages, abundances at Lake Faro were lowest in winter (January), at 3,312 ind. m^{-3}. From May to June, abundances increased exponentially, peaking in June at 116,512 ind. m^{-3}. Thereafter, from summer to winter, the abundances decreased gradually (Figure 5).

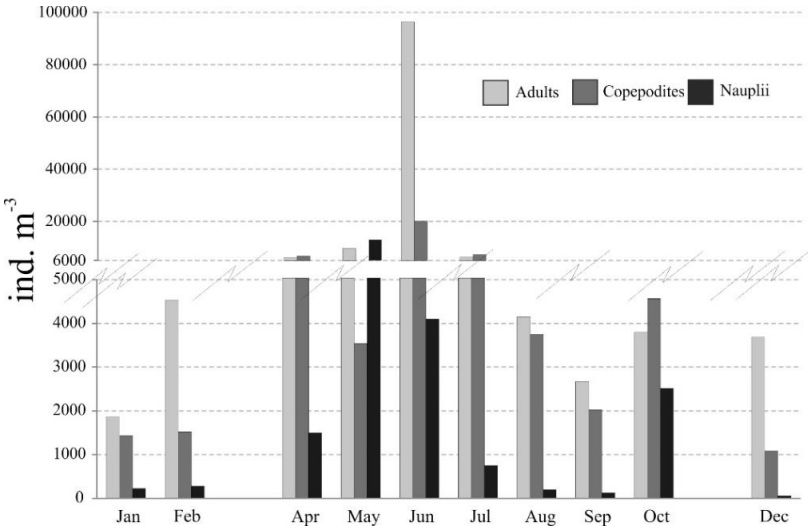

Figure 5. Seasonal variations in the abundance of *Oithona davisae* in Lake Faro.

For Lake Ganzirri, the abundances also increased from winter (970 ind. m^{-3} in January) to spring, when they increased exponentially through May to June, peaking at 211,617 ind. m^{-3}. Abundances remained high all summer, and then decreased rapidly beginning in September, to the lowest for the year in December, at 92.6 ind. m^{-3} (Figure 6).

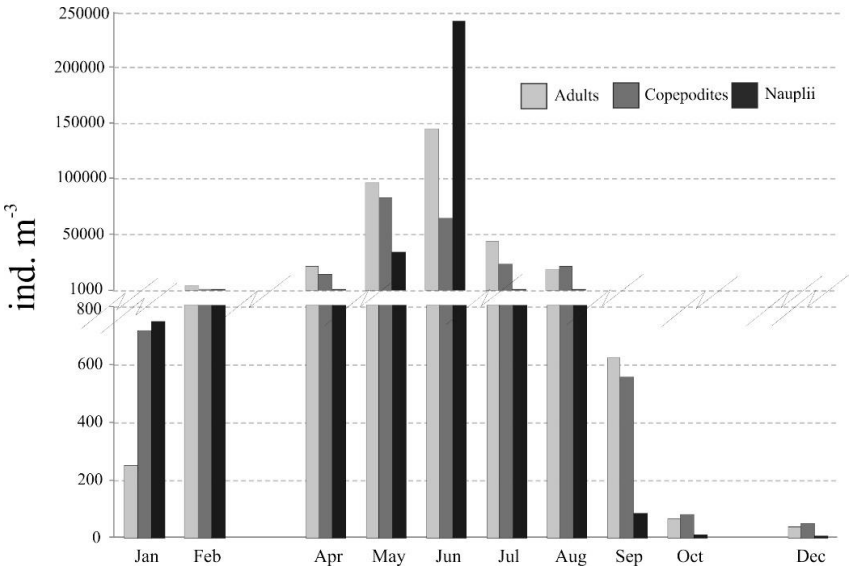

Figure 6. Seasonal variations in the abundance of *Oithona davisae* in Lake Ganzirri.

In both lakes, the naupliar abundances followed similar seasonal trends to the adults and copepodites. The nauplii abundance for Lake Faro peaked in May, at 13,000 ind. m^{-3}, and for Lake Ganzirri in June, at 242,692 ind. m^{-3}. Females always outnumbered males, with annual means of 85.4% females for Lake Faro and 87.2% females for Lake Ganzirri. Ovigerous females occurred throughout the year for both Lakes Faro and Ganzirri, although they showed

greater relative abundance in spring/early summer (28.7%, 30.2%, respectively) than in winter (16.7% and 17.4%, respectively). Overall, the total annual mean *O. davisae* population for Lake Faro (19,141 ± 34,423 ind. m^{-3}) was lower than for Lake Ganzirri (55,420 ± 78,470 ind. m^{-3}), although this difference was not statistically significant (paired Student's t-test, p=0.19; n=20). The contribution of *O. davisae* to the total mean copepod abundance was also lower for Lake Faro compared to Lake Ganzirri (Figure 7). During the periods of peak abundance of *O. davisae*, its relative abundance in terms of the total copepod densities reached 98.3% and 99.6% for Lakes Faro and Ganzirri, respectively.

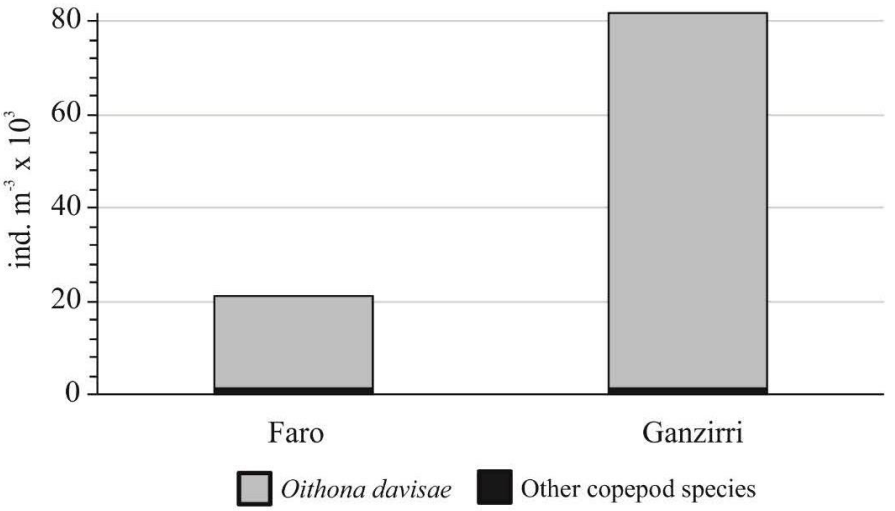

Figure 7. Mean annual contribution of *Oithona davisae* to the total copepod abundance in Lakes Faro and Ganzirri.

CONCLUSION

The passive dispersion of *Oithona davisae* due to synanthropic introduction in many regions of the world has rapidly widened its biogeographical distribution, with a circumglobal, though scattered distribution pattern. *O. davisae* occurs mostly in temperate waters of the boreal hemisphere. It is also found in both cold-temperate latitudes, as the northern Wadden Sea, and austral hemisphere, as southern Pacific Ocean (Chile).

The new records of *O. davisae* in Lakes Faro and Ganzirri confirm that this invasive species is typical of temperate environments. These lakes have physical characteristics similar to those of other locations where this species has been reported. The specimens identified as *Oithona nana* by Zagami and Guglielmo (1995) in these lakes were re-examined and confirmed to be only *O. nana*. In contrast, the specimens identified as *O. nana* and *Oithona brevicornis* by Brugnano (2006); Zagami and Brugnano (2013) and Pansera *et al.* (2014) in Lake Faro showed only a few specimens of *O. nana*, and high numbers of *O. davisae*. All of the *Oithona* specimens collected during the present study were identified as *O. davisae*. It thus appears that *O. davisae* has eliminated *O. nana* in Lakes Faro and Ganzirri.

Differences in abundances between Lakes Faro and Ganzirri can be ascribed to the different sampling methodologies employed: vertical and horizontal tows, respectively. The highest abundances were recorded in the sub-superficial layer (Pansera *et al*. 2014), so the vertical sampling throughout all the water column may have underestimated abundances.

In Lakes Faro and Ganzirri, the sex ratio of *O. davisae* is female skewed, with values close to those reported in literature (Uye and Sano, 1995; Ceballos and Kiørboe, 2011). Females can reproduce throughout the year, and thus across temperatures ranging from 12.5 to 29.0°C. The high abundance peaks of the adults, copepodites and nauplii, the relatively high proportions females carrying ovisacs, and the occurrence of brooding females and nauplii during winter, all indicate that this invasive species has become a permanent component of Lakes Faro and Ganzirri. As reported elsewhere (Uye and Sano, 1995), the specific egg production rate of *O. davisae* increases linearly with increasing temperature, up to ~22 °C, although at temperatures >22 °C this rate no longer increases, and can even decrease.

The density peaks of *O. davisae* in Lakes Faro and Ganzirri were recorded in late spring, when water temperatures were from 23 °C to 25 °C, in agreement with records from other temperate environments (Uye and Sano, 1998; Bollens *et al.*, 2011; Uriarte *et al.*, 2016). These also co-occurred with the seasonal peaks of flagellates, both autotrophic and heterotrophic (Giuffrè and Pezzani, 2005), and ciliates (Saccà and Giuffrè, 2013), so *O. davisae* might have an important role as a predator in the regulation of an eventual dystrophic crisis, due to recurrent summer blooms of flagellates. Conversely, *O. davisae* summer decreases were inversely correlated with temperatures over 25 °C.

Before 1992, *O. davisae* was not recorded from Lakes Faro and Ganzirri (Crisafi *et al.*, 1973; Zagami and Guglielmo, 1995). However, recent studies on the zooplankton community in Lake Faro in 2004-2005 showed gradual decreases eventually resulting in almost total disappearance of *O. nana*, with progressive replacement by *O. davisae*, although the latter was largely misidentified as *O. brevicornis* (Brugnano, 2006; Zagami and Brugnano, 2013). Therefore, *O. davisae* appears to have penetrated into Lake Faro between 1993 and 2004. However, it might have been present in Lake Faro earlier, but the absence of regular zooplankton monitoring in these lakes does not allow pinpointing the year of its introduction. After a few years of coexistence between *O. nana* and *O. davisae*, the non-indigenous *O. davisae* excluded the indigenous *O. nana*, which has not been reported since 2009 (Pansera *et al*., 2014; Zagami, personal observations). The replacement of *O. nana* by *O. davisae* agrees with reports from other locations (Altukhov *et al*., 2014; Isinibilir *et al*., 2016; Uriarte *et al*., 2015).

In Lakes Faro and Ganzirri, the low resilience of the *O. nana* native population to *O. davisae* invasion might have been caused by short anomalous high peaks in the water temperatures. These exceeded 32 °C in both lakes, with Lake Faro also showing upwelling of the bottom anoxic waters during the summer period for some of the years over the last decade. These effects resulted in the death of the entire zooplankton community, and also bivalve cultures (Zagami, personal observations). The resulting temporarily empty niche in the zooplankton community might have favoured *O. davisae* establishment in Lakes Faro and Ganzirri, also enhanced by human activities.

Coastal estuarine and marine environments are heavily subjected to non-indigenous species invasions. Ecological and evolutionary consequences of invasions can be observed at species, community and ecosystem levels. After its introduction, an invader must either find a

niche that is not occupied or it must compete for an occupied niche (Di Castri, 1990). In Lakes Faro and Ganzirri, *O. davisae* has become the most abundant species of the copepod assemblage in a short time. To date, no negative consequences appear to have been reported for the ecosystem functioning of these lakes, apart from the disappearance of the indigenous species *O. nana*. Small proportions of the *O. nana* and *O. davisae* numbers enter the planktonic foodweb as prey for the pelagic fish species *Atherina boyeri*, as seen from gut-content observations (Zagami personal observations), whereas the major proportions of *O. nana* and *O. davisae* are not consumed by pelagic predators, but instead flow into the benthic systems to maintain the microbial loop (Nakamura and Turner, 1997).

Lakes Faro and Ganzirri are shallow-water environments, closed to transoceanic ship traffic. Due to their shallow channels, and the weak *O. davisae* adaptation to the strong currents of the Messina Strait (Mosetti, 1988), it is improbable that this species was introduced by ship ballast waters. Furthermore, *O. davisae* has not been reported in recent samplings of the adjacent Ionian and Tyrrhenian Seas (Zagami, personal observations), and its introduction from adjacent basins is thus highly improbable. In addition, Lake Faro is an important importation centre for living bivalve**s** coming from many Atlantic and Mediterranean sites, thus it is more probable that aquaculture activities are responsible for *O. davisae* introduction. Monitoring of imported molluscs and further samplings in the Messina harbour would provide further evidence in support of this hypothesis. Recently, the identification of new copepod species for Lake Faro (Baviera *et al.*, 2007; Zagami *et al*., 2008; Brugnano *et al*., 2010) and reports of not only plankton species typical of different biogeographic regions (Zagami *et al*., 2005; Cosentino *et al*., 2009; Cosentino and Giacobbe, 2011; Saccà and Giuffrè, 2013; Sabia *et al.* 2014 and 2015) can probably be ascribed to the importation of these molluscs for aquaculture. Indeed the Thau Lagoon, a shallow water system on France's Mediterranean coast, is one of the major hotspots of marine species invasions in the world (Verlaque, 2001), and also an important exportation centre of living bivalves to other aquaculture sites. Consequently, the high numbers of non-indigenous species introduced into Thau Lagoon has a high probability of being transported to other shellfish farming sites in Europe. Thus, it is likely that *O. davisae* was introduced into Lakes Faro and Ganzirri via aquaculture acitivities. The passive dispersion of *O. davisae* by ballast water has already been widely demonstrated (Ferrari and Orsi, 1984; Nishida, 1985; Hirakawa, 1988; Cordell *et al*., 2009; Kaysan, 2010; Lawrence and Cordell, 2010) and the present study represents the first record of the introduction of *O. davisae* through aquaculture.

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Chapter 64

IMPACT OF THE INVASIVE SPECIES *ACARTIA TONSA* ON THE DISTRIBUTION OF AUTOCHTHONOUS ACARTIIDAE SPECIES IN ESTUARIES OF THE BAY OF BISCAY

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ABSTRACT

The impact of the settlement of the invasive copepod species *Acartia tonsa* on the spatial and temporal (seasonal and interannual) distribution of native Acartiidae species in two estuaries of the Bay of Biscay of contrasting morphology, hydrology, and level of pollution, namely the estuaries of Bilbao (polluted) and Urdaibai (weakly polluted), was assessed. Until the arrival of the non-indigenous species *A. tonsa* in 2001-2002 and its occurrence in high density in the inner zone of both estuaries in 2003, *Acartia clausi* dominated the assemblage of Acartiidae species in the estuary of Bilbao, whereas in the estuary of Urdaibai *A. clausi* dominated in the high salinity zone and the brackish species *Acartia bifilosa* in the low salinity zone. The settlement of *A. tonsa* caused (directly or indirectly) the spatial distribution of *A. clausi* to shrink in both estuaries, the latter species being almost driven out from the 30 salinity zone in the estuary of Bilbao. Low oxygen levels in the low salinity zone of the estuary of Bilbao may have helped the settlement of *A. tonsa*, which is more tolerant to hypoxic conditions than *A. clausi*. In the estuary of Urdaibai, which has a wider range of salinity habitats to sustain brackish planktonic copepods, the *A. tonsa* population optimum was at a lower salinity than in the estuary of Bilbao, and there was large overlapping of the spatial and temporal distributions of *A. tonsa* and *A. bifilosa*. Competitive pressure of *A. bifilosa* likely restricted the seasonal

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distribution of *A. tonsa*, and contributed to its decline from 2003 to 2005, a period during which temperature decreased. Conversely, the settlement of *A. tonsa* caused the displacement of the annual maximum of *A. bifilosa* from summer to late spring, as well as its spatial displacement seaward. The impact of *A. tonsa* on other Acartiidae species that are scarce in the estuaries of Bilbao (*Acartia discaudata* and *Acartia margalefi*) and Urdaibai (*Paracartia grani*) could not be clearly assessed. *A. discaudata* and *A. margalefi* almost disappeared when *A. tonsa* settled, but the concomitant deterioration of environmental conditions for the former species argues against the hypothesis of a biotic interaction with *A. tonsa* as the main cause for their decline. In contrast, the summertime species *P. grani* was the only one that showed highest abundance at the same time as *A. tonsa*, likely as a common positive response to temperature increase.

Keywords: *Acartia tonsa*, invasive species, Acartiidae assemblages, estuaries, Bay of Biscay

1. INTRODUCTION

After habitat loss or degradation, biological invasions are the major challenge for the conservation of biodiversity and natural resources (MEA, 2005). Non-indigenous species are able to become invaders because they may displace or replace indigenous species, and affect ecosystem structure and functioning, causing losses of native genotypes, habitat changes, and alterations in community structure, food web properties and ecosystem processes. They can thus prevent the provision of ecosystem services, resulting in negative effects on human health and large economical losses (Grosholz, 2002; Perrings *et al.*, 2002; Wallentinus and Nyberg, 2007; Molnar *et al.*, 2008; Vilà *et al.*, 2010). In a globalized world like today's, the fast increase in trading, travelling and transportation over the last decades has accelerated marine biological invasions, notably in estuaries due to their vulnerability (Nehring, 2006), through maritime transport (e. g. ballast water discharge, biofouling), channel construction for navigation, aquaculture and use of aquariums (Hulme 2009; Katsanevakis *et al.*, 2013).

The Convention on Biological Diversity (CBD, 2000) has recognized the need to compile and spread the information about non-indigenous species which threaten ecosystems, habitats, or native species, in order to use them in a context of prevention and mitigation actions. CBD (2000) has also issued a call to intensify research on the impact of non-indigenous species on biological diversity. In the case of the European Union, the Marine Strategy Framework Directive (EU, 2008) considers non-indigenous species as the main threat to biodiversity and ecosystems health in Europe.

Acartia tonsa is among the listed non-indigenous species that can become invasive in European marine systems and can have a high impact on ecosystems services or diversity, although both positive and negative impacts have been attributed to this species (Katsanevakis *et al.*, 2014). *A. tonsa* competes with native copepods, especially congenerics, and may become dominant in zooplankton communities. It may also modify food webs and trophic flows within invaded ecosystems (Katsanevakis *et al.*, 2014), as it can be a significant prey for pelagic fish (Detwyler and Houde, 1970) and it can control algal blooms (Leppäkoski *et al.*, 2002).

This species has become widespread across the world, but it is reported to be native to American and Indo-Pacific waters (Leppäkoski and Olenin, 2000). *A. tonsa* has been colonising new coastal areas and estuaries by self-propagation and/or anthropogenic

introduction, due mainly to its ability to cross geographic barriers owing to its capacity to develop resistance stages (Belmonte and Potenza, 2001) and to tolerate wide ranges of environmental factors (Holste and Peck, 2006). This species has become a well-known non-indigenous species in European waters since the early 1900s (Brylinski, 1981).

In the Bay of Biscay, *A. tonsa* has been reported since 1983 in the Gironde estuary (David *et al.*, 2007), the largest estuary flowing into this bay. In the small estuaries of the Basque coast, however, it was detected for the first time only in 2001 in the estuary of Bilbao, where it has become the dominant zooplankton calanoid in the inner estuary since 2003 (Aravena *et al.*, 2009). *A. tonsa* was observed for the first time in the nearby estuary of Urdaibai later than in the estuary of Bilbao, in 2003 (Aravena, 2009). In addition, *A. tonsa* has shared from the beginning the occupation of the upper reaches of both estuaries with *Oithona davisae*, a typical coastal and estuarine species that has been found to be a rapid invader of transitional environments in the Mediterranean (Zagami *et al.*, this volume), and since 2010 with *Pseudodiaptomus marinus* in the estuary of Bilbao (Uriarte *et al.*, 2016). The last two non-indigenous species are both native to the Indo-Pacific region (see Mihneva and Stefanova, 2013; Sabia *et al.*, 2015).

In this chapter we aim to show the impact of the arrival of the non-indigenous *A. tonsa* in the estuaries of Bilbao and Urdaibai on the spatial and temporal distribution of the other Acartiidae species inhabiting these areas. Since these two estuaries differ largely in morphological and hydrological properties, as well as in the level of human pressure and system health, we have also dealt with the role of system inherent characteristics in constraining or enhancing the impact of the non-indigenous species on co-occurring Acartiidae species.

2. MATERIAL AND METHODS

2.1. Study Area

The estuary of Bilbao (also known as Ibaizabal-Nerbioi estuary or Nervión estuary; 43º23'N, 03º07'W) and the estuary of Urdaibai (also known as Gernika estuary, Mundaka estuary or Oka estuary; 43º22'N, 02º43'W) are located in the Basque coast (inner Bay of Biscay) (Figure 1). Because of their proximity to each other, they share a temperate-oceanic climate. However, they differ largely in geomorphology, hydrodynamic characteristics, and level of anthropogenic impact, which result in differences in their water environments (Iriarte *et al.*, 2016).

2.1.1. Estuary of Bilbao

The estuary of Bilbao has two clearly different areas: a funnel-like harbour (23 km long, 3.8 km wide (on average) and 10-25 m deep) called Abra, and a man-made channel (15 km long, 50-150 m wide and 2–9 m deep) that extends from this harbour area up to the Ibaizabal river. The estuary is partially mixed in the outer area and highly stratified in the inner area. High salinity waters (>30) usually penetrate up to the inner reaches at the bottom, whilst freshwater flows seaward at surface and is progressively mixed with seawater. This results in a two-layer circulation with landward net flux in bottom layers and seaward net flux in surface layers, and much higher residence time in waters below the halocline (3-12.6 days)

than in waters above the halocline (0.5-3 days) (Uriarte *et al.*, 2014). The original features of the estuary were dramatically modified due to (i) land reclamation for urban, industrial and port developments, and (ii) channelization and dredging works to make navigation easier. As a result of these, the estuary has lost most of its original intertidal areas (Cearreta *et al.*, 2004). By the 1970s, the channelled area was extremely polluted with high heavy metal and organic matter concentrations, and hypoxic/anoxic conditions in waters and sediments (Cearreta *et al.*, 2000), which gave rise to extensive benthic areas devoid of fauna (González-Oreja and Saiz-Salinas, 1998). Since 1979 the estuary is in a rehabilitation process, as a consequence of the industrial decline in the area surrounding the estuary, the treatment of wastewaters from the Metropolitan Area of Bilbao and new environmental protection policies, resulting in a significant decrease in heavy metal, ammonia and organic matter loadings, and an increase in oxygenation and biodiversity (García-Barcina *et al.*, 2006; Borja *et al.*, 2010; Pascual *et al.*, 2012; Villate *et al.*, 2013).

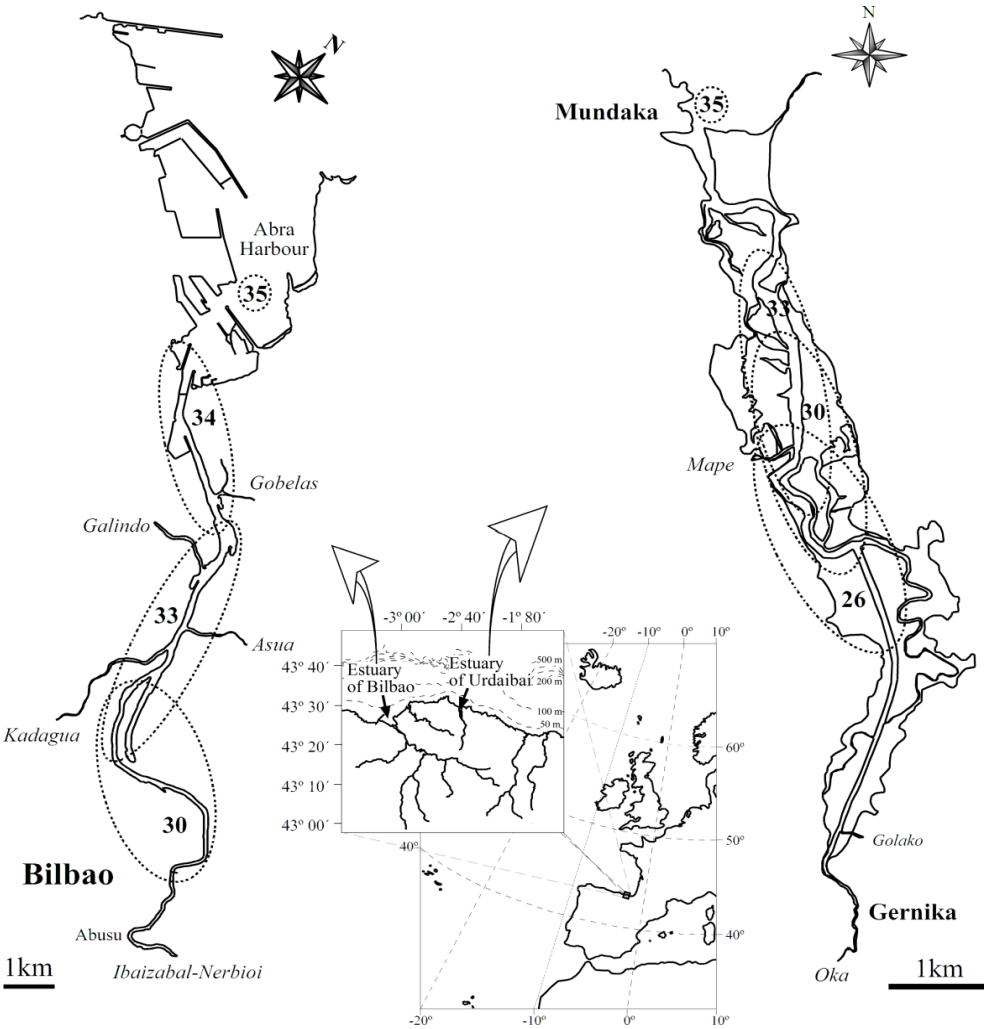

Figure 1. Map and location of the estuaries of Bilbao and Urdaibai, showing the 26, 30, 33, 34 and 35 salinity zones where samplings were carried out in each estuary.

The first plankton studies in this system, carried out around 1980, were restricted to the Abra harbour, where both phytoplankton biomass and neritic zooplankton abundance where shown to strongly decrease toward the inner-eastern zone due to the effect of the polluted estuarine plume coming from the channel mouth (Villate, 1991a and 1994). In these studies, *Acartia clausi* was the only species of Acartiidae found. Later studies initiated in 1996 also included the channelled zone and reported the occurrence in low numbers of two more Acartiidae species, *i.e.*, *Acartia margalefi* and *Acartia discaudata*, which were observed landward from the area occupied by *A. clausi* (Uriarte, 2001).

2.1.2. Estuary of Urdaibai

The estuary of Urdaibai, with a maximum and minimum width of 1.2 km and <20 m in the outer area and the inner channel respectively, is shorter (12.5 km), shallower (mean depth of 3 m) and physically less modified (*e.g.*, less channelized, with less land reclamation) than the estuary of Bilbao. Reed beds and salt marshes at its upper and middle reaches border the central channel, and relatively extensive intertidal flats of mainly muddy (in the inner part) and sandy (in the outer part) sediments occupy the outer half of the estuary. The watershed area is small and river inputs are usually low when compared to the tidal prism. As a consequence, most of the estuary is seawater-dominated at high tide, while a stronger axial gradient of salinity is only found in the upper reaches, where it receives freshwater inputs from its main tributary, the Oka river (Villate *et al.*, 2008). In the outer zone, tidal flushing is so high that waters of salinities >34 are flushed out of the estuary at each tidal cycle. The outer half of the estuary remains well mixed most of the time, while the inner half is partially stratified. In the upper reaches, the estuary receives relatively large amounts of nutrients and organic matter from an old primary waste water treatment plant (Franco *et al.*, 2004). Nevertheless, this estuary is among the healthiest coastal systems of the Basque coast, and constitutes the most valuable natural resource of the Biosphere Reserve of Urdaibai.

The first plankton studies in this system, in the early 1980s, showed the presence of three Acartiidae species segregated along the salinity gradient: the neritic *A. clausi*, dominant in the highest salinity waters; the brackish *Acartia bifilosa*, dominant in <33 salinity waters; and *Paracartia grani* (*Acartia grani* in previous publications), which was less abundant than the former two species, and occurred in an intermediate spatial position (Villate, 1991b and 1995). The abundance of the latter species has been shown to be much lower since 1996 when studies on zooplankton were resumed (Uriarte and Villate, 2005).

2.2. Data Source

Zooplankton and environmental data shown in this chapter come from an ongoing monitoring program carried out in the estuaries of Bilbao and Urdaibai since 1997, and correspond to the time-series of the period 1998–2005, for which zooplankton identification and counting has been completed for all salinity sites in both estuaries at present. Data were obtained from monthly samplings (usually during the last week of the month) conducted at high tide during neap tides at the 35, 34, 33 and 30 salinity zones of the estuary of Bilbao, and at the 35, 33, 30 and 26 salinity zones of the estuary of Urdaibai (Figure 1), using a Lagrangian type of sampling strategy. This consisted in obtaining the biological and environmental information in water masses of a given salinity instead of at spatially fixed

sampling sites (see Kimmerer *et al.*, 1998; Modéran *et al.*, 2010). The salinity zones were selected according to previous studies (*e.g.*, Villate, 1991b; Uriarte, 2001), which allowed to define the main salinity habitats within each estuary on the basis of the distribution of main zooplankton species. Water masses of around 30 salinity constitute the innermost salinity habitat occupied by mesozooplankton copepods in the estuary of Bilbao, while water masses of around 26 salinity represent the optimal inner limit for brackish mesozooplankton copepods in the estuary of Urdaibai (Villate *et al.*, 1993).

Zooplankton samples were obtained from below the halocline by 2–3 min. horizontal tows using a 200 µm mesh size net (mouth diameter 0.25 m) equipped with a Digital Flowmeter, and preserved in 4% buffered formalin. Zooplankton counting and identification to the lowest possible taxonomic level was made under a stereomicroscope Olympus IX70. In Acartiidae species, female, male and copepodite stages were distinguished, but for the purposes of the chapter only the total abundance of each species was taken into account. Vertical profiles of salinity, water temperature and dissolved oxygen saturation (DOS) were measured *in situ* using a WTW LF 197 thermosalinometer and a YSI 55 oxymeter, but only data from the depth of zooplankton sampling are presented in this chapter. Water samples were collected also at the depth of zooplankton sampling using a Niskin bottle for measuring chlorophyll *a* (Chl *a*), which was determined spectrophotometrically in triplicate samples according to the monochromatic method with acidification (Jeffrey and Mantoura, 1997). Secchi disk depths (SDDs) were also recorded to assess water turbidity at each sampling site.

Salinity stratification was calculated from salinity profiles obtained at each sampling site in each sampling day as the maximum difference in salinity at 0.5 m depth intervals in the water column (Δ salinity). This calculation has been put forward as an index to reflect the sharpening of the salinity gradient associated to the narrowing of the halocline layer, and was found to explain dissolved oxygen saturation dynamics below the halocline in the estuary of Bilbao better than a stratification index calculated from the difference between bottom and surface salinities (Villate *et al.*, 2013).

River flow data for the Ibaizabal-Nerbioi river (main tributary of the estuary of Bilbao) and for the Oka river (main tributary of the estuary of Urdaibai) were obtained from the Provincial Council of Bizkaia. In the case of the Oka river, missing values were filled with values obtained using a regression model ($y = 0.3892x$, $R^2 = 0.9333$) performed with data from the nearest station (Oleta (LE02) hydro-meteorological station) with a complete series. Daily values of river flow were averaged for each month.

2.3. Data Treatment

The impact of *A. tonsa* and the effect of environmental variables on Acartiidae species were assessed. To that purpose, the spatial (in relation to salinity) and temporal (seasonal and interannual) variations of abundance of all Acartiidae species, together with the niche breadth and overlap based on distribution of the dominant species (*A. clausi, A. bifilosa* and *A. tonsa*) were compared for three periods in the 1998-2005 data time series. The first two periods correspond to years prior to the large increase of *A. tonsa* abundance, but with different environmental conditions, *i.e.*, the 1998-2000 and the 2001-2002 periods. The third period extends from 2003 to 2005 and was characterized by the dominance of *A. tonsa* in the low

salinity waters of both estuaries at least during one month of the year. Prior to data analysis, Acartiidae species abundances were log-transformed (log (x+1)).

Principal components analysis (PCA) was used to assess the influence of environmental factors on spatial and seasonal changes in Acartiidae species abundance. The eigenvectors allowed identification of the variables contributing most to the principal components of environmental variability. The three first principal components were used to describe the main environmental gradients in each estuary. The plot of Acartiidae species within the space configured by such environmental gradients was used to describe the segregation of Acartiidae populations along environmental gradients and determine the ecological niche of these species in each estuary.

Data normality was tested by means of the Kolmogorov-Smirnov test. Data comparisons were carried out by Kruskal-Wallis and Mann-Whitney U tests. Relationships between variables were assessed by Spearman rank order correlations and regressions (linear and quadratic).

Spatial (salinity sites) and seasonal (monthly) uniformity of distribution of individuals of the dominant Acartiidae species for each time period was estimated by Levins' standardized niche breadth (B_A), suggested by Hurlbert (1978):

$$B_A = \frac{B-1}{n-1} \text{ being } B = \frac{1}{\sum p_j^2} \tag{1}$$

where:

B_A = Levins' standardized niche breadth
n = number of possible resource states
B = Levins' measure of niche breadth
p_j = proportion of individuals found in or using resource state j

Additionally, to measure spatial (salinity sites) and seasonal (monthly) niche overlap between *A. tonsa* and *A. clausi* in the estuary of Bilbao, and *A. tonsa*, *A. clausi* and *A. bifilosa* in the estuary of Urdaibai for each time period, the simplified Morisita index proposed by Horn (1966) was calculated:

$$C_H = \frac{2\sum_i^n p_{ij} p_{ik}}{\sum_i^n p_{ij}^2 + \sum_i^n p_{ik}^2} \tag{2}$$

where:

C_H = simplified Morisita index of overlap between species j and species k
p_{ij} = proportion of individuals *i* of species *j* found in resource state
p_{ik} = proportion of individuals *i* of species *k* found in resource state
n = total number of resource states

3. RESULTS

3.1. Environmental Conditions

Table 1 summarizes the abiotic and biotic data considered to describe and compare the environmental conditions in the estuaries of Bilbao and Urdaibai during the 1998-2005 study period. None of the variables showed normal distribution (Kolmogorov-Smirnov; $p < 0.001$).

The variations of river flow and salinity stratification index were similar in both estuaries (Figure 2), showing the typical seasonal patterns of river flow and stratification, with highest values in late autumn-winter and lowest values in summer. However, a clear anomaly in the autumn-winter period of 2001-2002 occurred, when river flow and salinity stratification were unusually low. In addition, summer river discharge and stratification were higher (Kruskal-Wallis test; $p = 0.005$) in 2002 than in the other years of the series. Between-estuary differences were evident in terms of fresh water discharges into the estuary and degree of stratification, both being much higher in the estuary of Bilbao than in Urdaibai (Table 1) due to the characteristics of the tributaries (much extensive watershed in the estuary of Bilbao) and morphological features of the estuarine basin (deeper and channelized in the estuary of Bilbao).

Table 1. Mean (± standard deviation, SD), minimum and maximum values of environmental variables obtained for the estuaries of Bilbao and Urdaibai during 1998-2005. *P*-values of Mann-Whitney U tests for between-estuary differences are also shown

	Estuary of Bilbao			Estuary of Urdaibai			
	Mean±SD	Min.	Max.	Mean±SD	Min.	Max.	*p*
River flow (m^3 s^{-1})	21.48±19.83	2.57	74.56	0.64±0.55	0.06	2.08	<0.001
Stratification (Δ salinity)	7.67±5.92	0.10	31.90	2.28±3.55	0.00	20.50	<0.001
Salinity	32.92±1.84	28.10	35.50	30.99±3.40	23.30	35.60	<0.001
Temperature (ºC)	16.08±3.32	10.20	24.20	16.34±4.36	8.00	26.30	0.947
DOS (%)	72.01±31.33	0.30	152.90	86.95±16.93	29.80	137.60	<0.001
SDD (m)	1.79±1.45	0.20	11.00	3.10±2.56	0.20	8.00	<0.001
Chl *a* (μg l^{-1})	3.02±4.53	0.00	31.33	2.53±3.59	0.13	25.78	0.583

Figure 3 shows the spatiotemporal variation of abiotic (salinity, temperature, DOS and SDD) factors and chlorophyll *a* (a variable that indicates food availability for zooplankton) () in the two estuaries during the period 1998-2005. Salinity values were almost constant at each sampling site over the study period as a result of the Lagrangian-type of sampling method. Salinity ranged from 35.5 at the 35 salinity site to 28.1 at the 30 salinity site in the estuary of Bilbao, and from 35.6 at the 35 salinity site to 23.3 at the 26 salinity site in the estuary of Urdaibai. Temperature showed the typical seasonal pattern in temperate areas with summer maxima and winter minima (Figure 3 and Table 1), but with a wider range of variation in low salinity waters (14.1ºC and 18.3ºC in the estuary of Bilbao and Urdaibai, respectively) than in high salinity ones (11.2ºC and 14.3ºC in the estuary of Bilbao and Urdaibai, respectively).

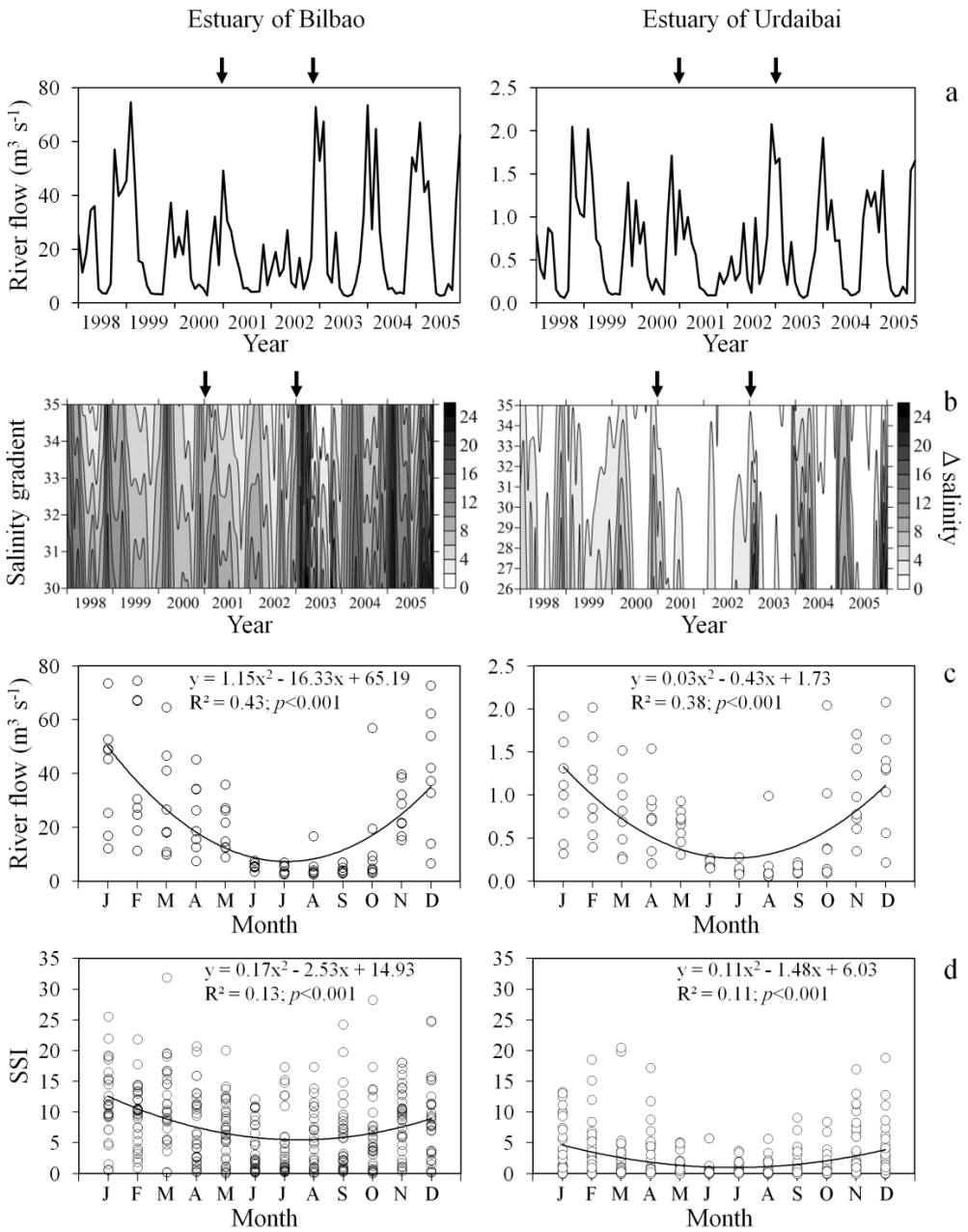

Figure 2. (a) Temporal variations of the monthly mean river flow ($m^3 s^{-1}$) of the main tributaries of the estuary of Bilbao (Ibaizabal-Nerbioi river) and Urdaibai (Oka river), and (b) spatiotemporal variations of the salinity stratification (Δ salinity) along the salinity gradient of the estuaries of Bilbao and Urdaibai, for the 1998-2005 period. Arrows indicate the division between the three periods distinguished in the series. (c) Seasonal pattern of the river flow ($m^3 s^{-1}$) of the main tributaries of the estuary of Bilbao (Ibaizabal-Nerbioi river) and Urdaibai (Oka river) and (d) seasonal pattern of the salinity stratification index (SSI) in the estuaries of Bilbao and Urdaibai. Results of the quadratic regressions are showed.

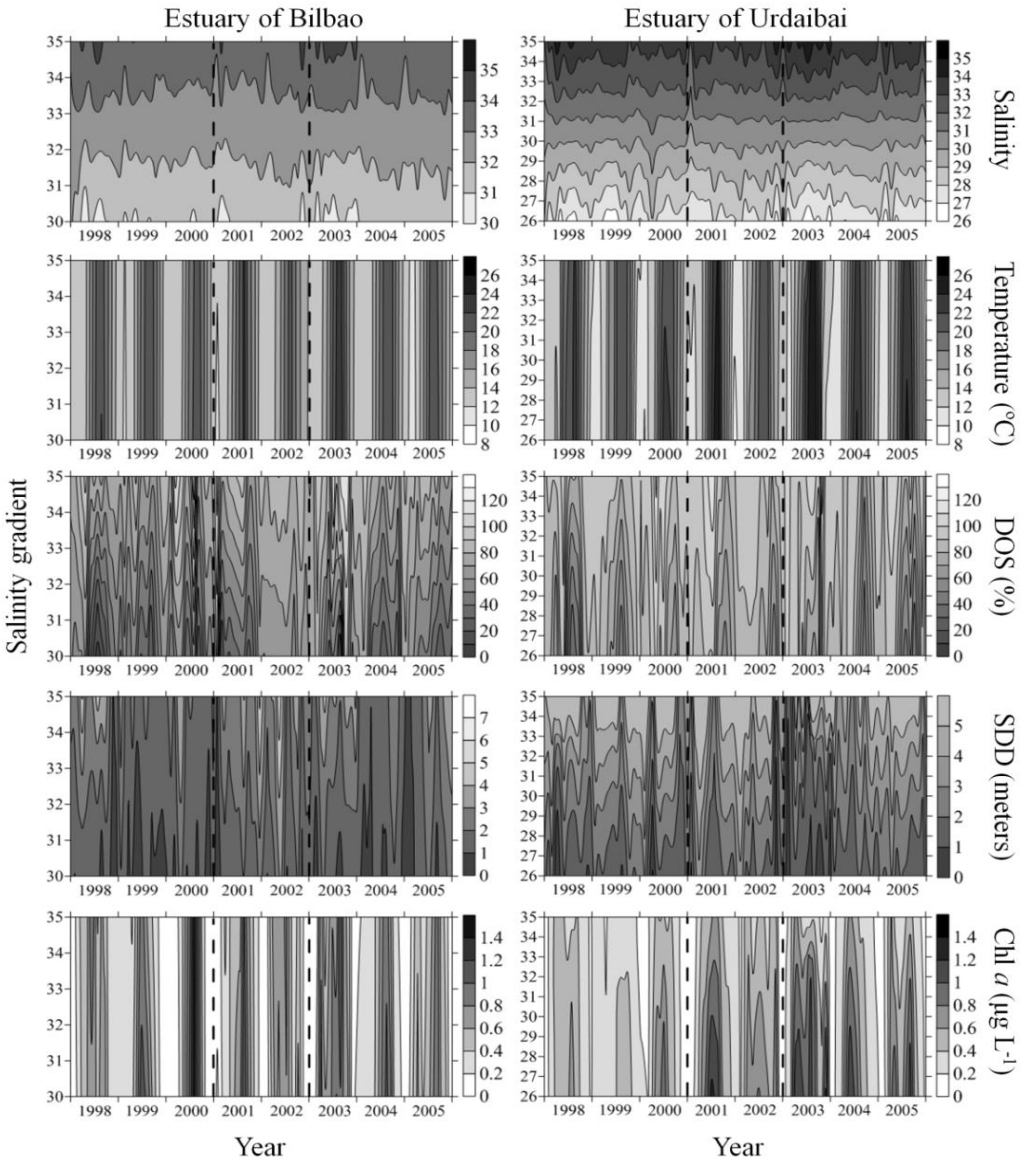

Figure 3. Spatiotemporal variations of salinity, temperature (ºC), dissolved oxygen saturation (DOS; %), Secchi disk depth (SDD; meters) and concentration of chlorophyll *a* (µg L^{-1}) along the salinity gradient of the estuaries of Bilbao and Urdaibai for the 1998-2005 period. Vertical dashed lines indicate the limits between the three periods distinguished in the series.

Between-year differences were similar in both estuaries. In the period 1998-2000 temperature showed smaller differences between years (range of variation – maximum temperatures: 0.7°C and 1.6°C; minimum temperatures: 1.2°C and 1.6°C, in the estuaries of Bilbao and Urdaibai, respectively) than in the second period (range of variation – maximum temperatures: 2.1°C and 4.2°C; minimum temperatures: 1.7°C and 3.6°C, in the estuaries of Bilbao and Urdaibai, respectively), the latter being characterized by an increase of summer temperatures in 2001 followed by a decrease in 2002. The highest summer temperatures of the series were found in the last period, with maximum values of 24.2°C and 26.3°C in the

estuaries of Bilbao and Urdaibai, respectively, in 2003. DOS showed higher values and lower differences between salinities in the estuary of Urdaibai (with a range from 29.8% to 137.6%) than in the estuary of Bilbao (with a range from 0.3% to 152.9%). DOS values were more homogenous in the estuary of Urdaibai than in the estuary of Bilbao. Hypoxic conditions (<30% of DOS) were frequent during the warm season in the 30 salinity site of the estuary of Bilbao in the first (1998-2000) and last (2003-2005) periods. In the middle period, at the lowest salinity site of this estuary the highest values of DOS were observed in 2002. Turbidity increased, in general, with decreasing salinity in both estuaries (Spearman rank correlation: R = -0.603, $p < 0.001$ in the estuary of Bilbao and R = -0.698, $p < 0.001$ in the estuary of Urdaibai), but was clearly higher in the estuary of Bilbao (Table 1). Chlorophyll *a* showed a clear decreasing trend with increasing salinity in the estuary of Urdaibai (Spearman rank correlation: R = -0.309, $p < 0.001$), whereas in the estuary of Bilbao its distribution was rather uniform along the salinity gradient (Spearman rank correlation: R = -0.094, $p = 0.065$) and showed seasonally well defined cycles with maxima in summer. In addition, chlorophyll *a* fluctuated from year to year without a clear trend in the estuary of Bilbao ($R^2 = 5\times10^{-5}$, $p = 0.881$), but showed a slight but significant increasing trend over the study period in the estuary of Urdaibai ($R^2 = 0.029$, $p = 0.001$).

3.2. Spatial and Temporal Distribution of Acartiidae Species

The densities of Acartiidae species (*Acartia clausi*, *Acartia discaudata*, *Acartia margalefi*, *Paracartia grani*, *Acartia bifilosa* and *Acartia tonsa*) in the estuaries of Bilbao and Urdaibai in each salinity zone and in the three periods distinguished in the time series are summarized in Table 2 and Table 3, respectively.

Table 2. Mean±standard deviation of the density (ind. m^{-3}) of the Acartiidae species at the 35, 34, 33 and 30 salinity sites in the estuary of Bilbao and the 35, 33, 30 and 26 salinity sites in the estuary of Urdaibai

	Estuary of Bilbao			
Species	35	34	33	30
A. clausi	793.7±1267.5	777.6±1563.4	213.6±539.4	50.4±120.1
A. discaudata	3.1±9.2	9.2±32.3	3.7±12.9	0.6±2.3
A. margalefi	0.5±1.9	5.8±20.1	4.4±13.1	3.1±12.1
P. grani	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
A. bifilosa	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
A. tonsa	64.9±617.7	29.5±158.5	318.8±1649.3	618.2±2379.4
	Estuary of Urdaibai			
Species	35	33	30	26
A. clausi	684.4±1571.2	372.9±2488.7	47.2±103.6	106.5±891.1
A. discaudata	0.3±2.3	0.1±1.0	0.1±1.1	0.03±0.3
A. margalefi	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
P. grani	0.03±0.3	4.5±28.6	6.3±46.9	0.2±2.1
A. bifilosa	4.2±20.4	258.5±743.6	1541.1±6392.2	1380.0±3175.9
A. tonsa	0.0±0.0	37.3±159.5	131.4±778.1	1251.5±10289.8

Table 3. Mean±standard deviation of the density (ind. m^{-3}) of the Acartiidae species of the estuaries of Bilbao and Urdaibai for each of the three periods distinguished in the time series

	Estuary of Bilbao		
Species	1998-2000	2001-2002	2003-2005
A. clausi	442.6±1159.9	569.6±1290.1	392.5±834.0
A. discaudata	3.8±22.1	6.9±18.9	2.5±12.0
A. margalefi	2.9±15.2	8.3±18.4	0.7±3.4
P. grani	0.0±0.0	0.0±0.0	0.0±0.0
A. bifilosa	0.0±0.0	0.0±0.0	0.0±0.0
A. tonsa	0.0±0.0	0.9±4.4	700.1±2407.7
	Estuary of Urdaibai		
Species	1998-2000	2001-2002	2003-2005
A. clausi	343.7±2159.7	322.9±1325.5	248.4±789.6
A. discaudata	0.1±0.9	0.2±2.1	0.1±1.1
A. margalefi	0.0±0.0	0.0±0.0	0.0±0.0
P. grani	0.03±0.3	0.03±0.02	7.4±44.7
A. bifilosa	1335.3±5651.1	137.8±544.8	695.4±1644.4
A. tonsa	0.0±0.0	0.0±0.0	946.8±8421.4

The dominant neritic species *A. clausi* showed a decrease of abundance with decreasing salinity in both estuaries (Spearman rank correlation: R = 0.425, $p < 0.001$ in the estuary of Bilbao and R = 0.410, $p < 0.001$ in the estuary of Urdaibai) (Figures 4 and 5). In addition, the seasonal cycle of this species showed the annual maximum earlier (in March) and was more constrained to the spring period in the estuary of Urdaibai than in the estuary of Bilbao, where it peaked between April (during the first and the third period) and June (in the second period) (Figure 6). Throughout the study period, *A. clausi* fluctuated from year to year without a clear temporal trend until 2003 in both estuaries, but showed a clear decrease during the last period (2003-2005) in waters of salinities below 35 (Kruskal-Wallis test; $p < 0.001$, for both estuaries), this decrease being more evident at the lowest salinity zone of the estuary of Bilbao (Figure 4).

A. discaudata and *A. margalefi* appeared in low abundances in the estuary of Bilbao during the first period (1998-2000). They reached highest densities, mainly *A. margalefi*, during the second period, in 2002, and were rarely observed since 2003 (Figure 4). In most of the years, *A. discaudata* and *A. margalefi* showed the highest density in late winter-early spring (February-March), although the seasonal distribution of both species was extended throughout the year during the second period, in 2002, when secondary peaks in early summer or autumn were also observed (Figure 6). Spatially, both species peaked at lower salinities than *A. clausi*, but the distribution of *A. discaudata* in the estuary skewed toward high salinity, whereas that of *A. margalefi* skewed toward low salinity (Figures 4 and 5). *A. discaudata* was also found in the estuary of Urdaibai, but only in one occasion during each period of the series (Figure 4).

P. grani was only recorded in the estuary of Urdaibai: in the first two periods it was found occasionally, but in the last period it was found in relatively high abundance in 2003 and in much lower abundance in 2004 (Figure 4). Seasonally, the occurrence of *P. grani* was restricted to the warmest period, mainly to July and August (Figure 6), showing a spatial preference for intermediate salinity (33-30) sites (Figure 5).

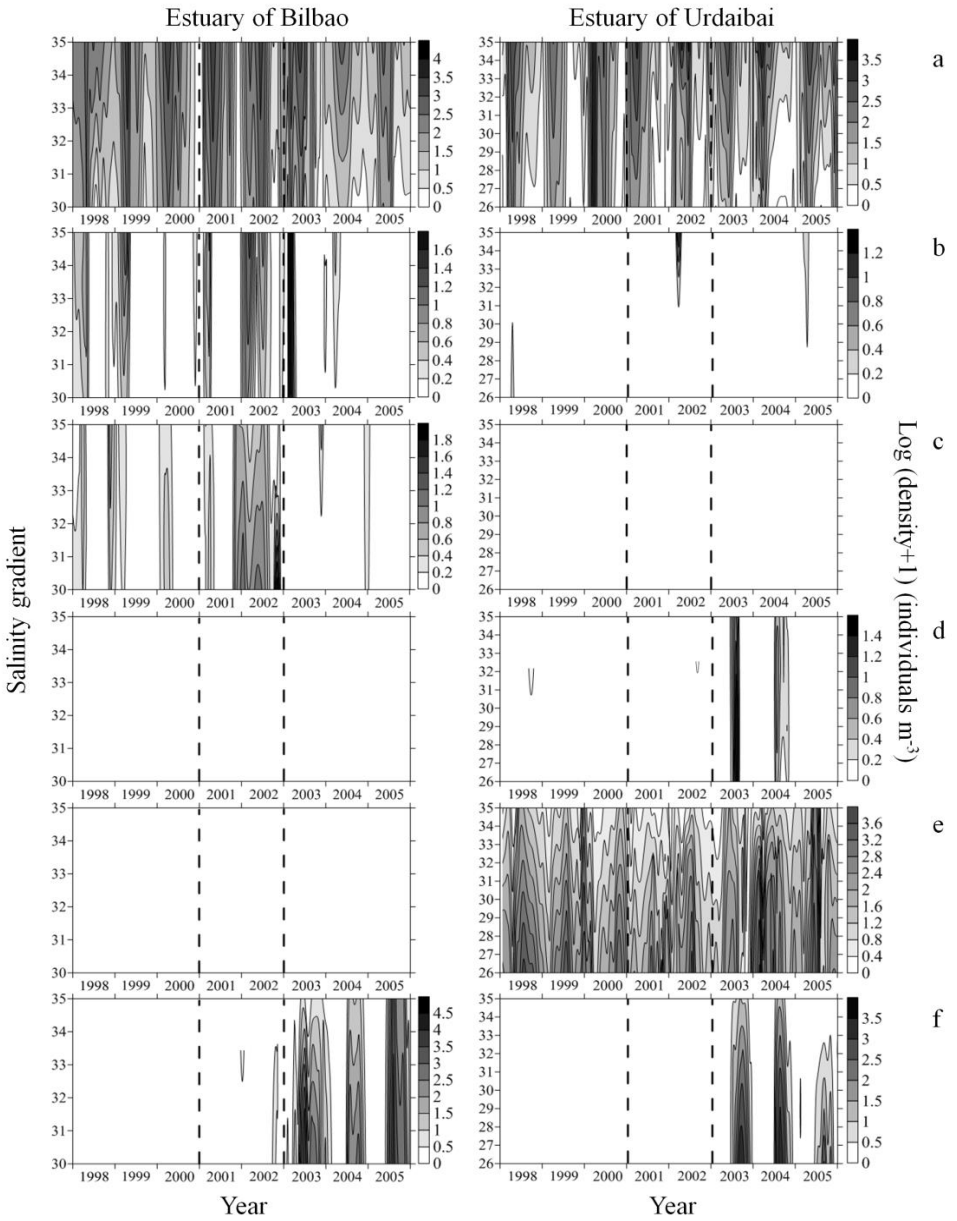

Figure 4. Spatiotemporal distribution of the density (log (x+1), individuals m^{-3}) of (a) *Acartia clausi*, (b) *Acartia discaudata*, (c) *Acartia margalefi*, (d) *Paracartia grani*, (e) *Acartia bifilosa* and (f) *Acartia tonsa* along the salinity gradient of the estuaries of Bilbao and Urdaibai from 1998 to 2005. Vertical dashed lines indicate the division between the three periods distinguished in the time series.

The brackish species *A. bifilosa* was only observed in the estuary of Urdaibai, where it dominated the assemblage of Acartiidae species in low salinity waters (Figures 4 and 5). *A. bifilosa* showed a rather variable seasonal pattern during the period of study (Figure 6) and marked interannual variations, with lowest abundances and atypical seasonal distributions in the second period (Figure 4). The seasonal increase and annual maximum were found to occur earlier in the last period, from 2003 to 2005.

A. tonsa was recorded for the first time in autumn 2001 in the estuary of Bilbao, and from 2003 onward it became very abundant in this system (Figure 4). In the estuary of Urdaibai it also occurred in high density in 2003, but in contrast with observations in the estuary of Bilbao it showed a marked decrease in 2005. The population of this species decreased with increasing salinity in both systems (Spearman rank correlation: R = -0.368, $p < 0.001$ in the estuary of Bilbao and R = -0.294, $p < 0.001$ in the estuary of Urdaibai), where the highest densities were observed at the lowest salinity sites of 30 and 26 in the estuaries of Bilbao and Urdaibai, respectively (Figure 5). Seasonally, *A. tonsa* showed maxima in the warm period, but with a wider seasonal distribution in the estuary of Bilbao (Figure 6).

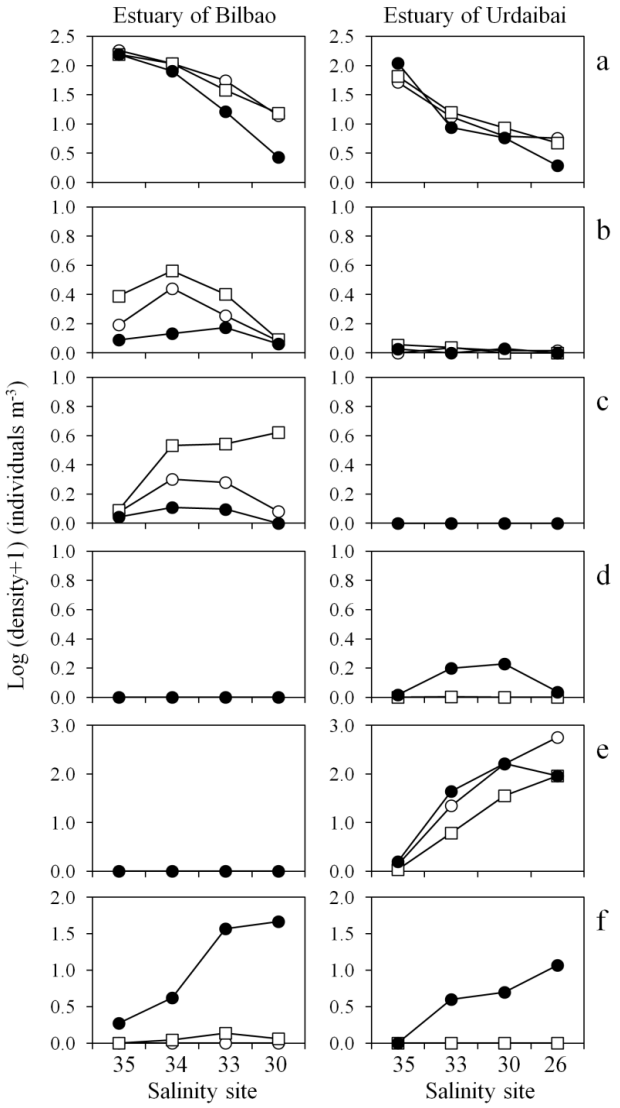

Figure 5. Mean density (log (x+1), individuals m^{-3}) values of (a) *Acartia clausi*, (b) *Acartia discaudata*, (c) *Acartia margalefi*, (d) *Paracartia grani*, (e) *Acartia bifilosa* and (f) *Acartia tonsa* at the 35, 34, 33 and 30 salinity sites in the estuary of Bilbao and the 35, 33, 30 and 26 salinity sites in the estuary of Urdaibai for the three different periods of the series: 1998-2000 (open circles), 2001-2002 (open squares) and 2003-2005 (full circles).

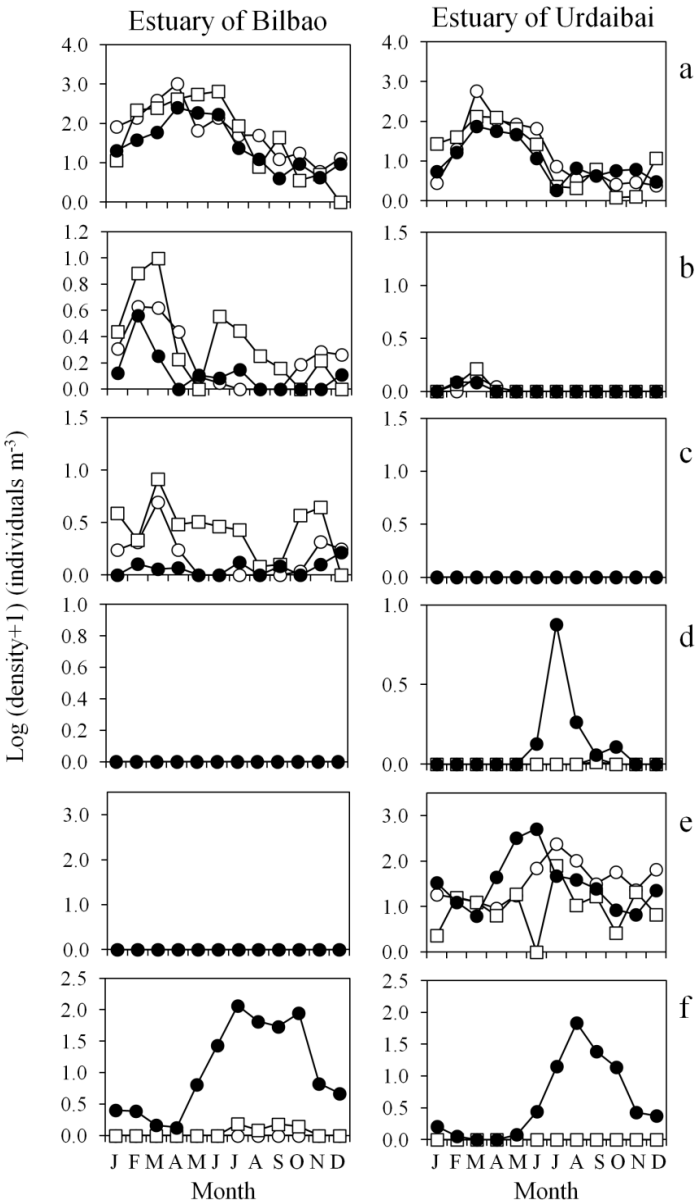

Figure 6. Monthly mean density (log (x+1), individuals m^{-3}) values of (a) *Acartia clausi*, (b) *Acartia discaudata*, (c) *Acartia margalefi*, (d) *Paracartia grani*, (e) *Acartia bifilosa* and (f) *Acartia tonsa* in the estuaries of Bilbao and Urdaibai for the three different periods of the series: 1998-2000 (open circles), 2001-2002 (open squares) and 2003-2005 (full circles).

3.3. Species Segregation in Environmental Gradients

Three main modes of environmental variation were extracted by the PCA of environmental variables in each estuary (Table 4). The first one was mainly explained by salinity, DOS and SDD in opposition to stratification in the estuary of Bilbao (37.07% of

variance), and by salinity and SDD in opposition to stratification in the estuary of Urdaibai (30.85% of variance), reflecting the spatial gradient of salinity in both estuaries. The second one was mainly explained by river flow in opposition to temperature in the estuary of Bilbao (27.51% of variance) and by temperature and chlorophyll *a* in the estuary of Urdaibai (22.35% of variance), while the third one was mainly explained by chlorophyll *a* in the estuary of Bilbao (14.94% of variance) and by river flow in opposition to DOS in the estuary of Urdaibai (17.38% of variance). Both the second and the third factors reflected temporal environmental variations.

The plot of species abundance in the space configured by the three main factors of environmental variability in the two estuaries showed the spatial and temporal segregation pattern of Acartiidae species along environmental gradients (Figure 7). In the estuary of Bilbao, the segregation of species along the spatial gradient determined by salinity, DOS, stratification and turbidity showed the succession *A. clausi – A. discaudata – A. margalefi – A. tonsa* from the outer to the inner estuary; however, *A. clausi* and *A. tonsa* showed a wider distribution along this gradient than *A. discaudata* and *A. margalefi*. Along the seasonal gradient related to river flow and temperature, *A. clausi*, *A. discaudata* and *A. margalefi* were all similarly positioned under conditions of higher river flow and lower temperature than *A. tonsa*, which was more restricted to low river flow and high temperature conditions. By contrast, *A. clausi* showed a wider distribution along this environmental gradient than the other three species. Similarly, *A. clausi* had a wider distribution than the other congeneric species along the trophic gradient accounted for by chlorophyll *a* concentration.

Table 4. Loadings of environmental variables for the three main rotated components extracted by PCA in the estuaries of Bilbao and Urdaibai. Percentage of variance explained by each component is shown in parentheses

	Estuary of Bilbao			**Estuary of Urdaibai**		
	Component			Component		
	1 (37.07%)	2 (27.51%)	3 (14.94%)	1 (30.85%)	2 (22.35%)	3 (17.38%)
River flow ($m^3\ s^{-1}$)	-0.049	**0.896**	-0.080	0.022	-0.225	**0.828**
Stratification (Δ salinity)	**-0.737**	0.431	-0.058	**-0.676**	-0.291	0.165
Salinity	**0.895**	0.041	-0.042	**0.874**	-0.143	-0.035
Temperature (ºC)	0.004	**-0.830**	0.363	0.140	**0.919**	0.081
DOS (%)	**0.895**	0.238	0.151	0.369	-0.348	**-0.668**
SDD (m)	**0.669**	-0.263	-0.355	**0.811**	-0.180	-0.059
Chl *a* ($\mu g\ l^{-1}$)	0.005	-0.349	**0.868**	-0.353	**0.640**	-0.214

In the estuary of Urdaibai, the segregation of species along the spatial gradient determined by salinity, turbidity and stratification showed the succession *A. clausi – A. discaudata – P. grani – A. bifilosa – A. tonsa* from the outer to the inner estuary. The spatial segregation between the outermost species *A. clausi* and the innermost species *A. tonsa* was more marked than in the estuary of Bilbao. Along the environmental gradient driven mainly by temperature and secondarily by chlorophyll *a* concentration, *A. bifilosa* showed the widest distribution, whereas more restricted distributions and a clear segregation along temperature gradients were found for *P. grani* and *A. tonsa*, associated to highest temperatures, and *A. discaudata*, associated to low temperatures.

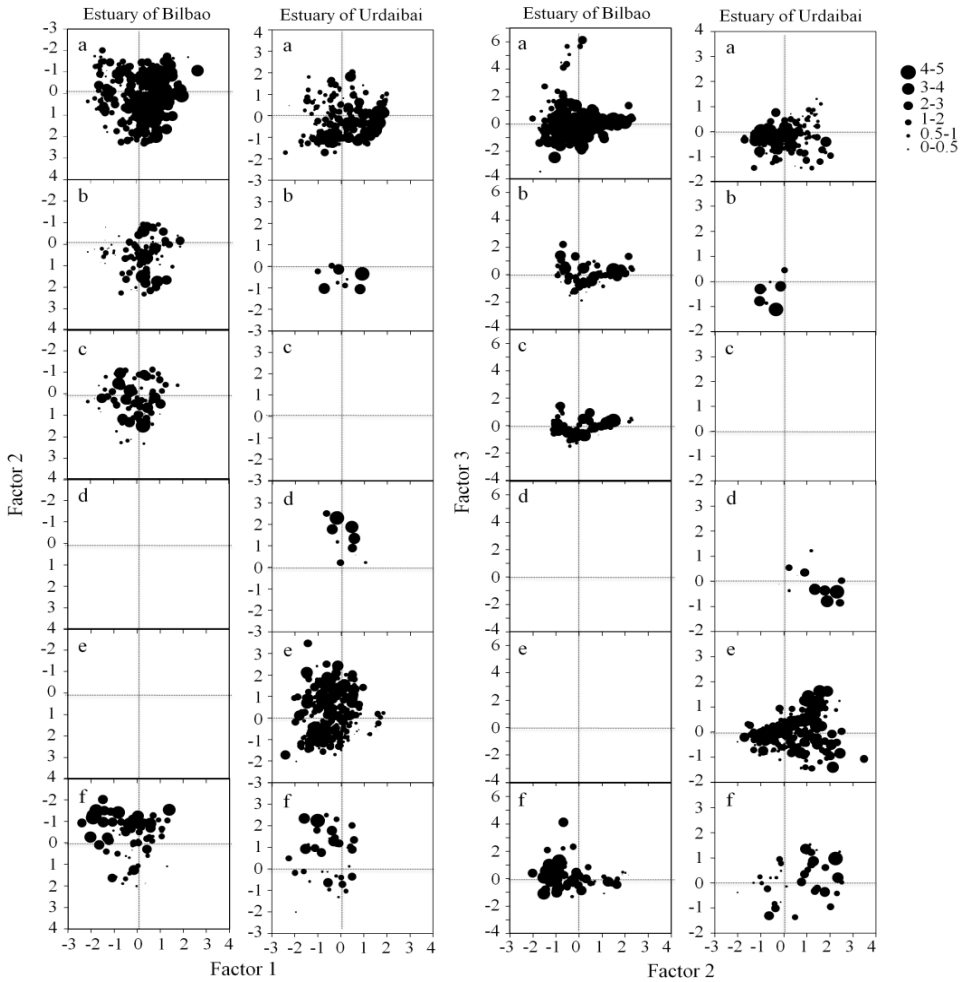

Figure 7. Plot of density values (log (x+1), individuals m^{-3}) of (a) *Acartia clausi*, (b) *Acartia discaudata*, (c) *Acartia margalefi*, (d) *Paracartia grani*, (e) *Acartia bifilosa* and (f) *Acartia tonsa* in the space configured by the first and second factors (left figures) and the second and third factors (right figures) of environmental variability in the estuaries of Bilbao and Urdaibai during the 1998-2005 period.

3.4. Niche Breadth and Overlap

The niche breadths estimated in relation to salinity sites (spatial) and months (seasonal) of the three dominant species *A. clausi*, *A. bifilosa* and *A. tonsa* in the estuaries of Bilbao and Urdaibai for each of the three periods distinguished in the time series are shown in Table 5.

The spatial niche of *A. clausi* was reduced from the first two periods to the third in both estuaries. The spatial niche breadth of *A. bifilosa* in the estuary of Urdaibai did not differ largely between periods, but it was lowest in the second period, this coinciding with the highest spatial niche breadth of *A. clausi*. In the third period, when *A. tonsa* was established in both estuaries, the spatial niche breadth of *A. tonsa* was smaller than of *A. clausi* in the estuary of Bilbao and than of *A. bifilosa* in the estuary of Urdaibai.

Table 5. Levin's niche breadth (B_A) of *Acartia clausi*, *Acartia bifilosa* and *Acartia tonsa* in relation to salinity (spatial) and months (seasonal) in the estuaries of Bilbao and Urdaibai for each of the three periods distinguished in the time series

	Period	Niche breadth (B_A)					
		Spatial			Seasonal		
		A. clausi	*A. bifilosa*	*A. tonsa*	*A. clausi*	*A. bifilosa*	*A. tonsa*
Estuary of Bilbao	1998-2000	0.997	---	---	0.985	---	---
	2001-2002	0.960	---	---	0.951	---	---
	2003-2005	0.706	---	0.652	0.887	---	0.687
Estuary of Urdaibai	1998-2000	0.501	0.702	---	0.771	0.878	---
	2001-2002	0.622	0.607	---	0.763	0.876	---
	2003-2005	0.273	0.679	0.539	0.870	0.916	0.451

Table 6. Simplified Morisita Index for spatial niche overlap (C_H), in relation to salinity (spatial) and months (seasonal) between *Acartia clausi* ($_c$), *Acartia bifilosa* ($_b$) and *Acartia tonsa* ($_t$) in the estuaries of Bilbao and Urdaibai for each of the three periods distinguished in the time series

	Period	Niche overlap (C_H)					
		Spatial			Temporal		
		C_{Hcb}	C_{Hct}	C_{Hbt}	C_{Hcb}	C_{Hct}	C_{Hbt}
Estuary of Bilbao	1998-2000	---	---	---	---	---	---
	2001-2002	---	---	---	---	---	---
	2003-2005	---	0.53	---	---	0.64	---
Estuary of Urdaibai	1998-2000	0.41	---	---	0.68	---	---
	2001-2002	0.42	---	---	0.71	---	---
	2003-2005	0.22	0.16	0.95	0.87	0.53	0.52

The seasonal niche of *A. clausi* diminished from the first two periods to the third in the estuary of Bilbao, the niche breadth in the third period being higher than that of *A. tonsa*. In the estuary of Urdaibai, however, the seasonal niche of both *A. clausi* and *A. bifilosa* expanded in the third period, when the niche breadth of *A. tonsa* was much lower than those of the two former species.

Results of niche overlap between the three dominant species *A. clausi*, *A. bifilosa* and *A. tonsa* in the estuaries of Bilbao and Urdaibai for each of the three periods distinguished in the time series, in relation to salinity (spatial) and months (seasonal) are shown in Table 6. The spatial niche overlap between *A. clausi* and *A. tonsa* was much higher in the estuary of Bilbao than in Urdaibai, and that between *A. bifilosa* and *A. tonsa* in the latter estuary was the highest found between the dominant *Acartia* species in any of the two estuaries. Seasonally, the highest niche overlap was obtained between *A. clausi* and *A. bifilosa* in the estuary of Urdaibai, and the overlap between *A. tonsa* and *A. clausi* was higher in the estuary of Bilbao than in Urdaibai. In addition, in the 2003-2005 period the spatial overlap between *A. clausi* and *A. bifilosa* decreased while the seasonal overlap increased in the estuary of Urdaibai.

CONCLUSION

Before the arrival of the non-indigenous species *Acartia tonsa* in the estuaries of Bilbao and Urdaibai, the Acartiidae assemblage was constituted by the perennial neritic species *Acartia clausi* and the occasional species *Acartia discaudata* and *Acartia margalefi* in the estuary of Bilbao, and by the perennial neritic species *A. clausi*, the perennial brackish species *Acartia bifilosa* and the occasional species *A. discaudata* and *Paracartia grani* in the estuary of Urdaibai. This indicates that each estuary, in spite of the geographic proximity, was inhabited by different assemblages of Acartiidae species. Such differences cannot be attributed only to differences in the health status, but also to the extent of salinity habitats able to support perennial brackish species populations. The role of system features in the composition of the Acartiidae assemblage is corroborated by the variety of species combinations reported in different types of estuarine systems of the Bay of Biscay and nearby European Atlantic coasts. Without taking into account the invasive *A. tonsa*, for example, *A. clausi*, *A. bifilosa* and *A. discaudata* have been reported in the Ems estuary (Baretta and Malschaert, 1988), *A. clausi*, *A. bifilosa*, *A. discaudata* and *A. margalefi* in Southampton Water (Muxagata, 2005), *A. clausi*, *A. bifilosa*, *A. discaudata* and *A. grani* in the Marennes-Oléron Bay (Sautour and Castel, 1993), and *A. clausi*, *A. discaudata*, *A. margalefi* and *A. grani* in the Ría de Vigo (Alcaraz, 1983).

A. tonsa reached high abundances in the estuaries of Bilbao and Urdaibai at the same time, in 2003. This may have been favoured by a common environmental event in both systems, such as the marked increase of temperature in 2003 after the dry period of 2001-2002. In fact, already by this period *A. tonsa* was occasionally found in the estuary of Bilbao. The most plausible hypothesis is that *A. tonsa* arrived in this estuary (maybe even repeatedly) via ballast water from ships that brought it to the outer port area of the Abra harbour. In 2002 the penetration of this species to the upper reaches was presumably favoured by the decrease of river flow, which enabled a higher penetration of marine waters upstream, a mechanism reported for this species in the Gironde estuary too (David *et al.*, 2007). There is no commercial shipping transport in the estuary of Urdaibai but small boat circulation between this estuary and that of Bilbao is frequent, being likely responsible for carrying plankton organisms between them. This has been claimed to be an important way of secondary spread for crustacean zooplankton (Kelly *et al.*, 2013). Although the exact mechanism by which *A. tonsa* arrived in the estuaries of Bilbao and Urdaibai cannot be known for certain, genetic analysis of individuals from both estuaries suggested a secondary invasion from a European source to Basque estuaries (Albaina *et al.*, 2016).

A. tonsa reached the highest population growth in the innermost salinity habitats of both estuaries in spite of the differences in salinity range, which was wider in the estuary of Urdaibai. *A. tonsa* is found to be an extremely euryhaline species well adapted to instantaneous variations of salinity (Svetlichny *et al.*, this volume), with high reproductive success over wide ranges of temperature and salinity (Holste and Peck, 2006) and optimal adaptation to salinities between 15 and 22 (Cervetto *et al.*, 1999). It is abundant from oligohaline (0.5-5 salinity) to polyhaline (18-30 salinity) waters, with usual maxima in the mesohaline (5-18 salinity) region of large estuaries like Chesapeake Bay and the Gironde (Kimmel and Roman, 2004; David *et al.*, 2007). Thus, *A. tonsa* population settled preferentially in the available innermost salinity habitat of the estuaries of Bilbao and

Urdaibai which were in the limit between euhaline and polyhaline waters (around 30 salinity) in the former, but in polyhaline (18-30 salinity) waters in the latter. This shows the ability of this species to adapt to different salinity habitats in transitional waters of estuarine systems, depending on their availability.

The colonization of *A. tonsa* seems to be facilitated by environmental conditions close to its temperature and salinity optima (Chaalali *et al.*, 2013). However, although salinity conditions in the estuary of Urdaibai were nearer to the optimal, the settlement of *A. tonsa* was more successful in the estuary of Bilbao than in Urdaibai, where this species showed a more restricted seasonal presence and decreased from 2003 to 2005. This may be the result of the combined effect of biotic and abiotic factors, such as competition, temperature and ecosystem health. The lack of perennial brackish *Acartia* species in the inner estuary of Bilbao, in contrast to the development of a large population of *A. bifilosa* in the inner estuary of Urdaibai, suggests a stronger competitive pressure for *A. tonsa* in the estuary of Urdaibai, which could be enhanced when environmental conditions were less favourable for this species than for *A. bifilosa*. *A. tonsa* is well known as a warm-water species (Katajisto, 2006) which reaches its maximum abundances and dominance in summer-early autumn in a wide variety of estuaries and coastal systems (Soetaert and Rijswijk, 1993; Mouny and Dauvin, 2002; David *et al.*, 2005; Purcell and Decker, 2005; Marques *et al.*, 2007; Feike and Heerkloss, 2008; Mackas *et al.*, 2012; Rowshan *et al.*, 2014), and usually disappears from the water column in winter or winter-spring for long periods in which it stays on the bottom as resting eggs (David *et al.*, 2005; Katajisto, 2006; Milligan *et al.*, 2011). The absence or lower abundance of this species during the cold season may be caused by low water temperature, high river discharge and high water export from the estuary (Mortazavi *et al.*, 2000). River flow is also reported to govern *A. tonsa* dynamics in the inner Mondego estuary by advective transport and turbulence (Marques *et al.*, this volume). *A. bifilosa*, however, has been found to have a wide thermal niche, and it is usually a perennial species which may peak from spring to summer depending on the year (our own results; Hernroth and Ackefors, 1979; Viitasalo, 1992; David *et al.*, 2005). *A. tonsa* decreased as temperature decreased from 2003 to 2005 in the estuary of Urdaibai but not in the estuary of Bilbao, this suggesting that the temperature decrease which can be unfavourable in itself for this species could have an additional negative effect by enhancing the competitive pressure by *A. bifilosa*. In the estuary of Bilbao, on the contrary, *A. tonsa* increased from 2003 to 2005, likely due to the lack of brackish competitors and to the deterioration of oxygen conditions in the estuary of Bilbao, which were found to confer a competitive advantage to *A. tonsa* over the neritic species *A. clausi* in this system (Aravena *et al.*, 2009). At very low dissolved oxygen concentrations, *i.e.*, 0.5 mL L^{-1}, *A. tonsa* shows high mortality, but it can tolerate dissolved oxygen concentrations as low as 1.0 mL L^{-1} and adapt well to hypoxia (Decker *et al.*, 2003; Kimmel *et al.*, 2009). The differences observed in the colonising success of *A. tonsa* in the inner estuarine habitats of the estuaries of Bilbao and Urdaibai corroborate the finding that both natural and anthropogenic forcings can explain the successful settlement of this species in estuaries, as reported in the Gironde estuary (David *et al.*, 2007). Our results also exemplify the plasticity of this species, a property which helps to explain why *A. tonsa* is a key species in many estuaries around the world (Derisio *et al.*, 2014). Once this species is established, its replacement by other *Acartia* species might require strong environmental changes such as reported for Berre lagoon, where rehabilitation processes resulting in an increase of salinity have led to the replacement of *A. tonsa* by *A. clausi* (Delpy and Pagano, this volume). The ongoing rehabilitation processes in

the estuary of Bilbao may also lead to changes in the relative abundances of these species, but possibly linked to modifications in trophic and/or water quality parameters rather than to unlikely changes of salinity due to anthropogenic activities in the system.

Another difference in the distribution of *A. tonsa* attributable to the peculiarities inherent to the system was that its abundance decreased seaward with increasing salinity in both estuaries, but dropped sharply in 35 salinity waters of the estuary of Urdaibai, as the abundance of *A. bifilosa* did. This may be related to the much stronger effect of tides on the dynamics of water masses in the estuary of Urdaibai, where water masses of 35 salinity are removed from the estuary at low tide during each semidiurnal tidal cycle, thus enhancing dispersal of brackish species populations. The marked differences in environmental and plankton dynamics of the 35 salinity site of the estuary of Urdaibai compared to lower salinity sites have also been emphasized for dissolved oxygen and phytoplankton biomass (Villate *et al.*, 2008; Iriarte *et al.*, 2014).

The settlement of *A. tonsa* in the estuaries of Bilbao and Urdaibai caused an impact on the spatial and seasonal distribution of Acartiidae species that coexisted in these estuaries before its arrival, in different ways depending on the estuary characteristics and the brackish or neritic origin of species. The spatial distribution of *A. clausi* was displaced seaward and its salinity niche breadth shrank in both estuaries after the settlement of *A. tonsa*, but the direct effect was more evident in the estuary of Bilbao, where both the spatial and the seasonal niche overlaps between *A. clausi* and *A. tonsa* were higher than in the estuary of Urdaibai. This was the result of the smaller spatial salinity range available for the distribution of these two species in the estuary of Bilbao and of the wider seasonal distribution of both species as compared to the estuary of Urdaibai. Therefore, a strong spatial segregation can be expected when salinity habitat range increases and both species coexist in time, as it has been reported also for the Mondego estuary and the Ria de Aveiro (Azeiteiro *et al.*, 2005; Leandro *et al.*, 2014). In the estuary of Urdaibai, however, the seasonal occurrence of *A. tonsa* was more restricted than in the estuary of Bilbao, and *A. clausi* showed a longer period of coexistence with *A. bifilosa*. This latter species showed higher spatial and seasonal niche breadth, and higher spatial and seasonal overlap with *A. clausi* than *A. tonsa*. Consequently, a greater effect of *A. bifilosa* – than of *A. tonsa* – in the spatial and seasonal distribution of *A. clausi* can be expected in the estuary of Urdaibai. Since both the spatial and seasonal distributions of *A. bifilosa* changed when *A. tonsa* settled in the estuary of Urdaibai, the likely impact of *A. tonsa* on *A. clausi* could be better understood as an indirect effect via the impact of *A. tonsa* on *A. bifilosa*. Our results indicate that *A. bifilosa* population maximum was displaced seaward along the salinity gradient, from 26 to 30 salinity, and the seasonal maximum came earlier, being displaced from summer to late spring. This was due to the fact that *A. tonsa* occupied preferentially the lowest salinity habitat and reached the annual maximum in summer when it colonized the estuary of Urdaibai. The spatial segregation pattern of *A. tonsa* and *A. bifilosa* observed in this estuary agrees with that observed in other estuaries where both species coexist after the colonization by the former. For instance, Soetaert and Rijswijk (1993) reported that *A. bifilosa* occupied preferentially the central part and *A. tonsa* the upstream part of the Westerschelde estuary, and that *A. bifilosa* could not penetrate more upstream in summer because of the occurrence of *A. tonsa* in this area. Seasonally, *A. bifilosa* also showed a phenological change upon *A. tonsa* establishment in the Gironde estuary, with the production period occurring one month earlier than before, which resulted in a clear seasonal segregation between the two species (David *et al.*, 2007; Selleslagh *et al.*, 2012). In

an interannual context, after the occurrence in large numbers of *A. tonsa* in 2003, this species showed a decline in the estuary of Urdaibai, while the congeneric brackish species *A. bifilosa* recovered from lower abundances during the period prior to the occurrence of *A. tonsa*. This is in contrast with observations in the Gironde estuary, where the colonization process of *A. tonsa* was more progressive and consisted in a succession of long phases from its initial occurrence in low numbers in 1983 to the point of becoming more abundant than *A. bifilosa* from 1999 onward (Chaalali *et al.*, 2013). In any case, the period of coexistence of *A. tonsa* and *A. bifilosa* analyzed in the estuary of Urdaibai is still short to be able to explain the pattern of joint population dynamic of these species in this system, and an extension of the study to include more years is needed. Changes in environmental conditions may also play a key role in the variations of brackish species in the estuary of Urdaibai as inferred from the strong decline of *A. bifilosa* prior to the occurrence of *A. tonsa*, in the period characterized by the anomalous river flow regime during 2001-2002 and low summer temperatures in 2002.

The impact of *A. tonsa* on the much less abundant and occasional Acartiidae species, such as *A. discaudata* in both estuaries, *A. margalefi* in the estuary of Bilbao and *P. grani* in the estuary of Urdaibai, could not be easily assessed on the basis of our data. These species show an intermediate spatial position in the salinity gradient between the neritic species *A. clausi* and the brackish species *A. bifilosa* and *A. tonsa*, but they also appear spatially (*A. discaudata* and *A. margalefi* in the estuary of Bilbao) or seasonally (*A. discaudata* and *P. grani* in the estuary of Urdaibai) segregated among them. In the estuary of Bilbao, both *A. discaudata* and *A. margalefi* increased in the period prior to the settlement of *A. tonsa*, coinciding with an improvement of water environmental conditions in the inner estuary, and they almost disappeared after the settlement of *A. tonsa*. The arrival of *A. tonsa* has been suggested as the cause for the disappearance of the formerly abundant *A. margalefi* in some Mediterranean systems (Sei *et al.*, 1996; Sei and Ferrari, 2008) and the Black Sea (Gubanova *et al.*, 2014). However, it is difficult to attribute the strong decline of *A. discaudata* and *A. margalefi* to the negative impact of *A. tonsa* in the estuary of Bilbao when it coincided with a general deterioration of environmental conditions, namely a decrease in dissolved oxygen and an increase in river flow and temperature. Such conditions were similar to those found in the period 1998-2000, in which *A. tonsa* was not present but *A. discaudata* and *A. margalefi* were also scarce. The sensitivity of *A. margalefi* to environmental changes is corroborated by results from other studies. In the lagoon of Venice *A. margalefi* density also declined with the introduction of *A. tonsa*, but the reported simultaneous disappearance of this species from other Italian estuarine and lagoon systems, as discussed in Ribera d'Alcalà *et al.* (2004), suggested that larger scale environmental changes might be responsible (Bandelj *et al.*, 2008).

The case of *P. grani* in the estuary of Urdaibai was clearly different, as this species showed a very weak presence until 2003 when it showed the highest abundance at the same time as *A. tonsa* did. This only allows concluding that the occurrence of *P. grani* was linked to high temperatures, and that it responded to this factor in the same way as *A. tonsa* did. The presence of *P. grani* in the plankton also appears restricted to the summer period in Mediterranean lagoon systems (Boyer *et al.*, 2013). At an interannual scale, the occurrence of *P. grani* in the estuary of Urdaibai was erratic and with long periods of absence. Its highest abundance and seasonal presence were recorded in previous studies during the years 1981-1982 and the summer of 1990 (Villate, 1982; Uriarte *et al.*, 2000), but it was almost absent from 1997 until 2003. Similarly, this species was reported in the 1960s but not in more recent studies (2001-2002) in Southampton Water (Muxagata, 2005). This indicates that this species

might be undergoing a long term decline in these systems. However, the present scarcity of *P. grani* in the estuary of Urdaibai precluded the analysis of competitive relationships with other Acartiidae species, as is the case of the invading *A. tonsa*.

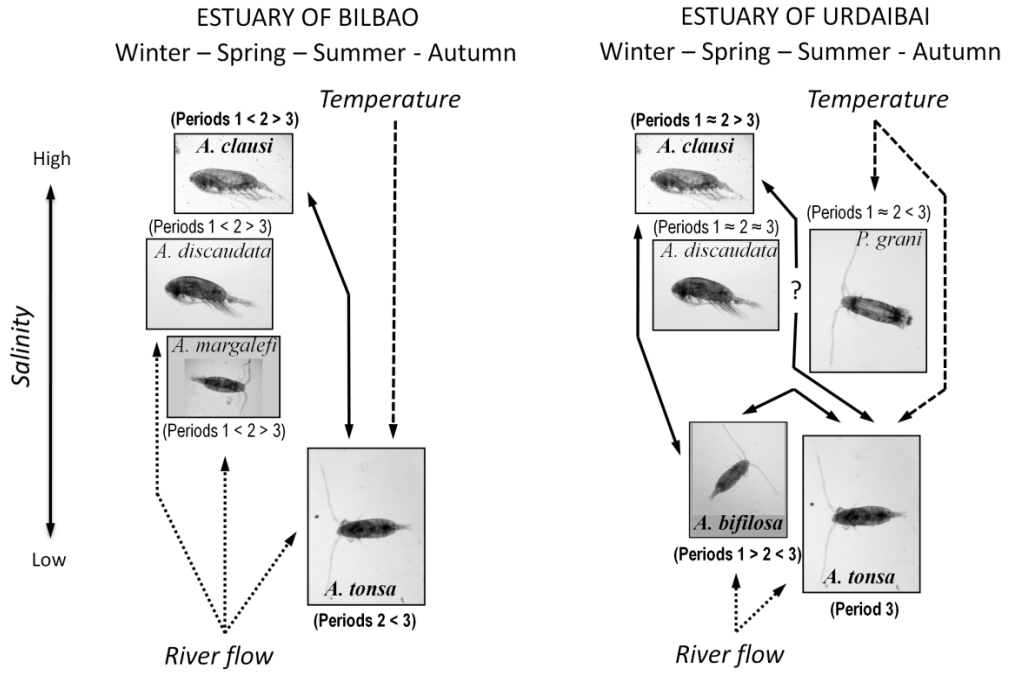

Figure 8. Synoptic diagram of the spatial and temporal distribution of Acartiidae species in the estuaries of Bilbao and Urdaibai, with indication of the observed effects of species competition and hydroclimatic factors in the variations in abundance of *Acartia clausi*, *Acartia discaudata*, *Acartia margalefi*, *Paracartia grani*, *Acartia bifilosa* and *Acartia tonsa* during the 1998-2005 period. Continuous lines: competitive interactions resulting in reduction or displacement of spatial and/or seasonal distributions, or changes in abundance between periods (1: 1998-2000, 2: 2001-2002 and 3: 2003-2005). Dashed lines: negative effect of river flow and positive effect of temperature.

In summary, the colonization of the inner salinity habitats of the estuaries of Bilbao and Urdaibai by the non-indigenous copepod *A. tonsa* since 2003 was found to be directly or indirectly responsible for spatial and temporal changes in the distribution of the autochthonous dominant neritic species *A. clausi* in both systems, and the autochthonous dominant brackish species *A. bifilosa* in the estuary of Urdaibai. No clear impact of *A. tonsa* on the less abundant and occasional species *A. discaudata*, *A. margalefi* and *P. grani* was observed, presumably because the development of these species was primarily limited by environmental constraints. Differences in the spatial, seasonal and interannual distribution of *A. tonsa* between the two estuaries showed that population dynamics were affected by biotic and abiotic features specific to each system, and that *A. tonsa* benefited from the worse environmental conditions and the lack of a dominant brackish species in the inner estuary of Bilbao. The synoptic scheme shown in Figure 8 summarizes the role of specific competition and hydroclimatic factors in the changes observed for Acartiidae species throughout the 1998-2005 period in the estuaries of Bilbao and Urdaibai. Results are preliminary because they cover a short period of time after the occurrence of *A. tonsa* in the estuaries of Bilbao and

Urdaibai. An extension of the study encompassing more years would be advisable to obtain a more robust picture of changes in the Acartiidae congenerics, and determine the invasive character of *A. tonsa* in these systems. For this last purpose, future studies should be expanded to address also the impact of this species at the ecosystem level, considering ecosystem services too. Comparative studies covering a wide variety of systems colonized by *A. tonsa* would also be desirable in order to determine how system typology and level of anthropogenic disturbance affect the invasive character of this species. In addition, long term studies based on ongoing time series to analyse the response of *A. tonsa* to current modes of climate variation should be promoted. They could provide useful information to predict future impacts of this species under different climate change scenarios.

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Chapter 65

CAN CHANGES IN THE DISTRIBUTION OF TWO CONGENERIC COPEPODS (*ACARTIA CLAUSI VS. ACARTIA TONSA*) CONSTITUTE A SIGN OF RECOVERY FOR THE ANTHROPIZED BERRE LAGOON (FRANCE, MEDITERRANEAN SEA)?

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ABSTRACT

The impact of rehabilitation processes on *Acartia* distributions in the anthropized Berre Lagoon was investigated comparing a study performed in 2010-12 to ones achieved before the rehabilitation period. In 1966, the opening of a hydroelectric powerplant led to the establishment of a strong unidirectional salinity gradient. The invasive copepod *Acartia tonsa*, introduced in the 1980's, dominated a low-diversity zooplankton community with another common brackish species, the rotifer *Brachionus plicatilis*. At that time, *Acartia clausi* was restricted to the adjacent coastal area. Initiated since the mid 1990's, the rehabilitation processes have managed to reduce salinity fluctuations and maintain it above 15. The time and space partitioning of both *Acartia* species was modified, since *A. tonsa* and *A. clausi* coexisted over the whole lagoon. A seasonal succession pattern was then outlined throughout the year, with *A. clausi* dominant from winter to spring and *A. tonsa* from summer to autumn. Likewise, a spatial segregation was observed in the entire lagoon, as *A. clausi* remained in more marine areas. *A. tonsa* was less abundant, highlighting that a balance seemed established between these two congeneric species. However, environmental variables did not display any clear direct relationship with these distributions, suggesting more complex mechanisms, such as trophic interactions. Nevertheless, these changes in the distribution of *Acartia* species following the rehabilitation processes constitute a sign of a hoped recovery.

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Keywords: anthropized lagoon, salinity gradient, rehabilitation processes, *Acartia tonsa*, *Acartia clause*

1. INTRODUCTION

Due to their short generation time, copepods, as other zooplankton organisms, quickly respond to environmental changes, a response that could be considered useful to appreciate the health status of aquatic ecosystems and assess their variability at distinct space and time scales (Cairns *et al.*, 1993; Beaugrand, 2005; Fernández de Puelles *et al.*, this volume; Wootton *et al.*, this volume). Community structure modifications (*e.g.*, species composition, size spectra) following perturbations, as well as changes in the spatial distribution of 'steno' species depending on different types of gradients (haline, trophic, pollution, *etc.*), are often considered tools to acknowledge the variability of marine, coastal and brackish water environments (Etilé *et al.*, 2009; Delpy *et al.*, 2012; Serranito *et al.*, 2016; Svetlichny *et al.*, this volume). For instance, at large scale, alterations in copepod communities have been associated to hydroclimatic changes in the North Atlantic Ocean (Beaugrand and Reid, 2003). At the regional scale, the impact of pollution, water quality, eutrophication and brief climatic events have been evidenced on copepod communities (Vincent *et al.*, 2002; Etilé *et al.*, 2009; Delpy *et al.*, 2012; Serranito *et al.*, 2016). Their monitoring may thus represent a powerful tool for a sustainable management of coastal lagoons, which are major sites of socioeconomic interests (*e.g.*, artisanal fisheries, aquaculture, tourism, various aquatic leisure activities). They represent ~13% of worldwide coastlines and are highly impacted by various anthropogenic pressures (*e.g.*, urbanization, chemical industries, agriculture, marine commercial traffic) which combine with natural forcing such as temperature and salinity variations matching seawater and/or freshwater inputs. Coastal lagoons are then very selective to copepods, and particularly to species which may be considered reliable bio-indicators (Capuzzo, 1980; Bianchi *et al.*, 2003; Marcus, 2004; Beaugrand, 2005; Etilé *et al.*, 2009; Delpy *et al.*, 2012; Falcão *et al.*, 2012; Serranito *et al.*, 2016).

Among the noteworthy copepod families, Acartiidae represent typical coastal and brackish water copepods that have been used to characterize water quality and hydrological changes in coastal ecosystems (*e.g.*, Alcaraz, 1983; Bianchi *et al.*, 2003; David *et al.*, 2007; Aravena *et al.*, 2009; Etilé *et al.*, 2009; Marques *et al.*, this volume; Villate *et al.*, this volume). In this chapter, we focus on the only two *Acartia* species (*Acartia tonsa* Dana, 1849 and *Acartia clausi* Giesbrecht, 1889) observed in a French Mediterranean coastal lagoon (Berre Lagoon), which has been particularly impacted by major hydrological changes. Berre Lagoon has been subject to strong anthropogenic pressures for several decades (Warner, 2012). The development of chemical and petrochemical industries (1920-70), as well as a massive urbanization of surrounding cities (1973-90), led to large inputs of organic and inorganic pollutants. This was in addition to the contribution of three small tributaries (*i.e.*, Touloubre, Durançole and Arc) which drained a watershed of ~1,300 km², bringing annually 275 tons of $N\text{-}NO_3$ and 36 tons of $P\text{-}PO_4$ into the lagoon (Gouze *et al.*, 2008; Warner, 2012). In 2005-06, floods were responsible for supplying up to 33% of nitrate, 53% of phosphate and 99% of suspended matter (Gouze *et al.*, 2008). Moreover, strong winds (*i.e.*, Mistral and East wind) contributed to the release of stored phosphorus within the sediment. In 1966, a

derivation canal of the Durance River into the northern part of the lagoon was built to supply a hydroelectric powerplant. Releases of freshwater (3.3×10^9 m^3 y^{-1}, 3.7 times Berre volume) and silt (520,000 t y^{-1}) were realized with no or very few controls causing intense eutrophication (Minas, 1976a; Gaudy *et al.*, 1995; Gouze *et al.*, 2008) and significant anoxia events (Minas, 1976b; Nerini *et al.*, 2000). In 1994, the rehabilitation processes were initiated with a reduction in freshwater (1.2×10^9 m^3 y^{-1}) and silt (100,000 t y^{-1}) inputs through Barnier Plan. However, these restrictions were not enough as large fluctuations of salinity and eutrophication were still observed. In 2006, a litigation of the European Union imposed stricter limitations with a further reduction of silt inputs (< 60,000 t y^{-1}) and a mandatory smoothing of both freshwater and silt releases over a weekly basis. The objective was the maintenance of salinity above 15.

As other coastal environments, Berre Lagoon was also impacted by the introduction of several alien species probably *via* ballast waters of commercial ships (Gaudy and Vinas, 1985; Delpy *et al.*, 2012 and 2016). Among them, two species (the copepod *A. tonsa* and the ctenophore *Mnemiopsis leidyi*) were known to be particularly invasive, modifying food webs drastically (Gaudy *et al.*, 1995; Katsanevakis *et al.*, 2014; Albaina *et al.*, 2016; Delpy *et al.*, 2016). Thus, *A. tonsa* can alter trophic fluxes to higher trophic levels competing with native copepods, and exert a potential control on primary producers (Chaalali *et al.*, 2013; Katsanevakis *et al.*, 2014; Marques *et al.*, this volume; Villate *et al.*, this volume). In Berre Lagoon, *A. tonsa* was observed for the first time in the 1980's (Gaudy and Vinas, 1985) and then co-dominated the zooplankton community with another euryhaline and eurythermal species, the rotifer *Brachionus plicatilis* (Cervetto, 1995; Gaudy *et al.*, 1995). Conversely, *A. clausi* was an autochtonous species observed in Berre Lagoon before 1966 when it had the characteristics of a marine environment (Blanc *et al.*, 1967). Afterwards, the introduction of *A. tonsa* and the powerplant-induced freshwater discharges drove it out from the lagoon, limiting it to the adjacent coastal area where it represented one of the most abundant species (Cervetto, 1995; Cervetto *et al.*, 1995). While *A. tonsa* and *A. clausi* exhibited an opposite spatial distribution in the lagoon and its adjacent coastal waters before any rehabilitation effort (Cervetto, 1995; Gaudy *et al.*, 1995 and 2000), they now coexist in both areas (Delpy *et al.*, 2012; unpublished data).

Several environmental parameters may be responsible for the maintenance of *A. tonsa* within invaded areas. Temperature, salinity and diet (*i.e.*, type and amount of available food) can differently influence the metabolism of congeneric species, particularly in terms of reproductive success and feeding activity (Jeffries, 1962; Gaudy *et al.*, 2000; Calliari *et al.*, 2006; Boyer *et al.*, 2013; Marques *et al.*, this volume; Svetlichny *et al.*, this volume; Villate *et al.*, this volume). For instance, *A. clausi* and *A. tonsa* are known to accept a wide range of salinity (1-65 and 1-72, respectively), but with different optimal values (24-30 and 15-22) (Cervetto *et al.*, 1995 and 1999). Therefore, the presence of many *Acartia* species in the same area generally matched a seasonal succession (Jeffries, 1962; Lee and McAlice, 1979; Wooldridge and Melville-Smith, 1979) and/or a spatial segregation especially in brackish environments presenting a unidirectional salinity gradient (Greenwood, 1981; Alcaraz, 1983; Azeiteiro *et al.*, 2005; Aravena *et al.*, 2009; Falcão *et al.*, 2012; Leandro *et al.*, 2014). These segregations, whether temporal and/or spatial, highlighted that *A. tonsa* presents competitive advantages compared to other species, regarding the production of resistance stages

(Belmonte and Potenza, 2001; Svetlichny *et al.*, this volume) and its large tolerance to low oxygen concentrations (Decker *et al.*, 2003; Kimmel *et al.*, 2009).

In this context, the present chapter, based on a comparison of our current results and studies achieved before the rehabilitation period (Gaudy *et al.*, 1995; Cervetto, 1995), aims at: (1) analyzing the relationships between *A. clausi* and *A. tonsa* abundances and their responses to environmental conditions, and (2) evaluating the effects of the rehabilitation processes on the respective distribution of these species. This comparison shows in particular that, linked to strong environmental changes after the rehabilitation, the two *Acartia* species are now cohabiting within the lagoon, representing a sign of recovery, but display seasonal succession and spatial segregation.

2. METHODS

2.1. Study Site and Sampling Strategy

Located northwest off Marseille (southeast France), Berre Lagoon is a large (155 km²) and shallow (7 m average depth) brackish water basin composed of two parts: the main lagoon and Vaïne Lagoon in the east (Figure 1). Freshwater inflows are concentrated in the northern part with three small rivers (*i.e.*, Touloubre, Durançole and Arc) and the man-made Durance River derivation canal supplying a hydroelectric powerplant at Saint-Chamas. In the southwestern part, Caronte Canal is its only connection to the Mediterranean Sea.

Between 1966 and 1994, releases of freshwater by the hydroelectric powerplant into Berre Lagoon were realized with no or very few controls leading to large fluctuations of salinity (4.0 to 28.0). Since 1994, rehabilitation processes (*i.e.*, gradual reduction and smoothing of inputs) were performed to maintain the salinity above 15. To evaluate the responses of *Acartia clausi* and *Acartia tonsa* distributions to these different environmental conditions, we compared a recent study we performed in 2010-12 (referred to "after rehabilitation") to previous ones by Gaudy *et al.* (1995) and Cervetto (1995) conducted in 1985 and 1992-93, respectively just before the first rehabilitation efforts (referred to "before rehabilitation").

In our study, monthly sampling was realized from January 2010 to January 2012 (39 samplings) at three mooring stations in Berre Lagoon (SA1, SA2 and SA3) and at Port de Bouc (PdB) (Figure 1). Located in the northern part, SA1 (43°29.735'N / 5°2.953'E, 6.9 m deep) is impacted by freshwater inflows. In the middle, SA2 (43°27.598'N / 5°5.404'E, 7.8 m deep) represents an intermediate situation. In the southern part, SA3 (43°25.300'N / 5°6.219'E, 9.5 m deep) is influenced by bottom inflows of marine water through the Caronte Canal. Situated at the mouth of the Caronte Canal, PdB (43°23.944'N / 4°59.299'E, 6.5 m deep) presents almost typical marine characteristics of the Mediterranean Sea with a minimal influence of brackish water surface inflows from the Berre Lagoon.

In Gaudy *et al.* (1995), data were collected in February, June, October and November 1985 at stations close to SA1, SA2 and SA3 (G1, G2 and G3, Figure 1). In Cervetto (1995), sampling was performed between March 1992 and March 1993 (22 samplings) at a station slightly southeast of SA3 and another one close to PdB (C3 and C4, Figure 1). Thus, Gaudy *et al.* (1995)'s dataset allowed a seasonal comparison of the spatial distribution of *Acartia*

assemblages over the whole lagoon, while Cervetto (1995) was used to evaluate the impact of the rehabilitation processes on their higher-frequency temporal distribution in the south of the lagoon and its adjacent coastal area.

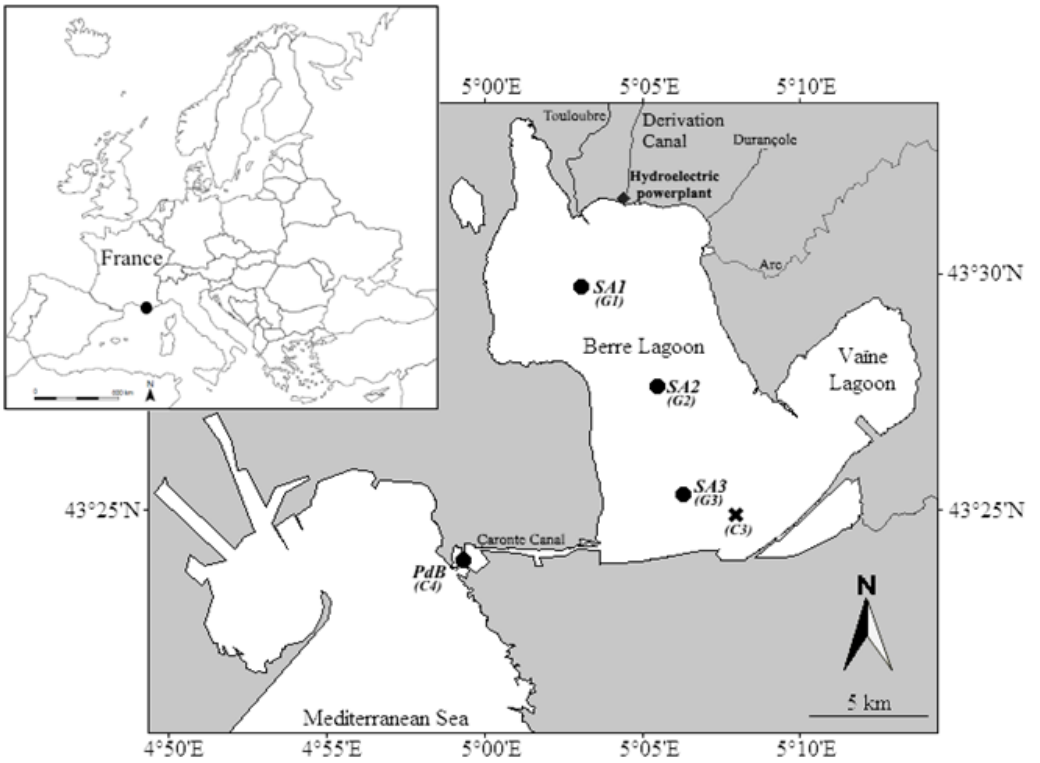

Figure 1. Map of Berre Lagoon and its adjacent coastal area. Station location: SA1, SA2, SA3 and PdB sampled in 2010-12; G1, G2 and G3 in 1985 (Gaudy *et al.*, 1995); C3 and C4 in 1992-93 (Cervetto, 1995).

2.2. Environmental Parameters

In our study, each mooring station in Berre Lagoon was equipped with multiparametric probes CTD SBE37 (Seabird) located at ~1 m from the surface and the bottom. Daily averages of temperature and salinity were computed from a high frequency sampling (96 times per day) dataset. At PdB, vertical profiles of temperature and salinity were realized with the multiparametric probe CTD SBE19+ (Seabird) at each sampling date.

To estimate chlorophyll *a* (Chl *a*) concentrations, water was collected at sub-surface (0.5 m) and at ~1 m from the bottom using an 8 L Niskin bottle. Triplicate samples of 20-100 mL were filtered directly onboard onto GF/F filters (0.7 µm), then frozen with liquid nitrogen and stored at -20°C until further analysis. Chlorophyll *a* content was extracted using acetone (90%) in the dark, at 4°C, for 24 h. Measurements were performed by fluorimetry (Turner Design – TD700 fluorimeter) according to Lorenzen (1967). Chlorophyll *a* concentrations were obtained by averaging triplicate values.

Temperature and salinity were measured by Gaudy *et al.* (1995) in the surface and deeper layers using a salinograph YTECH, whereas Cervetto (1995) realized vertical profiles with a multiparametric probe (ME Hydrodata). In both studies, chlorophyll *a* concentrations were evaluated at the same depths using a fluorimetric method.

2.3. Metazooplankton Community

In our study, to sample metazooplankton (defined as planktonic metazoans), a haul was performed at each station with a modified WP2 plankton net (1.2 m long, 50 cm diameter of opening area and 80 µm mesh size). The net was towed vertically, from bottom to surface, at a speed of ~1 m s^{-1}. The cod-end content was immediately fixed with a formaldehyde - buffered seawater solution (4% final). Sub-samples (1-20%) were realized with micropipettes (1 and 5 mL) and analyzed using a dissecting microscope (Leica MZ6). An average of 948 individuals were counted per sample and the enumeration error was estimated at ± 6.5% (Postel *et al.*, 2000). The metazooplankton community of Berre Lagoon presented only two *Acartia* species: *A. clausi* and *A. tonsa*. Adults of these two congeneric copepods were identified and counted according to Rose (1933) and Razouls *et al.* (2005-2017). *Acartia* copepodite stages were counted, but they could not be discriminated at the species level. Abundances of remaining metazooplankton were also estimated and expressed as ind. m^{-3}.

In Gaudy *et al.* (1995) and Cervetto (1995), metazooplankton was collected in the surface and deeper layers with a Clarke Bumpus type net (80 µm) and a WP2 plankton net (60 µm), respectively. Data were averaged over the water column to allow relevant comparisons.

2.4. Data Analysis

Only the dataset obtained from Cervetto (1995) allowed a higher-frequency comparison matching our dataset and was used for statistical analyses.

Non-parametric Mann-Whitney U tests (MWU thereafter; Mann and Whitney, 1947) were used to evaluate differences between studied periods, stations and depths. Environmental parameters (*i.e.*, temperature, salinity and chlorophyll *a* concentrations) and biological data (*i.e.*, abundance and relative abundance) were not normally distributed (Shapiro-Wilk test) and considered independently of one another.

The relationships between environmental variables (*i.e.*, temperature, salinity and chlorophyll *a* concentrations) and zooplankton (*A. clausi* and *A. tonsa*, other - *i.e.*, non-*Acartia* - copepods, meroplanktonic larvae and other zooplankton organisms) were assessed using principal component analyses (PCA) performed on log-transformed data of 1992-93 and 2010-12 for the two shared stations (C3/SA3 and C4/PdB) using ADE4 software (Thioulouse *et al.*, 1997). Partial correlation analyses were also carried out on log-transformed data to explore the relationships between the two *Acartia* species and the same environmental and zooplankton variables. In these multivariate analyses, abundances of *Acartia* species were estimated by cumulating abundance of adults and copepodites. As *Acartia* copepodite stages could not be discriminated at the species level, their abundance in each species was estimated at the *pro rata* of adult abundance.

Furthermore, two dimensional surface plots were drawn to show the effects of salinity and chlorophyll *a* concentrations on *A. clausi* and *A. tonsa* abundances. They were performed on pooled datasets of 1992-93 and 2010-12 with least square adjustment using Statistica software 7.1.

3. RESULTS

3.1. Environmental Parameters

Before and after the rehabilitation processes, temperature displayed comparable range values (4.7 to 26.2°C in Berre Lagoon, 6.5 to 23.8°C at Port de Bouc) and similar seasonal pattern with higher values in summer and lower ones in winter (Figures 2 and 3). These observations corresponded to the typical seasonal cycle in temperate areas.

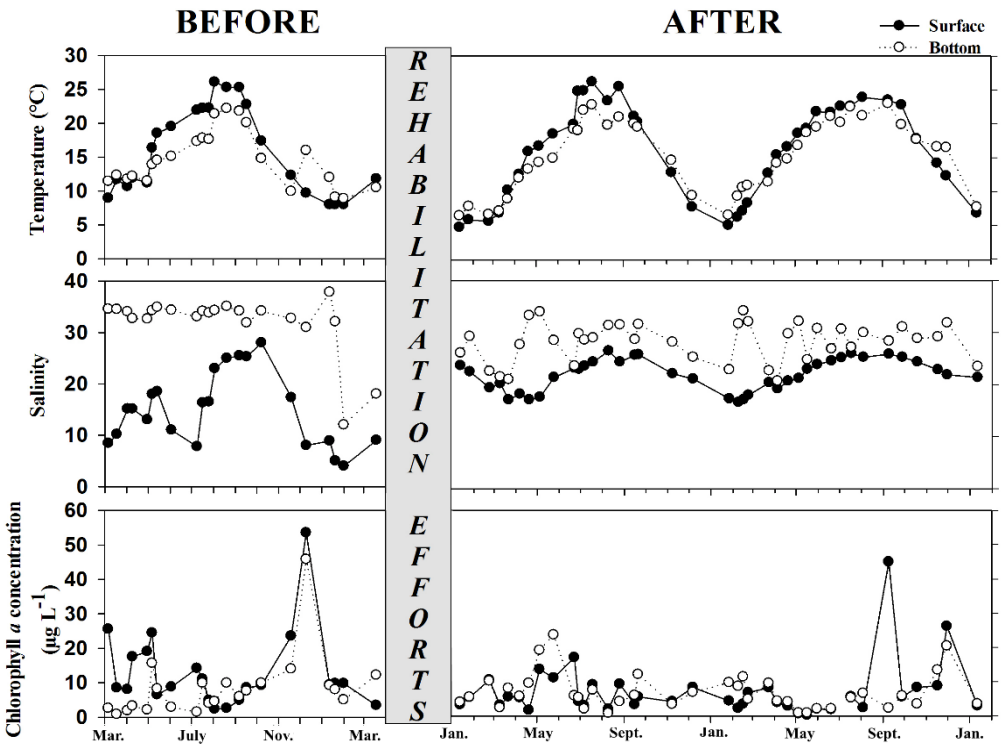

Figure 2. Temporal variations of temperature (°C), salinity and chlorophyll *a* concentrations (µg Chl *a* L^{-1}) in the south of Berre Lagoon (C3/SA3) before (1992-93 – Cervetto, 1995) and after (2010-12 – this study) the rehabilitation efforts.

For both periods, salinity displayed a clear seasonal pattern that matched the temperature pattern (Figures 2 and 3). Lower values were observed in winter corresponding to higher freshwater inputs related to increased activity of the hydroelectric powerplant to fulfill heating requirements (Warner, 2012). However, in the lagoon (C3/SA3 stations), this temporal variability was significantly lower after the rehabilitation efforts, particularly

regarding surface values (4.0 to 28.0 in 1992-93 *vs.* 16.6 to 26.5 in 2010-12; MWU, $p<0.001$) (Figure 2). A lower vertical stratification was also observed after the rehabilitation compared to before (difference bottom-surface: 6.2 to 29.0 in 1992-93 *vs.* 0.4 to 17.1 in 2010-12; MWU, $p<0.001$). At Port de Bouc (C4/PdB stations), surface salinity significantly increased after the rehabilitation (13.0 to 32.2 in 1992-93 *vs.* 21.0 to 35.4 in 2010-12; MWU, $p<0.001$) (Figure 3). This salinity change matched the gradual reduction in freshwater released by the powerplant since 1994 and the mandatory smoothing over a weekly basis realized since 2006 (Delpy *et al.*, 2012; Warner, 2012). They led also to lower brackish water outputs through the Caronte Canal into the adjacent coastal area.

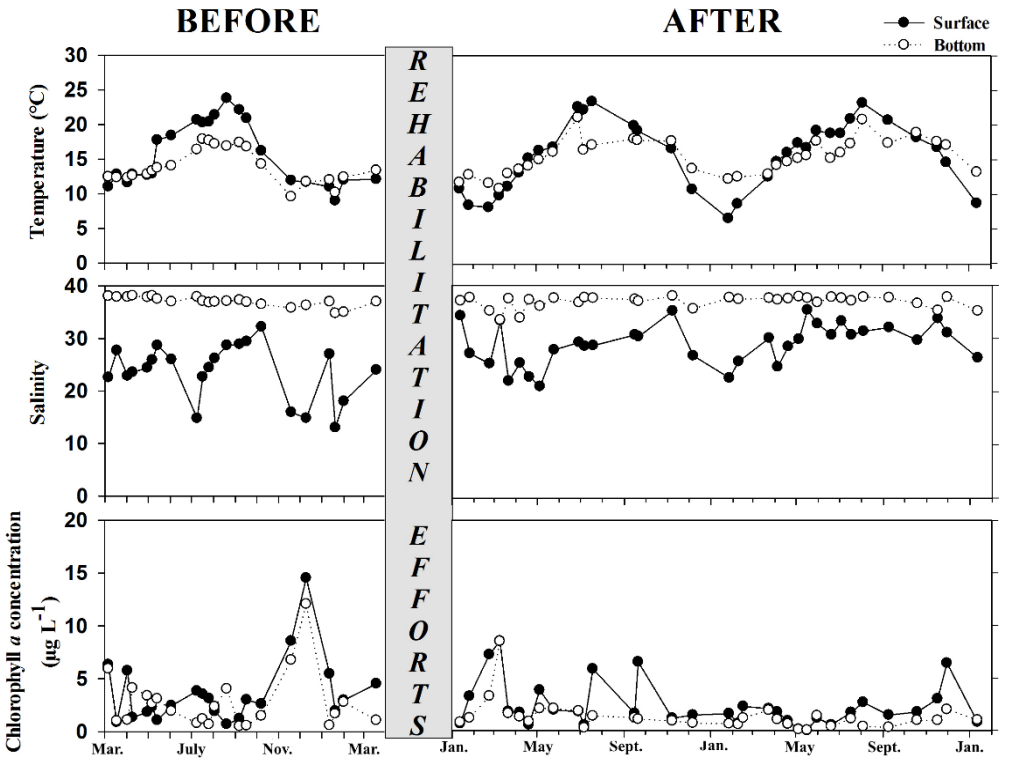

Figure 3. Temporal variations of temperature (°C), salinity and chlorophyll *a* concentrations (µg Chl *a* L^{-1}) at Port de Bouc (C4/PdB) before (1992-93 – Cervetto, 1995) and after (2010-12 – this study) the rehabilitation efforts.

Chlorophyll *a* concentrations presented no clear seasonal pattern during both periods. In Berre Lagoon, significantly lower values were observed after the rehabilitation (7.0 ± 6.8 µg Chl *a* L^{-1} in 2010-12) compared to before (10.7 ± 10.5 µg Chl *a* L^{-1} in 1992-93) (MWU, $p<0.05$) (Figure 2). However, very high concentrations were occasionally observed in 1992-93 (53.5 µg Chl *a* L^{-1} in December 1992), and even in 2010-12 (45.0 µg Chl *a* L^{-1} in September 2011). At Port de Bouc, chlorophyll *a* concentrations presented a significant decrease after the rehabilitation processes with mean values of 1.8 ± 1.9 µg Chl *a* L^{-1} in 2010-12 *vs.* 3.2 ± 2.9 µg Chl *a* L^{-1} in 1992-93 (MWU, $p<0.001$) (Figure 3). The maximum value observed in 1992-93 was 14.5 µg Chl *a* L^{-1}, whereas it was only 8.5 µg Chl *a* L^{-1} in 2010-12. Accompanying the drastic changes in salinity conditions, a modification of the trophic status

was then observed with lower chlorophyll *a* concentrations in 2010-12. It is worth noticing that the persistence of high peaks of chlorophyll *a* concentrations is typical of coastal and shallow ecosystems. In this type of environments, development of phytoplanktonic blooms depended also on short-term episodic weather events like heavy rainfall and strong winds (*i.e.*, Mistral and East wind in the studied area), and not only on traditional seasonal variations in plankton succession (Cloern, 1996; Pinazo *et al.*, 2004; Cook *et al.*, 2010; Liess *et al.*, 2016).

3.2. Spatiotemporal Variations of Zooplankton Community and *Acartia* Populations

At all stations, total zooplankton abundance followed a clear seasonal pattern before and after the rehabilitation efforts. Minimal values were observed in winter, followed by a progressive increase in spring to maximal values reached in early summer (from 0.8 to 200.6 x 10^3 ind. m^{-3}) (Figure 4), matching seasonal variations of temperature previously described (Figures 2 and 3). Before the rehabilitation, peaks of total zooplankton abundance were characterized by an increase of *Acartia* abundance (copepodites, *A. clausi* and *A. tonsa* adults) (Figure 4). After the rehabilitation, *Acartia* assemblages were particularly abundant in winter/early-spring, as well as in summer but to a lesser extent.

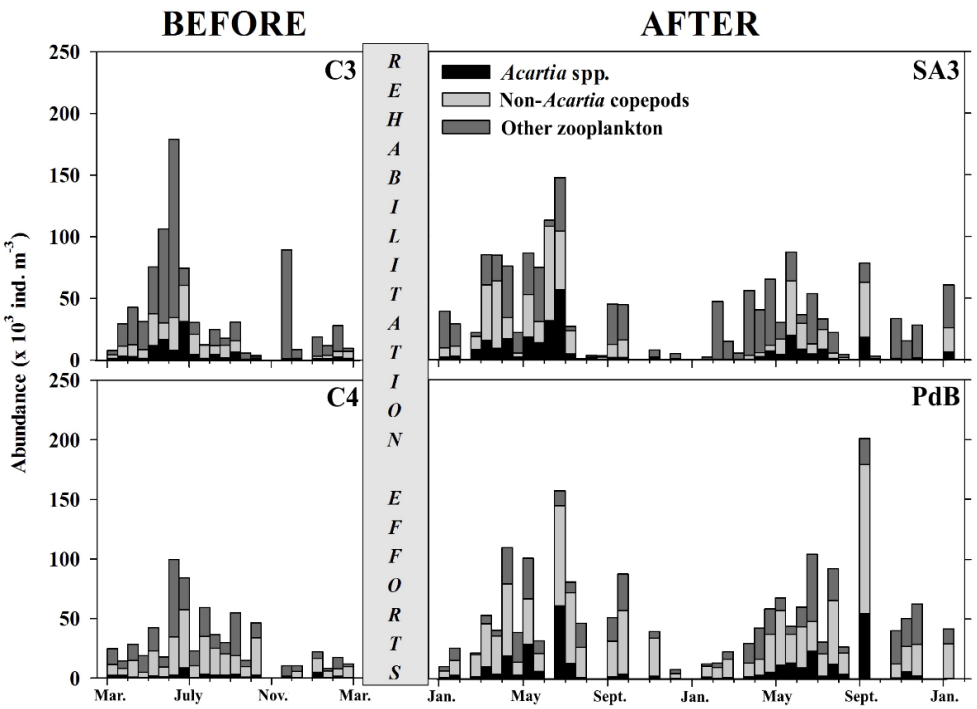

Figure 4. Temporal variations of abundances (x10^3 ind. m^{-3}) of *Acartia* spp. (black), non-*Acartia* copepods (grey) and other zooplankton organisms (dark grey) before (1992-93 – Cervetto, 1995) and after (2010-12 – this study) the rehabilitation efforts, in the south of Berre Lagoon (C3/SA3) and at Port de Bouc (C4/PdB).

In the lagoon (C3/SA3 stations), seasonal pattern and range of abundance were not significantly different between both periods (40.0 ± 43.1 x 10^3 ind. m^{-3} in 1992-93 *vs.* 42.0 ± 34.6 x 10^3 ind. m^{-3} in 2010-12; MWU, $p>0.05$). Zooplankton communities presented comparable structures with comparable relative abundances for copepods (43.6 ± 26.0% in 1992-93 *vs.* 40.5 ± 28.8% in 2010-12) and other zooplankton organisms (56.4 ± 26.0% in 1992-93 *vs.* 59.5 ± 28.8% in 2010-12) (MWU, $p>0.05$). At Port de Bouc (C4/PdB stations), the same seasonal pattern was observed whereas total zooplankton abundance nearly doubled after the rehabilitation processes (32.1 ± 24.8 x 10^3 ind. m^{-3} in 1992-93 *vs.* 56.0 ± 41.7 x 10^3 ind. m^{-3} in 2010-12; MWU, $p<0.01$). Maximal value was 99.7 x 10^3 ind. m^{-3} in 1992-93 and reached 200.6 x 10^3 ind. m^{-3} in 2010-12. Compared to the lagoon, copepods contribution to total abundance was significantly higher (54.8 ± 17.6% in 1992-93 *vs.* 68.3 ± 17.1% in 2010-12) and occurred at the expense of other zooplankton organisms (45.2 ± 17.6% in 1992-93 *vs.* 31.7 ± 17.1% in 2010-12) (MWU, $p<0.05$).

The relative contributions of *A. tonsa* and *A. clausi* to total Acartiidae revealed a clear change before and after the rehabilitation processes (Figure 5). In 1992-93, *A. tonsa* was almost the only *Acartia* species in the lagoon (C3) representing up to 100% almost year round. Conversely, at Port de Bouc (C4), *A. clausi* and *A. tonsa* successively dominated the *Acartia* population. In 2010-12, a similar temporal pattern was observed at both stations (SA3 and PdB). At the spatial scale, *A. clausi* presented a higher relative abundance at Port de Bouc (63.1 ± 41.6%), while *A. tonsa* displayed a correlative higher one in the lagoon (65.1 ± 39.4%) (MWU, $p<0.05$). Nevertheless, at Port de Bouc, it is worth noticing that in 2010-12 the main predominance periods of each species (winter to spring for *A. clausi vs.* summer to autumn for *A. tonsa*) were longer than in 1992-93 exhibiting alternation of five dominance peaks for each species.

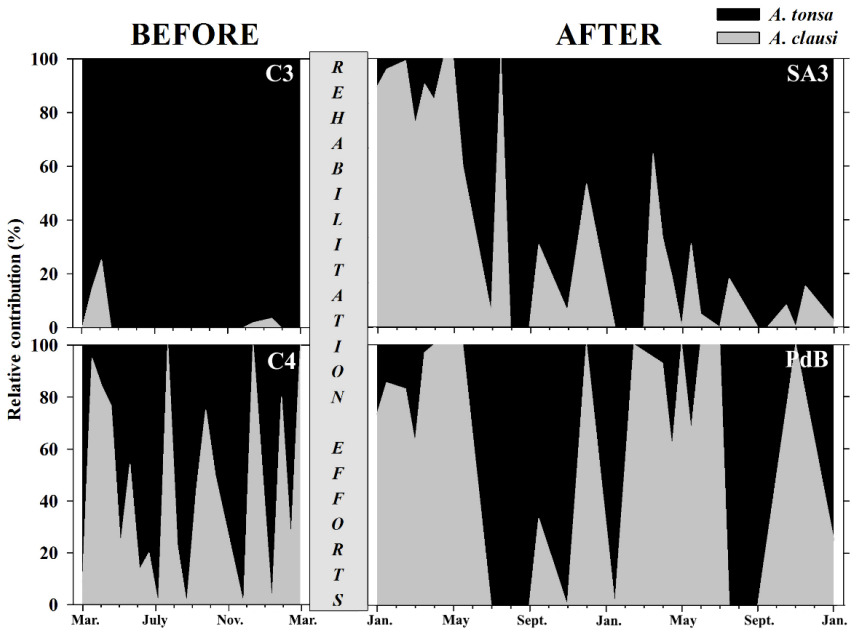

Figure 5. Temporal variations of relative abundance (% of total Acartiidae) of *A. tonsa* (dark) and *A. clausi* (grey) adults before (1992-93 – Cervetto, 1995) and after (2010-12 – this study) the rehabilitation efforts, in the south of Berre Lagoon (C3/SA3) and at Port de Bouc (C4/PdB).

The spatial distribution of *Acartia* populations in Berre Lagoon at both periods was compared to salinity variations in Figure 6. Before the rehabilitation, the salinity presented a clear seasonal variation with lowest values (~6) observed in winter in the northern part of the lagoon and highest ones (~18) in summer in the southern part (Figure 6 bottom). *A. clausi* was already absent from Berre Lagoon (G1-G2-G3), whereas adults of *A. tonsa* dominated the zooplankton community with a mean abundance of 3,458 ± 4,352 ind. m^{-3} from June to November. In February, low abundance (2.1 ± 3.2 ind. m^{-3}) corresponded to the development of a large population of the rotifer *Brachionus plicatilis* (Gaudy *et al.*, 1995). After the rehabilitation, the salinity presented the same seasonal variations, but to a lesser extent, with a range of values from 17 to 28 (Figure 6 bottom). The mean abundance of *A. tonsa* was twice higher than the one of *A. clausi* (469 ± 1,632 ind. m^{-3} and 221 ± 536 ind. m^{-3}, respectively). Despite the inter-annual variability, our study emphasizes that highest abundances of *A. clausi* occurred in winter/early-spring, and those of *A. tonsa* in summer following the same trend as in 1985 (Figures 4 and 6). Even if the two *Acartia* species were present over the whole lagoon, a spatial segregation was generally observed with a predominance of *A. tonsa* in the northern and intermediate parts (SA1-SA2) and a strong presence of *A. clausi* in the southern area (SA3). A reverse trend was only observed from January to April 2010.

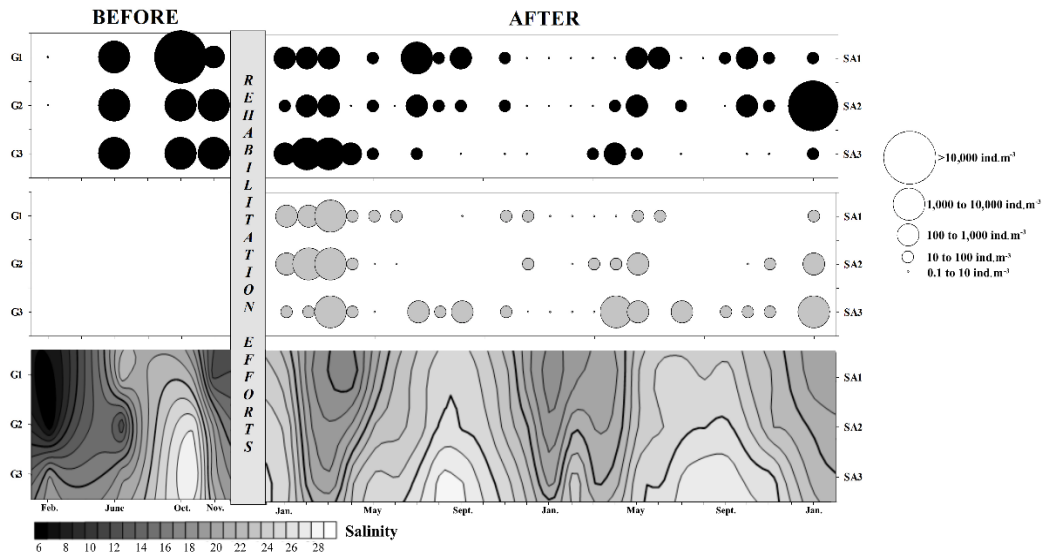

Figure 6. Bubble plots representing abundances of *A. tonsa* (black) and *A. clausi* (grey) adults before (1985 – Gaudy *et al.*, 1995) and after (2010-12 – this study) the rehabilitation efforts from North (G1/SA1) to South (G3/SA3) of Berre Lagoon. Size of bubbles is proportional to the range of abundance (ind. m^{-3}). The bottom graph represents the temporal variations of the salinity (average of surface and bottom values) gradient within the lagoon.

3.3. Effects of Environmental Factors on *Acartia* Distribution

The PCA analyses on the lagoon data before and after the rehabilitation processes explained 57.5% and 54.6% of the variance, respectively (Figure 7 top). Before the rehabilitation, *A. clausi* was poorly correlated with the first axis (33.5%) contrarily to *A.*

tonsa. These two congeneric species, together with other copepods and meroplankton, were opposed to high chlorophyll *a* concentrations. On the second axis (24.0%), both species, together with the other zooplankton groups and chlorophyll, were opposed to high temperature and salinity. After the rehabilitation, the first axis (30.2%) opposed the two *Acartia* species to high temperature and salinity, whereas the second axis (24.3%) opposed *A. tonsa*, temperature and salinity to chlorophyll.

The PCA on Port de Bouc data before and after the rehabilitation explained 60.5% and 58.2%, respectively (Figure 7 bottom). Before the rehabilitation, the first axis (42.3%) opposed high abundances of meroplankton and copepods (including both *Acartia* species) to high chlorophyll, whereas the second axis (18.2%) opposed *A. clausi* to *A. tonsa*. After the rehabilitation, on the first axis (34%), non-*Acartia* copepods and other holoplankton were associated to high temperature and opposed to *A. clausi* and chlorophyll. The second axis (24.4%) clearly opposed the two *Acartia* species with *A. tonsa* associated to high salinity. Thus, the role of temperature, salinity and chlorophyll on the two *Acartia* species did not appear very clearly on the basis of the PCA analyses.

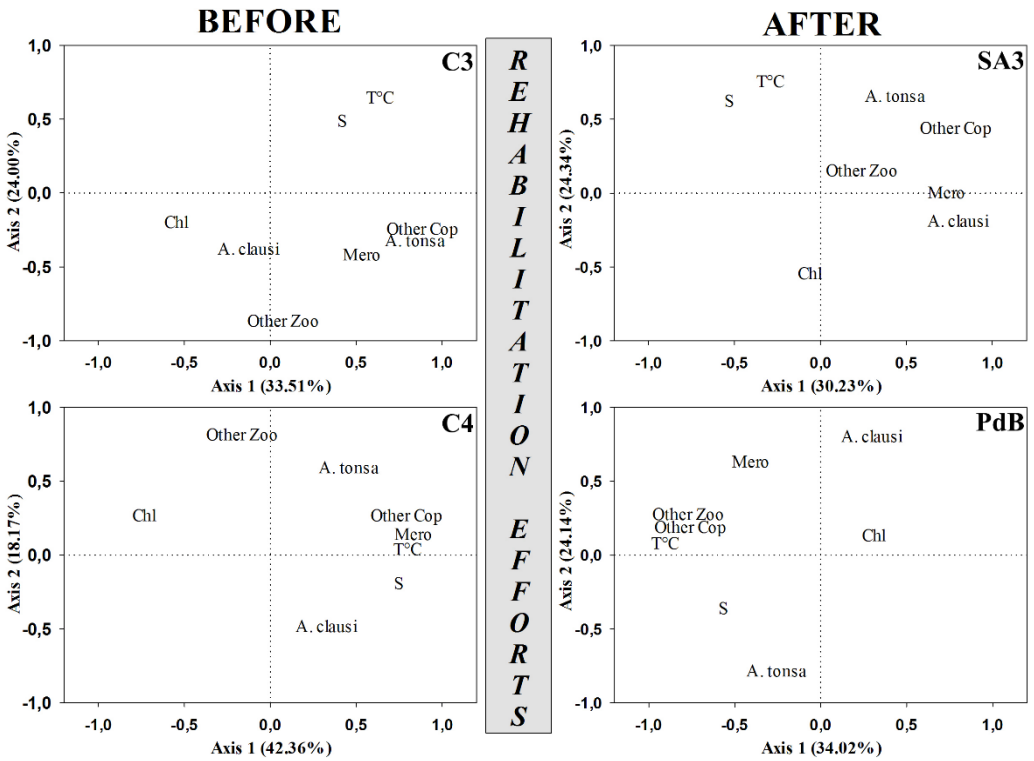

Figure 7. Principal component analyses (PCA) realized on datasets obtained before (1992-93 – Cervetto, 1995) and after (2010-12 – this study) the rehabilitation efforts in Berre Lagoon (C3/SA3) and at Port de Bouc (C4/PdB). T°C: temperature, S: salinity, Chl: chlorophyll *a* concentrations, *A. tonsa*: *A. tonsa* adults and copepodites, *A. clausi*: *A. clausi* adults and copepodites, Other Cop: non-*Acartia* copepods, Mero: meroplanktonic larvae and Other Zoo: other zooplankton organisms.

The partial correlation analysis confirmed this tendency as no correlation was found with temperature, salinity or chlorophyll for both species in the lagoon at the two periods (Table 1). However, in Port de Bouc, after the rehabilitation, both species were negatively correlated with temperature, whereas *A. tonsa* was also positively correlated to salinity. Furthermore, the analysis showed high positive correlation between *Acartia* and non-*Acartia* copepods in the lagoon, for the different situations considered (before and after the rehabilitation and pooled data of the two periods) in the case of *A. tonsa* and only after the rehabilitation processes in the case of *A. clausi.* The two *Acartia* species were negatively correlated in both sites (Berre and Port de Bouc) after the rehabilitation or when considering pooled data of the two periods. Before the rehabilitation, negative correlation between the two species was only found when considering pooled data of the two sites, which illustrated the reverse spatial trend at this period with *A. clausi* only present in PdB and *A. tonsa* quite exclusively present in Berre.

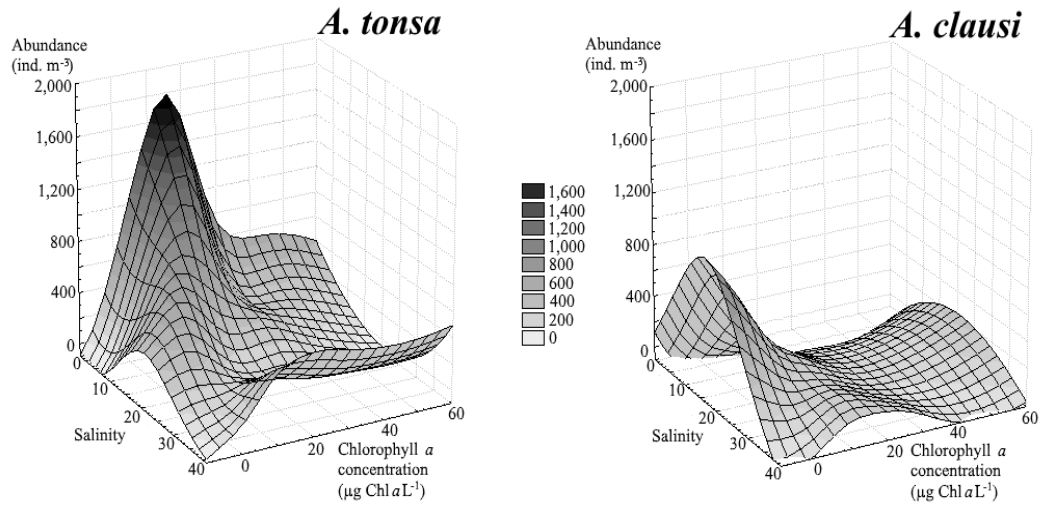

Figure 8. Surface plots of *A. tonsa* and *A. clausi* abundances (ind. m^{-3}) depending on salinity and chlorophyll *a* concentrations (µg Chl *a* L^{-1}). Datasets of 1992-93 (C3 and C4 – Cervetto, 1995) and 2010-12 (SA3 and PdB – this study) were pooled in these analyses.

In summary, if the negative relationship between the two *Acartia* species illustrated their space and/or time partitioning, the absence of clear direct relationship with environmental variables suggests complex mechanisms as interaction or competition to explain these distributions.

To better explore this issue and better understand the effects of salinity and trophic changes after the rehabilitation, we represented, on the same surface plots, the *Acartia* abundances relative to salinity and chlorophyll *a* concentrations by combining the data obtained in 2010-12 and in 1992-93 (Figure 8). The two species showed different patterns. *A. tonsa* presented clear optimal ranges with higher abundances at low salinity (< 10) and medium chlorophyll *a* concentrations (5 to 25 µg Chl *a* L^{-1}). The abundance of *A. clausi* was also dependent on these two environmental parameters with a peak outlined at medium salinity (5 to 25) and low chlorophyll *a* concentrations (< 10 µg Chl *a* L^{-1}). The start of another slight peak can be observed at medium salinity (5 to 25) and very high chlorophyll *a* concentrations (> 50 µg Chl *a* L^{-1}).

Table 1. Partial correlation coefficients between the two *Acartia* species, environmental and zooplankton variables. n= number of data. Significance values ns = non-significant, * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$

	Acartia tonsa						*Acartia clausi*					
	Berre		Port de Bouc		Total		Berre		Port de Bouc		Total	
Before												
n	*22*		*22*		*44*		*22*		*22*		*44*	
Temperature	0.000	ns	-0.249	ns	0.007	ns	-0.422	ns	-0.439	ns	**-0.371**	*
Salinity	-0.011	ns	-0.052	ns	-0.108	ns	0.379	ns	-0.301	ns	0.235	ns
Chlorophyll	0.050	ns	-0.368	ns	-0.062	ns	-0.377	ns	-0.366	ns	**-0.449**	**
Other copepods	**0.897**	***	0.262	ns	**0.358**	*	0.111	ns	0.463	ns	0.252	ns
Meroplankton	-0.080	ns	0.264	ns	0.133	ns	-0.217	ns	0.368	ns	0.083	ns
Other holoplankton	0.240	ns	0.074	ns	0.095	ns	0.309	ns	-0.273	ns	0.063	ns
Other Acartia	-0.138	ns	-0.488	ns	**-0.452**	**	-0.138	ns	-0.488	ns	**-0.452**	**
After												
n	*39*		*32*		*71*		*39*		*32*		*71*	
Temperature	0.045	ns	**-0.523**	*	-0.043	ns	-0.084	ns	**-0.515**	*	-0.238	ns
Salinity	-0.125	ns	**0.521**	*	**-0.272**	*	-0.251	ns	0.347	ns	-0.165	ns
Chlorophyll	-0.355	ns	0.296	ns	-0.036	ns	0.036	ns	0.254	ns	0.069	ns
Other copepods	**0.566**	**	0.397	ns	**0.506**	***	**0.657**	***	0.316	ns	**0.598**	***
Meroplankton	0.164	ns	-0.047	ns	0.077	ns	0.222	ns	0.215	ns	0.195	ns
Other holoplankton	-0.034	ns	0.270	ns	-0.094	ns	-0.067	ns	0.190	ns	-0.112	ns
Other Acartia	**-0.425**	*	-0.732	***	**-0.574**	***	**-0.425**	**	**-0.732**	***	**-0.574**	***
Before + After												
n	*61*		*54*		*115*		*61*		*54*		*115*	
Temperature	-0.047	ns	-0.225	ns	-0.050	ns	**-0.297**	*	**-0.341**	*	**-0.307**	**
Salinity	-0.067	ns	0.137	ns	-0.167	ns	0.056	ns	-0.038	ns	0.028	ns
Chlorophyll	**-0.284**	*	0.042	ns	-0.022	ns	-0.178	ns	-0.043	ns	-0.079	ns
Other copepods	**0.644**	***	0.257	ns	**0.444**	***	**0.548**	***	**0.315**	*	**0.478**	***
Meroplankton	0.042	ns	0.130	ns	0.154	ns	0.239	ns	**0.362**	*	**0.277**	**
Other holoplankton	0.069	ns	-0.013	ns	-0.082	ns	-0.101	ns	-0.151	ns	-0.165	ns
Other Acartia	**-0.501**	***	**-0.588**	***	**-0.549**	***	**-0.501**	***	**-0.588**	***	**-0.549**	***

CONCLUSION

In the anthropized Berre Lagoon, the rehabilitation processes did not seem to have any significant effect on seasonal pattern of zooplankton abundance. Thus, the rough community structure remained unchanged with similar relative contributions of copepods and other zooplankton organisms, despite the lower chlorophyll *a* concentrations observed after the rehabilitation processes. Conversely, at Port de Bouc, total zooplankton abundance increased significantly in 2010-12 reaching values as high as in the lagoon. Copepods gained prominence in the zooplankton community reaching relative contributions (*ca.* 70%) that are currently observed in coastal marine conditions (Siokou-Frangou *et al.*, 2010). The effects of the rehabilitation processes were then perceived as far as the adjacent coastal area, confirming a lower influence of surface brackish water originating from Berre Lagoon. Environmental restorations have already shown positive impacts on plankton communities, and particularly on copepod assemblages. For instance, in a coastal marine system of Tunisia, the removal and containment of phosphogypsum has allowed a diversification of several plankton compartments (Kobbi-Rebai *et al.*, 2012; Rekik *et al.*, 2015). Likewise, the taxonomic composition of zooplankton has drastically changed in Berre Lagoon after the rehabilitation, with a more diverse community including several typical coastal marine species as evidenced by Delpy *et al.* (2012) for the copepods *Centropages typicus*, *Paracalanus parvus* and *Acartia clausi*. The lower variability in salinity likely allowed the development of these marine species brought into the lagoon through the Caronte Canal. Even if both *Acartia tonsa* and *A. clausi* were present in the entire studied area, they tended to succeed one another over time and space.

A clear succession of predominance periods was observed in Berre, as well as at Port de Bouc, with *A. clausi* dominating from winter to spring and *A. tonsa* from summer to autumn. Thus, the reappearance of *A. clausi* in Berre Lagoon and its predominance in winter/spring coincided with the disappearance of the rotifer *Brachionus plicatilis* which had been reported missing after the rehabilitation processes (Gaudy *et al.*, 1995; Delpy *et al.*, 2012). These observations corresponded to the well-defined influence of temperature on the population dynamics of both species (Jeffries, 1962). *A. clausi* is preferentially present in winter/spring as its growth rate increased with increasing temperature and food level (Klein Breteler and Schogt, 1994). In summer, egg production of *A. clausi* was negatively impacted by high temperature (Boyer *et al.*, 2013). This matched the development of the warm-water species *A. tonsa*, mainly depending on production and hatching time of its resting eggs (Katajisto, 2006; Mackas *et al.*, 2012; Svetlichny *et al.*, this volume).

At both periods, a spatial segregation of both *Acartia* species occurred along the salinity gradient from the northern part of Berre Lagoon to Port de Bouc. Before the rehabilitation processes, *A. clausi* was restricted to the marine coastal area, while *A. tonsa* occupied the entire brackish lagoon. This opposite distribution was highlighted in many estuarine systems with *A. clausi* observed downstream and *A. tonsa* upstream (Alcaraz, 1983; Azeiteiro *et al.*, 2005; Aravena *et al.*, 2009; Leandro *et al.*, 2014; Marques *et al.*, this volume; Villate *et al.*, this volume). After the rehabilitation, increasing salinity allowed *A. clausi* penetration into Berre Lagoon, as highlighted in the Mondego estuary by Marques *et al.* (this volume). *A. tonsa* dominated *Acartia* assemblages in most of the lagoon, except in the southern part where *A. clausi* was more abundant, matching optimal salinity range of both species (Cervetto *et al.*,

1995 and 1999; Svetlichny *et al.*, this volume). In the adjacent coastal area, *A. clausi* was still predominant, but followed closely by *A. tonsa*. Falcão *et al.* (2012) showed that restoration measures can lead to a relative homogenization of zooplankton community downstream of the Mondego estuary.

A focus on the two environmental factors modified by the rehabilitation processes (*i.e.*, salinity and chlorophyll *a* concentration) highlighted the first signs of ecological niche for *A. tonsa* and *A. clausi*. After the rehabilitation processes, *A. clausi* co-dominated *Acartia* assemblages with the non-indigenous species *A. tonsa*. The drastic change in salinity conditions may partly explain this evolution, although the salinity effect was not shown in the multivariate analyses in Berre Lagoon. Higher salinity (mean value of 23.6) was less favorable for *A. tonsa*, whose optimal adaptation for metabolism (respiration and excretion) was found at 15-22 (Cervetto *et al.*, 1999; Gaudy *et al.*, 2000; Calliari *et al.*, 2006). The decrease in *A. tonsa* abundance in 2010-12 also corresponded with the installation of *A. clausi* population throughout Berre Lagoon. Its penetration into the lagoon was also linked to salinity increase as *A. clausi* was shown to exhibit reduced ingestion and highly elevated cost of growth at low salinity (< 20) (Calliari *et al.*, 2006). Its optimal salinity range was found to be 24-30, matching the new salinity conditions observed in the lagoon (Cervetto, 1995; Gaudy *et al.*, 2000). Short-term variations of salinity may also explain the spatial segregation observed in Berre after the rehabilitation processes. Following this hypothesis, due to its higher tolerance to sudden salinity variations (Cervetto *et al.*, 1999; Hubareva *et al.*, 2008) and its ability for osmoregulation (Svetlichny *et al.*, this volume), *A. tonsa* would be better adapted to the northern part, which suffered influences of freshwater releases in surface. *A. clausi* would prefer the southern part with marine conditions closer to that observed in the adjacent coastal area.

Concerning chlorophyll, Boyer *et al.* (2013) showed that this factor played a far lower importance than salinity and temperature on egg production of *Acartia* species in another Mediterranean coastal lagoon (Thau Lagoon). However, the following development stages of *A. tonsa* selected their prey according to their physiological need: P-rich prey for the rapid growth of nauplii and N-rich prey for the slower growth of copepodites (Meunier *et al.*, 2015). For *Acartia* adults, the feeding behavior is even more complex since they are omnivorous (Kleppel, 1993; Tiselius, 1989). Even if the peaks of *A. tonsa* and *A. clausi* abundances appeared in Berre Lagoon at medium and low chlorophyll *a* concentrations respectively, the quality of prey could be more important than its quantity. While the feeding regime of *A. tonsa* was more oriented toward proteinic food than *A. clausi*, no relationship between ingestion and food concentration was observed for both species (Gaudy *et al.*, 2000). Thus, *A. tonsa* could be present in less eutrophic environments as it exhibits a fast response to food and a strong efficiency to remain inside ephemeral and thin patches (Tiselius, 1992). Likewise, *A. clausi* also occurred in hyper eutrophic environments in African sites (Arfi *et al.*, 1989). Nevertheless, in 2011, the succession of predominance periods was less evident with a higher relative contribution of *A. tonsa* from April. This predominance coincided with chlorophyll *a* concentrations twice higher in surface waters (MWU, $p<0.05$), values close to those observed before the rehabilitation processes (Cervetto, 1995; Gaudy *et al.*, 1995). Increased abundances of *A. tonsa* have often been associated to increase in chlorophyll and turbidity in other coastal systems (Derisio *et al.*, 2014; Biancala *et al.*, 2014).

The role of temperature, salinity and chlorophyll appeared not to be so obvious, as described in other coastal ecosystems (Jeffries, 1962; Alcaraz, 1983; Gaudy *et al.*, 2000;

Calliari *et al.*, 2006; Boyer *et al.*, 2013; Marques *et al.*, this volume; Svetlichny *et al.*, this volume; Villate *et al.*, this volume). Kimmel *et al.* (2012) showed that environmental and food web changes can be involved together to explain the decline in *A. tonsa* population in the central Chesapeake Bay. Then, a combination of site-specific factors has to be considered to explain modifications in *Acartia* assemblages (Lakkis, 1994; Calliari *et al.*, 2006). Trophic interactions, like competition and predation, can move out species from their optimal temporal and/or spatial distribution. Villate *et al.* (this volume) emphasized a restricted seasonal distribution of *A. tonsa* due to a competitive pressure with *Acartia bifilosa* in the estuary of Urdaibai.

In conclusion, the rehabilitation processes have managed to reduce salinity fluctuations and maintain it above 15. These modifications led to a space and time partitioning of *A. tonsa* and *A. clausi* along the salinity gradient as described in other brackish water systems. Changes in the distribution of *Acartia* species following the rehabilitation processes constitute a sign of a hoped recovery. Thus, even if Acartiidae constitute an important link to higher trophic levels in brackish environments (Alcaraz, 1983; Azeitero *et al.*, 2005; Werbrouch *et al.*, 2016; Villate *et al.*, this volume), the contribution of both *Acartia* species to the food web may be different. Werbrouch *et al.* (2016) highlighted that the replacement of *A. clausi* by *A. tonsa* is detrimental for higher trophic levels as *A. tonsa* presents a lower content of fatty acids (*i.e.*, DHA and EPA) in membrane lipids for the same body size. In Berre Lagoon, the cephalothorax length of *A. tonsa* was significantly lower than the one of *A. clausi* with mean values of 0.98 ± 0.12 cm (n = 272 ind. for the 2010-12 survey) and 1.08 ± 0.06 cm (n= 419 ind. for the 2010-12 survey), respectively (MWU, $p<0.001$; data not shown). Before the rehabilitation processes, the energy transfer was then likely less effective since *A. tonsa* was the only Acartiidae observed in the lagoon. The arrival of *A. clausi* in Berre Lagoon, resulting from salinity increase, could eventually drive to a rich and diverse pelagic ecosystem which is actually very damaged by the anthropogenic pressures of these last decades. For the moment, a positive influence on higher trophic levels is still expected as food web is dominated by gelatinous zooplankton considered as a trophic dead end, particularly the indigenous scyphozoan *Aurelia* sp. (Marques *et al.*, 2015) and two alien species (*i.e.*, the ctenophore *Mnemiopsis leidyi* (Delpy *et al.*, 2016) and the hydrozoan *Gonionemus vertens* (F. Delpy, June 2013, pers. obs.). Nonetheless, the presence of some planktivorous fish whether inhabiting the lagoon (*e.g.*, goby and atherine) or coming from the Mediterranean Sea through Caronte Canal (*e.g.*, sardine and anchovy) can offer hopes for a next improvement (GIPREB, pers. com.). These results constitute a very first step in the long road to Berre recovery.

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Chapter 66

THE IMPACT OF CONSPICUOUS ENVIRONMENTAL CHANGES ON THE SPATIAL AND TEMPORAL DYNAMICS OF *ACARTIA TONSA* AND *ACARTIA CLAUSI*: A DECADAL STUDY IN A TEMPERATE ESTUARY (MONDEGO, PORTUGAL)

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ABSTRACT

This chapter aimed to identify the role of natural environmental factors in the distribution of the congeneric species *Acartia tonsa* and *Acartia clausi* in the Mondego estuary, central Iberian Peninsula, over the period 2003-2012. *A. tonsa* was the dominant species during the study period, representing 84% of the total abundance of *Acartia*. The distribution patterns of *Acartia* species revealed a spatial segregation along the estuary, with *A. tonsa* confined to upstream areas, while *A. clausi* was restricted downstream. Both species showed seasonal variation, peaking during warmer months. Since 2007, *A. tonsa* exibithed yearly averages consistently above the long-term mean abundance. The results identified a clear effect of temperature warming on the ecosystem, favoring and accelerating the settlement of the non-indigenous species *A. tonsa* at an unexpectedly rapid rate.

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STATICO analysis revealed that 2007, 2010 and 2011 were years with strong temporal patterns, which best fitted the compromise analyses between *Acartia* abundance and environmental parameters. *A. tonsa* showed an overall positive relationship with temperature, salinity, and total suspended solids (TSS), while *A. clausi* presented a positive association with chlorophyll *a*. Local variability of freshwater flow was the dominant signal in the present time-series, and explained the observed dynamics in *Acartia* populations structure at shorter temporal scales. However, this effect could be masked by larger time-scale phenomena occurring simultaneously, such as a rise in water temperature, leading to a higher abundance of opportunistic tolerant-species, as *A. tonsa*.

These results suggest that copepods living in highly dynamic ecosystems are prone to shifts in community equilibria that show complex, non-linear responses to climatic oscillations.

Keywords: *Acartia tonsa*, *Acartia clausi*, Mondego estuary, environmental variability

1. INTRODUCTION

Zooplankton constitutes the ocean's essential secondary producers, representing a key link between primary producers and higher trophic levels (Turner, 2004). Their spatial and temporal variability are known to be linked with hydrological patterns, seasonal fluctuations, trophic status and pollution (*e.g.*, Kimmerer, 1993; Lawrence *et al.*, 2004; Uriarte *et al.*, 2005; Aravena *et al.*, 2009). Often, zooplankton communities are reported as more sensitive indicators of change than environmental variables themselves (Taylor *et al.*, 2002, Hays *et al.*, 2005), acting as integrators of hydroclimatic forcing and providing an accurate diagnosis of the ecosystem state (Beaugrand, 2005). Zooplankton is highly sensitive to short-term environmental variability, with studies reporting changes in zooplanktonic communities according to seasonal, tidal, and diurnal variations (*e.g.*, Kimmerer, 1993; Roman *et al.*, 2001; Lawrence *et al.*, 2004; Marques *et al.*, 2009; Menéndez *et al.*, 2012; Leandro *et al.*, 2014). Furthermore, zooplankton communities have been useful to track large-scale climate-driven environmental changes (Hays *et al.*, 2005; Richardson, 2008; Aravena *et al.*, 2009; Edwards *et al.*, 2013), and recent evidences of long-term studies indicate variations in the seasonal window and timing around the world (*e.g.*, Hinder *et al.*, 2012; Mackas *et al.*, 2012), as well as the reduction and redistribution of several species (*e.g.*, Hinder *et al.*, 2012; Edwards *et al.*, 2013; Gubanova *et al.*, 2014).

Global climate change has led to a sustained increase in ocean temperatures in the last decades. In the North Atlantic, for instance, sea surface temperature (SST) has increased by about 0.5 °C during the past 50 years (IPCC, 2013). Copepods play a fundamental role in the energy transfer to higher trophic levels, in the transport of organic matter to depth (*via* fecal pellets), in the dynamics of their main prey (phyto- and microzooplankton), and in the mineralisation of nitrogen and carbon *via* grazing (Turner, 2004). Thus, it is mandatory to know how these communities are affected by this increased climatic variability. Further, the phenotypic plasticity and tolerance to hydrological variability will also determine the zooplankton community structure in highly unstable ecosystems, such as estuaries (Dam, 2013).

Copepods clearly dominate the numerical abundance of zooplankton, particularly calanoid copepods (Mauchline, 1998). These small microcrustaceans graze upon phytoplankton and microzooplankton, including nauplii of their own class (Turner, 2004).

Among calanoids, members of the genus *Acartia* exhibit a clear supremacy in the plankton communities across several temperate and subtropical estuaries (Brylinski, 1981; David *et al.*, 2007; Marques *et al.*, 2007) and other semi-enclosed marine areas (Kimmerer, 1993; Roman *et al.*, 2001; Leandro *et al.*, 2014). Their spatial and temporal variability are known to be linked with hydrological patterns, seasonal fluctuations, trophic status and pollution (*e.g.*, Kimmerer, 1993; Lawrence *et al.*, 2004; Uriarte *et al.*, 2005; Aravena *et al.*, 2009). Recently, non-native species of *Acartia* are efficiently colonizing many coastal areas and estuaries, increasing the pressure on autochthonous copepod communities (*e.g.*, Comaschi *et al.*, 2000; Seuront, 2005; David *et al.*, 2007; Delpy *et al.*, this volume; Villate *et al.*, this volume). The increase in ocean temperature has been pointed out as an important factor for the successful establishment of these non-native species (Aravena *et al.*, 2009; Chaalali *et al.*, 2013; David *et al.*, 2007). Non indigenous *Acartia* species have beeen responsible for changing the seasonal distribution pattern of zooplankton leading to a phenological shift in the native copepod *Acartia bifilosa* production period at the Gironde estuary (David *et al.*, 2007), the decreased abundance of the autochtonous *Acartia clausi* in the estuary of Bilbao (Aravena *et al.*, 2009; Villate *et al.*, this volume), or the complete replacement of formerly abundant *Acartia margalefi* in the South Adriatic Sea (Brugnano *et al.*, 2011). Changes in the ecosystem also favoured the non-native *Acartia* species to the detriment of the indigenous copepods in the early 1970s in the Black Sea (Gubanova *et al.*, 2014): in this case, increased pollution and eutrophication have been identified as the main factors determining the replacement of native species, rather than the increase in temperature (Gubanova *et al.*, 2014). Also, changes in salinity proved to be unfavorable for the autochthonous species of *Acartia* congeners in the Gironde estuary (David *et al.*, 2007) and in the estuary of Bilbao (Aravena *et al.*, 2009). Non-native species are extremely efficient at colonizing brackish waters, which are more vulnerable to the establishment of alien species owing to the greatest natural 'indigenous species minimum' (Paavola *et al.*, 2005).

Relevant interannual variability observed in the weather conditions over the Iberian Peninsula attests to the decreasing trend in annual precipitation in Portugal (Rodrigo and Trigo 2007; Santos *et al.*, 2010). In the particular case of the Mondego estuary, located in the Portuguese Atlantic coast, lower annual precipitations have led to a reduction in river flow, influencing also the extent of the estuarine salinity gradient and of the spatial distribution of planktonic species (Marques *et al.*, 2008, 2014; Primo *et al.*, 2011, 2015). This highlights the strong influence of water exchanges with the Atlantic Ocean on the composition and dynamics of local pelagic communities (Bento *et al.*, 2016; D'Ambrosio *et al.*, 2016). As in other temperate estuaries, *Acartia tonsa* represents the main species of planktonic communities of this estuary, only outreached by its congener *A. clausi* (Marques *et al.*, 2006; Primo *et al.*, 2009). The congeners present different seasonal and spatial distributions patterns in the estuary and are generally linked with estuarine and marine water masses, respectively.

The main aim of this contribution was to identify the role of natural environmental factors in the populations of the coexisting species *A. tonsa* and *A. clausi* in the Mondego estuary over a 10-year period (2003-2012). To answer this objective, we analysed and described the influence of environmental factors (hydrology and biology) on *Acartia* species dynamics and assessed the effects of different environmental regimes on congeneric spatial segregation. Our approach reveals that variability of freshwater flow explained the observed dynamics in *Acartia* populations structure at shorter temporal scales. However, this effect

could be masked by an increase in water temperature, leading to a higher abundance of opportunistic tolerant-species as *A. tonsa*.

2. METHODS

2.1. Study Area

The Mondego estuary (40º 08' N, 8º 50' W) is located in a warm temperate region on the west coast of Portugal (Figure 1). This system experiences a Mediterranean temperate climate and is classified as a mesotidal estuary. About 7 km from the sea, the river branches into 2 arms (northern and southern), which converge again near the mouth. The two branches exhibit different hydrographic characteristics: the northern branch presents a low residence time (<1 day, Kenov *et al.*, 2012), is deeper (4–8 m during high tide), and is the location of the Figueira da Foz harbour, constituting the main navigation channel and being subjected to regular dredging activities. The southern branch is shallower (2–4 m deep, during high tide) with longer residence time (2–8 days) (Kenov *et al.*, 2012).

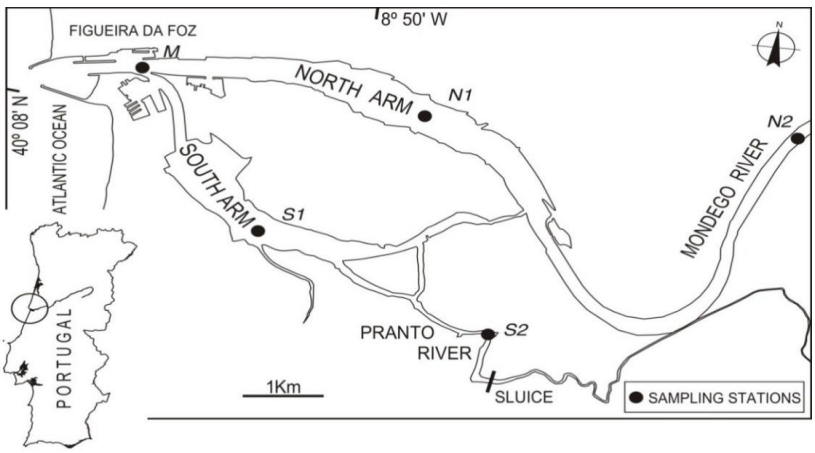

Figure 1. Map of the Mondego estuary showing the location of the sampling sites. M (mouth site), S1 and S2 (southern arm sites), N1 and N 2 (northern arm sites).

2.2. Environmental and Biological Data

Data on the abundance of *Acartia clausi* and *Acartia tonsa* cover a 10 year period (2003 to 2012) and were obtained from monthly samples at 5 stations distributed throughout both branches (M – mouth, N1 and N2 – northern branch, S1 and S2 – southern branch; M, S1 and N1– downstream areas, S2 and N2 – upstream areas) (Figure 1). This constitutes a unique time series dataset for an estuarine system of the West Iberian coast (which started in 2003), considering the consistency in the sampling methods and number of samples. Zooplankton samples were collected by subsurface horizontal hauls, using a 335 μm mesh bongo net (mouth diameter: 0.5 m). Subsequently, all collected organisms were fixed and stored with buffered formaldehyde (4%). The zooplankton analysis was carried out under a Leica M80

stereomicroscopice and subsamples (a minimum of 500 individuals were counted) were obtained for numerical abundance using a Folsom-splitter. Records of *Acartia* were only based on adults, since the mesh size used under-estimates the early life stages. Abundance data were standardized as the number of ind. m^{-3}.

In situ surface salinity and water temperature were recorded with appropriate sensors (WTW Cond 330i) simultaneously to zooplankton sampling. Water samples were also collected for determination of total suspended solids (TSS, mg l^{-1}; APHA, 1992) and chlorophyll *a* concentration (Chl *a*, mg m^{-3}; Parsons *et al.*, 1984) as a measure of total phytoplankton biomass.

2.3. Statistical Analysis

Interannual variability of biological and environmental variables was represented by standardised anomalies (z-scores), computed as deviations from the mean of the time-series divided by the respective standard deviation. Additionally, the relationship between environmental variables was determined using the Spearman's non parametric correlation ρ, using SigmaPlot v12.5 (Systat Software). When applicable, results were presented as mean ± standard deviation (STD).

In order to investigate the influence of the environmental conditions on *Acartia* populations, species abundance and environmental parameters (temperature, salinity, Chl *a* and TSS) for each year and sampling site were combined to generate two series of tables: one for the environmental parameters and another for *Acartia* species densities. Each pair of tables corresponded to the same sampling year (in rows). Therefore, both tables (biological and environmental) were composed of 10 matrices (2003-2012). Prior to calculations, *Acartia* abundance was log(x+1) transformed, in order to minimize the dominant effect of exceptional values, and environmental data were normalized.

In order to detect change points in *Acartia* abundance and environmental parameters, the cumulative sum (CUSUM) of the deviations from the mean of the reference period 2003 to 2012 were computed. The interpretation was based on the sign and steepness of the slopes, which reflect the deviation of a period from the time-series mean value (Ibañez *et al.*, 1993).

The STATICO method (Simier *et al.*, 1999; Thioulouse *et al.*, 2004) was carried out to analyze the two series of tables (Figure 2). In this chapter, the common structure between environmental and *Acartia* abundance tables and the stability of this structure over the sampling period were assessed. The samples, which must be the same for both paired tables but may vary between the pairs, correspond to a monthly sampling. The STATICO method proceeds in three steps: (1) the interstructure, which is achieved by performing a PCA for analyzing each table by a one table method (normed for the the environmental variables and centred for the species data); (2) the compromise analysis gives an ordination of the environmental parameters and an ordination of the *Acartia* species representing the average *Acartia*-environment relationship across the years; the compromise analysis links each pair of table Co-inertia analysis (Dolédec and Chessel, 1994) providing an average image of the co-structure (species-variables cross-table); and (3) the trajectories analysis involves the projection of the individual tables onto the axes of the compromise. This step allows visualisation of the similarities and differences amongst the years' structures, *i.e.*, environmental and *Acartia* cycles. A Partial Triadic Analysis (Thioulouse and Chessel, 1987)

was used to analyze the series of species and environmental variables cross-tables. STATICO allows building compromise maps composed of compromise axes on which observation (*i.e.*, monthly samples), species abundance and environmental factors from each original table are projected. Hence, it is possible to observe the correlation between species distribution and environmental factors. Calculations and graphs shown in this work were done using ADE-4 software (Thioulouse *et al.*, 1997; available at http://pbil.univ-lyon1.fr/ADE-4).

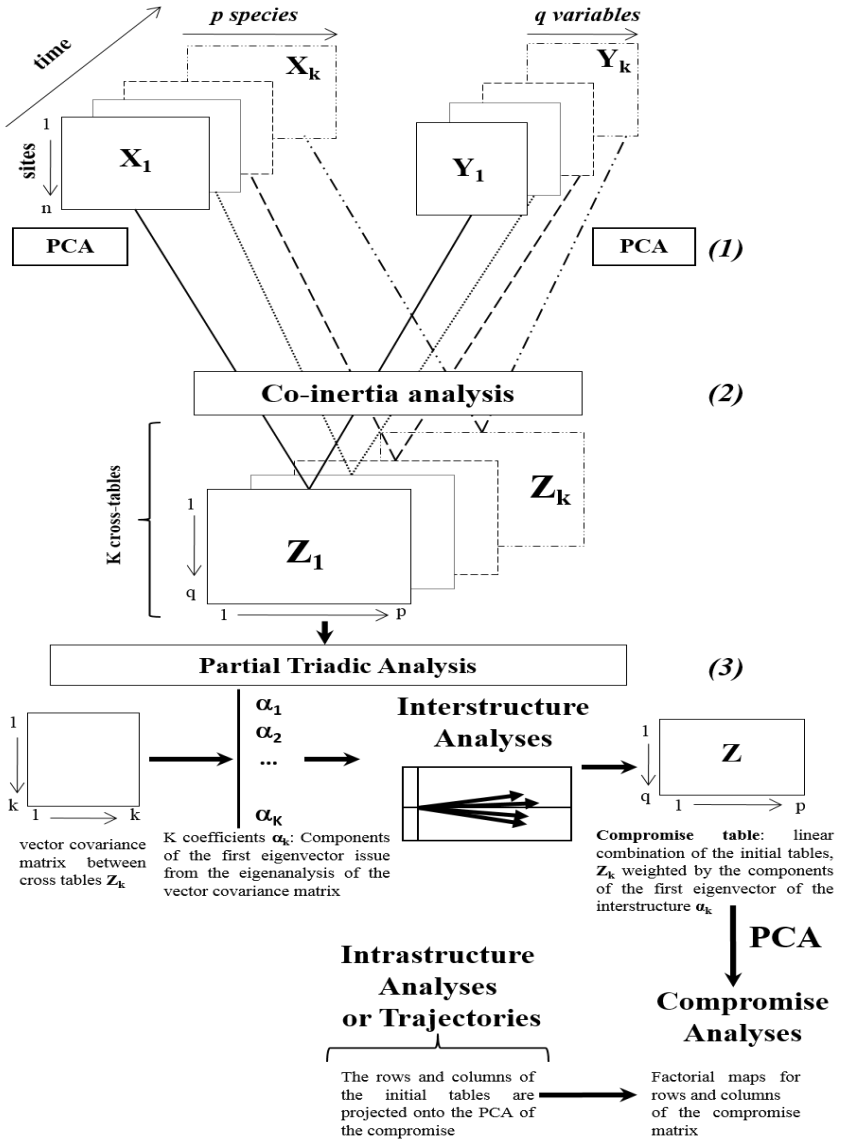

Figure 2. STATICO scheme. The data structure is a sequence of K paired ecological tables. X_k and Y_k are respectively the species (dimension n_k x p) and environmental (dimension n_k x q) tables in the pair. Z_k is the cross-table at k occurences; p is the number of species, q is the number of environmental variables, n_k is the number of rows at k different dates. (1) Basic analyses (PCA for species abundance and environmental tables) are performed on each table; (2) Co-inertia analyses allow linkage of the pairs of PCA-PCA, producing a sequence of K cross-tables; (3) PTA is finally used to analyze this sequence.

All data regarding precipitation and freshwater runoff were acquired from the Portuguese Water Institute (INAG, www.snirh.inag.pt) stations Soure 13/01G and Açude Ponte de Coimbra 12G/01A (nearby the city of Coimbra, since no meteorological station was present in the study area). In accordance with the reports of the Portuguese Institute for Sea and Atmosphere (IPMA, https://www.ipma.pt/pt/), three major drought events were considered during the study period: 2004-2005, 2007-08 and 2012 (García-Herrera *et al.*, 2007; Trigo *et al.*, 2013).

3. RESULTS

3.1. Variabilty of Environmental Parameters and *Acartia* Abundance

The years sampled included a very wide range of environmental conditions, encompassing extreme climate events (Figure 3). Compared to the mean precipitation regime for central Portugal during 1981-2010 (winter: 92.4 mm; spring: 58.3 mm; summer: 8.8 mm; autumn: 87.1 mm, http://www.ipma.pt/pt/oclima/normais.clima), it was possible to distinguish three periods where drought regimes prevailed, namely 2004-2005, 2007-2008 and 2012 (García-Herrera *et al.*, 2007; Trigo *et al.*, 2013). According to IPMA, 2009 was considered a regular to dry year, while 2010 and 2011 regular years. The drought episode of 2004-2005 was considered the most severe in terms of meteorological data, extent of the area affected, and impacts on different socio-economic and environmental sectors (https://www.ipma.pt/pt/).

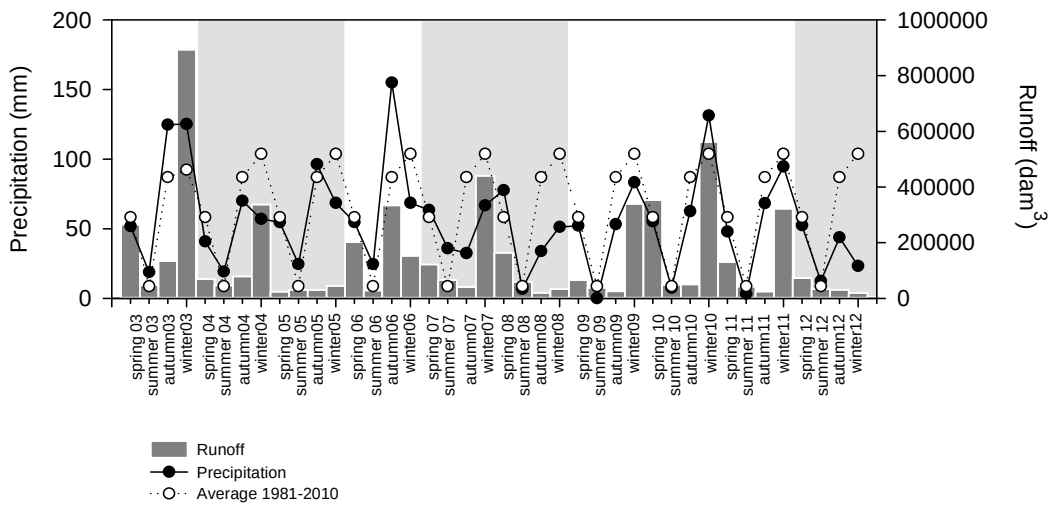

Figure 3. Seasonal precipitation (mm) and runoff (dam^3) for the 10-year study period (2003-2012) in the Mondego estuary. Shaded areas represent drought periods. Black circles – precipitation (mm), white circles – average precipitation for 1981-2010, and grey bars – runoff (dam^3).

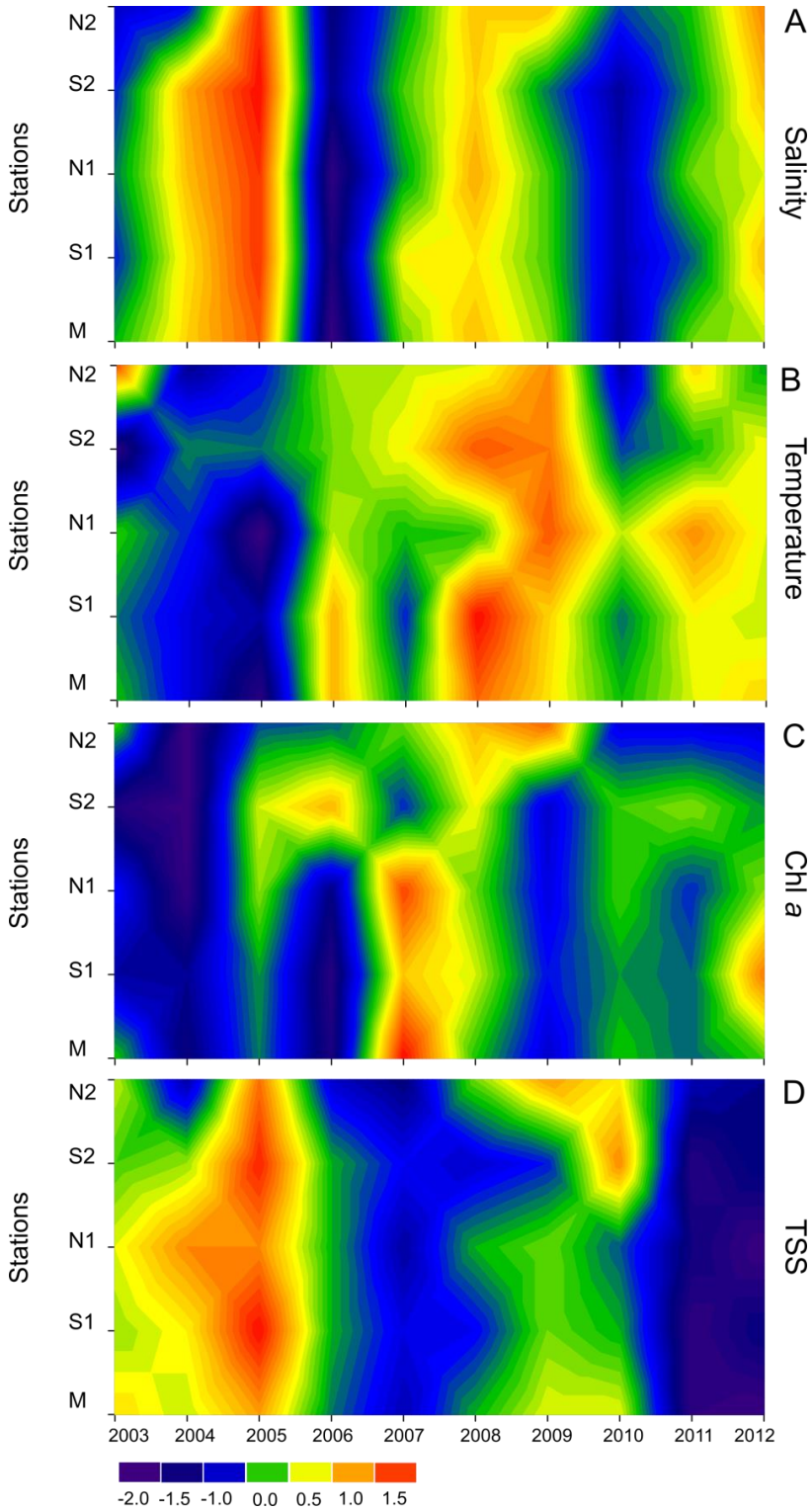

Figure 4. Standardized anomalies of mean annual (A) salinity (surface), (B) water temperature, (C) chlorophyll *a* (Chl *a*) and (D) total suspended solids (TSS).

Table 1. Spearman correlations coefficient between the environmental parameters anomalies: temperature (°C, Temp), salinity (Sal), chlorophyll a (mg m^{-3}, Chl *a*), total suspended solids (mg m^{-3}, TSS), precipitation (mm, Precip) and runoff (dam^3). Significant *p*-values are in bold; **p* > 0.05; **0.05 > *p* > 0.01; ***0.01 > *p* > 0.001

	Sal	Chl *a*	TSS	Precip	Runoff
Temp	-0.164	0.136	**-0.413*****	-0.233	-0.104
Sal		0.229	0.204	**-0.612*****	**-0.812*****
Chl *a*			-0.166	-0.164	-0.245
TSS				**0.368****	0.073
Precip					**0.673*****

The variations of freshwater discharge into the Mondego estuary were clearly influenced by precipitation (Figure 3), showing a highly significant positive relationship between these two parameters (Spearman rank, $p < 0.001$, R = 0.67, Table 1). Maximum salinity values were typically observed during the lowest runoff periods described above, especially in 2004-2005, due to a drought started in 2004 and extended to an extreme event in 2005 (Figure 4A). In comparison, low salinity values were observed in 2003, 2006 and 2010, concomitant with the years of highest river runoff registered during the study period. The salinity anomalies values exhibited a highly significant negative correlation with runoff ($p < 0.001$, R = -0.81, Table 1) and precipitation ($p < 0.001$, R = -0.61, Table 1). Dealing with water temperature, the ten years presented the typical annual pattern for temperate ecosystems, with lower values in winter (9.0-15.2ºC) and higher in summer (15.4-26.7ºC) (data not shown). Positive anomalies were recorded in 2006 (particularly downstream), with highest positive deviation in 2008-09 and 2011-12 (Figure 4B).

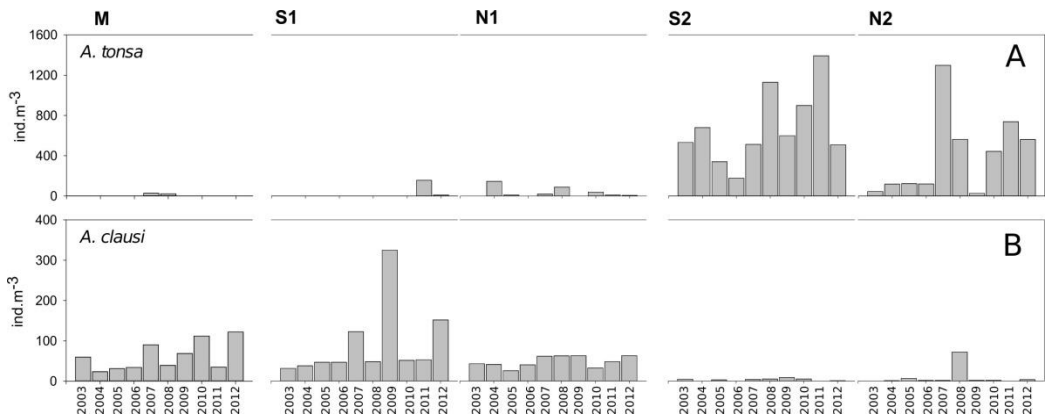

Figure 5. Variability of (A) *Acartia tonsa* and (B) *Acartia clausi* abundance (ind. m^{-3}) over the 2003-2012 period.

Chlorophyll *a* values varied between 0.9 and 29.9 mg m^{-3} (mean = 6.7 ± 5.4 mg m^{-3}) during the 2003–2012 period. This parameter was generally above the long-term average in 2007 (downstream), 2008 (upstream), and 2012 (downstream) (Figure 4C). Higher positive average of TSS were recorded between 2003 and 2005, and in 2009-2010, more pronounced at upstream, while the lowest were observed in 2007 and 2011-12. The correlation between TSS anomalies and water temperature anomalies was highly significant ($p < 0.001$, R = -0.41, Table 1).

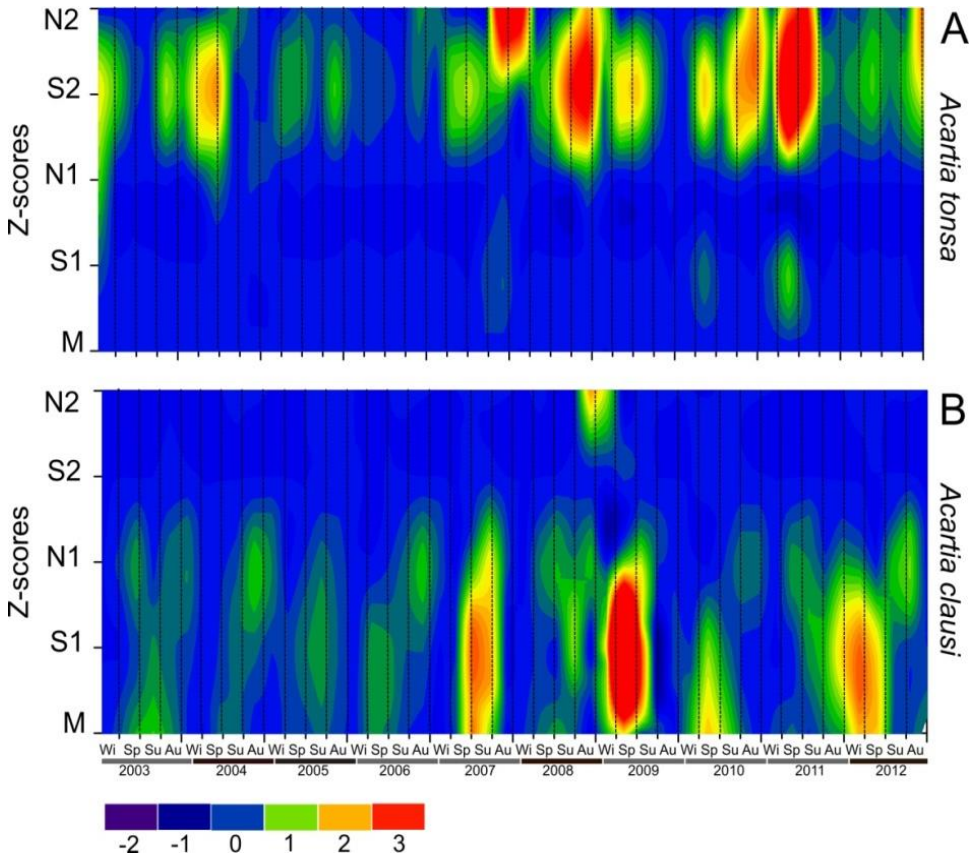

Figure 6. Z-scores of (A) *Acartia clausi* and (B) *Acartia tonsa*, abundance in the Mondego estuary over the 2003-2012 period (winter-Wi, spring- Sp, summer – Su, autumn-Au).

Over the 2003-2012 period, *Acartia tonsa* was the dominant species, making up 84% of the total abundance of *Acartia* (Figure 5). The distribution pattern of *Acartia* species revealed a spatial segregation along the estuary. On one hand, *A. tonsa* was confined to the upstream areas, with an average annual density of 540.5 ± 401.0 ind. m^{-3} (Figure 5A). The anomalies in *A. tonsa* abundance revealed a cyclical variation, with peaks during the warming seasons (Figure 6A). After 2007, *A. tonsa* abundance increased considerably, with yearly averages consistently above the long-term mean. In autumn 2007-2008, 2010 and 2012, the species exhibithed higher amplitude of its seasonal maxima compared to other years (max. 4.089, 2.618, 2.075, 1.605 ind. m^{-3}, respectively) and extended its period of presence until winter in 2010 and 2012. On the other hand, its congener *Acartia clausi* was more restricted to the lower estuary (mean = 67.2 ± 58.0 ind. m^{-3}). A clear general pattern was observed over time, with a bimodal increase of *A. clausi* abundance in spring and summer. The highest positive anomalies were detected in summer 2007 (377 ind. m^{-3}), spring 2009 (1,235 ind. m^{-3}), spring and winter 2012 (291 and 287 ind. m^{-3}, respectively) (Figure 6B). Noticeable changes also occurred in the phenology of *A. clausi*, with an earlier timing of seasonal peak after 2007, with the occurrence of major abundance peaks in late winter/early spring.

3.2. *Acartia* Variability in Relation to Environmental Parameters

3.2.1. CUSUM Analysis of Time-Series

Regarding environmental variability, water temperature showed two main periods, delimited by a breakpoint in 2008 (Figure 7A). The first period (2003-08) was characterized by lower seasonal values. Afterwards, an upward change in temperature was observed. Salinity showed higher variability: two main periods of positive slope, 2005-early 2006 and late 2007 until 2010, were highlighted by the cumulative sums (Figure 7B). Chl *a* presented a clear upward trend after 2007 (Figure 7C), while TSS showed positive slope in 2006-07 and during the period 2009-2012 (Figure 7D). As for *Acartia*, the abundance of both species exhibited a synchronized increase after 2006 and 2007, for *A. tonsa* and *A. clausi*, respectively (Figures 7E, F).

Overall, despite the variability in the CUSUM analysis, a significant positive correlation was observed between water temperature and Chl *a* ($R = 0.43$, $p < 0.001$), which may have also played a role in influencing the changes in *Acartia* abundance, since a positive correlation was also detected between them and Chl *a* (*A. clausi*, $R = 0.57$, $p = 0.001$ and *A. tonsa*, $R = 0.70$, $p = 0.001$). TSS showed negative significant correlations with *A. clausi* ($R = -0.59$, $p = 0.001$) and *A. tonsa* ($R = -0.53$, $p = 0.001$).

3.2.2. Interstructure Analysis of the Years

The first axis represented 94.5% of the total inertia (3.4% for the second axis, Figure 8A). Therefore, no structure inversion was evidenced from one sample to the other, and distribution patterns of *Acartia* were (at least partially) common to all sampling years. Weights (reflecting the contribution of each sub-matrix in the construction of the compromise) and $\cos^2$ (indicating of how much the compromise expresses the information contained in each table) values (Table 2) showed that each year shared characteristics with the compromise, despite this has some individual features. From the analysis of the correlation and weights (Table 1, Figure 8), the common temporal pattern appeared to be stronger in 2007, 2011, 2010 and 2006 (higher weight and longer arrows), exhibiting a strong association with PC1; this in turn indicated similar structures and meant that the compromise would be more influenced by these years. The years of 2004, 2005, 2008, 2009, 2012 presented lower weight (short arrows) indicating that their corresponding tables were less structured and consequently their contribution to the compromise was lower.

3.2.3. Compromise Analysis

The factor map of the first two axes are shown for the *Acartia* populations (Figure 9A) and environmental variables (Figure 9B). Axis 1 accounted for 53.9% of the explained variance and differentiated the samples with higher and lower abundance, whilst Axis 2 (10.63% of the explained variance) indicated a clear separation in the distribution patterns of *A. clausi* and *A. tonsa* within the estuary (Figure 9A). As can be seen from the environmental compromise analysis (Figure 9B), water temperature and salinity were displayed on the positive section of Axis 1, confirming that high abundance were typically observed in years of higher salinity and temperature. Overall, chlorophyll *a* was positively linked to Axis 2, whereas TSS was negatively associated to this axis.

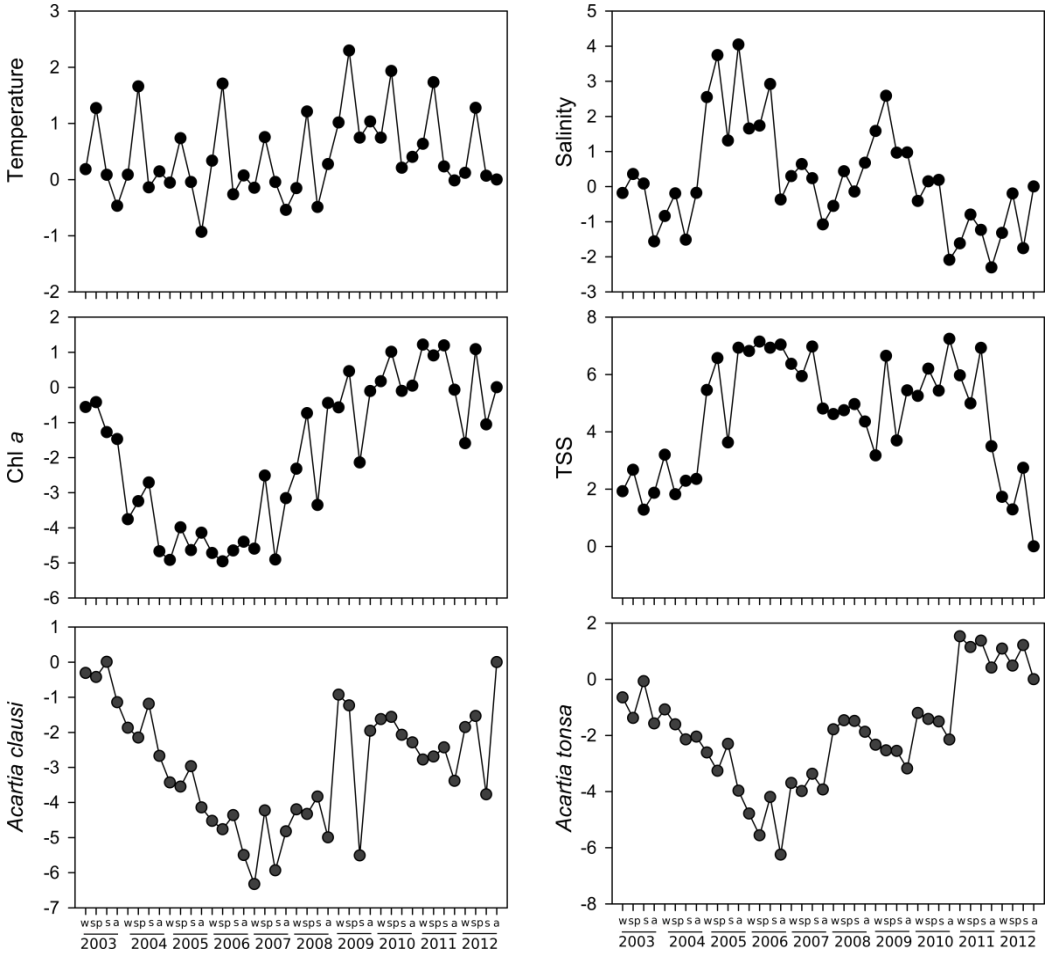

Figure 7. Cumulative sums of normal standard deviates of (A) water temperature, (B) salinity, (C) clorophyll *a* (Chl *a*), (D) total suspended solids (TSS), (E) *Acartia clausi* abundance (F) *Acartia tonsa* abundance; 2003 to 2012 (winter -w, spring - sp, summer-s and autumn-a).

Table 2. Correlation matrix and typological value indices: Weights = weights of tables in the compromise; cos² = square cosine between table and approximated compromise

Year											Weights	cos²
2003	1000										0.277	0.629
2004	230	1000									0.116	0.081
2005	712	293	1000								0.273	0.621
2006	601	65	571	1000							0.387	0.572
2007	623	61	611	496	1000						0.456	0.645
2008	434	70	378	383	412	1000					0.206	0.250
2009	440	200	447	524	354	175	1000				0.245	0.400
2010	573	306	571	509	509	410	592	1000			0.403	0.622
2011	589	269	638	503	666	393	420	501	1000		0.407	0.640
2012	476	247	537	462	555	241	422	569	525	1000	0.218	0.477

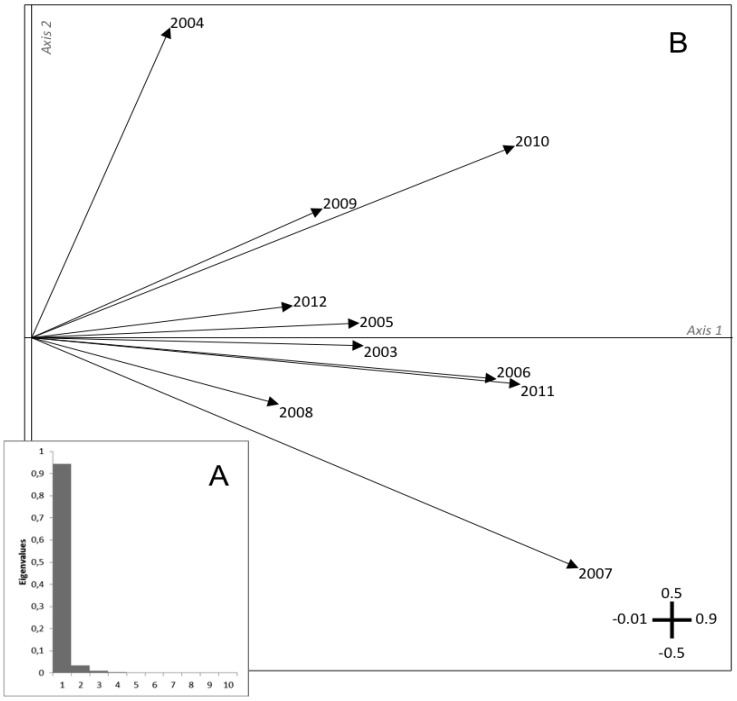

Figure 8. Results from the interstructure analysis. (A) Histogram of eigenvalues. (B) Factorial map for years. The contribution of sampled years to the compromise map is indicated by the strength of association with each principal component (PC1, x-axis; PC2, y-axis) extracted from the analysis. The y-axis is the second principal component (PC2). Scales for axes is given in the bottom right corner.

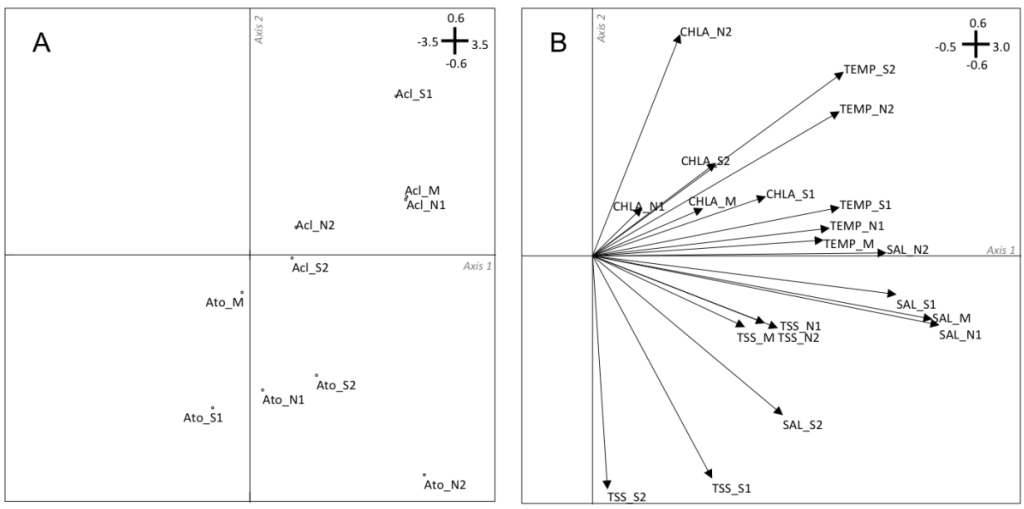

Figure 9. Compromise factor maps of the STATICO analysis. This plot represents the typical (A) *Acartia* population structure and (B) environmental structure in the Mondego estuary. Ato – *Acartia tonsa*, Acl– *A. clausi*, CHLA – chlorophyll *a*, TEMP – water temperature, SAL – Salinity, TSS – total suspended solids. The scales for axes are given in the right upper corner.

In the downstream areas of the estuary (sites M, S1, N1), the higher abundance of *Acartia* were associated to *A. clausi*, whereas in the upstream areas (sites S2 and N2) they were associated to *A. tonsa*. These results confirmed the positive relationship between *Acartia*

abundance with temperature and salinity. Axis 2 also permitted to evidence the positive association between Chl *a* and *A. clausi*, while *A. tonsa* was more related with TSS.

3.2.4. Trajectories Analysis

The projection of the environmental variables and *Acartia* abundance on the compromise axes is showed in the factorial map of trajectories (Figure 10). High interannual variability in the *Acartia* populations and environmental conditions was evidenced, and differences between years were predominantly driven by variablity of salinity and temperature. The characteristic structure of the species-environmental dynamics, revealed by the compromise analysis, was well expressed in 2007, 2010 and 2011, which corresponded to the highest abundance peaks of *A. tonsa*. A different scenario emerged in 2003, 2005 and 2009, when a spatial segregation in *Acartia* species abundance was not so evident.

The co-structure analysis (divided according to sampling years) clearly showed the dynamics of the *Acartia* species-environment relationships and highlighted seasonal differences (Figure 11). Whatever the date, the species points (circles) were more stable than the environmental points. This expresses the steady establishment of the *Acartia* assemblages, despite the high environmental variability (salinity and water temperature, in particular). The end of the arrows (environmental variables) had comparatively different values (generally presented separated) and simultaneously close *Acartia* abundances (the circles were in general in the same area). Indeed, considering the species, the sites were regularly projected on the right-hand side of the first axis, characterized by the highest *Acartia* abundances (see Figure 9A).

In general, and notwithstanding the strong dispersion of the environmental points and a poor fit between the *Acartia* species and environment parameters (long arrows), summer, autumn and winter were regularly grouped together, expressing a better consensus in the species-environment relationship. In fact, this was most clear (in general) for 2007, 2010 and 2011. Overall, in winter the environmental points (given by the end of arrows) were located on the left-hand side of the first axis, corresponding to “lower environmental values”. On the other hand, environmental points (end of arrows) corresponding to summer (followed by autumn) were located on the right-hand side of the first axis, which means “higher environmental values”. In sum, species and environmental parameters denote the absence of a clear coupling between environmental and copepod trajectories.

CONCLUSION

Estuaries are higly dynamic and variable environments, which promote not only a low level of species diversity, but also the co-existence of congeneric species. Our results revealed considerable complexity in the processes structuring the dynamics of the two congeneric *Acartia* species, *Acartia clausi* and *Acartia tonsa*, which represent nearly 60% of the total zooplankton abundance of the Mondego estuary (Marques *et al.*, 2006), a shallow coastal ecosystem under the influence of river discharge. In these ecosystems, zooplankton abundance is characterized by a high degree of spatial and temporal variability (Kimmel, 2011). Our results showed the existence of a spatial segregation between congeneric species: *A. clausi* was restricted to the downstream areas where salinity is relatively higher, while *A.*

tonsa occurred at upstream sections of the estuary. Environmental pressures characterized in the present chapter (*e.g.*, salinity, temperature and chlorophyll *a*), together with biotic and physiological aspects already observed by other authors (*e.g.*, Gaudy *et al.*, 2000; Leandro *et al.*, 2014), explain the spatial distribution pattern for these two species at the Mondego estuary.

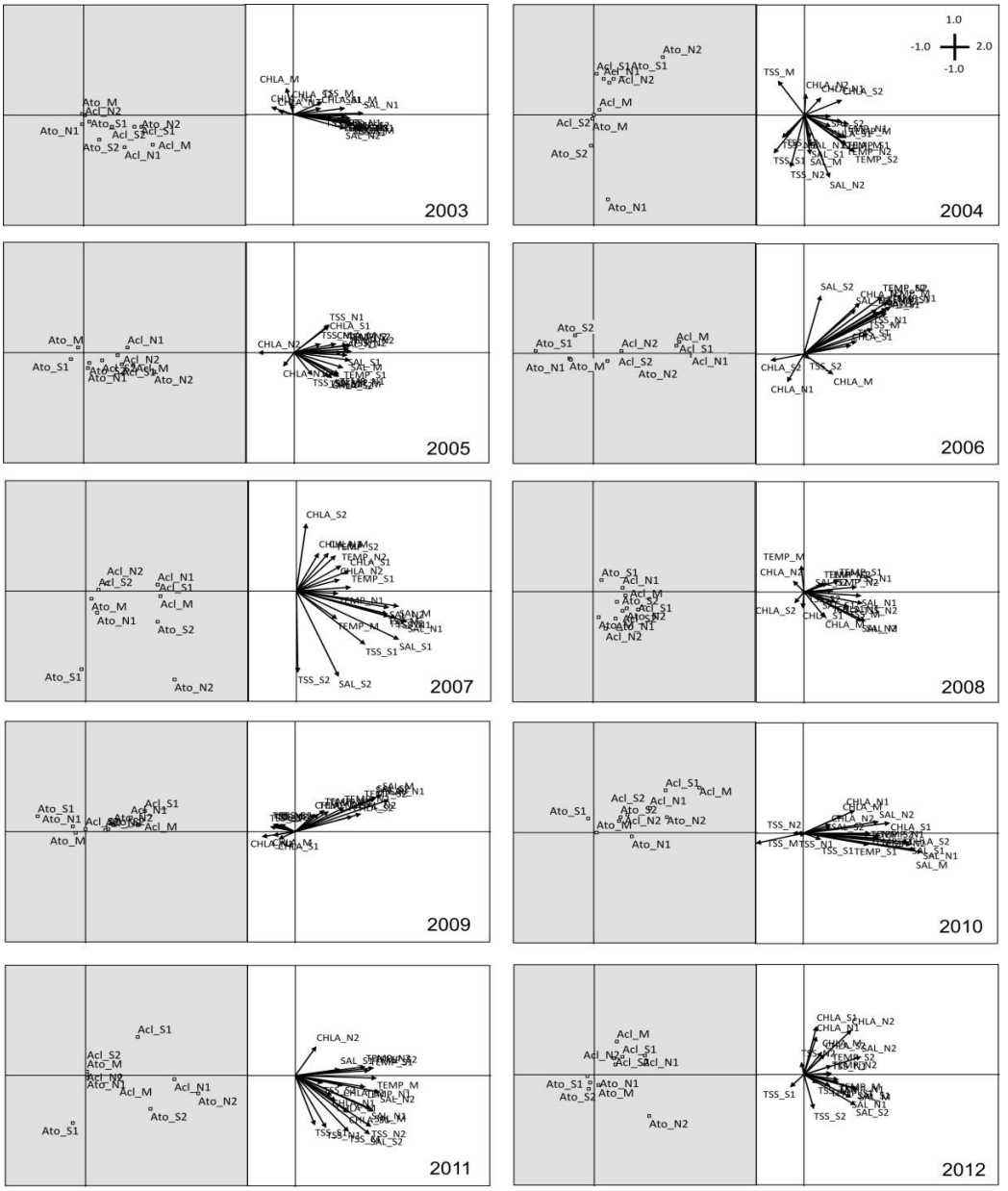

Figure 10. Factorial map of trajectories for species (grey) and environmental parameters (white). Depicted are annual projections of *Acartia* abundance and environmental variables along the two principal components of STATICO analysis. Scale for axes are given at the top right corner.

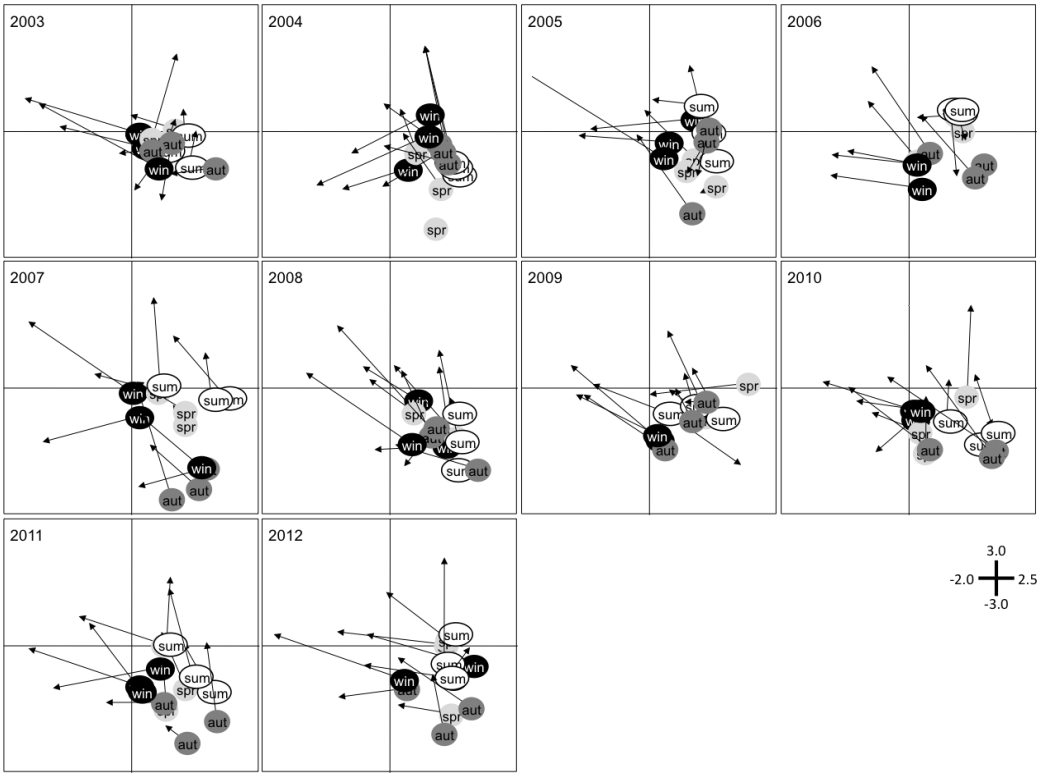

Figure 11. Trajectories factor plots of the STATICO analysis: projection of the samples in respect to seasons on the first factorial plan of the compromise analysis. Each sample is represented by two points: one is the projection of the row of the *Acartia* table (circle: origin of arrows), and the other is the projection of the row of the environmental table (end of arrows). The length of the connecting line reveals the disagreement or the consensus between the two profiles (*Acartia*–environment), *i.e.*, the length of the line is proportional to the divergence between the datasets. When the datasets strongly agree, the arrows will be short. Likewise, a long arrow demonstrates a locally weak relationship between the environment and *Acartia* features for that case. The scales for axes are given in the right lower corner.

A. tonsa was first observed in the estuary in 1994 by Azeiteiro *et al.* (1999), and since then persistent proliferation and swarming demonstrate the adaptive behavior of this species. In this sense, Gaudy *et al. (*2000) described the influence of temperature and salinity on the metabolism of *A. clausi* and *A. tonsa* and concluded that, for several temperatures tested, at the salinity of 35 psu, respiration rates were lower in *A. clausi* than in *A. tonsa*, with the contrary being observed at the lowest salinity. High respiration rates reflect the existence of some physiological constrains (*e.g.*, osmotic pressure) that are overtaken through costly energetic processes. If more energy is available for growth and reproduction, that will be reflected on the population structure of a given copepod species – higher abundance and high reproductive rate. A dense population of *A. tonsa* occurring at a specific location as a result of the combination of optimal environmental conditions (temperature, salinity and food) will have a negative effect on other pelagic copepod population like *A. clausi* given its predatory impact on other copepod species.

Results from the present work corroborate findings suggesting that *A. tonsa* is an opportunistic-tolerant species, which can take advantage of thermal increase. An increment in

water temperature has been suggested as a key factor to explain the introduction of *A. tonsa* in some estuarine ecosystems (Kimmel and Roman, 2004; David *et al.*, 2007). According to Leandro *et al. (*2006 a, b), *A. tonsa* has a stronger response to temperature than the majority of marine and estuarine calanoid copepods, including *A. clausi*, exhibiting higher growth rates and lower developmental times. By developing faster, *A. tonsa* populations will be able to dominate the estuarine zooplankton community. Coastal waters have warmed during the last century, leading to profound consequences for the dynamic regime of coastal ecosystems (Scavia *et al.*, 2002; Goberville *et al.*, 2010). In fact, the abundance of this species showed an increase after 2007-2008 in the inner upstream areas of the estuary, concomitantly with an increase in water temperature and chlorophyll *a* concentration. In the western Iberian coast, previous studies showed that hydrographic modifications covary with the secular trend of both sea surface temperatures and the North Atlantic Oscillation (NAO) (Pérez *et al.*, 2010). Indeed, the interannual changes of the NAO showed enhanced variance (twofold higher) after 2008 that encompassed a dramatic drop in the NAO during 2010–2011, followed by a marked reversal change of the signal (Marques *et al.*, 2017). In turn, the physical environment in the Mondego estuary showed prominent monthly variations of hydrological conditions that exhibited a larger variance around 2007–2010 as well. This suggests a close link between the environmental conditions at the Mondego estuary and the NAO through the influence of the later on regional atmospheric variables in the Northeast Atlantic coast, such as temperature, atmospheric pressure, wind and precipitation, and whose variability permeated into the environmental conditions in the estuary, as suggested by the temporal patterns of autotrophic and heterotrophic communities (Marques *et al.*, 2017). Overall, the observed structural change in the *Acartia* populations lies in the direction of pervasive structural changes in Northeast Atlantic coastal ecosystems. Indeed, increased abundances of copepod cosmopolitan species in the western English Channel have been shown to be linked to hydroclimate changes acting in the North Atlantic Ocean (Reygondeau *et al.*, 2015).

The STATICO approach used in this work proved to be an efficient tool to analyse sequences of paired ecological tables. This technique has previously been used by Simier *et al.* (2006) to study the spatial and seasonal variability of fish assemblages in Gambia estuary. Recently, Mazzocchi *et al.* (2012) applied this statistical method in planktonic communities in order to describe the variability of copepod assemblages in relation to local environmental dynamics. In both works, it was possible to visualize the variations in the species distribution and abundance patterns as a function of different environmental scenarios. As already demonstrated by Mendes *et al.* (2011), the STATICO advantage over other classical methods, such as canonical correspondence analysis (CCA) and redundancy analysis (RDA) commonly used in community ecology, is that it provides a complete and consistent analysis framework and the results presented are thus interesting and important for understanding estuarine dynamics. The findings reported in the STATICO compromise analysis suggested that water temperature and salinity were key factors determining the inter-annual occurrence and abundance of *Acartia* species in the Mondego estuary. In estuaries with a wide range of brackish habitats, salinity is thought to determine the spatial segregation of *Acartia* species (*e.g.*, David *et al.*, 2007; Aravena *et al.*, 2009; Leandro *et al.*, 2014). During regular years (in sense of precipitation) the Mondego Estuary presented a dynamic horizontal salinity gradient, with values increasing gradually from the inner to the lower estuary. These hydrographical conditions induced a spatial segregation in the distribution of *A. tonsa* and *A. clausi*.

A. clausi is a neritic species that occurs in the Mondego estuary as a result of water mass transport, namely advection during the flood, and its population dynamics is not directly dependent on the hydrological estuarine characteristics. The relatively stability of nertitic waters is reflected on the constant occurrence of *A. clausi* at the most downstream station of Mondego estuary between 2003-2012. Despite the evidence that *A. clausi* can tolerate a broad range of salinities (*e.g.*, Cervetto *et al.*, 1999; Gaudy *et al.*, 2000), our results indicate that a decrease in salinity had an unfavourable effect on *A. clausi* populations, which was revealed by their distribution restricted to the outer part of the estuary. This agrees with observations in other estuarine systems of the European Atlantic coast, where *A. clausi* populations reach higher densities at higher salinity areas (*e.g.*, Uriarte *et al.*, 2005; Albaina and Irigoien, 2007).

In Portugal, a high inter-annual variability and irregular distribution of precipitation was observed between 2003 and 2012, which was responsible for altered hydrological regimes in the Mondego estuary. As a result, drought conditions gave rise to prolonged periods of reduced freshwater inflow. This is in accordance with regional climate models that estimate sligh precipitation decreases for southern Europe by the end of the 21st century (Miranda *et al.*, 2006; IPCC 2007), including in the Iberian Peninsula (Lehner *et al.*, 2006). The reduced river flow, caused by the general evolution of climate conditions, was an important factor that induced the observed trends in *A. tonsa* and *A. clausi* dynamics in the Mondego estuary. First, it is well known that the diffusive and advective properties of freshwater discharge play a critical role in the population distribution patterns and richness, as well as in their temporal variability (*e.g.*, Licandro and Ibanez, 2000; Sundby, 2000; Roman *et al.*, 2001; Lindley and Daykin, 2005). During dry years, the lower advective transport also allowed stabilization of the environmental estuarine conditions, and the recolonization of important species such as *A. tonsa* (Marques *et al.*, 2007).

However, the success of an invader population over time cannot be linked solely to abiotic parameters such as temperature or salinity. Other important factors in the Mondego estuary were total suspended solids and clorophyll *a*. These can be helpful to explain the lower abundance of *A. tonsa* at the downstream stations. The low concentrations of appropriate food in seawater (Paffenhöfer and Stearns, 1988) and the qualitative nature of food (less proteinic material in marine seston) (Gaudy *et al.*, 2000) could explain the difficulties of *A. tonsa* to develop in coastal marine waters, because of its inability to filter sufficient food at lower concentrations. Therefore, it is reasonable to consider that processes such as interspecific competition and distinct feeding strategies might also have played a significant role in the observed distribution patterns of the two copepod species.

In sum, local variability of freshwater flow was the dominant signal in the present time-series, and explained the observed dynamics in *Acartia* populations structure at shorter temporal scales. However, this effect could be masked by larger time-scale phenomena occurring simultaneously, such as a rise in temperature, leading to a higher abundance of opportunistic tolerant-species as *A. tonsa*. Indeed, more than one mechanism was probably operating simultaneously, thus establishing the complex nonlinear relationships between climate variability and zooplankton dynamics (Hays *et al.*, 2005), an issue that needs to be assessed based on longer observation periods.

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Chapter 67

TEMPERATURE, SALINITY AND OXYGEN CONCENTRATION IN LIFE CYCLE TRAITS OF THE BLACK SEA COPEPODS

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ABSTRACT

In the Black Sea copepod species from marine and coastal areas (including Ponto-Caspian and boreal relicts, old Mediterranean immigrants and recent Atlantic and Indo-Pacific invaders), the salinity tolerance ranges and osmotic responses to salinity changes were estimated. Also, the effect of temperature and dissolved oxygen concentration on respiration rate, feeding, growth and locomotion activity was studied in these copepods. In comparison with native marine species, conspecific alien copepods possessed wider salinity tolerance ranges and were able to osmoregulate. Moreover, the Ponto-Caspian relict *Calanipeda aquaedulcis*, capable to survive as in fresh as in hypersaline water, was found to be an exceptional osmoconformer over the salinity range of 0.2 to 40 psu. A population strategy of overwintering (unknown for cyclopoid copepods) was first reported for the warm-water Indo-Pacific cyclopoid *Oithona davisae*. The unique specific adaptation to local temperature and oxygen regimes was shown in *Calanus helgolandicus* (dominating species in the open zone of the Black Sea). Due to diel vertical migrations from surface warm oxygenated layers to deep cold hypoxic zones, the late developmental stages of this species can decrease their energy requirements and increase the duration and efficiency of lipid accumulation in the form of wax esters. Therefore, the values of definitive body size, lipid amount and productivity in the Black Sea *C. helgolandicus* are as high as those in *Calanus* species from the more productive North Atlantic seas. This

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review aims to synthesize the understanding of the processes of behavioral and physiological adaptation of the copepod species to the highly diversificated Black Sea environment.

Keywords: Copepoda, effect of temperature, salinity, oxygen concentration, Black Sea

1. INTRODUCTION

The Black Sea is nowadays the world's largest brackish and anoxic semi-isolated basin. Mean salinity of the sea upper layers amounts to about 18 psu (nearly half than that of the surface layers of the World Ocean). As a rule, water salinity decreases near the river creeks down to 15 psu and increases up to 22 psu at the depths bordering upon the hydrogen sulfide zone. In the coastal areas, the temperature varies from 0°C in winter to 28 – 29°C at maximum summer insolation. However, even in summer the upper layer temperature of the Black Sea may change sharply by 15 – 20°C due to the winds (Zaitsev, 2006). In the deep-sea regions, the cold intermediate layer (CIL) with the temperature of 6 – 8°C is located permanently under the upper quasi-homogeneous zone at the depth of 30 – 100 m. Below the CIL, temperature and salinity increase slightly whilst oxygen concentration decreases dramatically down to zero at a depth of 100–200 m, where a permanent halocline separates oxygenated waters from the sulfide-rich deep waters (Sorokin, 1983).

Copepods constituting the major Black Sea mesozooplankton fraction play the key role in transferring the primary production to higher trophic levels. Modern taxonomic composition of the Black Sea copepods (Gubanova *et al.*, 2014) reflects the general geological processes and modern trends of ecosystem formation (Mordukhay-Boltovskoy, 1960; Zaitsev and Alexandrov, 1998; Zaitsev and Oztiirk, 2001). According to the origins of the species, copepods inhabiting open and coastal Black Sea regions can be divided into six groups:

1) Mediterranean-Ponto-Caspian relict *Calanipeda aquaedulcis* (Grindley, 1969 and 1984). The most ancient inhabitant and primitive form is found in the waters with low salinity.
2) Palaearctic halophylic species like *Arctodiaptomus salinus* (Boxshall and Defaye, 2008) living in salty and brackish inland lakes, common in the mouths of rivers, discharge areas and estuaries of the Black Sea (Mordukhay-Boltovskoy, 1972) and also in the hypersaline lakes of Crimea (Belmonte *et al.*, 2012).
3) Boreal-Atlantic immigrants: *Calanus helgolandicus* (*euxinus*)*, *Pseudocalanus elongatus* and *Oithona similis*, because in the Black Sea these three species form the united deep-sea complex of cold-water copepods (Nikitin, 1929). Probably, in the period of melting of glaciers, the cold waters brought by rivers from the northern seas filled the ancient Black Sea (Polischuk, 1984; Zaitsev, 2006). This fact is in agreement with the results of Unal *et al.* (2006) reporting the absence of substantial genetic differentiation between the Black Sea *C. helgolandicus* and *P. elongatus* and the same species from the English Channel. According to Papadopoulos *et al.* (2005), the divergences between the Mediterranean and the Black Sea populations of *C. helgolandicus* are much older than the estimated dates of colonization of the Black Sea. Perhaps, the last connection between the North and Black Seas could have been

about 2800 - 2700 BP in the Late Holocene (Polischuk, 1984). Nowadays, *C. helgolandicus, P. elongatus* and *O. similis* constituted more than 90% of the Black Sea mesozooplankton inhabiting open zones of the sea.

4) Mediterranean immigrants like *Paracalanus parvus*, *Centropages ponticus*, *Acartia clausi*, *Pontella mediterranea*, *Anomalocera patersoni* that have invaded and ultimately adapted to local environmental conditions after the connection of the Mediterranean and Black Seas. These species form up to 90% of the total mesozooplankton in the Black Sea coastal area. Most of them prefer warm upper layers of the sea. This group also includes *Acartia margalefi, Paracartia latisetosa* and *Oithona nana*, recently they have disappeared from the Black Sea (Gubanova *et al.*, 2014).
5) Freshwater species: mainly calanoid copepods of the family Diaptomidae (Samchyshyna, 2011) and Cyclopoida introduced by river discharges, which usually occur in the sea water during the maximum river run-off.
6) Alien species *Acartia tonsa* and *Oithona davisae* introduced by ship ballast waters from the countries where these species are distributed. *A. tonsa* was transferred to the Black Sea via Baltic-Black sea connection (Belmonte *et al.*, 2011) probably starting from the North American Atlantic coastal regions which are considered to be a native area of this species (McAlice, 1981). *O. davisae* is a representative of the Indo-West Pacific Oithonidae (Ferrari and Orsi, 1984) which are distributed widely all over the world after the synanthropic introduction, probably from Japanese coastal waters.

Since the Mediterranean Sea (which connects the Black Sea with the Atlantic Ocean) is a highly saline and oxygenated water body with moderate temperature regime, one could suggest that the cold, brackish and hypoxic Black Sea was inhabited by the eurybiotic species. This hypothesis may be tested by studying the adaptive potential of the Black Sea copepods and mechanisms of their physiological adaptation to salinity, temperature and oxygen changes.

Table 1. Capture location and sampling and experimental conditions of studied species

Species	Capture location	Habitual conditions		Experimental conditions		
		Temp. (°C)	Salinity (psu)	Temp. (°C)	Salinity (psu)	Dissolved Oxygen (mg O_2 L^{-1})
*Calanipeda aquaedulcis**	Coastal salt lakes of the Black Sea	20 - 22*	18*	20 - 22	0 - 60	0.15 – 9.8
*Arctodiaptomus salinus**		20 - 22*	18*	20 - 22	0 - 70	0.22 – 9.8
Calanus helgolandicus	Ionian Sea	15	39	20	18 - 39	7
	Marmara Sea	15 - 20	22 - 38	20	18 - 40	7 - 9
	Black Sea	8	18	2 - 24	10 - 40	0.15 - 10
Pseudocalanus elongatus	Black Sea	8	18	6 - 22	18	7 - 10
Acartia clausi	Marmara Sea	18 - 22	20 - 22	20 - 22	22 - 39	7 -9
Acartia clausi	Black Sea	18 - 25	17 - 18	20 - 22	2 - 45	6.5 - 10
Acartia tonsa		18 - 25	17 - 18	20 - 22	0 - 70	5 - 9
Oithona similis		8 - 10	18	8 - 10	7 - 33	8 - 10
Oithona davisae		18 - 25	17 - 18	6 - 28	0 - 60	5 - 9
Oithona nana	Marmara Sea	20 - 25	18 - 20	20 - 22	10 - 32	7 - 9
*Cultured for 4 years under laboratory conditions						

Nikitin (1926) was the first to determine the temperature preferences of mass copepod species of the Black Sea. Nikitin and Malm (1932) studied their tolerance to oxygen, hydrogen ions and carbon dioxide concentrations. Kovalev (1966) investigated the effect of salinity on survival of the mass Black Sea copepods. The influence of temperature on growth and development of copepods was investigated by Sazhina (1987). Svetlichny (1989) analyzed the locomotor activity of *C. helgolandicus* under different temperatures.

This review aims to synthesize the understanding of the processes of behavioral and physiological adaptation of the key copepod species to the highly diversified Black Sea environment. In particular, the impact of changing salinity, temperature and oxygen concentration on survival, respiration rate, moving behavior, egg formation and lipid accumulation will be discussed for native and introduced species. This work is based on laboratory and field studies carried out by the Department of Animal Physiology and Biochemistry, Institute of Biology of the Southern Seas (IBSS) from 1984 to 2014.The list of the studied species and experimental conditions used are given in Table 1.

2. Salinity Tolerance of the Black Sea Species and Their Congeneric Populations from the Marmara and Ionian Seas

Salinity tolerance was studied in females of all the species listed in Table 1 (except *Pseudocalanus elongatus*). The experiments were carried out on board of research vessels "Professor Vodyanitsky," "Bilim" and "Knorr" during the cruises and in the laboratories of the Institute of Biology of the Southern Seas (Sevastopol), Istanbul University (Turkey) and Marine Biological Station, University of Salento (Italy).

To study the effect of salinity on the survival of copepods, we placed 20-30 (for species with the body length greater or equal to 1 mm) or 40-50 (for species with the body length of about 0.5 mm) active females of every species (in 3-5 treatments) into transparent 100 mL beakers filled with filtered seawater and exposed (at natural habitat temperature) to a gradual decrease or increase in salinity over the periods ranging from 2 to 10 h at a rate of 2–3 psu h^{-1} (Hubareva *et al.*, 2008; Svetlichny *et al.*, 2012a and b; Hubareva and Svetlichny, 2016). The range of salinity changes was determined according to the results of preliminary experiments (Kovalev, 1966; Svetlichny *et al.*, 2006a). The salinity tolerance range of copepods was estimated basing on the values of the median lethal salinity (LS_{50}). To access the ability of copepods to shift the salinity tolerance range, in some experiments the females which had survived at the LS_{50} were exposed to a slow uniform increase in salinity due to natural evaporation of seawater. Salinity tolerance of *Calanus helgolandicus* was estimated taking into account behavioral and physiological response of separate individuals to salinity change.

2.1. Salinity Tolerance in *Calanipeda aquaedulcis* and *Arctodiaptomus salinus*

Calanipeda aquaedulcis and *Arctodiaptomus salinus* are representatives of two taxonomically and ecologically close families: Pseudodiaptomidae and Diaptomidae. According to Grindley (1984), "Pseudodiaptomidae have arisen from the sea and represent an intermediate stage in adaptation to the freshwater environment" while "Diaptomidae appear

equally well adapted to the fresh-water environment." Although *C. aquaedulcis* and *A. salinus* are not strictly marine copepods, these two species have been considered for comparison with the marine ones.

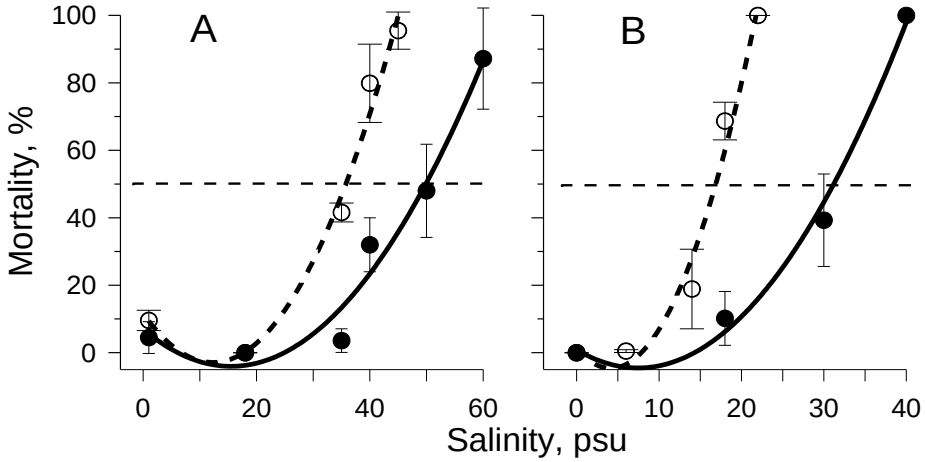

Figure 1. Salinity tolerance of *Calanipeda aquaedulcis* (A) and *Arctodiaptomus salinus* (B) reared at 0.2 psu (○) and 18 psu (●) in 5–10 d after gradual salinity acclimation. The salinity tolerance range in females and males of both species was between 0.1 psu; the horizontal long-dashed line denotes 50% mortality (from Svetlichny *et al.*, 2012a with changes).

According to our results, *C. aquaedulcis* and *A. salinus* are able to develop successfully as in fresh water, as in the Black Sea brackish water. These species have been kept for 4 years in the laboratory at a room temperature and salinity of 18 psu, and two months prior the experiment half of the culture was transferred to fresh water. For fresh water copepod generations, the salinity tolerance ranges were within 0.2 to 17 psu in *A. salinus* and 0.2 to 35 psu in *C. aquaedulcis*. For individuals of both species acclimated to 18 psu (Black Sea water) the borders of salinity ranges were shifted to 35-40 psu (*A. salinus*) and 50 psu (*C. aquaedulcis*) (Figure 1). In our experiments 50% of population of *C. aquaedulcis* could survive gradual salinity changes during 8 - 10 h up to 30 psu, whereas that of *A. salinus* tolerated only salinity alterations limited to a range of about 20 psu.

2.2. Salinity Tolerance of Native *Acartia clausi* and Alien *Acartia tonsa*

Copepods of the genus *Acartia* inhabit many coastal and offshore environments where they are usually among the most abundant zooplankton species. *Acartia tonsa* possesses a wide range of tolerance to salinity whilst *Acartia clausi* is considered to be a more stenohaline species (Stalder and Marcus, 1997; Calliary *et al.*, 2006 and 2008). *A. tonsa* adapted to high food concentration (Paffenhöfer and Stearns, 1988) usually is more abundant in the near-shore environments and estuaries. Similar trend in the distribution of these species is observed in the Black Sea. After the introduction of *A. tonsa* in the Black Sea presumably in 1976, both species can coexist (Gubanova, 2000); however, *A. clausi* dominates in the open zone of coastal areas, while *A. tonsa* occurs in semi-closed bays (Delpy and Pagano, this volume; Marques *et al.*, this volume; Villate *et al.*, this volume).

Salinity tolerance ranges for females of *A. clausi* and *A. tonsa* collected at 18 psu in Sevastopol Bay (Black Sea) were estimated as 10-35 and 3–30 psu, respectively (Figure 2). However, after 1 week of acclimation to 30 psu the salinity tolerance ranges were extended up to 50 psu in *A. tonsa*. Therefore, in comparison with *A. clausi*, *A. tonsa* introduced in the Black Sea possessed the salinity tolerance range shifted to lower salinity (up to 3 psu) and, at the same time, this species was able to acclimate to high- saline water (50 psu).

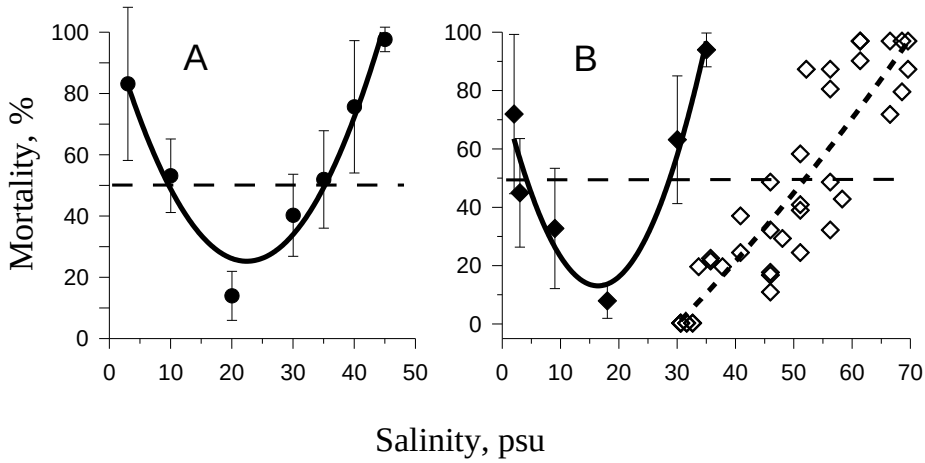

Figure 2. Mortality of *Acartia clausi* (A, ●) and *Acartia tonsa* (B, ♦) in 3 days after gradual salinity change of the individuals acclimated to 18–20 psu. ◊ - Effect of salinity on mortality of *A. tonsa* acclimated during one week to 30 psu. (Figure 2B from Svetlichny and Hubareva, 2014a with changes).

Consequently indigenous *A. clausi*, living at the quasi-permanent salinity of 18 psu in natural environment but demonstrating the ability to survive in the high-saline water, showed the "genetic memory" about the conditions of its oceanic origin. Salinity tolerance range of *A. tonsa* inhabiting the Black Sea was close to that of the same species from Øresund kept for several generations at *ca.* 33 psu in the laboratory (Calliari *et al.*, 2006), also being capable to sustain the decrease in the salinity down to 2 psu. Thus, *A. tonsa* may be considered as an extremely euryhaline species widely distributed in the eutrophic regions of the World Ocean.

2.3. Salinity Tolerance of the Copepods from the Genus *Oithona*

The effect of salinity on mortality of Oithonidae was studied (Figure 3) at 20 ºC for warm-water *Oithona davisae* and *Oithona nana* (Svetlichny and Hubareva, 2014a) and at 8-10 ºC for cold-water *Oithona similis* (Hubareva and Svetlichny, 2016). Salinity tolerance range (5–45 psu) of the Black Sea invader *O. davisae* (Ferrari and Orsi, 1984) in 3 days after the gradual salinity acclimation at a rate of 2-3 psu h^{-1} was significantly wider than that of indigenous copepods *O. similis* (10–30 psu) and especially *O. nana* (15–28 psu). Narrow salinity tolerance range of *O. nana* was known from the studies of Kovalev (1966) and, probably, may be one of the reasons of the elimination of this species from the Black Sea as the most vulnerable component of the zooplankton community which underwent dramatic changes during last decades (Gubanova *et al.*, 2014).

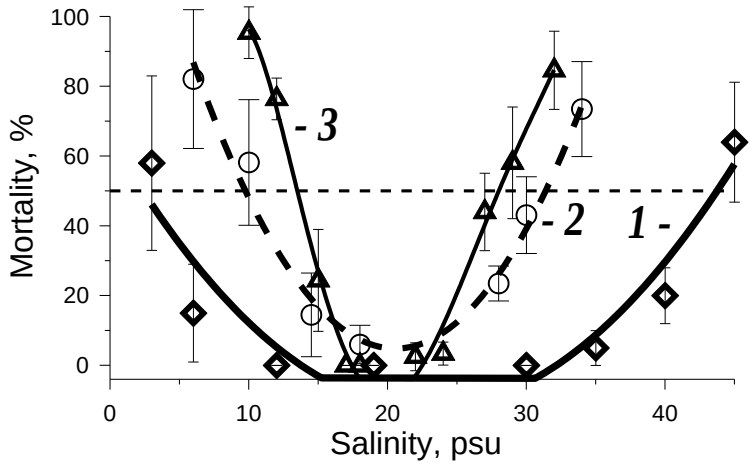

Figure 3. Salinity tolerance of *Oithona davisae* (1), *Oithona similis* (2) and *Oithona nana* (3) (from Isinibilir *et al.*, 2016).

On the contrary, high salinity tolerance of *O. davisae* seemed to facilitate the expansion of this species from Eastern Asia waters to the European and American seas.

An extraordinary sustainability of *O. davisae* was confirmed by our experiments (unpublished data) when 63.8 ± 20.5% of females were alive during 3 days after the transfer from 18 to 35 psu and 86.9 ± 9.7% of individuals survived after the transfer from 35 to 18 psu during the same period. After sharp increase in salinity the bodies of copepods shrunk and flattened. Such individuals descended to the bottom of the aquaria and kept the immobility for several hours. After that, their bodies reshaped and the copepods began to swim in the water again.

2.4. Salinity Tolerance of *Calanus helgolandicus*

Calanus helgolandicus is widely distributed in the North Atlantic seas. *Calanus euxinus* in the Black Sea is considered to be a phenotypic variation of *C. helgolandicus* (Papadopoulos *et al.*, 2005; Unal *et al.*, 2006; Yebra *et al.*, 2011). To evaluate the adaptive potential of this species, we studied the effect of gradual salinity changes on survival, locomotion and feeding (as mostly sensitive indicator of animal health) of *C. helgolandicus* living at the quasi-homogeneous salinity of about 38 and 18 psu in the Ionian and Black Seas, respectively, and in the Marmara Sea with a two-layer salinity structure (about 20 and 38 psu in the upper and lower layers, respectively).

In 0.5 L aquaria, the individuals of this species usually aggregate near the bottom whilst after salinity changes these copepods ascend to the surface and swim there. In *C. helgolandicus* collected in the Black Sea at 18 psu, a gradual decrease and increase in salinity at a rate of 2 psu h^{-1} within the range of 10-40 psu resulted in an increase in locomotion activity up to maximum values at 14 and 31 psu, and dramatic decrease at the following salinity change (Figure 4A). Although after salinity increase up to 40 psu the copepods remained alive, the muscles of these individuals became opaque indicating irreversible tissue changes (Svetlichny *et al.*, 2010b). More than 50% of studied copepods died in 3 – 5 days after gradual salinity increase higher than 30 psu (Figure 4B).

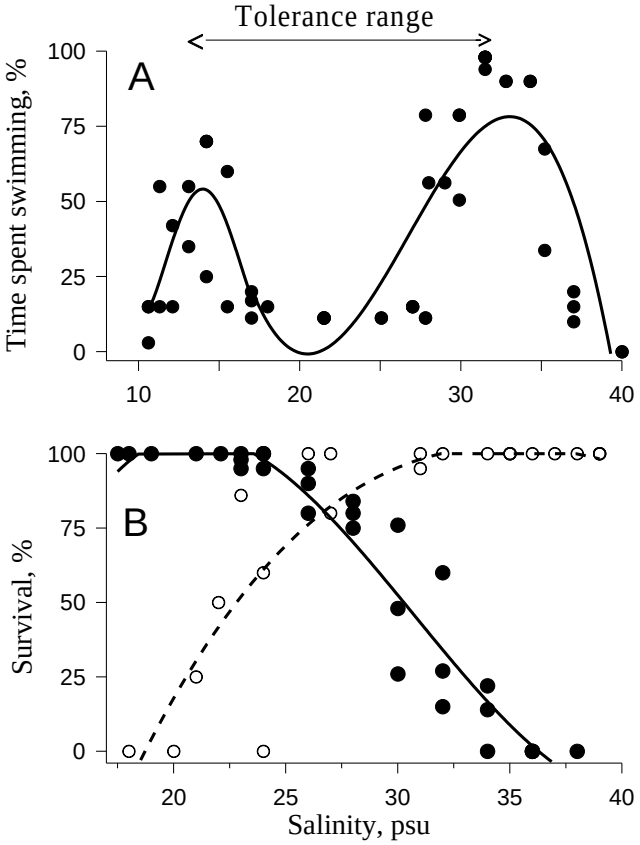

Figure 4. Time spent routing swimming (A) in the Black Sea *Calanus helgolandicus* as a response to gradual salinity changes and survival (B) of *C. helgolandicus* from the Black (black circle) and Ionian (open circle) Seas after 72 and 24 h of salinity changes, respectively (data on *C. helgolandicus* from the Ionian Sea from Isinibilir *et al.*, 2011).

In the feeding experiments (see details in Svetlichny *et al.*, 2010b) a gradual salinity increase from 17 to 25 psu during 1.5 h did not affect dinoflagellate *Prorocentrum minimum* consumption rate in females from the Black Sea. The anterior part of the gut was constantly full of algae in all individuals. During the following salinity increase, every change in the salinity (by 2–3 psu) brought to rapid gut evacuation and renewed feeding during the period of acclimation to new salinity. The duration of salinity acclimation period changed from 15-40 min at 17–25 psu to 80–120 min at 28-30 psu. When the salinity exceeded 31 psu, copepods completely stopped consuming algae. However, after the acclimation to 27 psu during about 3 days *C. helgolandicus* kept the ability to feed.

In *C. helgolandicus* inhabiting the Ionian Sea, the gradual salinity transition from 39 to 26 psu did not affect the survival of preadults and adults; however, after further reduction of the salinity down to 18 psu all animals died within 24 h (Figure 4B) (Isinibilir *et al.*, 2011). According to the LS_{50} criteria, the critical salinity for survival of population in the Ionian Sea was equal to 22 psu. Therefore, without the pre-adaptation the salinity tolerance range in *C. helgolandicus* living at constantly high salinity amounted to 22-40 psu whilst that for this species from the brackish Black Sea was about 15-30 psu (Svetlichny *et al.*, 2010b).

In the Marmara Sea with two types of water masses (brackish Black Sea and high-saline Mediterranean water), *C. helgolandicus* inhabit the whole water column performing diel vertical migrations (Svetlichny *et al.*, 2010b). From this perspective, the Marmara Sea can be considered as the zone of natural wide amplitude acclimation. In *C. helgolandicus* females sampled in deep high-saline layers of the Marmara Sea near the Prince Islands, moving activity did not change significantly after gradual (during 4 h) salinity increase from 22 to 40 psu and only at 40-50 psu we observed a pronounced depression of locomotion (Svetlichny *et al.*, 2010b). We found no regular trends in changing of locomotor activity of females kept for several days at 38.5 psu and then exposed to a short-term (2.5 h) gradual salinity decrease down to 22 psu. During gradual increase and further decrease in salinity within the range of 22–38.5 psu the copepods did not stop consuming food. The copepods were alive even after the acute transfer from 22 to 39 psu (Svetlichny *et al.*, 2010b).

2.5. Types of Osmotic Response in the Black Sea Copepods

According to Mauchline (1998), most marine copepod species are osmoconformers with the internal osmolality following the molality of the surrounding water. In this case, the mass body density of these animals should be proportional to the surrounding water density. It is advantageous for floating in the water because the buoyancy and sinking speed are constant, and energy losses to keep the body in the water are independent of the salinity. In the benthic copepod *Tigriopus brevicornis* mass density (determined in the formalin preserved individuals with the density test medium) linearly increased from 1.036 to 1.085 g cm^{-3} (McAllen *et al.*, 1998) with a salinity increase from 5 to 100 psu (Figure 5A). The authors considered this species to be a euryhaline osmoconformer. In the Black Sea, marine indigenous *A. clausi*, coastal *C. aquaedulcis* and *A. salinus* inhabiting the Crimean coastal hypersaline lakes the body mass density (measured in accordance with sinking speed of anesthetized individuals) varied in the range of 18–40, 0.2–43 and 0.2-40, respectively. This was directly in accordance with the theoretical expectation suggesting that copepod body volume is constant and that water content is iso-osmotic to the surrounding water (Figure 5A). In males, non-ovigerous and ovigerous females of *Eurytemora affinis* from Seine estuary (Seuront, 2006), sinking speeds did not change significantly within the salinity range from 0 to 35 psu, indicating that this estuarine species is also as strong osmoconformer, as the above mentioned Black Sea species. Therefore, an osmoconformic response to salinity changes can be attributed not only to marine copepods, but also to the eurybiotic species, such as *C. aquaedulcis* and *A. salinus.*

Nevertheless, some copepods may possess homeostatic mechanisms which permit physiological compensatory osmoregulation. The ability to regulate the inorganic ion content of hemolymph was found in the benthic copepod *Tisbe reticulata* (Battaglia and Bryan, 1964) and pelagic copepods *Calanoides acutus* and *Rhincalanus gigas* (Sartoris *et al.*, 2010). Osmoregulatory responses to salinity alterations in organic osmolyte content were reported for the marine copepod *E. affinis* (Roddie *et al.*, 1984) and estuarine copepods *T. californicus* (Goolish and Burton, 1989) and *Temora longicornis* (Tang *et al.*, 2000). The ability to keep a body fluid homeostasis allows species-osmoregulators to overcome abrupt salinity changes at rain, evaporation, river flow and tides. Jeffries (1962) suggested that estuarine *A. tonsa* had developed an efficient osmoregulatory mechanism. Nevertheless, Lance (1965) found no

clear evidence that *A. tonsa* could effectively control its water balance although in laboratory experiments the body fluid of this species was hyper-osmotic to the external medium salinities ranging from 90% to 15% sea water for 12 h.

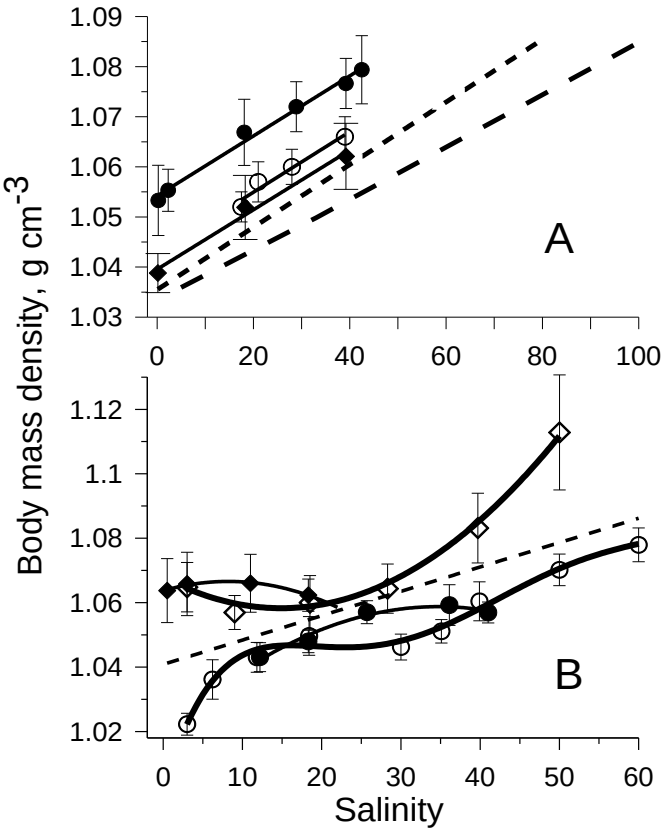

Figure 5. A: Effect of salinity on body mass density of females of *Arctodiaptomus salinus* (shaded diamonds) and *Calanipeda aquaedulcis* (shaded circles) reared at 18 psu for 4 years (from Svetlichny *et al.*, 2012a with additions and changes) and *Acartia clausi* (open circles) collected at 17.5 psu (unpublished data). Short-dashed line: theoretical change in body mass density in the case of ideal osmoconformity of copepods with a water content of 76% of the body volume. The long-dashed line shows the relationship between body mass density and salinity in *Tigriopus brevicornis* (McAllen *et al.*, 1998). B: Effect of salinity on body mass density of *Acartia tonsa* acclimated to 18 (◊, open diamonds) and 3 psu (♦, shaded diamonds), and *Oithona davisae* acclimated to 18 (○, open circles) and 41 psu (●, shaded circles). Short-dashed line shows the isometric changes of body mass density (from Svetlichny and Hubareva, 2014a with changes).

Our results (Svetlichny and Hubareva, 2014a) demonstrated the clear evidence of osmoregulation in *A. tonsa* from the Black Sea within the salinity tolerance range of 2–30 psu (Figure 5B). Females of *A. tonsa* collected at 18 psu and acclimated to 3 psu for 2 weeks, kept quasi-constant body density of 1.064 g cm^{-3}, however, responded hyper-osmotically at the salinity increase from 30 to 50 psu. It is worth noting that hyper-osmotic increase in body mass density of *A. tonsa* after the salinity increase from 30 to 50 psu in our experiments is not in accordance with the data on hypo-osmotic changes in free amino acid pool (Farmer and Reeve, 1978) and hemolymph Na (Farmer, 1980) in *A. tonsa* after the increase in external

salinity from 34 to 39 psu. Probably, the results of these authors may reflect not only the osmoregulation processes but also an inadequate metabolic response of the organisms under the extreme salinities because such experimental artifacts as starvation may affect *A. tonsa* body composition.

In *O. davisae* within the range 3–60 psu, three types of reaction to salinity changes were found: a) hypo-osmotic response: abrupt decrease in body mass density due to pronounced swelling and watering of the body after salinity changes from 6 to 3 psu; b) homeostatic regulation: maintenance of constant body mass density at salinity increase from 18.4 to 35.1 psu in females collected in the sea at 18 psu, and at salinity decrease from 41 to 25.7 psu in females acclimated for one week to 41 psu. This phenomenon may be due to low permeability of *O. davisae* integuments, providing an osmotic isolation from the external medium as a mechanism of physiological regulation of ion exchange in stressful habitats (Lee *et al.*, 2012); c) iso-osmotic response: body mass density changes equiproportionally to surrounding water salinity decrease from 18 to 6 psu and increase from 40 to 60 psu in the copepods collected in the sea at 18 psu.

Wide salinity range and osmoregulation ability in *A. tonsa* and *O. davisae* seem to be the features that facilitate the formation of self-reproducing populations of only these two species in the Black Sea (Gubanova *et al.*, 2014), despite the fact that a great number of copepods was brought with ship ballast waters from adjacent and distant seas (Selifonova, 2011).

2.6. Copepod Egg Salinity Tolerance

Despite the fact that females of some marine copepods (for example, *A. tonsa*) possess the ability to osmoregulate, the volume of their eggs immediately follows the salinity changes as a perfect osmometer (Calliari *et al.*, 2006; Hansen *et al.*, 2012), and the egg density alters as well (Miller and Marcus, 1994). At the decrease in salinity from 31 to 15 psu, mass density of eggs in *A. tonsa* from the Gulf of Mexico reduced from 1.087 to 1.066 g cm^{-3} (Miller and Marcus, 1994). In the Black Sea *A. salinus* acclimated to fresh water, a salinity increase from 0.2 to 18 psu resulted in an increase in the mass density of both resting and subitaneous eggs (Figure 6).

As a whole, in marine copepods hatching success of subitaneous eggs is in accordance with the parental salinity tolerance. Egg hatching success of widely euryhaline *A. tonsa* remained high as at extremely low salinity of 2 psu, as at high salinity of 39 psu (Calliari *et al.*, 2006; Svetlichny *et al.*, 2010a).

For copepods with narrower salinity tolerance range, egg hatching success is due to a parental shift in salinity acclimation. In *A. clausi* from the Black Sea almost all eggs died after the gradual salinity increase from 18 to 39 psu (Figure 7A); however, up to 50% of eggs of this species living in the Marmara Sea at changing salinity (22-39 psu) kept the ability to develop into nauplii at 39 psu (Svetlichny *et al.*, 2010a). On the contrary, egg hatching success in *A. clausi* maintained for several generations at a salinity of *ca.* 33 psu decreased dramatically at salinity lower than 20 psu (Calliari *et al.*, 2006).

Eggs laid by the Black Sea *C. helgolandicus* females at 17–18.7 psu died at salinities >30 psu, in contrast to 47 ± 11% of eggs laid by the Marmara Sea females which produced nauplii successfully after the salinity increase from 22 to 39 psu (Figure 7B).

Hansen *et al.* (2012) proposed that the embryo of *A. tonsa* can be protected from the salinity stress by its plasma membrane and that water exchange driven by osmosis was restricted to the perivitelline space of the egg. However, under extreme hypo-saline (0 psu) or hyper-saline (76 psu) conditions the eggs swelled or compressed, respectively, due to passive osmosis and killed the embryo.

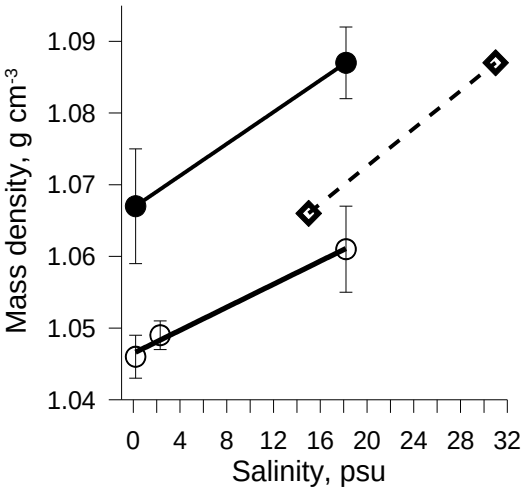

Figure 6. Effect of salinity on mass density of subitaneous (open circles) and resting eggs of *Arctodiaptomus salinus* (shaded circles) (data from Table 2 of Svetlichny *et al.*, 2012a) and subitaneous eggs of *Acartia tonsa* (open diamonds) (from Miller and Marcus, 1994).

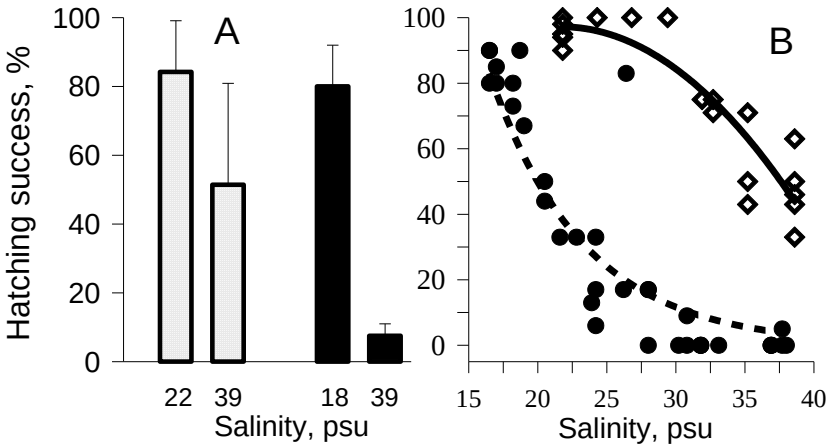

Figure 7. Hatching success of egg in *Acartia clausi* females from the Marmara (□) and Black (■) Seas (A) and *Calanus helgolandicus* females from the Marmara (◊) and Black (●) Seas (B) at different salinity (from Svetlichny *et al.*, 2010a with changes).

We observed similar effect of salinity on the eggs of *C. helgolandicus* from the Marmara Sea living at changing salinity. The eggs laid at 22 psu became wrinkled during gradual salinity up to 38.5 psu (Svetlichny *et al.*, 2010a) due to the loss of water (Figure 8). However, at long-term (1-2 days) keeping at this salinity the shape of survived eggs recovered and nauplii hatched successfully from these eggs.

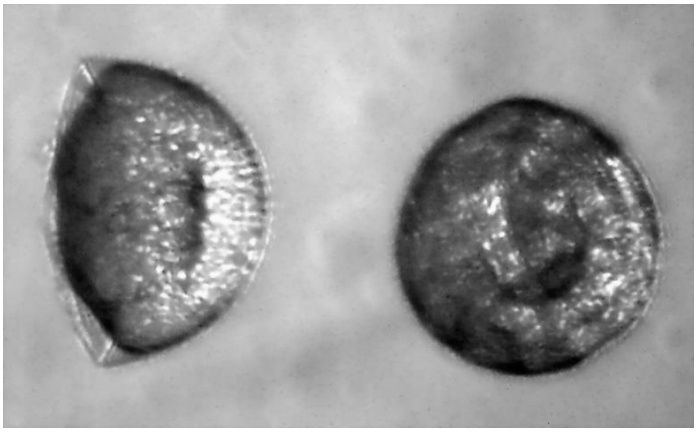

Figure 8. Eggs of *Calanus helgolandicus* from the Marmara Sea after salinity increase from 22 to 38 psu.

However, our results showed that copepod eggs are more sensitive to extremal salinities than the adults. Anufriieva (2014) found adults of *A. salinus* in hyper-saline lakes of Crimea at the salinity of about 300 psu. In our experiments, some *A. salinus* specimens survived for more than 14 d at the salinities of about 70 psu, and up to 15% of *C. aquaedulcis* females were alive for 10 d at 60 psu. However, we did not observe hatching of nauplii from the ovisacs of *A. salinus* and *C. aquaedulcis* females at salinities higher than 50 psu. Probably, this phenomenon was due to osmotic effects during hatching**.** According to Marshall and Orr (1972) and Davis (1959), nauplii burst out from eggs after cracking the outer membrane due to a sharp increase of pressure inside the inner case. The pressure increase seems to be caused by active absorption of water through the inner membrane. This suggestion may be confirmed by the increase in respiration rate of eggs at the hatching time (Nielsen *et al.*, 2007). According to Rokneddine and Chentoufi (2004), the reproductive potential of *A. salinus* from the Zima salt marsh in Morocco decreased 5-fold with an increase in salinity to the upper boundary of its tolerance range. In other estuarine copepods, the critical salinity for survival of their juvenile stages was also found to be lower than in adults of *E. affinis* (Ishikawa *et al.*, 1999; Lee *et al.*, 2007) and *P. annandalei* (Chen and Suzuki, 2006).

2.7. Effect of Salinity on Copepod Respiration Rate

Variations in respiration rate of copepods at the salinity changes are considered to be connected with additional energy losses for osmotic regulation (Gyllenberg and Lundqvist, 1978; McAllen and Taylor, 2001). Theoretically, the minimum osmotic work for ion transport constitutes only from 1 to 5% of the total metabolic energy requirements in brackish and freshwater animals (Potts, 1954). However, Goolish and Burton (1989) showed that the daily energy required for adjusting metabolism to hyperosmotic stress in *T. californicus* acclimated to constant salinity amounted to 11.6% of the total energy respired. In copepods metabolic rates of active individuals can exceed the basal metabolism by 6-fold (Buskey, 1998; Svetlichny and Hubareva, 2005); therefore, the energy of muscular activity associated with typical copepods avoidance response to unfavorable conditions may mask the losses for

osmoregulation. Probably, therefore, the results of numerous studies concerning the effect of salinity on respiration rates of copepods are very contradictory and are highly dependent on the functional mobility of the species.

For example, an increase in respiration rate of *A. clausi* and *A. tonsa* (Calliari *et al.*, 2006) and *Calanus finmarchicus* (Anraku, 1964; Marshall and Orr, 1972) followed to the increase in water salinity, while in *T. brevicornis* (McAllen and Taylor, 2001) an increase in salinity caused a decrease in oxygen consumption rate and activity as well. There was no evidence of salinity-associated respiratory distress in respiration experiments with *E. affinis* in the range of 0-40 psu (Roddie *et al.*, 1984). The respiration rate of *Pseudodiaptomus hessei* females also showed no differences within the salinity range of 3-31 psu (Isla and Perissinotto, 2004). According to Newrkla (1978), respiration rate and moving activity of *Arctodiaptomus spinosus* did not vary within the salinity range of 3–66 psu. In *C. helgolandicus* collected from the deep layers (38.5 psu, 15ºC) of the Marmara Sea and kept under laboratory conditions (20°C) at a salinity of 38.5 psu during 6 d and 22 psu over 13 d, weight-specific respiration rates did not differ significantly amounting to 1.62 ± 0.05 and 1.76 ± 0.2 μg O_2 mg^{-1} h^{-1}, respectively (Svetlichny *et al.*, 2010b). In the Black Sea for osmoconformers *A. salinus, C. aquaedulcis, A. clausi* and for osmoregulators *A. tonsa* and *O. davisae* there was also no significant difference in weight-specific respiration rates within the salinity tolerance ranges (Hubareva *et al.*, 2008; Svetlichny and Hubareva 2014a; Svetlichny *et al.*, 2012b).

3. Effect of Temperature

3.1. Effect of Temperature on Respiration Rate

Respiration rates of ectothermic animals are known to be affected by temperature with the Van't Hoff coefficient (Q_{10}) varying within the range of 2.0-2.5 (Prosser, 1973). According to Winberg, (1983), a mean Q_{10} value of 2.25 can be applied to the majority of hydrobionts within the range of tolerable temperatures. After the analyses of literature and own data on temperature dependence of respiration rate in planktonic copepods, Lee *et al.* (2001) concluded that most of them fitted well to Van't Hoff rule, however, the exceptions were found which may be interpreted as a dissimilarity of eurythermic and stenothermic responses in copepods (Conover, 1956).

Copepods inhabiting permanently the coastal zone of the Black Sea undergo mainly seasonal gradual changes in temperature (up to 30°C), whilst species from the open sea regions experience diel temperature variations during the warm season because nearly all these animals are able to migrate from warm (17 – 25°C) surface layers to cold (6-8°C) mixed strata. At the winter-spring homothermia, the temperature dependence of respiration rate for both copepod groups may be similar. In our study for cold-water *Calanus helgolandicus* and *Pseudocalanus elongatus* and overwintering (warm-water) *Oithona davisae*, respiration rate followed the Van't Hoff exponential rule (Figure 9) with the Q_{10} of 2.07 (Svetlichny *et al.*, 2009), 2.08 (unpublished data) and 2.06 (Svetlichny *et al.*, 2016), respectively. In the Marmara Sea *C. helgolandicus* the Q_{10} varied near 2 for all development stages from nauplii to adults (Svetlichny *et al.*, 2010b). The Q_{10} varying from 1.93 to 2.25 was found in *Acartia*

clausi for the range of 10 - 20ºC (Gaudy *et al.*, 2000). Similar Q_{10} values of about 2 were obtained for *Pseudocalanus newmani* (Lee *et al.*, 2001), *Calanus pacificus* (Vidal, 1980b) and other copepod groups (Ikeda *et al.*, 2001). However, in the experiments of some authors the temperature dependence of respiration rate in copepods was not found (*A. clausi*, see Anraku, 1964), or the Q_{10} was extraordinary high (about 6 in *Pseudocalanus* sp., see Isla *et al.*, 2008). Since the energy cost of swimming constitutes the main part of metabolism, these data discrepancies may be due to the specific behavioral response to temperature. Nevertheless, Hirche (1987) reported that in Arctic copepods respiration rates increased with temperature following the Arrhenius equation being independent of the activity. One should take into account that the trends in the temperature dependence of respiration rate of copepods may be affected by the combinations of methodical errors due to several factors including duration of experiment, capture and starvation stress (see review of Ikeda *et al.*, 2000).

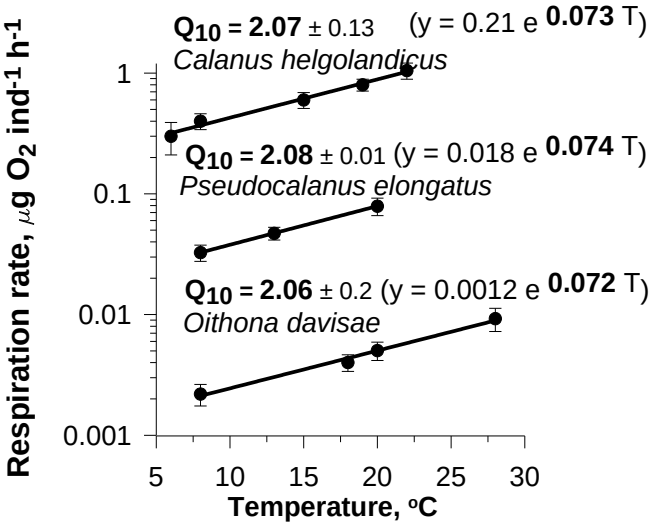

Figure 9. Effect of temperature on respiration rate of *Calanus helgolandicus*, *Pseudocalanus elongatus* and *Oithona davisae* (regression lines are constructed based on the tabular data from Svetlichny *et al.*, 2009 and 2016).

3.2. Effect of Temperature on Moving Activity

In calanoid copepods active swimming is realized by two principally different modes: uniform locomotion using the mouthparts limbs and unsteady jerk locomotion using mainly the thoracic limbs.

The effect of temperature on the parameters of swimming in copepods may be due to the temperature dependence of muscle contraction and neurogenic rhythm controlling muscle action (see a review of Lenz *et al.*, 2005). For example, the contractile rates of vertebrate skeletal muscle were temperature-dependent with the Van`t Hoff temperature coefficients Q_{10} of 1.95 – 2.42 (Bennett, 1985), and a pyloric rhythm in the intact crab increased significantly with the Q_{10} = 2–2.5 (Tang *et al.*, 2012).

Nevertheless, the Q_{10} values of locomotor parameters are predominantly lower than those mentioned above.

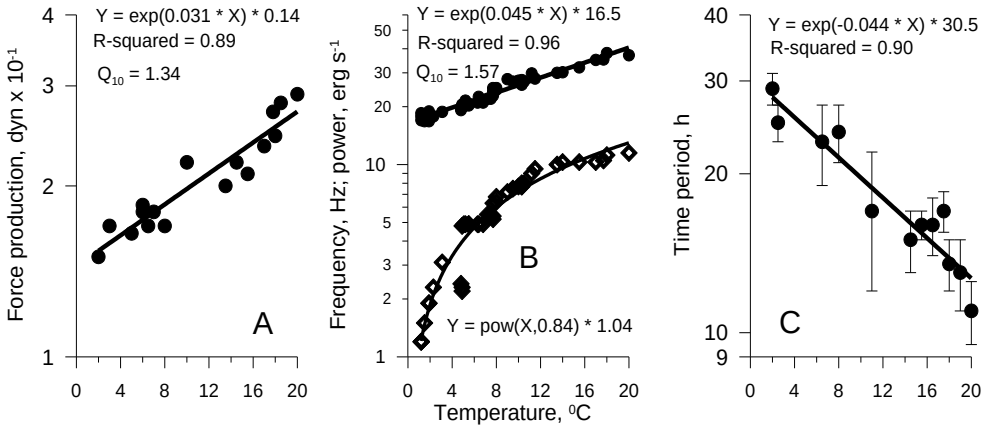

Figure 10. Effect of temperature on force production (A), beat frequency (●) and mechanic power (◊) of mouth limbs at routing swimming (B) and the period of circadian rhythm of activity (C) in *Calanus helgolandicus* (from Svetlichny, 1989).

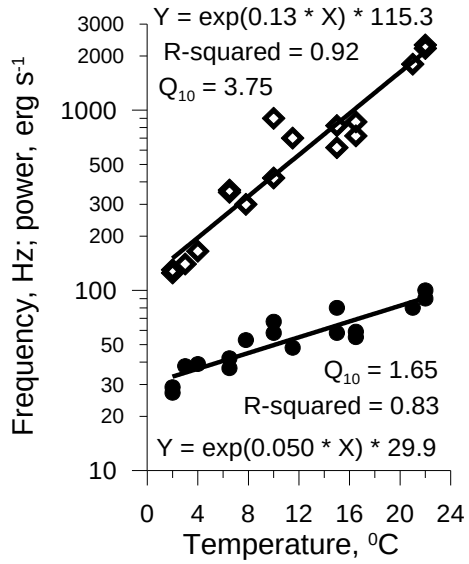

Figure 11. Effect of temperature on kick frequency (●) and maximum power (◊) of stroke phase of kicks during escape swimming of *Calanus helgolandicus* (from Svetlichny, 1989).

The frequency of locomotor patterns shows a strong effect of temperature on locomotor activity and behavior both in free swimming (Hirche, 1987) and tethered copepods (Lenz *et al.*, 2005; Gill and Crisp, 1985). In *Temora longicornis* frequency of beats by mouthpart limbs within the temperature range of 5-20ºC increased from 11.5 -15.4 to 23.8 – 30.2 Hz (Gill and Crisp, 1985) (our calculated Q_{10} = 1.66 ± 0.2). In the experiments of Larsen *et al.* (2008) the swimming velocity of *Acartia tonsa* in the temperature range of ~ 6 - 20ºC changed from 0.17 to 0.45 cm s^{-1} (Q_{10} = 1.7, calculated according to their equation in Figure 1). In behavior experiments of Lenz *et al.* (2005) with *Calanus finmarchicus* tethered to a force transducer, force transients produced by swimming legs and kick frequency during escape reaction increased with temperature with a Q_{10} value ranging from 1.23 to 1.86 and from 1.28 to 1.86, respectively.

In the Black Sea *C. helgolandicus*, force production and beat frequency of mouthpart limbs at routing swimming increased with the Q_{10} of 1.34 and 1.57 under strictly exponential law while the changes of power occurred in accordance with a power equation (Figure 10A, B). The period of the circadian rhythm of locomotor activity in copepod attached to a semiconductor force sensor in darkness was inversely related to the temperature described by the exponential equation (Figure 10C). It should be noted that the daily rhythm of 24 h was observed at a temperature of about 6^0C, which is close to the temperature of the cold mixed layer of the Black Sea where in total darkness *Calanus* spend the daytime during the warm season.

Parameters of quick and short-term escape reaction were also temperature-dependent with the Van`t Hoff temperature coefficients Q_{10} of 1.65 for the maximum jump frequency and 3.75 for mechanical power (Figure 11). Stronger temperature dependence of mechanical power both for routine and escape locomotion (Figure 10B and Figure 11) in comparison with the Q_{10} of respiration rate (about 2) seems to be due to the auxotonic type of muscle contraction during swimming when force, power and energy efficiency of muscles increase following the increase of their contraction. Therefore, one can suggest that maximum swimming speed should correlate with the temperature optimum for copepods.

3.3. Temperature Impact on the Life Cycle and Respiration Rate of the Black Sea Native and Alien Species

Due to lack of low-temperature tolerance in subitaneous eggs (capable to descend to the cold layers during development period) and nauplii (Svetlichny *et al.*, 2010a), *A. tonsa* population in the Black Sea inhabits shallow zones (mainly the bays) in summer and autumn, while in winter it endures cold period in the stage of resting eggs.

O. davisae is a perennial species. No records on production of diapausing eggs by cyclopoids are known (Alekseev and Starobogatov, 1996), and no evidence of any diapause stage exists for copepods from Oithonidae family (Marcus, 1996). Therefore, population of *O. davisae* cannot be temporarily intermittent but depends on year-round recruitment of mated females, and its abundance significantly depends on seasonal temperature alterations (Uye and Sano, 1995; Altukhov *et al.*, 2014; Svetlichny *et al.*, 2016). Our field observation and laboratory experiments (Svetlichny *et al.*, 2016) indicate that *O. davisae* population in the Black Sea can survive winter low temperature of about 8°C in the stage of pre-fertilized females similarly to *Cyclops strenuus* (Nsess and Nilssen, 1991). In our experiments, overwintering females transferred from the Black Sea to the laboratory began to lay viable eggs progressively to temperature higher than 10°C. The share of ovigerous females in *O. davisae* population increased in proportion to temperature reaching 100% at 24°C (Figure 12A).

In another experiment (Svetlichny *et al.*, 2016) with long-term exposures for *O. davisae* females (without males) fed *ad libitum* by heterotrophic dinoflagellates *Oxyrrhis* sp. during 34, 56 and 71 days at 8ºC, copepods began to lay eggs on the day 2 - 3 after the increase of temperature from 8 to 20ºC, and the viable nauplii hatched on day 5. The maximum number of ovigerous females was observed after 56 days of exposition at 8ºC (Figure 12B). The number of eggs in the ovisacs varied from 1 to 9 (mean value of 4.1 ± 1.5) and was independent of the exposition at 8ºC.

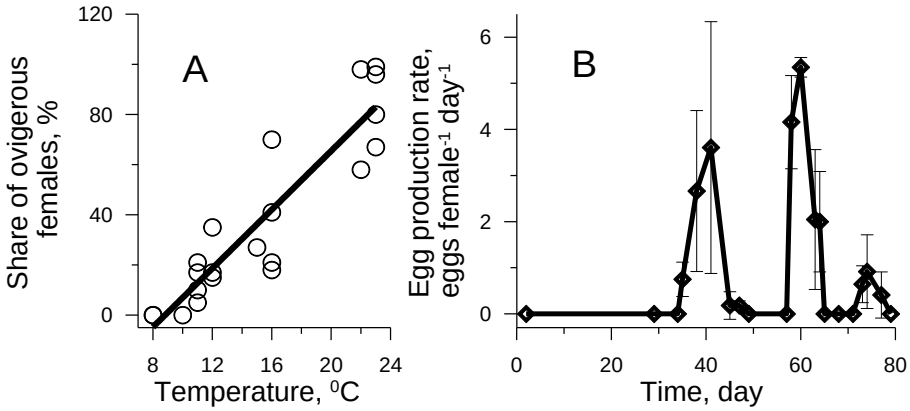

Figure 12. Effect of temperature on the reproductive activity of freshly collected overwintering females (A) (from Hubareva and Svetlichny, 2013) and egg production rate (B) of *Oithona davisae* females at 20ºC after the incubation during 34, 56 and 71 days at 8°C. All females used in the experiments were collected at the beginning of March.

Consequently, the maintenance of *O. davisae* population in the Black Sea during the cold period depends on the ability of females to keep the sperm in a spermatheca until the spring increase in temperature. However, in spite of high share of overwintering females which are capable to lay eggs, their clutch size was small, both in freshly collected from the sea and experimental *O. davisae*. The most probable explication to this phenomenon was the age of females affecting greatly the productivity of *O. davisae* (Ceballos and Kjorboe, 2011). On the contrary, in females developed in Sevastopol Bay in summer-autumn natural populations, the clutch size reached up to 19.4 ± 5.5 eggs female^{-1}.

Weight-specific respiration rate of active and anesthetized overwintering *O. davisae* females collected in spring at 8°C and acclimated to 20°C were significantly (about 1.5 times) lower than those of summer females at the 20°C (Figure 13). Probably, due to the temperature-induced shift in metabolic rates, *O. davisae* females can overcome the winter-spring decrease in number of small flagellates typical for the Black Sea (Uchima, 1988).

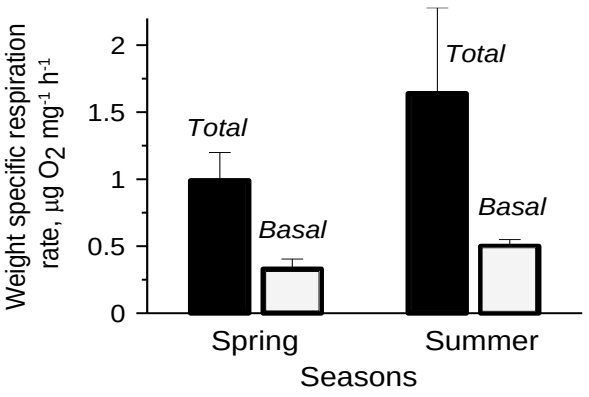

Figure 13. Weight-specific respiration rate of active and anesthetized *Oithona davisae* females collected during spring and summer. Values are presented as mean ± SD (constructed based on the data from Table 1 in Svetlichny *et al.*, 2016).

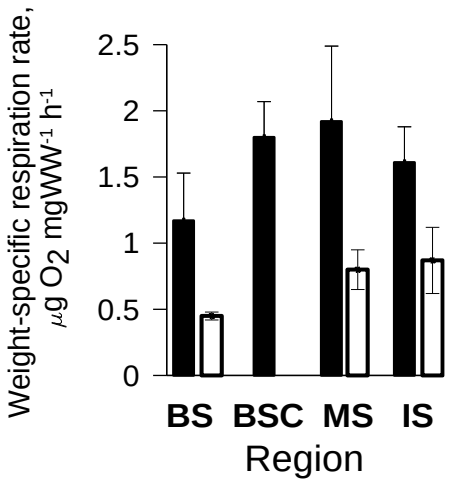

Figure 14. *Calanus helgolandicus.* Weight-specific respiration rate at 20°C of active (■) and anesthetized (□) females from the Black (BS), Marmara (MS), Ionian (IS) Seas and the Black Sea females reared in the laboratory at 18°C (BSC) (calculated from the data in Table 2 in Svetlichny *et al.*, 2010b).

Similar temperature shift was found in *C. helgolandicus* from the seas with different temperature regimes (Figure 14). In the Black Sea at the permanent temperature of daytime habitat (6 – 8°C), weight-specific respiration rate of *C. helgolandicus* females (1.2 ± 0.36 µg O_2 mg^{-1} h^{-1}) was 1.6-fold and 1.34-fold lower than those in the Marmara and Ionian Seas, respectively, where this species developed at 13-15°C. This value was also 1.5-fold lower than weight-specific respiration rate of the Black Sea females cultivated from eggs at 18°C.

Probably, this is due to the same patterns of changes in the basal metabolism when females inhabiting the seas with different temperature regimes have close metabolic energy losses for activity varying between 0.74 and 1.12 µg O_2 mg^{-1} h^{-1}.

However, it is necessary to take into consideration a higher salinity of the Marmara and Ionian Seas, which can accelerate the metabolism of copepods as well (Svetlichny *et al.*, 2010b).

4. TOLERANCE OF THE BLACK SEA COPEPODS TO OXYGEN DEFICIENCY STRESS

In the entire Black Sea below a depth of about 150 m, the oxygenated layers are replaced by hydrogen sulfide anoxic zone (probably, the largest one on the planet). The North-Western shelf of this basin and some shallow coastal areas are affected by seasonal hypoxia caused by the enrichment of seawater by nutrients from rivers.

Nikitin and Malm (1932) first estimated the lower habitat border for copepods in the Black Sea as 0.1 – 0.3 mL O_2 L^{-1} where *Calanus helgolandicus* aggregate during daytime. These authors reported that under conditions of experimental hypoxia the individuals of this species died at the close oxygen concentrations of 0.2-0.3 mL O_2 L^{-1}. Further studies (Flint, 1989; Vinogradov *et al.*, 1992; Arashkevich, 1996) showed that in summer-autumn season the daytime aggregation of *C. helgolandicus* had a two-layer structure. The lower layer

formed by diapausing CV (CV_d) was located where the oxygen concentration was near 0.2 mg O_2 L^{-1}. The upper layer consisting of migrating CV (CV_m) and adults was located at the oxygen concentration of about 0.8 mg O_2 L^{-1}.

Vinogradov *et al.* (1992) reported that the respiration rate of CV_d decreased about 3-fold from 0.13 to 0.043 μL O_2 mg^{-1} h^{-1} at the oxygen concentrations changing from 9.2 to 0.2 mg L^{-1}. They also found that CV_d were able to survive for some days under oxygen deficiency (up to 0.08 mg O_2 L^{-1}), however, the oxygen concentration of 0.06 mg L^{-1} was pointed as the lethal one.

4.1. Effect of Oxygen Concentration on Energy Metabolism of the Migrating and Diapausing *Calanus helgolandicus*

More detailed studies of the effect of dissolved oxygen on respiration rate and ammonia excretion in active and anesthetized (both diapausing and migrating copepods) *C. helgolandicus* were conducted in shipboard experiments (Svetlichny *et al.*, 1998, 2000 and 2002) with the individuals collected in offshore regions of the Black Sea.

In CV and adult females of *C. helgolandicus* performing daily vertical migrations, specific values of the total and basal metabolic rates, as well as the ammonium excretion rates as an indicator of protein catabolism, did not differ significantly. Therefore, the data obtained on migrating CV_m and females were joined and represented as one environmental group (CM).

It was shown that when the oxygen concentration decreased from 9 to 0.8 mg L^{-1}, in the CM collected at night in the sub-surface layers the weight-specific metabolic rate at 8°C reduced 2.7-fold from 7.0 to 2.6 J mg^{-1} h^{-1} (Figure 15A). At the same temperature, the energy of the protein catabolism was independent of the oxygen concentration varying randomly within the range from 3.1 to 4.6 J mg^{-1} h^{-1}. Constituting about 60% of total energy metabolism under the normoxia, protein catabolism exceeded the respired energy by 50% at the oxygen concentration of 0.8 mg L^{-1}. These data point to the great contribution of the anaerobic protein catabolism to the total energy metabolism in CM during daytime aggregation in the hypoxic layers.

In the anesthetized individuals from the CM group, both respired energy and the protein catabolism significantly decreased proportionally to the oxygen concentration. Due to this reason, in the individuals from the CM group, when the oxygen concentration reduced, the ratio between total metabolic energy of active and anesthetized CM increased from 2.5 under normoxia to 4.5 at 0.8-0.9 mg O_2 L^{-1} indicating a high cost of activity in CM under hypoxic conditions.

In CV_d collected at night in the hypoxic layers, the total energy metabolism also depended upon the oxygen concentration (Figure 15B). Higher metabolic rate of untreated CV_d in comparison with anesthetized individuals indicated that during diapause *C. helgolandicus* were able to show locomotor activity necessary for controlling the habitat depth near the hydroxide sulfide zone border. At the oxygen concentrations of 0.6 – 0.8 mg O_2 L^{-1} (minimum values for CM), the total energy metabolism in untreated CV_d was on average 2.3-fold lower than that in active CM, whilst the metabolic rates in anesthetized CM and CV_d were close. However, the ability of CV_d to live at the lower oxygen concentration

(about 0.3 mg O_2 L^{-1}) allows them to reduce 4-fold their metabolism in comparison with the possible minimum metabolism of active and anesthetized CM. As a result, the metabolism of CV_d aggregating in the active state in the hypoxic layers was 7-fold lower in comparison with the metabolic rate of active CM living at normoxia, whilst there was 28-fold difference between the metabolic rates of active CM and torpid CV_d under normoxic and hypoxic conditions, respectively. This difference was significantly higher than that between the specific metabolic rates in active CV and overwintering torpid CV_d of *C. finmarchicus* and *C. helgolandicus* (about 7 times) from the Norwegian fjords (Hirche, 1983), and *Calanoides carinatus* (factor of 11) from the Antarctic region of the Southern Ocean (Drits *et al.*, 1994) where the hypoxic zones are absent.

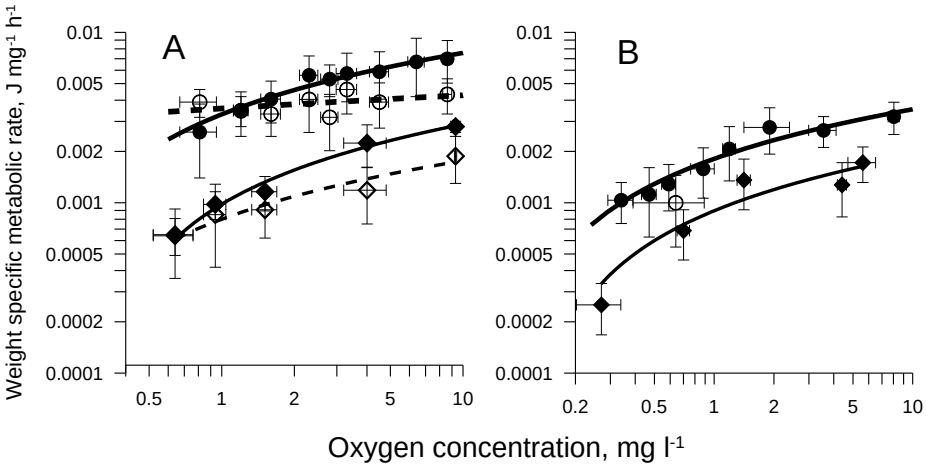

Figure 15. Energy equivalents (J mg^{-1} h^{-1}) of weight-specific respiration and ammonia excretion rates in CM (A) and diapausing CV_d (B) of *Calanus helgolandicus* at different oxygen concentrations and constant temperature of 8°C. A: respiratory energy of active (●) and anesthetized (♦) CM and ammonia excretion rate of active (○) and anesthetized (◊) CM; B: energy of total metabolism (●) and catabolized protein (○) of active diapausing CV_d and energy of basal metabolism in anesthetized CV_d (♦) (From Svetlichny *et al.*, 2002 with changes).

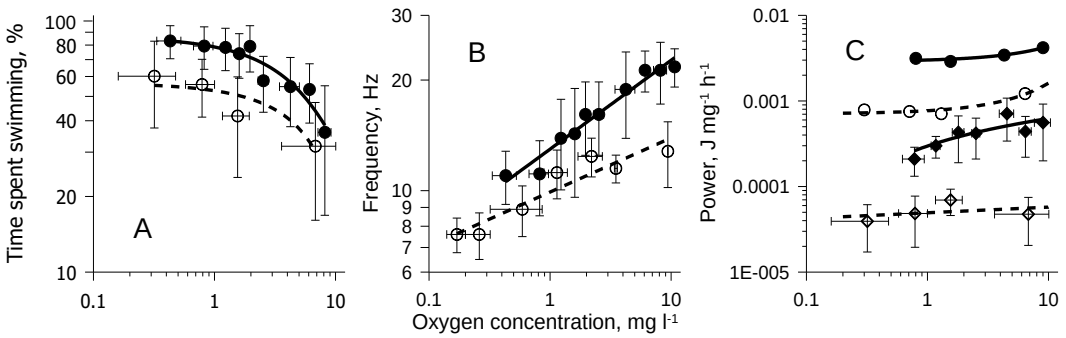

Figure 16. C*alanus helgolandicus.* Effect of oxygen concentration on locomotor parameters at 8ºC. A: time spent swimming (% of total time) and B: beat frequency (Hz) of mouthparts in CM (●) and CV_d (○), and C: active metabolism (J mg^{-1} h^{-1}) of CM (●) and CV_d (○) and mechanical power (J mg^{-1} h^{-1}) of CM (♦) and CV_d (◊) (From Svetlichny *et al.*, 2002 with changes).

The effect of the oxygen concentration on the parameters of locomotion activity was examined in the individuals attached to a semiconductor force sensor. In the experiments with CM, the oxygen concentration was decreased by bubbling the water with gaseous nitrogen during 2-3 hours imitating oxygen concentration changes during diel vertical migrations (Svetlichny *et al.*, 2000; Mutlu, 2003). In our study the individuals of CV_d collected at the depth by the plankton net experienced gradual oxygen concentration decrease in the similar mode. At every value of oxygen concentration, copepods were kept about 10 min when force production by mouth and swimming limbs, their beat frequency and duration of main behavioral acts were recorded.

Under experimental conditions, the decrease in oxygen concentration brought to an increase in the regularity of routine swimming of CM using mouth appendages (Figure 16A), until at about 1 mg O_2 L^{-1} the activity of copepods became nearly persistent. Such type of behavior in CM seems to be due to the necessity to control the habitat depth in the hypoxic layers located above the anoxic zone. These experimental results were confirmed by the numerous field acoustic studies of vertical migrations of *C. helgolandicus* in the Black Sea conducted by Mutlu (2003 and 2007). However, an increase in the regularity of swimming was followed by the decrease in beat frequency of mouthparts (Figure 16B) reflecting the speed of limb strokes. As a result of such adverse hypoxic effect, active metabolism of CM estimated from the difference in the energetic equivalents of active and anesthetized individuals insignificantly depended on the oxygen concentration, whilst the mechanical power of locomotion calculated from the force production by mouth and thoracic limbs and their circular speed (Svetlichny *et al.*, 1998) weakly decreased. Consequently, the total efficiency of the transformation of mechanical energy to biological one also reduced following the decrease in the oxygen concentration from 13 to 7% (Figure 16C).

The parameters of activity in CV_d showed similar trends of oxygen concentration dependence while their absolute values were significantly lower, especially the locomotion efficiency constituting only 3-4%. One should take into consideration that the absence of complete torpidity in CV_d may be due to the increase in their activity resulting from capture and maintenance in the oxygenated seawater.

4.2. Effect of Oxygen Concentration on Respiration Rate of *Calanipeda aquaedulcis* and *Arctodiaptomus salinus*

In contrast to *C. helgolandicus*, in *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* females a significant response of respiration rate to oxygen concentration changes over the range of 1 - 8 mg O_2 L^{-1} was not found. However, when oxygen concentration decreased from 0.7 mg O_2 L^{-1} to sublethal values (about 0.2 mg L^{-1} in *C. aquaedulcis* and 0.4 mg L^{-1} in *A. salinus*), which experimental copepods can tolerate only for 1 - 2 h, respiration rates fell dramatically (about 5 times) in both species (Figure 17).

Consequently, according to our results, the Black Sea deep-water *C. helgolandicus* and coastal *C. aquaedulcis* and *A. salinus* perform two types of reaction to oxygen concentration changes (see Prosser, 1973): oxyconformic response, when animals steadily reduce their energy metabolism with decreasing ambient oxygen concentration; and oxyregulation response, when animals keep nearly constant respiration rate up to critical value of oxygen concentration, after which oxygen consumption declines. In *C. aquaedulcis* and *A. salinus* a

critical oxygen concentration of 0.7 – 0.8 mg O_2 L^{-1} (or 0.5 – 0.6 mL O_2 L^{-1}) was found, lower than 1-2 mL O_2 L^{-1} which is the shift value for hypoxic and normoxic sea conditions (Middelburg and Levin, 2009). Nevertheless, these species may be considered as the oxyphilic copepods similar to *Acartia tonsa*, which experienced sub-lethal effects of hypoxia below the oxygen partial pressure of 3.1 mg L^{-1} (2.3 mL L^{-1}) (Elliott *et al.*, 2013) and died at the oxygen concentration of 0.7 mL L^{-1} (Marcus *et al.*, 2004).

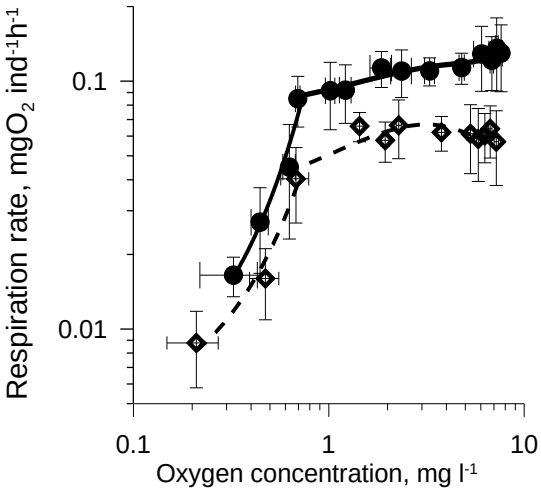

Figure 17. Effect of the oxygen concentration on respiration rate of *Arctodiaptomus salinus* (●) and *Calanipeda aquaedulcis* (◊) (Recalculated from Svetlichny *et al.*, 2012b).

4.3. Energy Benefits of the Development of *Calanus helgolandicus* in the Black Sea Environment

C. helgolandicus is widely distributed in the North Atlantic seas. Fleminger and Hulsemann (1987) were the first who put attention to the fact that the definitive body size of females from the Black Sea exceeds greatly that of females of *C. helgolandicus* from the West, Mid-North, East–North Atlantic and Mediterranean Sea. Only in the Celtic Sea (Williams and Robins, 1982) and North Sea (Hirche, 1983), the body length of *C. helgolandicus* approximates to that of the Black Sea individuals. CVs and adults from the Black Sea population are able to accumulate large amount of lipids, mainly wax esters (Yuneva *et al.*, 1997 and 1999) reaching up to 30% of body volume (Svetlichny *et al.*, 1998) which is close to lipid content of *C. helgolandicus* from the North-East Atlantic regions (Miller *et al.*, 2000). However, in the North-East Atlantic the development of *C. helgolandicus* population is timed with algae bloom at very high chlorophyll *a* (Chl *a*) concentration up to 7.6 μg L^{-1} (Ceballos *et al.*, 2004), while the Black Sea population develops at low algae concentrations (0.29–0.68 μg Chl *a* L^{-1}) (Yuneva *et al.*, 1997). We suggest that diel descending of *C. helgolandicus* from upper layers to cold hypoxic zone of the Black Sea facilitates the utilization of the energy of consumed food for growth and lipid accumulation. The ability to decrease energy expenditure is of great importance especially during summer season, when chlorophyll *a* concentration reduces to 0.22 μg L^{-1} (Yunev *et al.*, 2005).

To test this hypothesis, we conducted comparative studies of ontogenetic changes of size, lipid content, molting patterns and respiration rate of *C. helgolandicus* collected in the Black Sea (BS), Marmara Sea (MS) and Ionian (IS) Sea. The phases of molting cycle were determined in CV basing on changes in the mandibular gnathobase during tooth formation (Miller *et al.*, 1991; Arashkevich *et al.*, 2004; Svetlichny *et al.*, 2006b).

In all studied areas, the modal intervals of the egg diameter varied in the same range of 172–180 μm, which also was close to the egg diameter of this species in the English Channel (Marshall *et al.*, 1953, Guisande and Harris, 1995, Poulet *et al.*, 1995).

In *C. helgolandicus* from the BS, MS and IS the body sizes of nauplii and early copepodites were close as well. However, starting from CIII, the divergence of prosome length began to increase, and resulted in 20 - 25% excess of prosome length in CV and adults from the BS in comparison with the MS and IS (Figure 18A). In *C. helgolandicus* from the MS and IS, lipid accumulation in the oil sac occurred almost uniformly and reached maximum values in females (similar to the populations of this species in the North Sea, see Rey-Rassat *et al.*, 2002). By contrast, in the Black Sea *C. helgolandicus* CVs showed the maximum rate of lipid storage, which was spent then by females and males in the reproductive process (Figure 18B). In our opinion, the following reasons can determine the lipid accumulation in the Black Sea *C. helgolandicus*:

In our opinion, the following factors may contribute to a more efficient accumulation of lipids in the Black Sea *C. helgolandicus*: 1) an adaptation to periodic hypoxia during diel vertical migrations from subsurface to cold, hypoxic layers shifts the total energy metabolism of CVs to a lower level, as compared to non-migratory individuals (Figure 18C), due to lower level of the basal energy metabolism (Figure 14); 2) a decrease in temperature and oxygen concentration during vertical migration reduces the metabolic rates of CVs to the level of the basal metabolism due to the inhibition of their motor activity; both these factors lead to an increase in efficiency of use of food consumed for the formation of lipid reserves; 3) under hypoxic conditions during the daytime, the catabolism of lipids is depressed whilst the synthesis of fatty alcohols from non-lipid components in the oil sac may be facilitated (Sargent and McIntosh, 1974); 4) the development rate at the reduced metabolic level is decreased and CVs molted from CIVs have a longer period to accumulate lipids.

This phenomenon may be confirmed by the fact that, in contrast to CVs from MS and IS, in the BS CVs group during all seasons predominantly consisted of the postmolts (Figure 19A). In April 2003, during the cruise of the R/V "Knor," the postmolts with soft integument, low lipid content and small gonads and also the postmolts, intermolts and premolts with high lipid content and increased gonads were found simultaneously at night in the upper layers of the Main Rim Current of the BS (Figure 19B), whilst in the hypoxic layers diapausing postmolts without gonads and with high lipid content aggregated. Consequently, the population of *C. helgolandicus* concurrently included lipid-poor migrating CVs postmolts (just after molting), migrating CVs postmolts with medium lipid content and lipid-rich diapausing CVs postmolts (Svetlichny *et al.*, 2009). One can suggest that the main pool of reserved lipids is accumulated in the Black Sea *C. helgolandicus* in the phase of the postmolts of CVs. In any case, in the Black Sea the postmolts of CVs possessed 8-fold higher lipid content than CIVs (Figure 18B) collected during numerous cruises in 1996-2010 (Svetlichny *et al.*, 1998, 2006b, 2009; Svetlichny and Hubareva, 2014b).

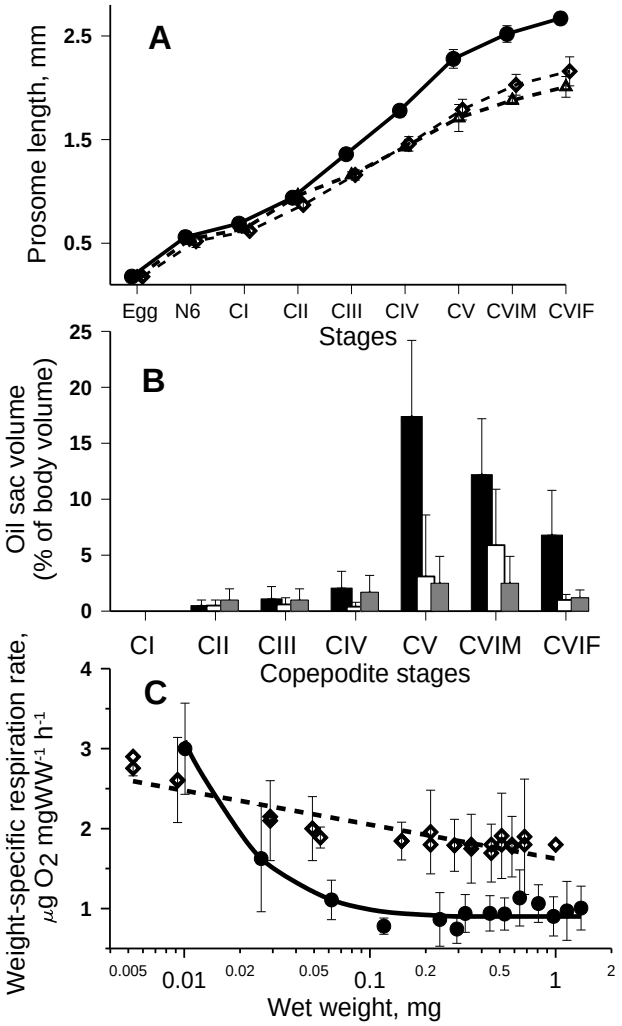

Figure 18. Ontogenetic changes of size (diameter of eggs, total length of nauplii and prosome length of copepodites) (A) in *Calanus helgolandicus* populations from the BS (●), MS (◊) and IS (▲), oil sac volume (B) in populations from the BS (■), MS (□) and IS (▒) and weight-specific respiration rate (C) of the BS and MS *C. helgolandicus* from nauplii to adults during winter-spring period (Recalculated and combined data from Isinibilir *et al.*, 2009; Svetlichny *et al.*, 2010b; Svetlichny and Hubareva, 2013).

To estimate the duration of the development stages and lipid accumulation in the Black Sea *C. helgolandicus*, a reconstruction of their age dynamics was made basing on the field studies (April 2003) of molt increments of the body weight, lipids content and specific growth rate as a function of stage specific metabolic rates and growth efficiency at 8°C (Svetlichny *et al.*, 2009). According to our estimates, the period of intensive lipid reserve formation from late premolts of CIVs to late postmolts of CVs took approximately 20 d while total duration of CVs is about 30 d. This is significantly higher than median development time (9.6 to 12.1 d) of CV in *Calanus* elsewhere at ~8°C (Vidal, 1980a; Thompson, 1982; Corkett *et al.*, 1986; Campbell *et al.*, 2001). Therefore, total development time from eggs to adults lasted about 66 d, which is in accordance with the development time only in subarctic *C. finmarchicus* (Miller and Tande, 1993; Jensen *et al.*, 2006).

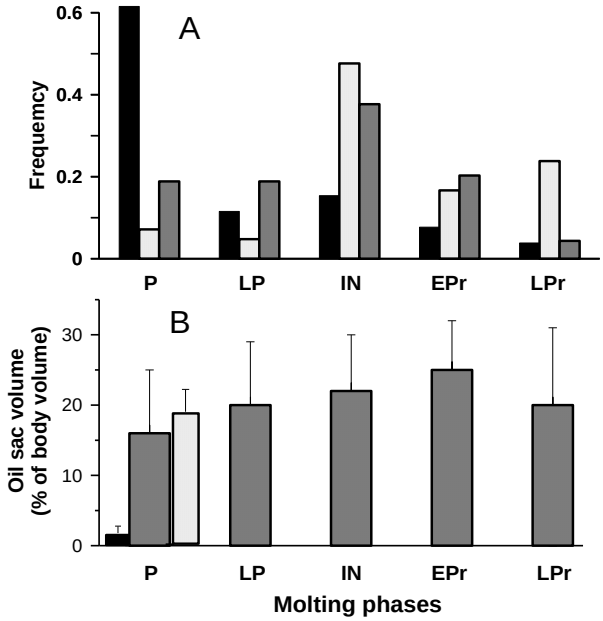

Figure 19. Frequency distribution of molting cycle phases (A) in CVs of *Calanus helgolandicus* from the BS (■), MS (□) and IS (▩) and mean oil sac volume of the Black Sea *C. helgolandicus* CVs molting groups (B) just after molting from CIVs (■), diapausing postmolts (□) and migrating (▩) postmolts (P), late postmolts (LP), intermolts (IN), early premolts (Epr) and late premolts (LPr) (Recalculated from Isinibilir *et al.*, 2009, and Svetlichny and Hubareva, 2014b).

Due to such characteristics of growth and development, the maximum daily production of 132 mg C m^{-2} day^{-1} (Svetlichny and Hubareva, 2011) for *C. helgolandicus* in the Black Sea was slightly lower than those for this species in the phytoplankton-rich North Sea (140 mg C m^{-2} day^{-1}) (Hay, 1995) and for *Calanus algulhensis* in the South Atlantic (164.4 mg C m^{-2} day^{-1}) (Hutchings *et al.*, 1995), and significantly lower in comparison with that for the North Sea *C. finmarchicus* (910 mg C m^{-2} day^{-1}) (Williams and Lindley, 1980).

CONCLUSION

In summary, when comparing the adaptive potentials of the Black Sea copepods, one should note the extraordinary euryhaline abilities in the Pontian relict *Calanipeda aquaedulcis* and Palaearctic *Acartodiaptomus salinus* when 50% of specimens of these species underwent salinity changes in the range of 0.2 – 50 and 0.2 – 35 psu, respectively. Both species collected in salt lakes of Crimea were able to be cultivated in the brackish and fresh water. Such osmotic relations of organisms with their environment characterize euryhaline amphiosmotic osmoregulators that originated from freshwater environments (Khlebovich and Aladin, 2010) although, according to our results, both studied species are osmoconformers as most marine copepods. It is important to emphasize that these abilities are genetically determined in both species as they were maintained at constant salinity of 18 psu for about 4 yr before the experiments. According to our results, up to 15% of *C. aquaedulcis* specimens survived at 60 psu during 10 d, and some individuals of *A. salinus* were alive for

more than 14 d at salinities up to 70 psu (Svetlichny *et al.*, 2012a). This fact may be considered as extraordinary because even the intertidal harpacticoid copepod *Tigriopus japonicus*, active in a salinity range of 0 to 60 psu, became dormant at salinities above 80 psu (Finney, 1979). Similar to harpacticoids, *C. aquaedulcis* and *A. salinus* are not sensible to Artenminimum barrier (5 to 8 psu) of Remane (1934), 'critical salinity' postulated by Khlebovich (1969), or 'the horohalinicum' (Kinne, 1971) dividing marine and freshwater species.

At the same laboratory conditions *C. aquaedulcis* (whose specific name means 'fresh water') withstood higher increase in salinity than *A. salinus* (whose specific name means 'salty'). In the Aral Sea *A. salinus* was a dominating species at low salinity (< 13 psu), however after the salinity increase up to 20 psu the invader *C. aquaedulcis* completely substituted for *A. salinus*. The effect of substitution can be related to the trophic factor, nevertheless when *C. aquaedulcis* disappeared from the Aral Sea after the increase in salinity up to 50 psu, *A. salinus* did not return to the sea from the adjacent lakes (Aladin *et al.*, 2004).

Widely euryhaline *C. aquaedulcis* is also an eurythermic species capable to develop within the temperature range from 3 to 30℃ (Bledzki and Rybak, 2016), whilst the lower temperature border for *A. salinus* was near 13℃ (Anufrieva and Shadrin, 2014), which is significantly higher than the winter temperature of the Black Sea. *A. salinus* survive cold season in the stage of diapausing eggs.

According to the response to oxygen concentration changes, both relict species are oxyphylic copepods.

A wide adaptive plasticity was found also in the alien species *Acartia tonsa* and *Oithona davisae* which was limited by critical salinity from 2 - 3 to 40 - 50 psu. According to Deaton and Greenberg (1986), the most pronounced changes in ionic composition of diluted seawater are attributed to the salinities lower than this value, therefore the salinity of 2 – 3 psu to a greater extent may be considered to be a physico-chemical barrier for brackish animals than "critical salinity" reported by Khlebovich (1969). Both of these marine species were able to osmoregulate in the salinity tolerance ranges.

Thermophylic *A. tonsa* and *O. davisae* used two different strategies of surviving at the low temperature during the assimilation in the Black Sea. Due to the absence of low-temperature tolerance, *A. tonsa* in the Black Sea survive winter in the stage of resting eggs, while population of *O. davisae* can overcome late winter to mid-spring period in the state of mating females.

Salinity tolerance range of the Mediterranean immigrants, the eurythermic *Acartia clausi* and the cold-water stenothermic *Oithona similis*, obtained in our experiments were typical for marine species. *A. clausi* collected in the Black Sea at 18 psu withstood gradual salinity changes from 10 to 35 psu, whilst the individuals collected in the Marmara Sea at 22 psu were not sensitive to salinity about 40 psu. Within that salinity range *A. clausi* performed the iso-osmotic response. For *O. similis* a salinity tolerance range turned out to be narrower (10 – 30 psu), however this fact may be due to the difficulties of keeping this species under laboratory conditions. According to Nikitin and Malm (1932), both species are able to survive at the oxygen concentration decrease to 0.17 mL L^{-1}, while only *O. similis* can descend to the hypoxic layers of the Black Sea.

The most abundant inhabitant of the open zone of the Black Sea, *Calanus helgolandicus* is well adapted to low temperature, oxygen concentration and salinity, although this species can tolerate a salinity about 40 psu after gradual salinity acclimation (Svetlichny *et al.*,

2010b). All these abiotic factors which decrease the metabolism, especially hypoxia, determine the successful development of *C. helgolandicus* in the Black Sea. The North Atlantic *C. helgolandicus* was considered to be transferred into the Black Sea after flooding of the Black Sea with Mediterranean waters and to form there a phenotypic isolated population which was recognized as a distinct species by Fleminger and Hulsemann (1987) with the name of *Calanus euxinus* (Hulsemann, 1991). However, the genetic divergences between the North Atlantic, Mediterranean and Black Sea populations are much lower than congeneric interspecific divergences in calanoid copepods (Papadopoulos *et al.*, 2005; Unal *et al.*, 2006). In addition, within the limits of revealed genetic variations in local populations in Swedish and Norwegian Fjords, Oceanic Inflow, North-East Atlantic, Adriatic Sea, Mljet Island, Aegean Sea and Black Sea the lowest level of genetic difference was obtained between the Fjords and Black Sea populations (Yebra *et al.*, 2011). Fjords, as a land-enclosed water bodies, have a long residence time, little water exchange and often exhibit temperature and oxygen depletion in deeper waters. Therefore, if the hypothesis of Polischuk (1984) about the penetration of of boreal species into the Black Sea directly from the northern seas is right, one can suggest that this species has been already pre-adapted for living in its highly stratified environment. In the Black Sea, *C. helgolandicus* is the most abundant copepod of the Main Rim Current system which is slightly sensitive to the global ecological trends. Therefore, the population of *C. helgolandicus* seems not to be affected by warming events so profoundly as the coastal copepod populations.

During several decades, coastal copepod species as *Paracartia latisetosa*, *Acartia margalefi* and *Oithona nana* were eliminated from the zooplankton community of the Black Sea. The ecological niches of *A. latisetosa* and *A. margalefi* were occupied by the invader *A. tonsa* (Gubanova, 2000), while since 2001 a new species *O. davisae* (Gubanova *et al.*, 2014) identified first as *Oithona brevicornis* (Zagorodnyaya, 2002) appeared instead of *O. nana* in the Black Sea.

Among our studied species, *O. nana* has extremely narrow salinity range (15 – 28 psu) indicating a narrow ecological specialization of this species, nevertheless this copepod is widely distributed in the high-saline and even hyper-saline Adriatic Sea (Razouls *et al.*, 2005 - 2017). At the beginning of the 1990s *O. nana* was eliminated from the Black Sea after the invasion of *Mnemiopsis leidyi*. According to Kovalev (2007), an inhabitant of the surface layers, broadcasting *O. nana* was subjected to the most severe elimination because females of this species were eaten by *M. leidyi* together with the brood. This copepod species survives until now in the brackish upper layers of the adjacent Marmara Sea (Isinibilir *et al.*, 2011) and Golden Horn Estuary (Dorak and Temel, 2015), probably due to lower abundance of *M. leidy*i in these regions. However, *O. nana* did not return to the Black Sea after the trophic balance restore in the late 1990s as a result of the appearance of another predator, the ctenophore *Beroe ovata* feeding on *M. leidyi*. The Bosphorus Strait as an ecological barrier may be one reason for this situation (Öztürk and Öztürk, 1996; Svetlichny *et al.*, 2006a; Oğuz and Öztürk, 2011). In order to return to the Black Sea, *O. nana* needs to use the Bosphorus bottom counter current with the salinity of about 38 psu which is harmful to this species. Another factor of its failure in the Black Sea may be the fact that its ecological niche from the early 2000s was occupied by the more competitive *O. davisae*.

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Chapter 68

THE BIOLOGY OF MYELIN IN CALANOID COPEPODS

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ABSTRACT

Myelin is an evolutionary innovation by the nervous system that greatly speeds nerve impulse conduction, thus reducing communication delays within an organism and enhancing its information processing capabilities. The discovery of myelin in approximately half of calanoid copepod taxa came as a surprise. The evolution of myelin is usually associated with large organism size and/or complex nervous systems, not small organisms with simplified nervous systems. Myelinate and amyelinate species occur in similar numbers, with both groups being highly abundant and key members of marine planktonic communities. Myelinates and amyelinates have similar size distributions, similar antennule to prosome length ratios, overlapping maximum escape speeds scaled to copepod length, and similar sensory and motor system organization. Nevertheless, the biogeographic distributions and functional ecological groups of myelinate and amyelinate taxa differ markedly, suggesting niche separation based on nervous system architecture. Behavioral differences between the amyelinate and myelinate forms are apparent: not only are myelinate copepodites quicker than amyelinates in responding to a sudden hydromechanical stimulus but they are also better at localizing and escaping away from its source. The enhanced performances conferred by nervous system myelination in calanoids in combination with biogeographic observations supports the conclusion that myelination provides extra protection in habitats characterized by high risk from visual predators. In contrast, amyelinate calanoids may depend on strategies that reduce encounter rates with predators, such as diel vertical migration, dormant eggs and reduced activity levels.

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1. INTRODUCTION

A fundamental paradigm shift in ecological theory came with the realization that major interactions such as the impact of predators on prey, competition for limiting resources, habitat use, and the nature of food webs could not be understood without incorporating behavior (Schmitz *et al*., 2008; Valdovinos *et al*., 2010; Sih *et al*., 2012). In turn, animal behavior is determined by the nervous system: information is acquired through sensory systems, which is transmitted for central processing prior to generating a behavioral output. The nervous system is critical to an organism's behavioral repertoire, and sets constraints on its niche. Thus, any major change to the nervous system in a group of organisms not only affects their behavior directly, but it has significant impacts on ecological patterns. The evolution of myelin in half of calanoid copepods is a prime example of such a change (Davis *et al*., 1999). Myelin is a radical innovation of the nervous system that increases the speed of nerve impulse conduction by an order of magnitude (Ritchie, 1984). This potentially endows a myelinate species with a multitude of advantages over similar amyelinate species, including greater success in escape from predators, greater success in predatory attacks, improved coordination in muscle contraction, greater compactness of nervous systems, more rapid processing of complex information, maintenance of timely communication over greater body distances (*e.g.*, whales and dinosaurs) and enhanced precision in event timing (Hartline and Colman, 2007; Hartline, 2008). Thus, the observed split of the calanoids into either amyelinate or myelinate taxa is of major significance not only to the animal's behavioral performance but also to its ecology.

How has the evolution of myelin affected calanoid copepods and planktonic communities in general? While it is unlikely that this question can be answered directly, behavioral studies in combination with analyses of plankton community structure and trophic cascades, placed against an understanding of the properties of myelinated nerves (Hartline and Colman, 2007; Rosenbluth, 1999), can provide insights into niche separation between myelinate and amyelinate taxa. Here, we review the biology of myelin in calanoids with two major goals: 1) to describe the current knowledge of the structure and function of myelin including its development; and 2) to discuss the significance of myelination to behavior and its potential role in the ecology of zooplankton communities. We propose that myelin plays an important role in the effectiveness of a copepod's escape response, which is key to niche separation between myelinate and amyelinate taxa.

2. STRUCTURE AND FUNCTION OF COPEPOD MYELIN

2.1. Copepod Myelin is an Axonal Sheath Composed of Multiple Concentric Layers of Membrane

Detailed anatomical studies of *Calanus finmarchicus* (myelinate) and *Epilabidocera amphritites* (amyelinate) show that the two species are similar in the overall organization of their central and peripheral nervous systems (Lowe, 1935; Park, 1966). The number and type of sensory setae on the antennules are similar in myelinate and amyelinate copepods (Huys

and Boxshall, 1991; Ohtsuka and Huys, 2001). Myelin stands out as the major difference between the nervous systems of myelinate and amyelinate calanoids.

The difference between a myelinated and an unmyelinated copepod axon is dramatic, as illustrated by the electron micrographs in Figure 1 (A, B). The unmyelinated axon consists of a single simple cell membrane surrounding microtubule-containing cytoplasm (termed "axoplasm"; Figure 1A). The myelinated axon is ensheathed in many concentric layers of membrane compacted together along one face (Figure 1B). The layers in the copepod are unusual in being without any gaps or seams, forming in mature myelin, concentric tubes surrounding the length of the axon and interrupted only in places by myelin-free patches termed "nodes" (Davis *et al*., 1999; Weatherby *et al*., 2000).

2.2. Copepod Myelin is Produced by Nerve Cells, Not Glia

Myelination of the nervous system is gradual and occurs throughout development. In the paracalanid *Bestiolina similis*, the first nauplius stage (NI) has been found to lack myelin completely (Wilson and Hartline, 2011a). Wilson and Hartline (2011a) were able to follow reidentifiable axons from one stage to the next and demonstrate how "naked" axons transition into myelinated axons through several intermediary stages as the copepod develops first through the six naupliar and then six copepodite stages. The first sign of myelin occurs in the second nauplius (NII) with the appearance of "partial" myelin in a single axon of the proto-ventral nerve cord. Two more axons commence myelination in the NIII stage. These three reidentifiable pairs of myelinated naupliar axons are likely involved in relaying sensory information from posterior mechanoreceptors to anterior centers involved in escape behavior. "Partial" myelin originates as a single internal cisterna adhering to a portion of the inner surface of the axon membrane (Figure 1C) (Wilson and Hartline, 2011a and b). As development progresses, additional cisternae are added internally against the layer previously laid down to form a stack. As the myelin develops further, the stack expands around the inside of the axon (Figure 1D) to eventually envelop it completely in a seamless multilamellar sheath of myelin (Figure 1B) (Wilson and Hartline, 2011a and b). The number of myelin layers surrounding individual axons is greater in larger fibers, as is the case with vertebrate myelin. The formation of myelin internally to the axon is a radical departure from all other known cases of myelin formation, in which it is derived from the surrounding glial cells (Raine, 1984). "Copepods break all the rules" (T.M. Weatherby, personal communication).

Large changes in form, including a reorganization of the nervous system, occur between the nauplius and the copepodite stages (Mauchline, 1998). The transition is characterized by the loss of the three pairs of large myelinated naupliar axons (Wilson and Hartline, 2011a). Myelination of the central nervous system and the antennular nerves is still at an early stage in the first copepodite, with just a few axons so ensheathed. The first axons to become myelinated in the early copepodite are the giant axons in the ventral nerve cord, which presumably are involved in escape responses (Lowe, 1935). Increases in both the number of myelinated axons and the number of layers are observed throughout the copepodite stages. Myelination is not complete until the final molt into the adult (Wilson and Hartline, 2011a). The overall pattern of myelin formation includes an anterior to posterior progression, large axons myelinating first, as happens also in vertebrates, and myelin developing earlier in the

central nervous system than in the peripheral nervous system (*i.e.*, antennules) (Wilson and Hartline, 2011a).

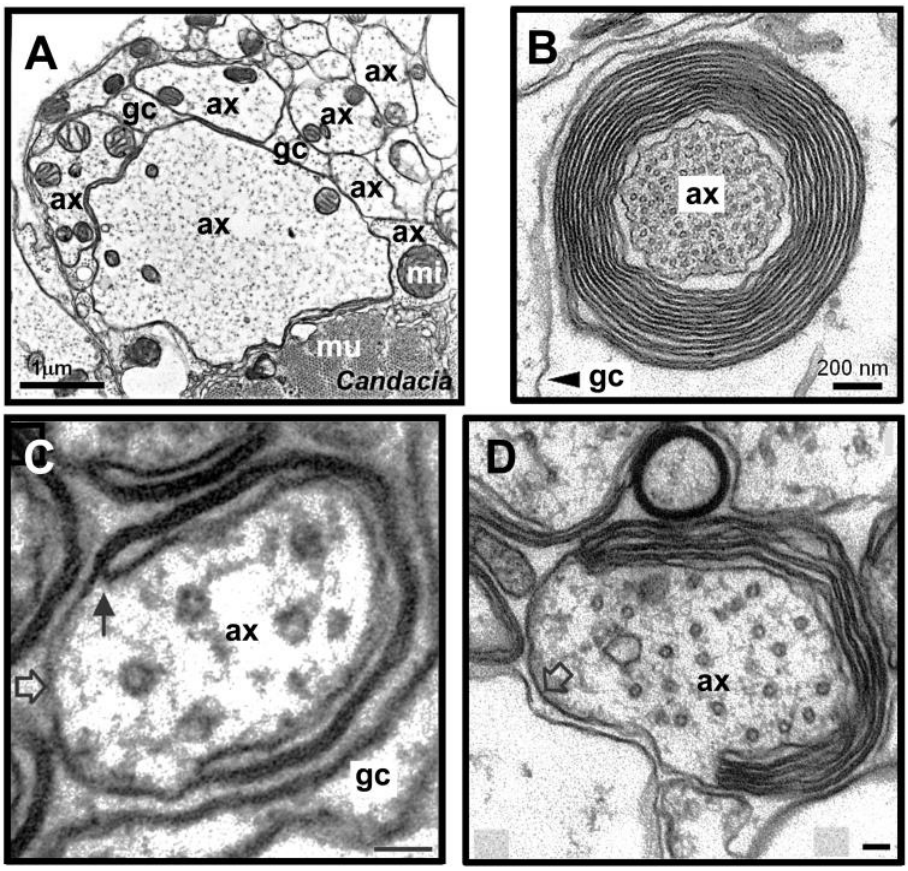

Figure 1. Ultrastructure of calanoid copepod myelin. A. Cross section through the antennular nerve of an amyelinate, *Candacia aethiopica*. Axons (ax) have at most a simple single-layer glial sheath (gc); other abbreviations: muscle (mu); mitochondria (mi). Note the large size of one mechanosensory axon presumed to mediate rapid responses to hydrodynamic disturbances. B. Cross section through a myelinated axon from the central nervous system of *Bestiolina similis*. Note the multiple continuous concentric layers of membrane tightly apposed along one face to neighboring membrane. Glial cells (gc) are not involved in forming the myelin. C. Cross section through an early stage in the development of a myelinated axon. A single internal cisterna (solid arrow) is apposed to the inner surface of the axon. The investment is incomplete, leaving a portion of the axonal membrane exposed on both intracellular and extracellular faces (open arrow). D. Cross section through an axon showing a somewhat later stage of myelin development: four cisternae are stacked together against the inner surface of the axon. Scale bars: A: 1 µm; B: 200 nm; C, D: 50 nm. A: from www.pbrc.hawaii.edu/~petra/copepod.html, with permission. modified from image by A. Davis, B-D: Modified from Wilson and Hartline (2011b), with permission.

These studies show that myelination, and by inference the conduction speed enhancement it provides, increases continuously throughout development. In adult calanoids, nearly all axons are myelinated, even very small, presumed chemosensory axons of the first antenna (Weatherby *et al.*, 2000). The advantages accruing to the organism from the faster conduction speed are expected to increase in parallel.

2.3. Myelin Functions by Electrically Insulating Axons

Myelin speeds nerve impulses by providing an insulating sheath around an axon that prevents leakage of electrical current needed to regenerate and propagate the impulses (Ritchie, 1984). Direct measurements of conduction speed have not been made in copepods owing to their small size. Nevertheless, the same principles are expected to govern its properties as for all other cases of myelin (Hartline, 2008). Consistent with properties of other myelinated nerve, extracellularly-recorded impulses in myelinate copepods are shorter in duration and smaller in amplitude than those in amyelinates (Lenz *et al*., 2000).

The better the insulation the faster the conduction. A nerve impulse is initiated when current through an axonal patch raises the trans-membrane voltage above a threshold level. Thereupon, voltage-gated sodium channels open sufficiently to cause regenerative entry of sodium ions that sustain the impulse. In unmyelinated fibers, the electrical current thereby produced expends itself charging near-by regions ahead of the travelling impulse to bring them to threshold. When those near-by regions are electrically insulated with myelin, the current passes unimpeded farther down the axon to the next uninsulated channel-bearing membrane (typically a node), where rapid charging to threshold results in a faster conduction speed. The seamless concentric leak-free construction of the copepod myelin along with the small-amplitude impulses observed suggests that the insulating properties may be better than those of other myelin sheaths.

3. COPEPOD MYELIN IS CONFINED TO MORE RECENTLY EVOLVED SUPERFAMILIES

Calanoid diversity is characterized by a nearly 50:50 split between amyelinate and myelinate taxa (Lenz, 2012; Lenz *et al*., 2000). The Calanoida have been organized into 10 super-families (Park, 1986). While this organization into super-families is generally supported by more detailed morphological analysis and molecular-based phylogenetic tree, it is likely to be an oversimplification of the evolution of the taxon (Blanco-Bercial *et al*., 2011; Bradford-Grieve *et al*., 2010). Nevertheless, the super-families provide a well-supported framework for the absence/presence of myelin (Davis *et al*., 1999; Lenz *et al*., 2000). Myelin has only been found in calanoids, where it is absent in taxa belonging to basal superfamilies, but is present in the more recently evolved ones (Figure 2). Amyelinates surveyed included representatives from the families Metridinidae in the Augaptiloidea, and Acartiidae, Candaciidae, Centropagidae, Pontellidae, Temoridae and Tortanidae (added since) in the Centropagoidea. Myelinate species were members of the families Calanidae, Megacalanidae (added since) and Paracalanidae in the Megacalanoidea; Eucalanidae in the Eucalanoidea, and Aetideidae, Clausocalanidae, and Euchaetidae in the Clausocalanoidea. Thus, myelinate taxa are largely absent from freshwater systems, which have been colonized primarily by centropagoidean (amyelinate) taxa (Boxshall and Jaume, 2000). So far no members of the superfamilies Bathypontioidea or Spinocalanoidea have been surveyed, but based on their phylogenetic placement, both are likely to be myelinate.

The identification of myelin (or lack thereof) requires examination of nerve tracts using transmission electron microscopy (Figure 1B-D), since myelin is poorly resolved at lower

magnification. Copepods that have been checked for myelin using TEM have either shown no evidence of myelin, or they have a fully myelinated nervous system with nearly all, if not all axons enveloped by myelin (Figure 2). For example, a detailed study of the antennule of *Euchaeta rimana*, a member of the Clausocalanoidea, has shown that motor, mechanosensory and chemosensory axons are all enveloped by myelin sheaths ranging from just a few layers to *ca*. 50 layers (Weatherby *et al*., 2000). Ultrastructural studies of harpacticoids, cyclopoids and siphonostomatoids have not found myelinated axons in these copepod orders (Fahrenbach, 1962 and 1964; Gresty *et al*., 1993). Thus, myelin appears to have evolved a single time in the Copepoda - in the Calanoida, most of which are free-living and planktonic. Some evidence suggests evolutionary trends within the different myelinate groups, for example a greater tendency for more complete membrane condensation in the Clausocalanoidea (*E. rimana*). No examples of copepods representing intermediate stages in myelin evolution have been found, nor any examples of myelin loss. However, the number of species that have been investigated is small, and additional studies are needed to either confirm or refute these generalizations.

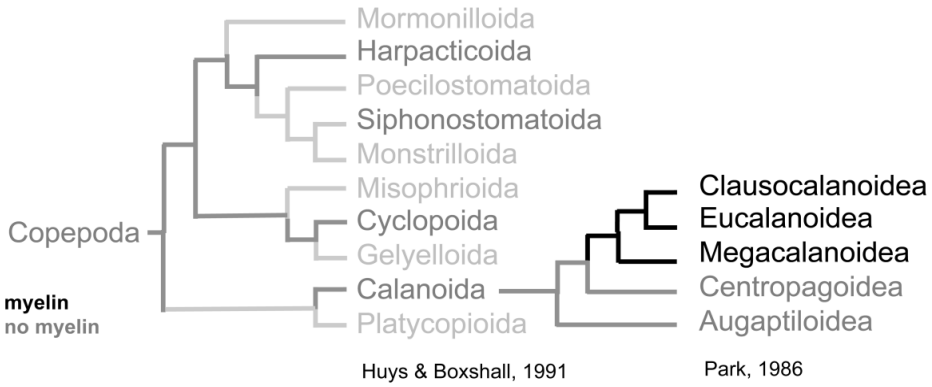

Figure 2. Phylogenetic tree of the Copepoda showing the hypothesized relationships between amyelinate (medium grey) and myelinate (black) taxa. Myelin appears only in the more recently-evolved superfamilies of the Calanoida. Light grey indicates unknown. Phylogeny of the order Copepoda from Huys and Boxshall (1991); that of the Calanoida from Park (1986).

4. BODY SIZE DOES NOT CORRELATE WITH MYELINATION

Since one major benefit of myelin is a ten-fold increase in nerve impulse conduction speed, it has been suggested that the emergence of myelin in the vertebrates was an important contributor to the evolution of larger size (Castelfranco and Hartline, 2016; Salzer and Zalc, 2016; Zalc, 2016). By analogy, one might predict that myelin would lead to the evolution of larger copepods. An analysis of total length of myelinate and amyelinate taxa does not support this prediction (Figure 3A, B). The mean lengths of myelinate and amyelinate species in a broad selection of calanoids from the North Pacific figured in a plankton atlas (Yamaji, 1976) were 2.4 mm (n = 36) and 2.3 mm (n = 57), respectively (Student's t-test; $p = 0.74$). A similar analysis based on data from copepods in the Mediterranean Sea (Benedetti *et al*., 2016) confirmed no difference in size between the two groups (myelinate taxa: average length = 2.2 mm, n = 68; amyelinate taxa: average length = 2.2 mm, n = 54; Student's t-test, p =

0.86). However, calanoid copepods were significantly larger than non-calanoid copepods (cyclopoids, poecilostomatoids and mormonilloids: average length = 1.4 mm, n = 69; Student's t-test, $p = 1 \times 10^{-4}$; data from Benedetti *et al*., 2016). While the calanoid body is quite compact, the antennules are long, typically as long or longer than the prosome length. Longer sensor-bearing antennules provide a copepod with greater sensitivity to water-borne disturbances that signal potential predatory threats (Kiørboe and Visser, 1999), but at the expense of a longer delay in the arrival of that information in the central nervous system (CNS) where it must be acted upon to produce a behavioral response. Faster conduction of this sensory information would alleviate the disadvantage of long antennules. Thus, it might be predicted that antennular length is greater in myelinate than in amyelinate species. The data do not support this prediction (Figure 3C, D) – the median ratio of antennule length to prosome lengths is 1.3 (n = 38) and 1.2 (n = 58) in myelinate and amyelinate species, respectively (Wilcoxon-Mann-Whitney Rank Sum Test; $p = 0.50$). Thus, the general predictions of myelin leading to the evolution of larger bodies and longer communication distances between remote regions in calanoids are not supported.

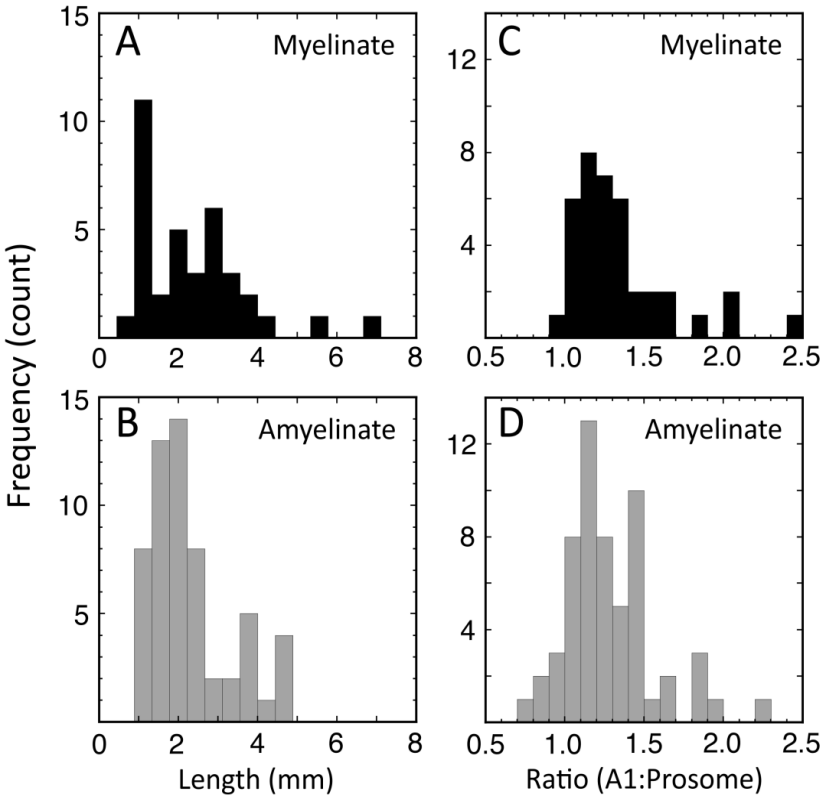

Figure 3. Histogram distributions of calanoid lengths (A, B) and antennular (A1) to prosome length ratios (C, D) of adult females in myelinates (black) and amyelinates (grey). Data were obtained from Yamaji (1976). Prosome and antennular lengths were measured from illustrations using ImageJ software. Calanoid lengths were obtained from descriptions, when a range of lengths were given a median value was used for the histogram. Calanoids were categorized into myelinate/amyelinate according to Lenz *et al*. (2000). Total number of observations – A: 36; B: 57; C: 38; D: 58.

5. MYELINATE COPEPODS HAVE SHORTER REACTION TIMES THAN AMYELINATES

While expanding body size does not appear to be a factor in copepod myelination, reaction times are a different matter. Two studies compared response latencies in amyelinate and myelinate species, with somewhat different results. Lenz *et al.* (2000) used brief controlled displacements of a small plastic sphere to produce a calibrated near-field dipole flow stimulus triggering escapes in copepods tethered to a force transducer, so that the time between the onset of the stimulus and that of the force transient of the escape was measured with sub-millisecond accuracy. In two myelinate (*Neocalanus robustior* and *Undinula vulgaris*) and two amyelinate (*Labidocera madurae* and *Pleuromamma xiphias*) species tested (> 2 mm in prosome length), minimum response latencies of myelinate taxa were significantly shorter than those of the amyelinate ones (Figure 4A). A study using an acoustic stimulus and high-speed video to record escapes in free-swimming copepods (myelinate: *Paracalanus parvus*, *Mesocalanus tenuicornis*, *Subeucalanus pileatus*; amyelinate: *Acartia spinata*, *Centropages typicus*, *Pontella marplatensis*, *Pontella* sp., *Pontellopsis brevis*, *Temora turbinata*) failed to find a similar pattern (Waggett and Buskey, 2008). Most of the measured average response latencies ranged between 2 and 4 ms irrespective of the state of myelination or copepod size (prosome length: 0.6 – 2.3 mm). However, the experimental set-up may not have been optimized for this type of analysis given the ambiguous nature of the acoustic stimulus, the temporal resolution (1 ms) and synchronization between the stimulus and the camera (± 0.5 ms). The predicted difference in response latency would be less than 1 ms in the small species tested and requires sub-millisecond resolution to determine reliably. In addition, the two outliers, one myelinate (*S. pileatus*) and one amyelinate (*T. turbinata*) species, responded with longer delays, suggesting that these escapes were mediated by a slower escape circuit, possibly an alternative to the giant fiber circuit (Park, 1966; Lowe, 1935). While not much studied in copepods, arthropods including crustaceans possess multiple neuronal escape circuits having different response latencies (Herberholz and Marquart, 2012). Additional measurements of minimum response latencies to precisely controlled stimuli with high temporal resolution are needed to resolve differences in reaction times among these small organisms.

6. MYELINATES LOCALIZE SUDDEN HYDRODYNAMIC DISTURBANCES BETTER THAN AMYELINATES

Another, more recent study examined the question of whether the presence of myelin might affect escape responses other than through response latencies (Buskey *et al.*, 2017; Figure 4). This study, motivated by the presence of exceptionally small myelinate calanoid copepods (< 1 mm total length) compared the directionality of the escape response between myelinate and amyelinate species (Figure 4B, C). Using a precisely controlled abrupt movement of a small plastic sphere to produce a rapidly rising deformation in the surrounding water, Buskey *et al.* (2017) discovered that myelinate copepodites and adults are better at localizing the stimulus and redirecting their escape away from it than amyelinates (Figure 4B, C). This difference was pronounced in copepodites that were initially oriented towards the

stimulus (Figure 4C), while those oriented away directed their escape away regardless of state of myelination (Figure 4B). While the difference in the ability to locate the stimulus was highly significant in copepodite stages (CI-CVI), it was not observed in the nauplii (Buskey *et al*., 2017). The absence of the long antennules and the early stage of myelination of the naupliar forms likely explain this difference.

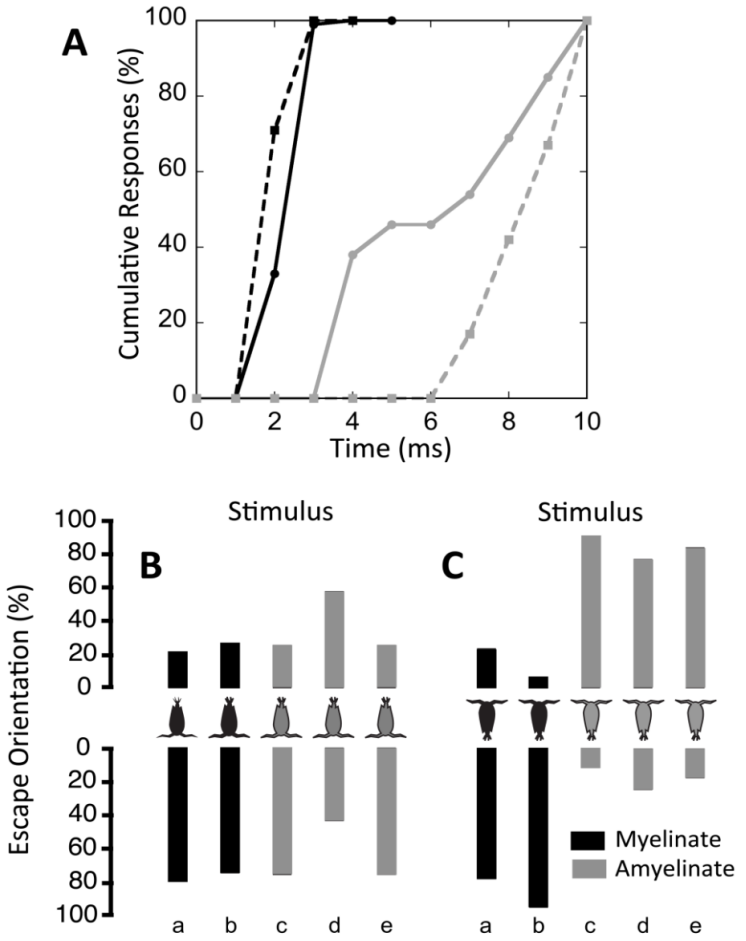

Figure 4. Calanoid copepod responses to a mechanosensory stimulus. A. Latencies to an abrupt stimulus. Cumulative distribution of the percent of responses within 10 ms after the stimulus was triggered. Black: myelinate species (*Undinula vulgaris* [circles, solid line; n = 77], *Neocalanus robustior* [squares, dashed line; n = 7]; grey: amyelinate species (*Labidocera madurae* [circles, solid line; n = 13], *Pleuromamma xiphias* [squares dashed line; n=12]). Response latencies measured for individuals tethered to a force transducer. Data redrawn from Lenz *et al*. (2000; Figure 5). Number of responses with delays greater than 10 ms of the stimulus trigger: *U. vulgaris* (n = 0), *N. robustior* (n = 0), *L. madurae* (n = 3), *P. xiphias* (n = 14). B & C. Direction of escape swims of myelinate and amyelinate free-swimming copepods oriented away from (B) or towards (C) the stimulus. Direction of escape response of copepodites (CI-CIII, and CVI) triggered by an abrupt stimulus located at the top of the observation chamber. Species tested: myelinate species - a: *Bestiolina similis*, b: *Parvocalanus crassirostris* (black bars); amyelinate species - c: *Acartia tonsa*; d: *Eurytemora affinis*; e: *Centropages hamatus* (grey bars). Data presented as percentages; number of observations for panel B - a: 14; b: 19; c: 12; d: 37; e: 4, number of observations for panel C – a: 73; b: 67; c: 54; d: 117; e: 35. Figure redrawn from Buskey *et al*. (2017), with permission.

7. DO MILLISECONDS MATTER?

How might the speed advantages of myelin play out in a predatory attack on a copepod? A number of behavioral studies have quantified various aspects of the copepod escape response including outcomes of interactions between copepod prey and fish predators (Bradley *et al*., 2013; Burdick *et al*., 2007; Buskey *et al*., 2002 and 2012; Clarke *et al*., 2005; Coughlin and Strickler, 1990; Coughlin *et al*., 1992; Gemmell and Buskey, 2011 and this volume; Gemmell *et al*., 2012; Jackson and Lenz, 2016; Waggett and Buskey, 2007). While these studies do not answer the question directly, they provide data that can be applied to hypothetical situations to estimate how differences in response time might affect the outcome of a predator-prey interaction. A copepod's peak escape speed matches that of a fish 10 to 30 times its body length (Lenz *et al*., 2004). Thus, a copepod 1 mm in body length can "outrun" a fish 1 to 3 cm in length. Pelagic fish larvae are in this size category and they are major predators of planktonic copepods (Sampey *et al*., 2007). If it takes 1 ms for a 1-mm copepod accelerating at a rate of 200 m s^{-2} (Buskey *et al*., 2002) to exceed the peak ram speed of ~200 mm s^{-1} of a 1-cm fish larva ($v_{(mm/s)} = 45 \times BL_{mm}^{0.68}$) (Lenz *et al*., 2004), the copepod can travel 0.1 mm in this time ($s = 0.5\ at^2$; s = distance; a = acceleration; t = time). However, there is an expected response-time delay of some 2 ms between detection and the initiation of the escape behavior by amyelinate copepods of this size (*Acartia*: Buskey *et al.*, 2002). The fish would thus have at least a 3-ms head start on the copepod in which time it can cover a distance of 0.9 mm if it is capable of the same acceleration. Thus, the copepod can out-distance the fish if the strike distance is > 1 mm, which is comparable to reported strike distances (China and Holzman, 2014; Coughlin, 1994). Nerve fibers in ~1 mm calanoid escape circuits are ~3 μm in diameter (Figure 1A; Wilson and Hartline, 2011a) and are expected to conduct at around 1.7 m s^{-1} if unmyelinated and around 8 m s^{-1} if myelinated (Bullock and Horridge, 1965). A myelinate calanoid of this size can thus shave ~1.2 ms off its conduction time along a 2.25 mm pathway (antennule + ventral nerve cord; see also Lenz *et al.* 2004). So the fish would have to reduce its strike distance to 0.2 mm for a successful capture (absent suction) – less easily achieved without alerting the copepod to the fish's approach.

Another important component of the escape is its direction. On a purely random distribution of escapes relative to the direction to the predator, half of escapes would be toward it and half away. While some of the escapes toward the predator might be sufficiently off the line of attack to elude capture (absent strong suction), those directed away are on average more likely to be successful. Thus, an effective escape often involves a reorientation by the prey (Figure 4B, C), but this adds an additional delay to the escape swim. *Acartia tonsa*, for example, takes 1 ms or more to turn 100° (Buskey *et al*., 2002). For a strike from a distance of 0.9 mm, a 1.2 ms faster reaction would allow a myelinate copepod to reorient by up to 120º without exceeding the time needed to achieve a successful escape speed. While the discussion here is speculative, it provides specific hypotheses that might be tested experimentally using high-speed videography to quantitatively compare predatory lunges with escape behavior of copepod prey.

8. ECOLOGY OF MYELIN

8.1. Myelinates Dominate over Amyelinates in Marine Environments with High Visibility

Anti-predator strategies can be divided into adaptations that either 1) lower encounter rates with predators, or 2) allow the prey to escape from a predator once an encounter has occurred (Langerhans, 2007). While calanoids possess numerous adaptations that decrease encounter rates with predators such as transparent and small bodies, diel vertical migration, "sit-and-wait" strategies, and dormancy (Bollens and Frost, 1991; Pasternak *et al*., 2006; Thuesen *et al*., 1998; Strickler *et al*., 2005), the behavioral studies described above support the conclusion that myelin is an adaptation that enhances escape performance, and that it might be a factor in niche partitioning. Since myelinate and amyelinate taxa are widespread and co-occur in all marine environments, this raises the question how this difference in escape performance affects ecological success, measured either by diversity or abundance. A biogeographic study of the distribution of myelinate and amyelinate taxa in oceanic and estuarine environments addressed this question, and concluded that myelinate taxa are more abundant and diverse in environments where a better escape response may effectively decrease risk from visual predators (Lenz, 2012).

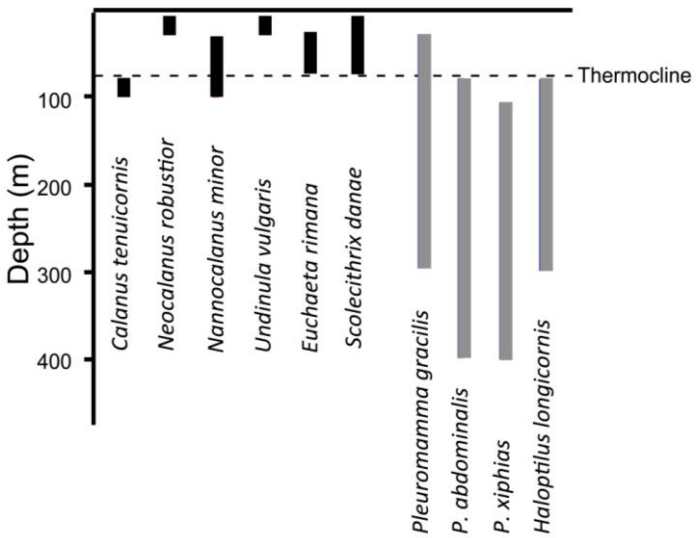

Figure 5. Vertical distribution of 10 abundant calanoids of the North Pacific gyre. Bars represent the range over which the indicated species are found. Myelinates (left set of short bars - black) show little diel vertical migration, hence their bars are short. Amyelinates (long, grey bars on the right) are found deeper and many undergo extensive diel vertical migration, so they are found at different depths at different times of the day, greatly expanding their vertical range. Data from Ambler and Miller (1987).

Epipelagic oceanic environments are characterized by high water transparency, low standing stocks of phytoplankton and an abundance of vertebrate and invertebrate predators. Resident calanoid copepods in the upper 100 m are typically dominated by myelinate taxa (Lenz, 2012; Fernández de Puelles *et al*., this volume). While amyelinates are important

members of these communities, most of them are predominately diel vertical migrators, which enter the upper water layers primarily at night, thus avoiding day-time encounters with visual predators (Lenz, 2012). Thus, an analysis of the vertical distribution of amyelinate and myelinate taxa in the North Pacific gyre shows a pattern of narrow vertical distributions for the dominant myelinate species, and broad distributions extending into mesopelagic depths for the amyelinates (Figure 5; data from Ambler and Miller, 1987).

Near-shore coral-reef-associated habitats, which are characterized by high transparency and low seasonality, have a predominance of myelinate taxa (Lenz, 2012). These habitats harbor diverse communities of planktivorous fishes, and hence present high risk from visual predators. This is in contrast to most estuarine environments, which are typically dominated by amyelinate calanoid copepods. Many of the latter environments are characterized by high turbidity, which reduces visual predation. Temperate estuaries also experience strong seasonality, so amyelinate calanoids in these environments produce overwintering eggs, a strategy that enhances survival during unfavorable environmental conditions including periods of high predation risk (Marcus, 1984 and 1996; Castro-Longoria and Williams, 1999).

8.2. Myelin is Correlated with Niche Separation between Co-Occurring Myelinate and Amyelinate Species

Niche separation between myelinate and amyelinate species within a zooplankton community is supported by a re-analysis of a study characterizing copepods by their ecological role in the Mediterranean Sea (Benedetti *et al*., 2016). The study found evidence for six distinct ecological niche groupings (= "functional groups") after clustering 191 copepod species by similarity using data on vertical distribution/migration, swimming behavior, feeding habits/trophic position and morphology. The analysis included 122 calanoids (68 myelinate and 54 amyelinate taxa), 61 cyclopoids/poecilostomatoids, six harpacticoids and two mormonilloids. While five of the six functional groups had a significant number of calanoid species, myelinate and amyelinate taxa did not contribute to these groups proportionally ($p \leq 0.001$; Chi-square test; Figure 6). Nearly half (49%) of myelinate taxa belonged to a single functional group (group 4), which consists of species that are mostly herbivorous, oceanic and contribute significantly to carbon flux. Of the remaining myelinate taxa 34% were assigned to functional group 6, which includes a large number of detritivores and small cruising herbivores. Amyelinate taxa were over-represented in three functional groups (70%, groups 1, 2 and 3), which include the large and small carnivores (groups 1 and 2, respectively) and mostly neritic species (group 3).

Metabolic studies of deep-living copepods provide additional evidence for niche separation based on the presence/absence of myelin. Calanoid taxa that occur in mesopelagic (200 – 1000 m depth) and bathypelagic habitats (1000 - 4000 m depth) include suspension feeders, detritivores and carnivores from both amyelinate and myelinate taxa. While in general deep-living calanoids have lower metabolic rates than epipelagic ones (Ikeda, 2008), adjusted metabolic rates (AMR), protein, energy content and condition factor index (CFI = dry weight/prosome length^3) were all significantly higher in myelinate calanoids compared with amyelinates (Ikeda *et al*., 2006a, 2006b and 2007). These indicators suggest that myelinates in these deep environments are more active than amyelinates.

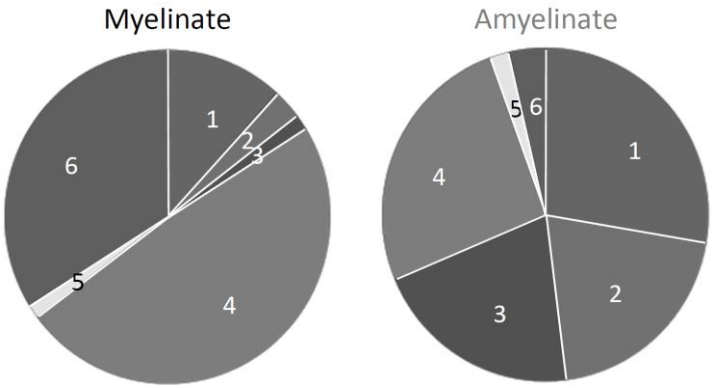

Figure 6. Distribution of myelinate vs amyelinate copepod species of the Mediterranean Sea among 6 functional groups defined by Benedetti *et al*. (2016). Myelinates dominate in groups 4 and 6; amyelinates in groups 1-3. Total number of species: myelinate, 68; amyelinate 54.

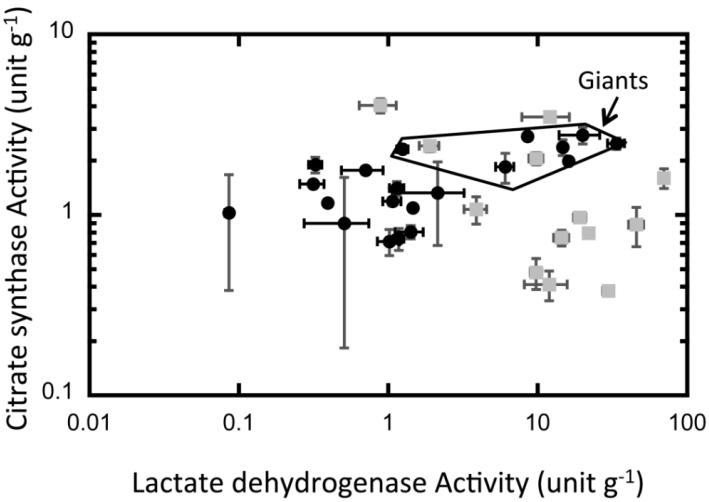

Figure 7. Relationship between citrate synthase activity and lactate dehydrogenase activity in copepods collected from depths between 200 and 2000 m. Black circles: myelinate taxa (*Onchocalanus magnus*; *Landrumius gigas*, *Gaetanus* spp., *Megacalanus* spp., *Bathycalanus* spp., Euchaetidae); grey squares: amyelinate taxa (*Metridia princeps*, *Pleuromamma abdominalis*, *Gaussia princeps*, *Arietellus plumifer*; Heterorhabdidae, Augaptilidae, Lucicutiidae). Giants: *Megacalanus* spp., *Bathycalanus* spp., *Gaussia princeps* (males, females). Data redrawn from Thuesen *et al*. (1998; Figure 8). Calanoids were categorized into myelinate/amyelinate according to Lenz *et al*. (2000). Error bars: standard errors.

Deep-living calanoids differ in their ATP metabolism and this difference correlates with myelin. Eukaryotes have two pathways for producing ATP: an aerobic one for which the activity level of citrate synthase is an indicator, and an anaerobic one, characterized by activity of lactate dehydrogenase (LDH). The anaerobic pathway is important when oxygen supply is insufficient to sustain high muscle activity. Using these two indicators, Thuesen *et al*. (1998) examined the relative importance of these pathways in calanoids. In general, they found that LDH activity levels are high in mesopelagic and bathypelagic calanoids compared with surface dwelling ones, while citrate synthase activity is low. Thus, they concluded that the deep-living calanoids have a higher dependence on anaerobic metabolism, which is

presumably important for episodic energy demands such as burst swimming. A re-analysis of Thuesen *et al*.'s (1998) data suggests that myelinates and amyelinates differ in LDH activity levels (Figure 7). With the exception of the giant calanoids, LDH activity was lower in myelinate calanoids than in amyelinates. Thus, deep-living myelinate calanoids, which are metabolically more active than amyelinate ones (Ikeda *et al*., 2006a), have a much less pronounced difference in the relative importance of aerobic and anaerobic energy production. By eliminating the need to restore sodium concentrations along the inactive insulated stretches of axons after an impulse, myelin is thought to greatly reduce demand on metabolic energy sources supporting neural activity. These phenotypic differences between myelinate and amyelinate calanoids may thus be indicators of differences in life history strategies related to optimizing survival. Reduction of activity level is one strategy to decrease encounter with and/or detection by potential predators (Langerhans, 2007). While this may be an important strategy for all mesopelagic and bathypelagic calanoids, it may be even more important for amyelinate taxa.

9. INVASION OF THE PELAGIC ENVIRONMENT AND EVOLUTION OF MYELIN

While copepods inhabit a variety of environments and niches that include interstitial, benthic, troglodytic and even terrestrial ecosystems, their importance as key members of pelagic communities in marine and freshwater habitats stands out. It has been proposed that planktonic taxa have evolved from benthic forms (Huys and Boxshall, 1991). The invasion of the pelagic may have occurred during a period of high turbidity during the Devonian (~400 million year ago [Mya], "Age of Fish"), which made the benthos a less favorable environment due to low light, decreasing food resources and low oxygen availability (Marcotte, 1999). High-speed miniaturization assured the success of many taxa including copepods, since it allowed them to selectively feed on small food particles (phytoplankton) mixed with inert ones (Marcotte, 1999). However, high turbidity also meant limited visibility, providing an opportunity for benthic forms to invade the water column during a period of relatively low risk from visual predators. The pelagic Arietelloidea (= Augaptiloidea) and Diaptomoidea (= Centropagoidea) may have evolved during this early period (Bradford-Grieve, 2002). High turbidity was followed by a period of high transparency during the Permian (~275 Mya). Based on the fossil record, diversity in pelagic species decreased dramatically, while there was an increase in benthic crustaceans, *i.e.* decapods (Marcotte, 1999). Increased predation risk from visual predators due to high transparency might have been a driving selective force. Thus, Bradford-Grieve (2002) hypothesized that the evolution of more-recent super-families such as the Calanoidea (Megacalanoidea) and Clausocalanoidea along with myelin occurred during this time.

CONCLUSION

The unexpected discovery of myelin in half of all calanoid copepods has raised questions regarding the advantages of this innovation in small organisms with simple nervous systems.

Ted Bullock (1996), a comparative neuroscientist and founding father of neuroethology, concluded that there was an inherent paradox in adaptations that increase conduction speeds in small organisms, since conduction delays are essentially negligible. The existence of taxa with and without myelin in the calanoids provides a unique opportunity to address the paradox and quantify the benefits of this innovation in small organisms. We have reviewed studies comparing escape reactions and predator-prey ecology between myelinate and amyelinate copepods, arguing that several lines of evidence support the hypothesis that the presence of myelin confers greater resistance to predation, especially from visual predators. The fact that there are few confounding differences between myelinates and amyelinates strengthens this conclusion: they share similar body forms, size distributions, antennule to prosome length ratios, maximum scaled escape speeds, and organization of sensory and motor systems.

Lower susceptibility to visual predators has led in turn to differences in biogeographic distributions and ecological functional groups of myelinate and amyelinate taxa, suggesting niche separation based on nervous system architecture. Early-stage development of myelin is observed in calanoid nauplii, and its presence may confer some benefit to these young stages. However, the extent of myelination of axons in the central and peripheral nervous systems is much greater in the copepodite stages and progressively increases to the adult stage. It is in the copepodites that behavioral differences between the amyelinate and myelinate forms become apparent. "Negligible" conduction delays notwithstanding, response latencies are shorter in myelinate copepodites than amyelinates, and myelinates are better at localizing and escaping away from a stimulus source.

Planktonic communities are characterized by high predation pressure and both phytoplankton and zooplankton exhibit a variety of adaptations that lower predation risk. Myelin is an adaptation that is likely to increase the chances of survival once an encounter has occurred. Thus, differences in escape performance are expected to lower predation risk in environments with high encounter rates, especially with visual predators that stalk unwary prey and then attempt capture with a sudden attack. The evolution of myelin is a key innovation in the Calanoida that undoubtedly promoted radiation within several superfamilies.

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Chapter 69

EVASION FROM PREDATION: UNDERSTANDING COPEPOD ESCAPE BEHAVIOR IN RELATION TO PREDATOR CAPTURE STRATEGIES

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ABSTRACT

Copepods are a key link in marine food webs and are consumed by a wide range of predators. As a result, copepods have evolved numerous adaptations for avoiding predation. The escape response of calanoid copepods is arguably one of the most important adaptations as it is well-developed in most species and developmental stages and functions against a variety of predatory modes. Copepods have evolved sensitive mechanoreceptors in the antennae to detect the presence of predators, and respond with powerful swimming strokes which can produce speeds in excess of 500 body lengths per second. Copepods also respond with one of the shortest response latencies of all aquatic organisms, and can react to a hydrodynamic disturbance in as little as 2 milliseconds. Yet many predators are capable of capturing copepods with high success. However, success in capturing copepods varies with predatory mode and developmental stages of the copepod. The great abundance of copepods within the marine environment and number of species that rely on them as food indicate the importance of understanding these interactions. Here we discuss the interactions between predators and their copepod prey, the ability of copepods to evade predators, and several of the mechanisms predators employ to capture copepods.

1. INTRODUCTION

Copepods are an evolutionarily successful group, as they are among the most numerous multicellular animals on earth (Humes, 1994). On a global scale copepods are estimated to

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process 7.5-10.5 gigatons of carbon per year (Calbet and Saiz, 2005). Therefore, this group of animals plays a key role in marine food webs which makes their behavioral adaptations to predation important to understand. Copepods in the order Calanoida are considered to be the most ecologically significant group, in part, because they often exceed other copepod groups within the neritic and pelagic zones in terms of abundance or biomass (Dagg and Turner, 1982). Calanoid copepods are important grazers on microplankton in marine food webs (Banse, 1995) and are, in turn, preyed upon by a wide range of predators with diverse feeding adaptations. In addition to their ecological roles, copepods are known to be a superior food source when compared to traditional feeds used in aquaculture such as enriched *Artemia* sp. (Shields *et al.*, 1999). Thus, understandings of copepod-predator interactions have both direct ecological and economic implications.

In nature, planktonic copepods are subject to predation from a wide array of different taxonomic groups. As such, they require a variety of adaptations that aid in minimizing detection, capture and ingestion from a host of different predation strategies. In this chapter many of these different predation modes will be explored with respect to a copepod's ability to detect, respond and ultimately survive encounters. Non-visual predators are the most taxonomically diverse and consist of cnidarian medusae, ctenophores, chaetognaths and even other copepods. There are also a host of benthic invertebrate predators that feed on copepods which include: bivalve molluscs (*e.g.*, mussels), crustaceans (*e.g.*, barnacles), cnidarians (*e.g.*, corals). Visual predators consist primarily of fish but also include cephalopods, large mammals (*e.g.*, baleen whales) and occasionally some bird species. Since fishes themselves are a highly diverse group, there is a variety of predation adaptations which result in high variation in predation success on copepods.

In order to survive in an ocean teeming with predators, copepods possess an array of adaptations to prevent detection, capture and ingestion (Figure 1). At each of these steps predators and prey are involved in an evolutionary arms race. Predator adaptions are aimed at increasing encounter, capture and ingestion rates while copepods possess adaptions that aid in minimizing these. The primary focus of this chapter will be on the factors that determine the outcome of post encounter interactions with predators. However it is worth identifying some of the other important factors that allow copepods to avoid succumbing to predators both pre-encounter and post capture.

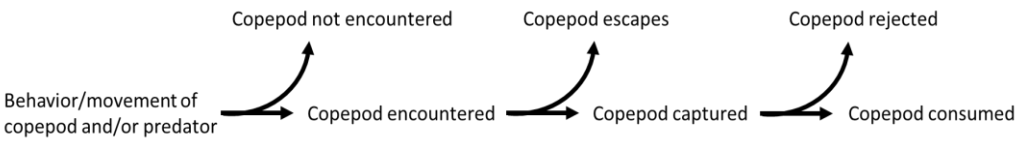

Figure 1. Potential outcomes of copepod interactions with a predator. The primary focus of the chapter will be on the factors that influence results of post-encounter situations that determine whether a copepod escapes or is captured.

The result of any predator-prey encounter will depend upon many factors. One central factor that emerges from the behavioral ecology of copepods is that there is no place to hide from a diverse assemblage of predators in open water. In order to limit encounters with predators, nearly transparent tissues help copepods lower their conspicuousness to visual predators. However this has implications for feeding because ingesting pigmented food

during daylight hours can partially mitigate this effect (Giguere and Northcote, 1987). A common behavioral mechanism to minimize encounters with predators is diel vertical migration. Copepods migrate downwards to depths with reduced light intensity during daylight hours to avoid the suite of visual predators that rely on light for prey detection (Bollens and Frost, 1989). In cases where non-visual invertebrate predators dominate and follow a similar migration pattern as copepods, a reverse diel vertical migration can occur where copepods move into surface waters during the day to minimize encounters with the dominate predator (Frost and Bollens, 1992). Another behavioral adaptation copepods can employ to minimize encounters is modifying their swimming patterns. Swimming kinematics that generate low hydrodynamic disturbance are likely to minimize encounters with rheotactic predators relying on the detection of flows created by moving prey (Titelman and Kiørboe, 2003; Tiselius *et al.*, 1997; Ohman, 1988).

If a copepod fails to avoid detection by a predator, it can still survive the encounter if it can promote rejection by the predator. Some copepods possess spines, which are known to cause rejection by small fish feeding on planktonic crustaceans (Barnhisel, 1991). Certain species of copepods can physiologically produce bioluminescence (Latz *et al.*, 1987; Herring, 1988) in response to a physical disturbance simulating the approach of a predator (Hartline *et al.*, 1999), and flashes of bioluminescence can trigger a startle response (Buskey and Swift, 1985) or potentially distract potential predators (Herring, 1988). However, the escape response which rapidly propels the animal away from a potential predator is arguably the most important mechanism in avoiding predation once an encounter has occurred.

2. DETECTION OF PREDATORS

In order to respond to a potential threat, there must be reliable mechanisms in place to detect the predator. Calanoid copepods have three sensory modalities by which they can potentially sense an approaching predator prior to executing an escape: photosensory, chemosensory and mechanosensory receptors. The visual system of copepods often consists of three pigment cups that combine to form a naupliar eye (Ong, 1970). This eye is not capable of forming images, only responding to rapid changes in light intensity such as flashes of light or shadows (Buskey *et al.*, 1986). Copepods can detect and respond to rapid changes in light with an escape response which may be an adaptive response to the presence of a diurnal predator overhead (casting a shadow) or to a bioluminescent predator such as a ctenophore (Buskey *et al.*, 1986). It therefore makes sense for both types of photic stimulation to elicit an escape response in copepods. Although copepods can detect sudden changes in light intensity, they appear unable to distinguish between sources. This is supported by observations that bioluminescent dinoflagellates, which are a food source of copepods, can elicit escape responses from copepods by producing their own bioluminescence (Buskey and Swift, 1983; Buskey *et al.*, 1986). Because these flashes of light from the dinoflagellate appear indistinguishable from those of a potential predator, the dinoflagellates may use this as a defense to disrupt the normal feeding behavior of the copepod.

Chemical stimuli can be detected via chemosensory cells located on the first antenna (Boxshall and Huys, 1998) but do not appear important in generating an escape response (Fields and Yen, 1997) or triggering vertical migration in the water column (Bollens *et al.*,

1994). Instead the chemosensory cells found in copepods likely function in prey and mate detection (Weissburg *et al.*, 1998; Yen *et al.*, 1998; Langhoff *et al.*, this volume). The first antennae is also lined with setae (or sensilla), which are small structures that are innervated by sensory cells (Figure 2; Strickler and Bal, 1973; Yen and Nicoll, 1990). Mechanical disturbances are detected from the deformations of fluid movement (Yen *et al.*, 1992). Depolarization causes the transmission of an action potential to a motor neuron which stimulates muscles and generates the escape response. The mechanosensory systems of pelagic adult copepods are well developed. The first antennae (antennules; A1) mechanoreceptors of the adults are highly sensitive (Hartline *et al.*, 1996) and have many microtubules (500 to 3000) which fill the distal dendrites of the mechanosensory neurons (Weatherby *et al.*, 1994). Each pair of dendrites is surrounded by a well-developed scolopale and by two sheath cells, one of which is firmly attached to the cuticle via microfilaments (Weatherby and Lenz, 2000). These characteristics make the system particularly rigid and thus contribute to its high mechanosensitivity (Hartline *et al.*, 1996).

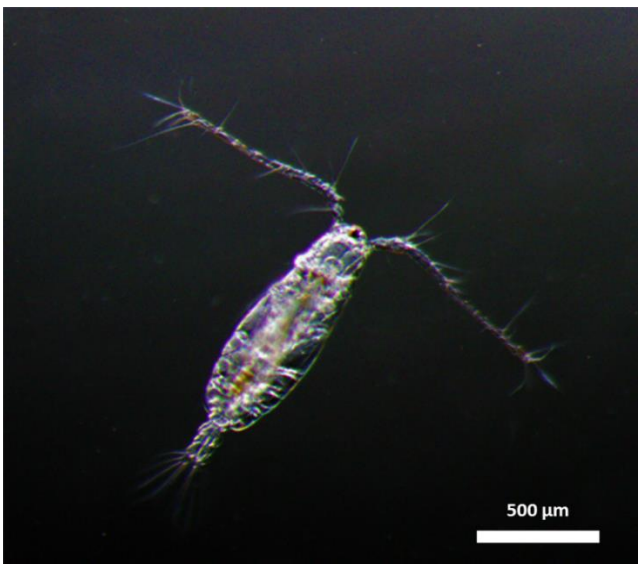

Figure 2. Photograph of the copepod *Acartia tonsa*. This species exhibits very short response latencies and high swimming velocities in response to hydrodynamic disturbances created by predators, which are detected using the large setae on the prominent antennule.

In order for a copepod to survive an attack from a predator, it must be able to detect the approach of a predator and perform an appropriate escape response. However, the strength of detection and escape vary depending on the developmental stage (Buskey, 1994). This is likely due to the fact that the setae on the distal tip of the antennae are primarily responsible for the detection of predators (Lenz and Yen, 1993), but the distal portion of the antennae does not fully resemble the adult until developmental stage N6 (Boxshall and Huys, 1998). This suggests that predator detection ability increases throughout each molt during the nauplii stages. During the transition from nauplii (N6) to copepodite (C1), the predator detection capabilities also increase (Buskey, 1994). This trend continues through each copepodite stage as the number of segments and setae proximal to the tip increase with subsequent molts (Boxshall and Huys, 1998), which provides a plausible mechanism for the continued

improvement in sensitivity with each molt. Although the distal tip resembles that of the adult at the N6 stage, sensitivity may also still improve at the distal tip due to continued development of the sensory neurons involved in detecting hydrodynamic disturbances, but little is known about the internal structures of antennae during development.

3. GENERATION OF AN ESCAPE JUMP

Rapid escape swimming may be the most important anti-predator adaptation copepods possess once encountered (Figure 3). During the peak of a typical escape, copepods exceed speeds of 100 body lengths per second (bl s^{-1}) and some species can achieve instantaneous speeds in excess of 500 bl s^{-1} (Trager *et al.*, 1994; Buskey *et al.*, 2002; Lenz *et al.*, 2004). Due to their small size, copepods perform rapid escape swimming under low/transitional Reynolds numbers (Re) from less than 100 (van Duren and Videler, 2003) to roughly 500 (calculated from Buskey *et al.*, 2002). In order to achieve such speeds, copepods must produce a large amount of force. Escaping copepods are capable of producing more than 100 dynes per jump (Lenz and Hartline, 1999) which is more energy per gram of body weight than almost any other animal. While achieving high relative swimming speeds through powerful swimming muscles is a clear advantage for surviving encounters with predators, the ability to react quickly to a perceived threat could be equally important. Copepods have one of the shortest reaction latencies known for aquatic organisms and can respond in as little as 2 ms to a hydrodynamic disturbance (Lenz and Hartline, 1999; Buskey *et al.*, 2002; Waggett and Buskey, 2007a).

Generally, there are six naupliar stages (N1-6) and five copepodite stages (C1-5) before a copepod molts into an adult (Lawson and Grice, 1970), and the escape response is present in all stages of copepod development (Buskey, 1994; Titelman, 2001; Green *et al.*, 2003). The escape response is produced by a different set of appendages in the youngest (naupliar) stages compared to the adult and copepodite stages (Gauld, 1959). In addition, many of the mechanoreceptors for hydrodynamic sensing of an approaching predator are missing in the youngest stages (Weatherby and Lenz, 2000). Interestingly, escape speed in terms of body lengths per second is similar for both nauplii and copepodites (Buskey *et al.*, 2002; Bradley *et al.*, 2012).

In the adult and copepodite stages, copepods are primarily propelled forward by anterior-to-posterior metachronal strokes of the thoracic pereiopods (Strickler, 1975). During the initial stages of the escape when the animal achieves maximal acceleration, the use of the telson as a thrust generating appendage is also very important (Figure 4). The sweeping motion of the telson creates a large jet directed posteriorly to the animal such that both pereiopods and telson motion contribute to forward thrust. Using these appendages, copepods accelerate within milliseconds to speeds up to 800 bl s^{-1} (Lenz *et al.*, 2005). However, the mechanism used for generating thrust for escapes is considerably different in the naupliar and copepodite stages. Nauplii lack pereiopods so in order to generate a rapid escape they beat the 1st antennae, 2nd antennae and mandibular palps sequentially (Gauld, 1959). In comparison, the 1st and 2nd antennae contribute very little to the propulsive forces that are generated during an escape for adults and copepodites as the antennae become folded against the body making the copepod more streamlined (Lenz *et al.*, 2004). The emergence of pereiopods in

copepodites results in an escape that is stronger and therefore allows the animals to propel themselves a greater distance from a potential predator (Landry, 1978). With each subsequent molt from C1 to C5, a new pair of pereiopods emerges and older ones become larger and presumably more powerful.

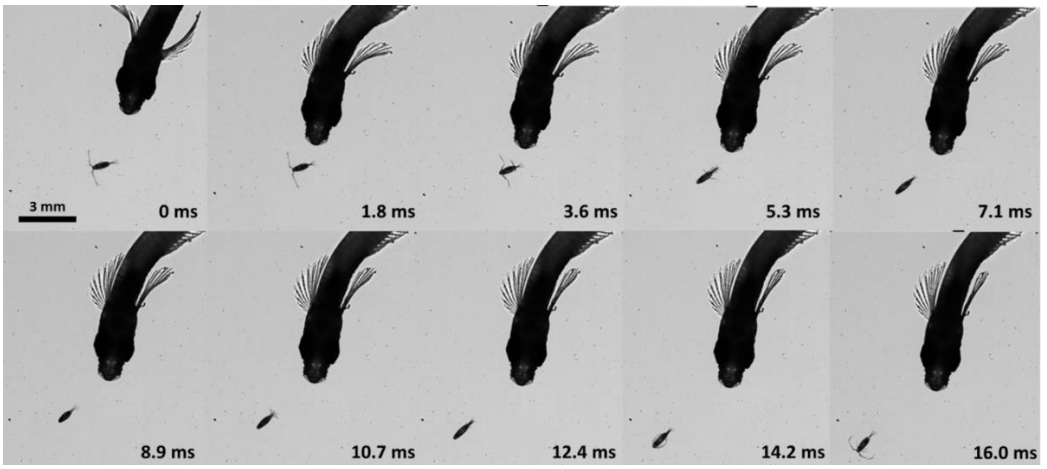

Figure 3. The copepod *Acartia tonsa* responding to the approach of a juvenile pinfish (*Lagodon rhomboids*) with rapid escape swimming.

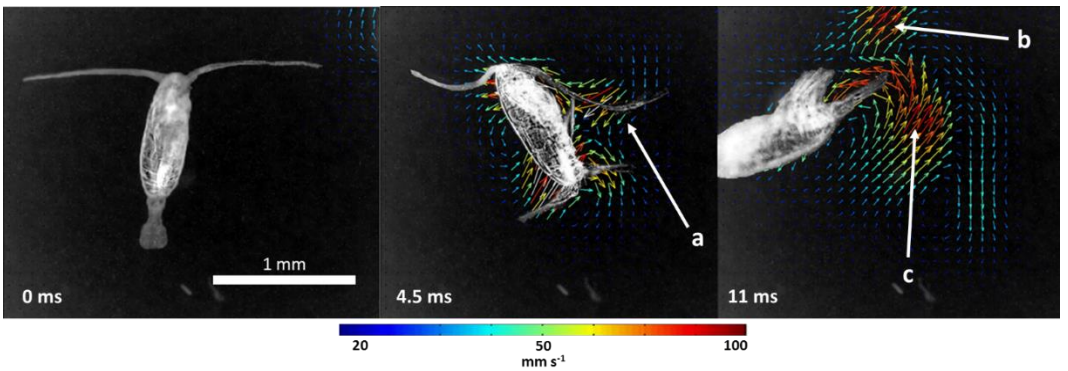

Figure 4. *Acartia tonsa* responding to a hydrodynamic disturbance. The left image is time zero, before initiation of the escape jump. In the center image, 4.5 ms later, the antennae is used to generate thrust and provide the initial reorientation away from the disturbance (a) and the telson is pulled back perpendicular to the prosome. In the right image at 11 ms, completion of the sequential movement of the pereiopods results in a jet of fluid ejected behind the copepod (b). However, the sweeping movement of the telson appears to generate most of thrust during the initial acceleration as evidenced by the much larger jet it produces (c).

Adult copepods are also able to change orientation of escape in response to an approaching predator (Buskey *et al.*, 2002). Escapes responses for adult *Acartia tonsa* usually begin with a rapid, random reorientation from the source of the stimulation which causes the escape, and the animals do this by turning at a rate of approximately 30° ms^{-1} (Buskey *et al.*, 2002). Adults reorient either by the asymmetrical sweep of the A1, or through a backward summersault produced by a combination of pereiopod and urosome movement. Directional capabilities are therefore likely to depend on differences in stimulus strength at the two A1

tips, as well as timing in the sensory neurons (Buskey *et al.*, 2017). The ability to produce a powerful escape with directionality is important, but another key to surviving an attack from a predator is being able to respond rapidly. Some species within the order Calanoida also possess myelination around neurons which, as in vertebrates, allows faster transmission of the action potential and thus faster responses to stimuli (Davis *et al.*, 1999). The reaction times of myelinated species can be 2 to 5 times faster than some non-myelinated species (Lenz *et al.*, 2000). This may provide an advantage by which myelinated species can respond more quickly to a hydrodynamic signal from a predator. However, non-myelinated species can also exhibit short response latencies, and some small non-myelinated copepods can exhibit minimum response latencies comparable to those of myelinated species (Waggett and Buskey, 2008). It was also observed that myelination did not result in increased survivorship when exposed to a visually hunting fish predator (Waggett and Buskey, 2007a). Because of this, it has been suggested that perhaps myelin functions more as an energy saving mechanism due to more efficient transfer of action potential which provides a likely advantage in low food oceanic habitats where myelinated species are most prevalent (Waggett and Buskey, 2008), or for more accurate detection of the direction of approach of a predator (Buskey *et al.*, 2017). This topic is discussed in more detail in the chapter by Lenz and Hartline (this volume).

The dominance of planktonic copepods within the mesozooplankton results in part from their highly developed detection and anti-capture adaptations. As with many species, predation risk is not uniform with age or developmental stage, and in copepods predation is greatest on the younger developing stages compared to the adults (Sell *et al.*, 2001). One reason is likely that younger stages are less capable of detecting a potential predator (Buskey, 1994), and even when an approaching predator is detected in time to generate an escape response, the escape of a young copepod is often much less effective than an adult's (Sell *et al.*, 2001; Titelman, 2001). However, with respect to relative escape speeds (*i.e.*, bl s^{-1}) copepods outperform fish by an order of magnitude, suggesting that an escaping copepod can keep ahead of a pursuing fish up to 30 times longer than the copepod itself (estimated from Lenz *et al.*, 2004). The changes in the escape system from nauplius (stages N1 to N6) to copepodite (C1 to C5) to adult in response to hydrodynamic and bioluminescent stimuli are substantial, but poorly understood. Most focus has been on research involving external changes to appendages, but behavioral studies are required to determine the significance in relation to interactions with predators which will have adaptive and evolutionary significance on both predator and prey.

4. NON-VISUAL PREDATORS

Predators of copepods can be classified into two main groups; visual and non-visual predators. The non-visual class includes a wide variety of organisms from entangling predators, such as medusae and ctenophores, to suspension feeders, such as bivalve mollusks and even other copepods. Chaetognaths (arrow worms) are raptorial predators on copepods and locate prey using the hydrodynamic disturbances created by copepod swimming (Feigenbaum and Reeve, 1977). Corals (Sebens *et al.*, 1996), barnacles (Trager *et al.*, 1994) and even larger copepods (Landry, 1980) are known to prey on smaller copepods. For

avoiding non-visual predators, mechanisms such as transparency are not effective; instead, reducing hydrodynamic signals to limit detections and exhibiting high sensitivity to hydrodynamic disturbance created by predators is vital.

Some cnidarian medusae create fluid motion during swimming to entrain prey and bring them in contact with tentacles (Costello and Colin, 1994) which contain immobilizing nematocysts. This flow over the bell creates a hydrodynamic regime different from surrounding water, and this shear stress potentially provides a signal for detection by copepods. The strength of the flow field is a function of medusa bell diameter. Medusae with bell diameters less than 7 cm produce weaker flow fields, and therefore highly evasive prey such as adult copepods will be negatively selected for, while prey which exhibit low escape velocities will be captured frequently (Costello and Colin, 1994). Thus, adult copepods should only be captured at high rates by larger medusae which can create high flow velocities which could exceed escape velocities of copepods. Suchman and Sullivan (2000) found that when *Acartia hudsonica* encountered the scyphomedusae *Aurelia aurita* and *Cyanea* sp.*, less than 1% of encounters resulted in ingestion suggesting that copepods could successfully detect and escape from these predators. This result is interesting as* several studies have concluded that gelatinous predators such as medusae can exert top-down control on copepod populations (Lindahl and Hernroth, 1983; Matsakis and Conover, 1991; Behrends and Schneider, 1995; Omori *et al.*, 1995). Perhaps turbulence in nature may increase encounter rates or mask the hydrodynamic signals available for copepods to detect a potential predator. Also, the impact on copepod populations may not be occurring strongly at the adult stage but instead on developing ones, which are known to have lower sensitivity to hydrodynamic disturbances (Buskey, 1994). Indeed developing copepods were found to be captured at a higher rate than adults (Suchman and Sullivan, 2000), which may explain the results suggesting an ability of medusae to exert top-down control of copepod populations under certain conditions.

Ctenophores have been found to be more effective predators on copepods than most cnidarian medusae, with clearance rates 1.2 times greater by volume and 3 times greater by carbon biomass (Purcell and Decker, 2005). The feeding mode of lobate ctenophores such as *Mnemiopsis* sp. is different than that of medusae, and appears specialized to capture evasive prey such as copepods. Studies by Waggett and Costello (1999) revealed two major routes by which prey are encountered and captured. The first is through feeding currents generated by the auricular cilia. This is the predominant mechanism producing encounters with smaller prey such as copepod nauplii (Waggett and Costello, 1999). The second mechanism is by entrapment on the broad oral lobes which is most effective with the larger, rapidly swimming prey such as adult *Acartia tonsa*. Entrainment through the tentillae selects for prey whose swimming speeds are less than the flow field velocities generated by the auricular cilia (Waggett and Costello, 1999). Nauplii rarely attempt to escape while being carried by the auricular flow towards the auricles and tentillae. Therefore the nauplii are thought to often fail to detect the predator's presence. This is supported by experiments by Fields and Yen (1997) which find that *A. tonsa* nauplii are much less sensitive to shear in flows than older stages and escape much less frequently in a suction flow. In contrast, adult *A. tonsa* are more active and stronger swimmers (Buskey, 1994) and, if entrained, should respond as shear rates increase near the tentillae and auricles. However, the use of cilia by lobate ctenophores creates a mechanism capable of processing large volumes of water but creating virtually no hydrodynamic signal (Colin *et al.*, 2010). This mechanism is likely responsible for

ctenophores being able to consume large quantities of copepods, as they can successfully avoid triggering escape response.

Bivalve mollusks such as clams and mussels are generally considered herbivorous suspension feeders consuming mainly phytoplankton, and are known to occur in high densities in the benthos (Meadows *et al.*, 1998). Because many bivalves can process large volumes of water (Davenport and Woolmington, 1982), they are known to substantially affect the overlying planktonic community in shallow water and can be important in determining not only phytoplankton dynamics (Cloern, 1982 and 1991; Møhlenberg, 1995; Dolmer, 2000), but also zooplankton communities (Green *et al.*, 2003; Davenport *et al.*, 2000; Maar *et al.*, 2008). Adult copepods and copepodites are occasionally captured by bivalves but nauplii are captured more frequently (Zeldis *et al.*, 2004). However, even nauplii often responded to the fluid deformation created by the feeding current of the bivalve siphon (Green *et al.*, 2003). N1 nauplii of *Temora longicornis* are captured more often than those of *A. tonsa*, which reflect their sensitivity to shear, and both species are captured more frequently when they are located furthest away from exhalent siphon where the fluid deformation rate is lowest. In this region nauplii are assumed to be unable to detect the hydrodynamic disturbance in time to escape (Green *et al.*, 2003).

Chaetognaths are small planktonic predators which often remain motionless suspended in the water column. Among the mesozooplankton, they are often second only to copepods in abundance and biomass (Froneman and Pakhomov, 1998; Fernández de Puelles *et al.*, this volume). Chaetognaths are one of the main sources of predation on the copepod community and have a substantial influence on the population structure of lower trophic levels (Pearre, 1980; Feigenbaum and Maris, 1984). Because chaetognaths are small, travel with ambient flow and do not actively swim while hunting, copepods are unlikely to detect the presence of a chaetognath unless an attack occurs. Copepods produce hydrodynamic disturbances when they swim or feed and these stimuli are detected by chaetognaths through sensory hairs (Feigenbaum and Reeve, 1977). Chaetognaths lie motionless in the water column until a copepod comes to within range of 1-3 mm (Horridge and Boulton, 1967). The rapid strike of a chaetognath provides only a brief time period in which to react before grasping spines are used to secure prey. Therefore, in order to escape from these non-visual predators, a short response latency is likely to be the most important adaptation and perhaps species exhibiting myelinated nerves may have a slight advantage for surviving these encounters.

Some copepod species exhibit carnivory and will readily feed on other copepods (Greene, 1988). This is a rather interesting scenario from the perspective of the copepods escape since the sensory and motility structures used to avoid predators are the same ones being used to detect and pursue them. While the structures (antennae and pleopods) may be present on both predator and prey, it has been argued that prey perception depends on the absolute magnitude of the fluid velocity generated by the moving prey, while predator perception depends on the magnitude of one or several of the components of the fluid velocity gradients (deformation rate, vorticity, acceleration) generated by the predator (Kiørboe and Visser, 1999).

To successfully avoid detection by carnivorous copepods, the prey species must minimize the hydrodynamic disturbance generated (Kiørboe and Visser, 1999; Kiørboe *et al.*, 2014). One strategy for doing this is to decouple feeding and swimming (Tiselius *et al.*, 1997). Species in which feeding and swimming are separate processes are able to generate hydrodynamic disturbances with a much faster dissipation rate and are able to display ‘quiet’ propulsion (Kiørboe *et al.*, 2014). If detected, prey copepods must have a very short response

latency and/or be capable of generating very high swimming speeds. Since speed is often proportional to body size, it is not surprising that predatory copepods capture higher proportions of small-bodied copepods (Yen, 1983) and nauplii (Lonsdale *et al.*, 1979). Cannibalism is also prevalent among copepods and occurs primarily on naupliar stages, even when sufficient phytoplankton is present (Hada and Uye, 1991). For omnivorous species, cannibalism makes up less than 10% of the minimum daily food requirement but can still result substantial mortality of naupliar stages (Hada and Uye, 1991).

5. VISUAL PREDATORS

Predators that hunt visually upon copepods are most commonly fish (Confer *et al.*, 1978) although other visually hunting species also consume copepods. Some species of birds are known to consume copepods (Hunt and Harrison, 1990). Birds have been documented feeding on large copepods which accumulate at oceanographic fronts (Hunt and Harrison, 1990), but no information exists on the success rates of birds feeding on copepods. Investigations into the interactions between these visual predators and copepods are needed to determine potential ecological effects and trophic interactions. The fact that copepods are known to react with escape jumps to shadows (Buskey and Hartline, 2003) suggest that perhaps this may be effective in limiting predation from birds feeding from above, but further investigation is necessary.

Larval squid also consume copepods which can make up a significant portion of their diet, and predation success rate increases as larval squid age and learn about capture of evasive prey (Chen *et al.*, 1996). Copepods are also used in aquaculture to raise *Loligo* sp. (Yang *et al.*, 1983). Some predatory zooplankton species such as mysids may use vision to assist with prey capture but are probably unable to capture evasive copepods (Viitasalo *et al.*, 1998). Whales (baleen) and some large sharks (basking, whale), although visual animals well known to consume large quantities of zooplankton including copepods (Lowry *et al.*, 2004), are not considered visual zooplanktivors due to the fact that they filter their food in massive volumes and do not visually track and locate individual copepods.

Teleost fishes are the most well-known visual predators on copepods (Confer *et al.*, 1978). They possess many sophisticated sensory systems including vision, chemoreception, hearing, and a lateral line. Because most fish are visual hunters, the color, size, and motion of prey should significantly affect the prey's chance of survival. Transparency, which is ineffective against non-visual hunters, acts to reduce contrast and reduce visual conspicuousness to visually hunting predators (Aksnes and Giske, 1993; Buskey, 1994). However, susceptibility can increase in species which bear highly visible clutches of eggs, as females have the highest encounter rates with fish and the egg-clutch is a major determinant of their susceptibility, while males are least successful in escaping once encountered (Svensson, 1992). It was hypothesized that this difference in escape reaction may have evolved because of sex-specific requirements in mate encounter and mate location, with male copepods using hydrodynamic disturbances to locate mates possibly leading to higher signal thresholds for initiation of escape behavior (Svensson, 1992). Another important aspect to consider when transparency is used to avoid visual predators is that feeding can increase visual conspicuousness by concentrating pigmented food in the gut (Giguere and Northcote,

1987). Copepods have been shown to exhibit a nocturnal feeding pattern (Stearns, 1986) which may be an adaptive strategy to limit conspicuousness during daylight hours. Brightly colored parasitic copepods visible through the cuticle of their amphipod secondary host can also alter visual conspicuousness and lead to more encounters with visual predators, including their primary fish host (Bakker *et al.*, 1997).

Diel vertical migration, where copepods move into deeper waters during daylight hours and back into surface water during darkness, is a mechanism to avoid visually hunting fish predators (Zaret and Suffern, 1976; Pearre, 1979; Bollens and Frost, 1989). Some copepods do not vertically migrate and remain in the brightly lit surface waters during the day. Pontellid copepods are one family containing several species that reside in the neustonic environment. These animals are often highly pigmented to reduce the effects of damaging UV radiation (Byron, 1982; Hansson *et al.*, 2007), and are larger in comparison with many other copepod species. This large size, combined with pigmentation, makes these copepods more visually conspicuous, and thus should be preferred by visual fish predators (Brooks and Dodson, 1965; Morgan and Christy, 1996). This would appear to put these copepods at a significant disadvantage, but at least two species, *Anomalocera ornata* and *Labidocera aestiva*, have been shown to make aerial escapes, whereby the animals break the surface tension of the water and travel many body lengths through the air, reentering the water beyond the perceptive field of the fish predator (Gemmell *et al.*, 2012) (Figure 5). The energetic cost of breaking the surface tension is high (copepods lose up to 88% of their kinetic energy), but the vastly lower density of air allows them to travel many times further from a predator than they could underwater.

Fish create a fluid disturbance when feeding, and copepods can receive a signal to alert them to the presence of a predator. To counter this, many fish exhibit adaptive morphology and behavior of their own. Many planktivorous fish feed by suction (Coughlin and Strickler, 1990; Coughlin, 1994; Holzman and Wainwright, 2009). In order to capture copepods in this manner, a fish must get sufficiently close to their prey to allow the suction flow to overwhelm the prey and draw it into the mouth. Both swimming towards the prey and suction flow create a hydrodynamic disturbance, which can elicit an escape response by the copepod. To overcome the bow wave created by swimming towards a copepod, the fish rapidly opens its mouth creating suction, thereby reversing fluid deformation (Holzman and Wainwright, 2009). Therefore, during a strike many fish can be detected only by its suction-induced disturbance, rather than the disturbance from the bow wave. These fish are able to produce a more subtle disturbance than expected based on their flow speeds and mouth size alone. Jaw protrusion and the rapid opening of the mouth during the strike both help to minimize the signal available to the prey (Holzman and Wainwright, 2009). It is likely that the jaw protrusion observed in many planktivorous fish is an adaptation to minimize the initiation of copepod escape responses.

Fish can also limit their hydrodynamic conspicuousness prior to the strike, during the approach phase. It is crucial for fish to get close to evasive copepods prior to initiating a feeding strike, given that suction-based feeding produces flows that are exceptionally short lived, lasting only 10–50 ms, and are restricted to an area very close to the mouth (Ferry-Graham *et al.*, 2003; Van Wassenbergh and Aerts, 2009; Day *et al.*, 2005). To avoid detection by copepods prior to a feeding strike, fish can mask their hydrodynamic signatures by utilizing either morphology or behavior. Seahorses are an example of fish that can overcome a copepod's sensory defense through morphology as the narrow, elongated snout, separated

from the blunt head is associated with minimal fluid deformation, which allows these fish to approach evasive copepods to within 1 mm (Gemmell *et al.*, 2013b). Other fish appear to behaviorally minimize the fluid deformation profile in front of the mouth by creating minor suction to offset the water being pushed forward (Gemmell *et al.*, 2014). Both of these strategies reduce the fluid deformation in the strike zone of the fish to levels just below detection limits of copepods, and illustrates the evolutionary "arms race" occurring and this important trophic link.

In the absence of modifications to minimize fluid disturbances, high strike speeds can also be successfully employed by some planktivorous fish. Small reef dwelling fish (blennies, *Acanthemblemaria* sp.) that live within small holes on coral reefs, wait for potential meals to swim or drift by in the current. Once a copepod is located visually the blenny attacks its prey by lunging forward, mouth agape to ingest its prey. The speed of attack is ≈230 mm s^{-1} (Waggett and Buskey, 2007a). Although rapid, this strike velocity is lower than the maximum escape velocity of *Acartia tonsa* (≈500 mm s^{-1}). This results in most copepods being able to sense and escape successfully from blennies under still water conditions (Clarke *et al.*, 2005). It is only under moderate turbulence that the reduced ability to hydrodynamically sense a predator results in higher capture success by the fish (Clarke *et al.*, 2009).

Copepods exhibit both continuous to intermittent modes of swimming. Species that maintain a relatively smooth swimming pattern and nearly constant frontward motion are termed 'continuous cruisers.' Species that only swim intermittently are known as either 'hop-and-sink' swimmers, where brief forward jumps are followed by a short period of sinking when appendage motion ceases, or 'cruise-and-sink' swimmers, which exhibit longer periods of forward swimming followed by short periods of sinking (Bainbridge, 1952). The pauses during intermittent swimming are believed to increase the perceptual abilities of the copepod by reducing any self-generated hydrodynamic noise (Bundy and Paffenhöfer, 1996; Yen, 2000; Kramer and McLaughlin, 2001). However, an intermittent swimming pattern can act to increase conspicuousness to a visual predator (Peterson and Ausubel, 1984; Buskey *et al.*, 1993). Even within a species, male and females can have different swimming patterns and this can translate into higher capture rates of males (Saito and Kiørboe, 2001). Alteration of swimming patterns behavior can also be used by fish such as herring to increase their encounters with prey. At low copepod densities, fish will increase speeds by approximately 100% compared to speeds at high copepod densities (Munk and Kiørboe, 1985). It is also noteworthy that fish, like copepods, appear to have increased perceptive abilities when not actively swimming. In cod larvae 94% of copepod prey are perceived during glide events where the fish are not actively swimming (Hunt von Herbing and Gallager, 2000). This has important implications for determining realistic encounter rates.

Due to their smaller size, nauplii should be less visually conspicuous and therefore be less vulnerable than larger stages to visual predators such as fish (Eiane *et al.*, 2002). This is in contrast to most findings on encounters with non-visual predators, where nauplii are often captured at significantly higher rates than later developmental stages (Costello and Colin, 1994; Waggett and Costello, 1999; Suchman and Sullivan, 2000). As copepods molt and become larger, this should make them more conspicuous to a visual predator which may translate into increased frequency and distance of attacks from a predator. However later stages have greater sensory abilities and greater escape responses (Buskey, 1994) which may act to offset the increase in visual conspicuousness.

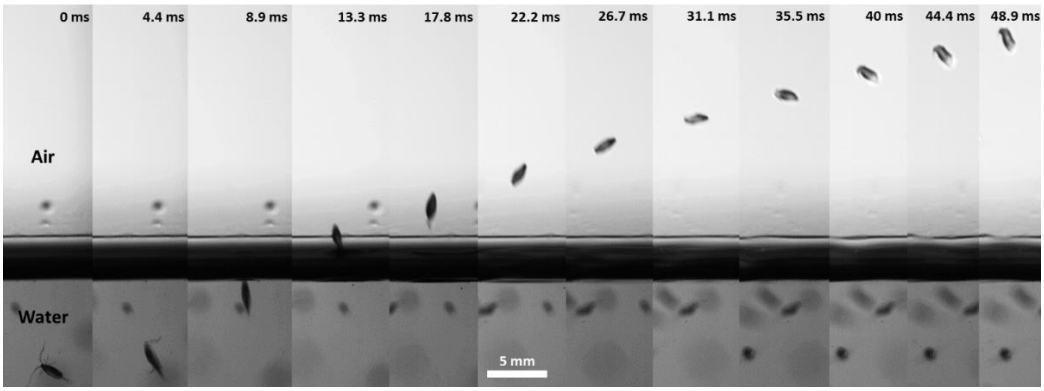

Figure 5. Aerial escape behavior of the pontellid copepod *Labidocera aestiva*. This is one of only a few species known to break the surface tension of the water during an escape and travel many times their own body length through air before re-entering the water.

6. Effect of Water Motion

The escape response involves rapid acceleration which is energetically costly, using over 400 times the normal energetic expenditure (Strickler, 1975; Alcaraz and Strickler, 1988). Therefore copepods must maintain a balance between being able to successfully avoid predators and conserving energy. In order to conserve energy, copepods of all developmental stages display escape behaviors only when a stimulus is detected above a certain threshold. This prevents a copepod from performing energetically costly escape responses when they are not necessary. However, when copepods are constantly stimulated above the threshold for escape, for instance in a turbulent environment, they have the ability to habituate to these stimuli which may reduce their ability to detect an approaching predator and result in a greater likelihood of capture (Costello *et al.*, 1990; Hwang *et al.*, 1994).

Most behavioral studies with marine copepods have been done under still water conditions, but turbulence has been shown to play an important role in determining how predator-prey interactions involving copepods operate in more realistic conditions (Clarke *et al.*, 2005 and 2009; Robinson *et al.*, 2007; Finelli *et al.*, 2009). Turbulence was originally thought of as a mechanism that simply increased encounter rates between predators and copepods (Rothschild and Osborn, 1988), but turbulence can impact capture and escape success of both predator and prey (Clarke *et al.*, 2009). The exact impact turbulence has on capture success is often difficult to predict because it has two opposing effects. Turbulence can create erratic movement patterns of prey particles making a fish (predator) more likely to miss during an attack or abort a pursuit completely (MacKenzie and Kiørboe, 2000).

Turbulent water motion can also mask the signals that copepods use to avoid capture making their reaction distances to stimuli shorter (Robinson *et al.*, 2007), which will increase the predation risk for evading a visual predator (Clarke *et al.*, 2009). The escape response and capture rates of the copepod *Acartia tonsa* were examined in laboratory flumes that created both unidirectional and oscillatory flow conditions. The reactive distance to a siphon remained the same as still water in low-flow conditions, but was reduced by 25% at elevated flow speeds, indicating a decline in the copepods' ability to detect velocity gradients formed

by the siphon (Robinson *et al.*, 2007). Turbulence appears to make it more difficult for a copepod to respond to a potential threat and may also affect the strength of the response. Escape speed of copepods can be slower under turbulent conditions (Lee *et al.*, 2010) but may not impact the total escape distance (Waggett and Buskey, 2007b). Copepod developmental stage and predator species are also important to consider in the context of water motion. Some planktivorous fish exhibit similar capture success rates under both calm and flow conditions, whereas other fish species display strong differences both within and across copepod developmental stages. For example the dwarf seahorse, *Hippocampus zosterae*, captures both copepodites and nauplii with very high success (>90%) under calm conditions compared to the blenny, *Acanthemblemaria paula*, which captures nauplii at a significantly higher rate than copepodites (Gemmell and Buskey, 2011). Under flow conditions however the blenny is able to maintain similar capture rates on both nauplii and copepodites but the seahorse success rate plummets to nearly zero.

7. EFFECT OF TEMPERATURE AND VISCOSITY

In addition to turbulence, physical characteristics of water such as viscosity and temperature can affect escape performance in copepods. As water becomes cooler, it also becomes denser and more viscous. For example, a decrease of temperature from 20°C to 10°C increases viscosity from 0.0109 Pa s to 0.0139 Pa s (**Bolton and Havenhand, 2005**). Therefore, the change in temperature alters not just the metabolic rate of organisms but also the physical characteristics of the ambient fluid, which in turn affects the ability of very small organisms to feed, move or escape within the water column. These effects of temperature are very important at hydrodynamic scales where viscous forces dominate motion ($Re < 1$) because low Re has a marked influence on the drag that operates against the feeding and swimming structures of small aquatic ectotherms such as copepods (Koehl and Strickler, 1981; Lagergren and Stenson, 2000).

This inverse relationship of water temperature and viscosity has particular importance for copepods living in temperate and sub-tropical coastal environments, where they are subject to large fluctuations in water temperature throughout the course of a year (from 5-30°C). Here, the escape response will be subject to water temperature variations from local weather (*e.g.*, cold fronts), seasonal variations, and large-scale global patterns (*e.g.*, climate change). Because viscosity fluctuates inversely with temperature, the impact of high viscous drag on small organisms' routine locomotion has substantial effects on swimming speeds (Bolton and Havenhand, 1997; Podolsky and Emlet, 1993; Fuiman and Batty, 1997; Muller *et al.*, 2000; Larsen *et al.*, 2008). However, the effect on escape behavior is unknown because non-escape (routine) locomotion occurs at $Re = 0.1$ (for copepod nauplii) based on body length and cruising speed, and escaping nauplii exhibit unsteady motion and their flow environment can rapidly transit to $Re = 6$ (Gemmell *et al.*, 2013a).

Although altering viscosity will undoubtedly influence locomotion, the effect on predator and prey will not be equal. This is because larger bodies operate at a higher Re and exhibit less viscous drag than smaller ones. Because predators (*e.g.*, fish larvae) are often much larger (an order of magnitude or more) than the nauplii themselves, the predator will be less affected by viscosity (Herbing, 2002) and should capture nauplii more effectively when viscosity

increases. Small organisms should escape less effectively at colder temperatures (Fuiman, 1986). However, field studies on copepod nauplii predation have shown no evidence that nauplii are captured more successfully at colder temperatures (Paul, 1983; MichaudI *et al.*, 1996) suggesting an ability of nauplii to compensate for viscosity disadvantages at reduced temperature. Gemmell *et al.*, (2013a) showed that the early nauplii of *Acartia tonsa* can indeed compensate to a certain degree and maximize the effectiveness of escape swimming at both ends of their natural thermal range. This is accomplished by a shift in timing of appendage motion which creates an increase in power stroke duration relative to recovery stroke duration at low temperatures. The shift in power stroke duration relative to recovery stroke duration is found to be regulated by the temperature dependence of swimming appendage muscle groups (Lenz *et al.*, 2005; Gemmell *et al.*, 2013a), not a dynamic response to viscosity change. While some copepod nauplii have natural adaptive mechanisms to compensate for viscosity variations with temperature, it would not appear to function in situations in which viscosity varies independent of temperature, such as in some phytoplankton blooms (Seuront *et al.*, 2006).

CONCLUSION

A copepod's success in escaping from predators depends on its ability to detect the predator's approach and to respond quickly and effectively, and the result of any predator-prey encounter will depend upon many factors. There is no place for copepods to hide from a diverse assemblage of predators in the pelagic ocean, and they have evolved escape performances matched by few other organisms, vertebrate or invertebrate.

Copepods provide model organisms to investigate the kinematics of escape in the aquatic environment, correlate it with physiological and morphological changes through development, and compare these results to measurements of predator susceptibility and changing environmental conditions. Previous studies have examined the changes in copepod vulnerability to predators through developmental stages, although changes in escape kinematics with development were not considered. By studying the developing stages of calanoid copepods, their ability to avoid predation at various stages of development and how environment affects these interactions, we can begin to understand which stages of copepods are most susceptible to different types of predators. Through the use of emerging techniques such as micro-Particle Image Velocimetry (μPIV) (Gemmell *et al.*, 2014) and 3D methods (Gemmell *et al.*, 2013a and 2014; Malkiel *et al.*, 2003) we are beginning to understand the role that the physical fluid environment plays in mediating these interactions. Eventually this may help to understand the factors that control plankton community structures, localized abundances or deficiencies of both predator and prey in the marine food webs, and predict the robustness of species to seasonal and long-term changes in climate.

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Chapter 70

CHEMOSENSATION AND A POTENTIAL NEURONAL MECHANISM OF RATIO DETECTION IN A COPEPOD

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ABSTRACT

Male copepods of the species *Temora longicornis* are able to follow a pheromone trail laid out by a female. Moreover, the male is able to change the direction of its movement if it initially follows the trail in the wrong direction. Previously, we proposed that the female pheromone may be blend of multiple compounds with different chemical properties and that the male senses their ratio rather than an absolute concentration. This allows for a better method to decide in which direction to move. Here we implement a simple, yet efficient design for the olfactory apparatus using the Leaky Integrate-and-Fire neuronal model. We implement a Simulated Annealing algorithm for the selection of optimal synaptic weights and show that the circuit enables ratio detection over a wide array of input signals. Our results encourage further research on similarities of brain organization in copepods and airborne arthropods in which ratio detection plays an important role.

Keywords: chemosensation, ratio detection, *Temora longicornis*, neuronal model

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ABBREVIATIONS

LIF	Leaky Integrate-and-Fire.
LNs	Local Neurons.
ORNs	Olfactory Receptor Neurons.
SA	Simulated Annealing.

1. INTRODUCTION

At a population density of about one individual per liter of water in the world ocean, copepods are the most abundant class and the greatest reservoir of organic carbon among all animals. They constitute a key link between autotrophic phytoplankton and higher trophic levels in freshwater and marine food webs. Thus their ecology has tremendous implications on other life forms, all the way to land and air based animals. Current challenges to their well-being include anthropogenic acidification and chemical pollution of their natural habitats. It is imperative to understand their ecology, in particular their mating behavior, to predict whether and how these challenges may be met by the world's copepods.

Copepods are sexual animals and their mating behavior has been a fascinating research topic for two centuries. Katona (1973) proposed that copepods use chemical signaling mechanisms to find each other in the dark and three-dimensional water column. This view was substantiated in a series of works at the turn of the 21st century (Bagøien and Kiørboe, 2005; Doall *et al.*, 1998; Seuront, 2013; Tsuda and Miller, 1998; Weissburg *et al.*, 1998; Yen *et al.*, 2004). Males often display swimming adaptations aimed at increasing the encounter success with conspecific females (Nihongi *et al.*, 2004; Uttieri *et al.*, 2007; Yen and Lasley, 2011). A key observation by Doall *et al.* (1998) was that when a male *Temora longicornis* finds the trail laid by a female, but begins the pursuit in the wrong direction, he is able to turn around and to continue until he has reached the female. This happens within a remarkably short time, after only 1-2 s of pursuit in the wrong direction (Figure 1). The observations of Doall *et al.* (1998) do not indicate that the males know the direction of the female at the moment of finding the trail.

In earlier work (Hinow *et al.*, 2017) we presented a simple mathematical description of how the directional information can be encoded in the pheromone trail left behind by the female. Even in quiet and undisturbed water, any compound emitted by the female is subject to diffusion and chemical decay reactions. In the fixed frame of the moving female, the concentrations of the compound(s) form an idealized one-dimensional trail of concentrations that decrease with the distance from the female. A searching agent (the male) can be equipped in one of two ways. On the one hand, it can attempt to detect a change in the absolute concentration of a single compound. If the concentration is increasing in the direction of the motion, it should continue in that direction; if the concentration is decreasing in the direction of the motion, it should turn around. On the other hand, the female can emit two compounds at roughly the same rate that differ however in their decay or diffusion rates. At a distance from the female, the male will encounter these compounds in a ratio that differs from 1. The direction of the motion should be maintained if the ratio approaches 1 and it should be reversed otherwise. Using mathematical modeling and simulation we found evidence that an

agent able to detect a ratio outperforms an agent that is dependent on the gradient of a single compound (Hinow *et al.*, 2017). Indeed, if the initial direction of motion is opposite to the direction of the moving female, it is possible to enter the trail further away from the female and still make the turn. Moreover, the threshold in the signal change required for turning can be chosen bigger.

Extracting directional information from chemical cues is an important task for a large number of animal species though the precise mechanisms often remain only partially understood. A well-known example are dogs (*Canis familiaris*) with special breeds for tracking purposes (Hepper and Wells, 2005; Gadbois and Reeve, 2014; Wells and Hepper, 2003). Ratio detection of chemicals has been investigated in a number of species (Clifford and Riffell, 2013; Wyatt, 2010), most notably in moths and other lepidopterans (Belmabrouk *et al.*, 2011; deBruyne and Baker, 2008; Zavada *et al.*, 2011). In various moth species where the chemical components of the pheromone blends have been identified, sympatric sister species use identical components of the mixture, albeit in different ratios. Zavada *et al.* (2011) proposed a simple competition-based neuronal model that is capable to compare the strengths of two signals and to lead a search towards a region where the signals are in the desired ratio. Their model is composed of sets of Olfactory Receptor Neurons (ORNs) that excite Local Neurons (LNs) of different types. The local neurons, in turn, are connected by inhibitory synapses. The presence of multiple compounds in the moth pheromone blend indicates that species-specific information is also conveyed. This can help to maintain species integrity, provided that the ratios of the components remain stable in the plume. Hinow *et al.* (2017) postulated that the ratio of components needs to change over time so that the trail can point toward the animal that laid it.

The goal of this chapter is to revisit the observations of Doall *et al.* (1998) in light of newer insights gained from chemically modulated mating behavior in airborne insects. We begin by reviewing in part the Schlieren optical technique pioneered by Töpler (1866) and its application to the capture of small translucent animals in water in Section 2. In Section 3 we consider a simplified version of the neuronal model proposed in Zavada *et al.* (2011). Instead of modeling the neurons by sophisticated models such as the one by Hodgkin and Huxley (1952), we use the Leaky Integrate-and-Fire (LIF) model (Gerstner *et al.*, 2014). This model can be traced back to Louis Lapicque (1907) from a time long before mechanisms generating neuronal action potentials were known (Abbott, 1999; Brunel and van Rossum, 2007). The LIF model is widely used today. Its main advantage, the computational simplicity, allows a focus on questions of design of circuitry. In Section 4 we implement the Simulated Annealing (SA) (Press *et al.*, 2007) algorithm to optimize the synaptic weights of the network. We find that a simple network model consisting of LIF neurons with conductance-based synaptic weights can implement a ratio detection mechanism. The optimal synaptic weights for the model indicate a strong mutual inhibition of the specialist and generalist local neurons. The relative simplicity of the network topology lends credibility to the thesis that ratio detection evolved early on in the ancestry of today's marine copepods.

2. OBSERVATIONS OF *TEMORA LONGICORNIS*

Most if not all photographs taken by the public at large are of "amplitude objects". In such an object, spatial differences in color values make it visible on the image. To image a clear wine glass submerged in clear water is one of the biggest challenges in photography. Special lighting at special angles may reflect from the surfaces to show parts of the glass. What has not been used in this case is the fact that the glass has a different refractive index than water. In such an environment, the glass should be looked at as a "phase object", and the optical techniques of making phase objects visible would render a distinct image of the glass. Phase objects with their refractive index slow down the light, creating a difference between light passing through the object and light from the background. Schlieren and shadowgraph techniques (Settles, 2001) render images based on differences in refractive indices. To employ these optical techniques became easier with the advent of single-line lasers and optics that allow collimated light beams carrying the information of an object for a long distance. For example, in our case, copepods swimming in a 1.5 L volume of water (10×10×15 cm) were imaged by a camera 2.16 m away with a resolution of 0.1 mm (Doall *et al.*, 1998; Strickler, 1977 and 1998; Strickler and Hwang, 1999).

Using collimated laser beams emitting laser light at one wavelength adds another component that can be used to observe particles of different sizes in 3D and suspended in volumes of water. Schlieren optical pathways were employed using white spectrum point sources, as well as laser in the visible and near-infrared emission range (Strickler, 1977; Strickler and Balázsi, 2007; Strickler and Hwang, 1999). Doall *et al.* (1998) used the advantage of the long distance between the object and the image to split the original collimated laser beam in two beams crossing each other perpendicularly in the vessel of the animals. The subsequent combination of the two beams in one gave us a beam that carried the information of the front and side view of each phase object swimming around in the vessel. The result was then a dark-field picture of the vessel with white to light grey images of the objects. A single video camera was used to register the events. The task to evaluate the videos in the late 20th century was to click on the object frame-by-frame, giving us the 3D coordinates at 60 Hz.

Figure 1 shows the time before a mating event as observed by Doall *et al.* (1998). The female starts at position *a'* and the male a little later at position *a*. The male meets the track of the female at position *b*, takes a turn and follows the track of the female. However, the male follows the track in the wrong direction. After 1.2 s it turns and back-tracks to catch up with the female at position *c*. In 27 of 67 pursuit observations (40%) the male started in the wrong direction, which does not suggest a good method to determine the direction at the moment of finding the trail. In 22 out of 27 observations when the initial swimming direction was incorrect the male turned around (81%). Thus, in a total of 92% of cases, the male eventually followed the female in the correct direction, a remarkable achievement for any member of the animal kingdom. Back tracking with similar time components was also observed in presence of trails simulating tracks from swimming zooplankters (Yen *et al.*, 2004).

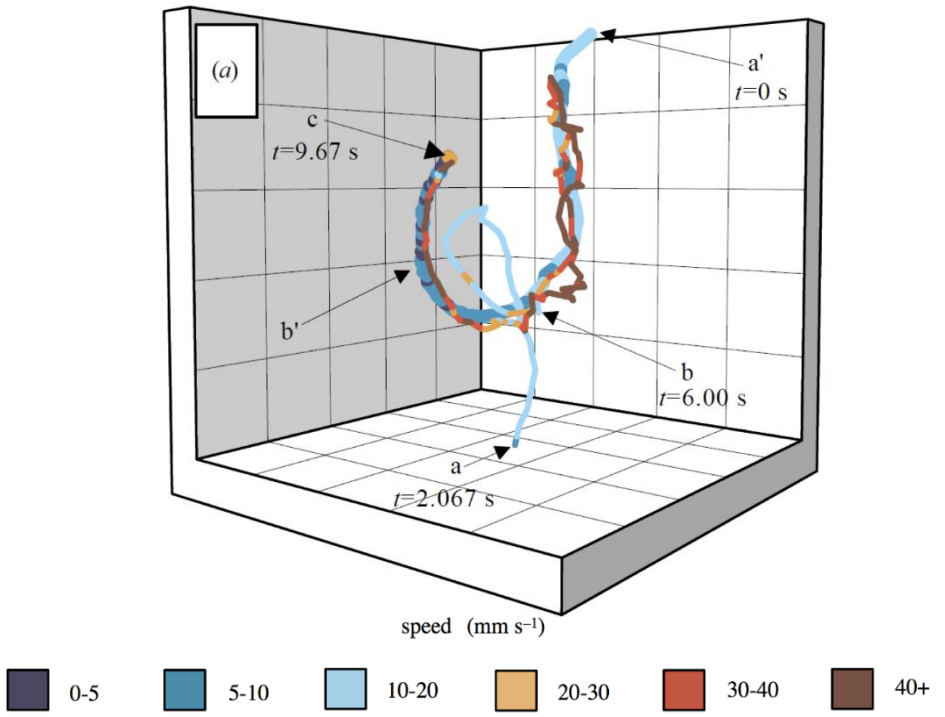

Figure 1. Trails of a female and a male from Doall *et al.* (1998). The fat and thin trails are that of the female and the male, respectively, while colors indicate swimming speeds. The male encounters the female's trail at position *b* and proceeds for the next ≈1.2 s in the wrong direction before turning around and finally capturing the female at position *c*. The grid unit is 1 cm. Reprinted from (Doall *et al.*, 1998) with permission from the Royal Society.

3. The Spiking Neuron Model for Ratio Detection

Simple phenomenological spiking neuron models are very useful for investigation of neural coding, memory and other functions, as they are easier to analyze than detailed electrophysiological neuron models; see Gerstner *et al.* (2014) for a thorough introduction to both classes. The simplest form of the LIF model for a single neuron is given by the following differential equation for the membrane potential v

$$\tau \frac{dv}{dt} = -v(t) + RI(t), \tag{1}$$

where τ is the membrane time constant, R is the resistance of the membrane and I is an external current, if present. Spikes are generated whenever the membrane potential reaches a threshold ϑ. Then the membrane potential is reset to the resting potential v_r,

$$\text{if } v(t-) = \vartheta \text{, then } v(t+) = v_r \text{,}$$

which also defines t as the spiking time of the LIF neuron. A schematic output of a LIF neuron with a single excitatory input is shown in Figure 2. For simplicity, a spike of the presynaptic neuron causes an immediate increase in the membrane potential.

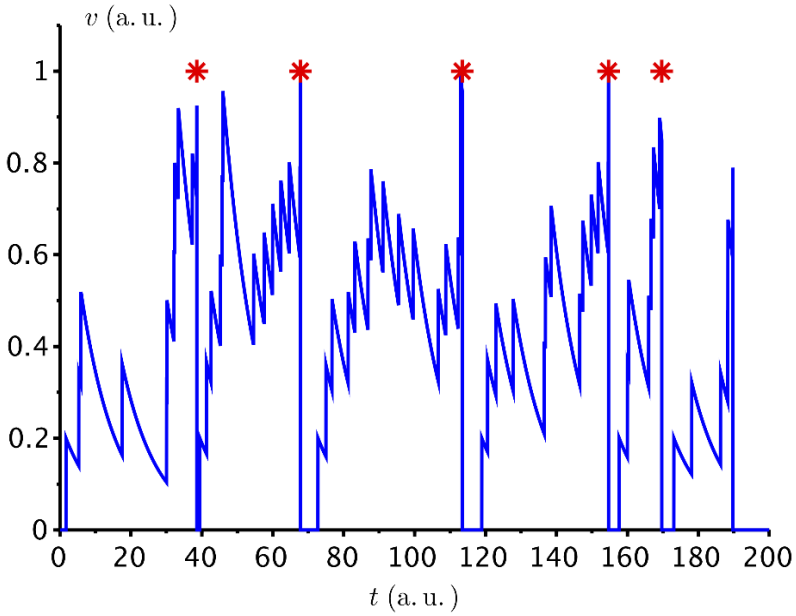

Figure 2. The membrane potential v of a LIF neuron governed by Equation (1) with I ≡ 0 that receives a single excitatory input ("a.u." = auxiliary unit). At each incoming spike, v is increased by 0.2. The spiking times of the neuron are indicated by red dots.

To model the synaptic connections between neurons we use the conductance-based model as described by Vogels and Abbott (2005). The sub-threshold membrane potential V and the excitatory, respectively inhibitory conductances g_{ex} and g_{inh} are governed by

$$\tau \frac{dV}{dt} = (V_r - V) + g_{ex}(E_{ex} - V) + g_{inh}(E_{inh} - V), \tag{2}$$

$$\tau_{ex} \frac{dg_{ex}}{dt} = -g_{ex}, \tag{3}$$

$$\tau_{inh} \frac{dg_{inh}}{dt} = -g_{inh}, \tag{4}$$

as long as no spiking takes place. We choose the resting potential V_r=-60 mV, the relaxation time constant for the membrane τ=20 ms and the synaptic time constants τ_{ex}=5 ms and τ_{inh}=10 ms, respectively (Vogels and Abbott, 2005). The key difference in the influence of the excitatory and inhibitory conductances is that the two reversal potentials are chosen to be E_{ex}=0 mV and E_{inh}=-80 mV, respectively. Once the membrane potential reaches the threshold of ϑ=-50 mV, the time is recorded as a spiking time and V is reset to V_r. Upon spiking of a presynaptic neuron at time t, all postsynaptic neurons have their excitatory respectively

inhibitory conductances changed by a certain amount, depending on the nature of the synapse. If the synapse is excitatory, the excitatory conductance of the postsynaptic neuron is increased,

$$g_{ex}(t+) = g_{ex}(t-) + w_{ex}, \tag{5}$$

if the synapse is inhibitory, the inhibitory conductance of the postsynaptic neuron is increased,

$$g_{inh}(t+) = g_{inh}(t-) + w_{inh}. \tag{6}$$

The amounts of increase in the respective conductances of the postsynaptic neuron are called the weights of the synapse. Their selection will be discussed in greater detail in Section 4. Note that these weights are non-negative in both cases and that only one of them characterizes a synapse.

A network topology for a ratio detection mechanism was proposed by Zavada *et al.* (2011). ORNs of types *a* and *b* are excited upon binding of their respective ligand. These are modeled as Poisson sources with firing rates r_a and r_b, respectively. Precisely, the probability that there are *k* spikes in a time interval Δt is

$$P\{k \text{ spikes during } \Delta t\} = \frac{e^{-r\Delta t}(r\Delta t)^k}{k!}.$$

Each ORN's firing rate grows linearly with respect to the logarithm of the ligand concentration *u*. This has been shown to hold for several orders of magnitude in the moth *Antheraea polyphemus* (Kaissling, 1996; Zack, 1979). For lack of better resources, we use the relationship

$$r = 48\lambda + 400$$

where λ is the logarithm of the concentration of the compound, see Figure 3 in Kaissling (1996), ranging from -8 to -2, and *r* is the firing rate in Hz. This is the hypothetical response curve for both components of the mixture.

The ORNs are connected to two types of LNs by excitatory synapses. Each ORN of type *a*, respectively *b*, is connected to a specialist LN of the same type and these receive excitatory input only from the corresponding ORNs. Simultaneously, the ORNs also are connected to a generalist LN, that receives excitatory input from both types of ORNs. In both cases, there is a convergence ratio of N ORNs feeding a single LN. For simplicity, we choose this convergence ratio to be the same for all excitatory connections. The LNs of all three types are connected by inhibitory synapses. We pick the smallest number possible, namely just one LN_a, one LN_b and one LN_{gen}. The synaptic connections are characterized by five weights where we make the following symmetry assumptions for the target ratio 1:1

1. the connections $ORN_x \rightarrow LN_x$ have the same weights for $x=a$ and $x=b$,
2. the connections $ORN_x \rightarrow LN_{gen}$ have the same weights for $x=a$ and $x=b$,

3. the mutual inhibition between LN_a and LN_b is symmetric,
4. $LN_{a/b}$ act the same way on LN_{gen}, and
5. LN_{gen} acts the same way on $LN_{a/b}$.

The network is depicted schematically in Figure 3. The output of the network is the firing rate of LN_{gen}. Note that by Dale's principle this output is necessarily inhibitory, as LN_{gen} already inhibits LN_a and LN_b. This signal is processed by further local intermediate neurons and projection neurons that we do not include in our model.

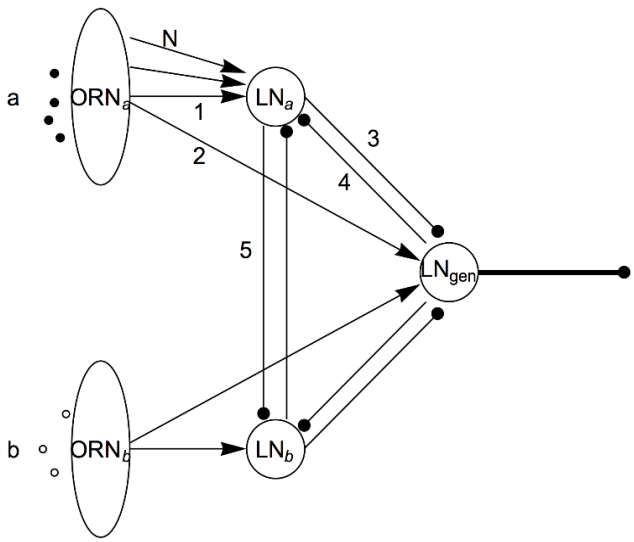

Figure 3. The topology of the ratio detection mechanism adapted from Zavada *et al.* (2011). Ligands of type *a* and *b* bind to the respective ORNs. The convergence from ORNs to LNs is indicated only once for clarity. Pointed and blunt arrows indicate excitatory respectively inhibitory relationships; labels indicate the independent weights.

The output of the generalist neuron LN_{gen}, *i.e.* its firing rate r_{gen} is the output of the mechanism, as a function of the firing rates r_a and r_b. If the mixture components *a* and *b* are present at the ratio 1:1, both LN_a and LN_b inhibit each other. Thus the ORNs excite directly LN_{gen}. If, however, component *a* is present at a much higher concentration than component *b*, then LN_a will silence both LN_b and LN_{gen}, similarly if *b* is present at a much higher concentration than component *a*.

4. SYNAPTIC WEIGHT SELECTION

The spiking neuron model in Equations (2)-(6) is almost complete except for the choice of the synaptic weights. Recall that the weights are non-negative numbers associated with each synapse. Thus the problem of choosing weights can be viewed as an optimization problem, where we optimize the network's behavior with respect to the desired output as a function of the five numerical weights. For each such weight vector we simulate the network behavior for selected ratios of r_a:r_b. Specifically, we use rates

$$r_a^i = r_0 \cdot 1.3^i \,,\; r_b^j = r_0 \cdot 1.3^j \,, \tag{7}$$

for i, j=0,..., 9 and r_0=10 Hz. This corresponds to logarithmic concentrations ranging from 10^{-8} to 10^{-6}. While the ORN firing rates span a much larger range, we chose this range to demonstrate ratio detection at very low concentrations. The firing rate of LN_{gen} is recorded for each such simulation in a 10×10 response matrix **R**. The numerical cost for each weight vector is defined to be the negative of the Frobenius inner product of the response matrix **R** with a convolution kernel **T** as in Figure 4,

$$C_T(w) = -\sum_{i,j=0}^{9} R_{i,j} T_{i,j} \,. \tag{8}$$

Note that for example negative off-diagonal entries in **T** strongly penalize against positive entries in the corresponding positions in **R**.

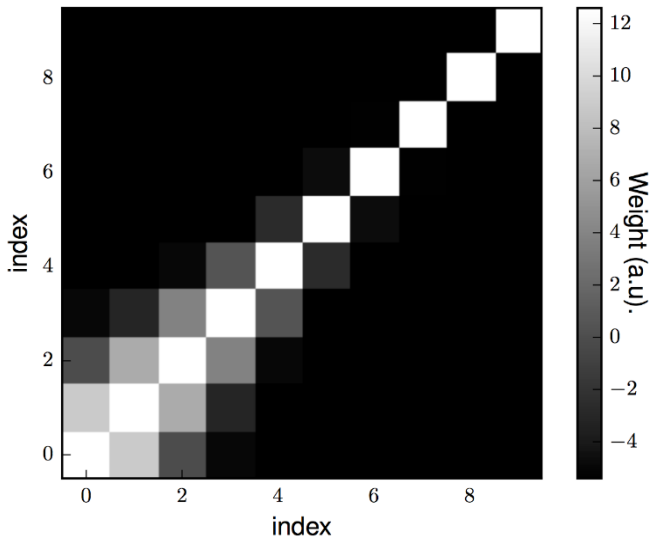

Figure 4. The convolution kernel **T** to determine the "cost" of the response matrix **R** used in Equation (8). The indices *i* and *j* are those from Equation (7).

We have implemented a SA algorithm (Press *et al.*, 2007, Section 10.12) to optimize the weights using the cost function in Equation (8). The PYTHON code is available from the github repository (Langhoff, 2017). The SA algorithm is built on an analogy from thermodynamics, namely the freezing and crystallization of liquids. Provided that the liquid cools sufficiently slowly, the constituents are able to align and to form ordered structures many orders of magnitude larger than the typical particle size. This amounts to a global minimization of the energy, as opposed to a rapid cooling that results only in a "quenched" or "amorphous" state corresponding to a local minimum. In practice, if a location x has been found, a random perturbation Δx is added. If $\Delta f = f(x+\Delta x) - f(x) < 0$, then x+Δx is chosen as the next point of the iteration. If $\Delta f = f(x+\Delta x) - f(x) > 0$, then x+Δx is chosen with a certain

probability that is proportional to $\exp\left(-\frac{\Delta f}{T}\right)$, where T is the quantity analogous to temperature. Thus any improvement in the cost function is taken, while a worsening is sometimes accepted, but less and less likely as the temperature decreases. The main choices to be specified are the generator for the random perturbations Δx and the method for decreasing T, called the "annealing schedule". Here we select Δx from a uniform distribution and enforce the constraint that all synaptic weights are non-negative. The temperature is multiplied by 0.85 every fifth iteration.

5. RESULTS

We choose the convergence ratio of N=100 ORNs feeding onto a single LN, for all possible ORN → LN connections. The optimal synapse weights are listed in Table 1.

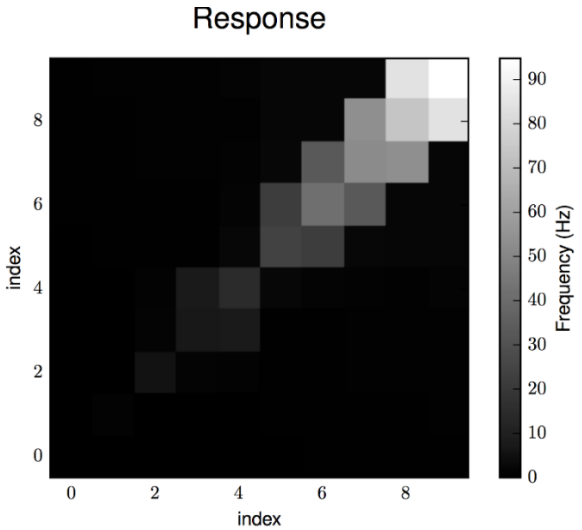

Figure 5. Optimal output of the networkm defined as the LN_{gen} firing frequency. The *(i, j)*-entry corresponds to ORN firing frequencies of $10 \cdot 1.3^i$ Hz and $10 \cdot 1.3^j$ Hz respectively.

Table 1. Optimal synaptic weights determined by the SA algorithm

Connection	Weight (μS)
$ORN_{a/b} \rightarrow LN_{a/b}$	0.14025 (w_{ex})
$ORN_{a/b} \rightarrow LN_{gen}$	0.06980 (w_{ex})
$LN_{a/b} \rightarrow LN_{b/a}$	0.77945 (w_{inh})
$LN_{a/b} \rightarrow LN_{gen}$	0.64391 (w_{inh})
$LN_{gen} \rightarrow LN_{a/b}$	0.59438 (w_{inh})

We note that the excitatory connections from the ORNs to the specialist LNs are stronger than to the generalist LN. Moreover, the mutual inhibition relations between the local neurons are roughly of similar strength. The model is somewhat limited in its discriminatory power at the lower end of the concentration ranges which may be caused by its strong simplification. In

future work one may increase the number of LNs in the mutually inhibitory groups in the triangle in Figure 3 and include the subsequent intermediate LNs and projection neurons.

CONCLUSION

It is difficult for us humans to imagine how challenging pelagic copepod life must be. They live at low Reynolds number, at low population densities, and in the dark and three dimensional ocean. With the adult males of some species even lacking functional mouthparts, finding mates can absolutely not be left to chance. It is therefore natural for the males to respond to specific chemical signals (Yen and Lasley, 2011). To the best of our knowledge it is still an open question whether the copepod sex pheromones are specifically produced by the female or whether they are incidental byproducts of naturally occurring metabolism as, say, would be CO_2. Another important open question is how the well defined trails that were observed in water at rest are deformed and perhaps torn in the actual oceanic habitat of *Temora longicornis*.

We have shown that even a minimalistic, simplified neuronal model is capable of ratio detection. This represents a significant simplification from the previous model (Zavada *et al.*, 2011), where a full Hodgkins-Huxley model in addition to a rate-based Hodgkins-Huxley model of the neuron were used. Phenomenological models like the LIF model considered here do not explicitly model the individual ion channels in a neuron and treat spikes as formal events (Abbott, 1999). By omitting some of the biological details, we can gain insight into the behavior we seek to understand. The simplification is of course more relevant for much more complex networks, containing for example 10^5 neurons. In our case we see the relative weight of the neural pathways used for ratio detection. In reality, the ratio detection network will consist of more than just three LNs. At present little is known about the neuroanatomy of copepod brains and peripheral nervous systems, but there is evidence from the species *Tigriopus californicus* that it is endowed with a complex brain (Andrew *et al.*, 2011). In the future it will be valuable to investigate and to model the "spatial structure" that arises from the presence of ORNs on both antennas of the copepod sending signals to a pair of LN structures. We anticipate that further impulses for research will come from comparison with airborne arthropods due to the high level of conservation of neural circuitry in the pancrustaceans (the clade comprising crustaceans and hexapods).

So far only few semiochemicals used by copepods have been identified. One example is isophorone which is used by the parasitic sea louse *Lepeophtheirus salmonis* to locate its host, the Atlantic salmon (*Salmo salar*) (Ingvarsdóttir *et al.*, 2002); see Figure 6. It has a molar mass of 138.21 g/mol and is used by airborne arthropods as semiochemical as well (El-Sayed, 2017). Very recently Selander *et al.* (2015) identified so-called "copepodamides" that signal to phytoplankton the presence of their zooplankton predators and thereby induce the production of toxins as a defense. Identification of such chemicals in seawater can be done by coupled liquid chromatography and mass spectrometry (LC-MS), and an electro-antennographic detector (EAD) to confirm the response of the animal's antenna at precisely the moment that the chemical is detected. If there are candidates for the pheromone components, behavioral assays such as the Y-tube assay (Ingvarsdóttir *et al.*, 2002) can be used. Selander *et al.* (2016) present a list of 87 exudates from male and female *T. longicornis*.

Their list contains nine compounds that are produced mainly or even exclusively by the females. These compounds did not initiate the pursuit reaction in the male, but this can be because there were other volatile compounds that were not retained. Future research is needed to investigate the decay and diffusion rates of the compounds and to locate potential differences. The number of compounds, their relatively large molecular masses (300-700 g/mol) and their likely complicated chemical structures indicate that a host of information should be available from their combined presence for the trained "observer".

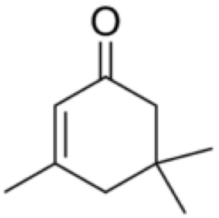

Figure 6. Isophorone is a kairomone used by the parasitic sea louse *Lepeophtheirus salmonis* to locate its host (Ingvarsdóttir *et al.*, 2002). Structural formula from Wikipedia (2017).

All marine fauna are currently challenged by increasing chemical pollution and decreasing oceanic pH values, both of human origin. Changes in the background chemical landscape have harmful effects on olfactory-mediated behaviors in fish and crustaceans (Olsén, 2011). Due to their critical linking position in the marine food web, it is of high value to identify the chemical components of copepod sex pheromones and how their mating behavior is affected in the presence of pollutants.

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Chapter 71

PLANKTONIC CALANOIDS EMBARK INTO THE "OMICS ERA"

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ABSTRACT

Since the very first DNA fragment was sequenced in early 70s and the 1980s Nobel Prize was awarded to Sanger and Gilbert "for their contributions concerning the determination of base sequences in nucleic acids," both biology and medicine have taken great steps forward. In particular, marine biologists have taken advantage of technical development and are increasingly applying molecular techniques to address biological and ecological questions. Recently, the growing interest in marine and freshwater organisms expressed by private companies involved in pharmaceuticals, nutriceutics, and bioproduction have boosted interest and applications of genomics, transcriptomics, proteomics and metabolomics. In parallel, molecular techniques like genetic transformation, genome modification, and gene silencing have been developed for non-model species. This improvement of molecular biology-related approach has enhanced academic research in biology, ecology and physiology of a plethora of metazoan non-model species. Copepods are the most species-rich class among the marine arthropods, are globally distributed and inhabit every environment from coastal to oceanic waters, where they represent the dominant group of zooplankton, and can also live as symbionts or parasites. This diverse array of life strategies and environments make copepods highly interesting as target species for genomic and transcriptomic studies. In the present contribution, we provide an overview of the state of the art about 'omics' studies in calanoid copepods, focusing on marine free-living species. Finally, we will suggest some future perspectives for possible applications of functional genomics studies in copepods.

Keywords: calanoid copepods, transcriptomics, metabolomics, proteomics

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1. INTRODUCTION

The phylum Arthropoda is one of the most successful among animals on our planet, with more than one million species (Telford *et al.*, 2008) inhabiting every environment. Among Arthropoda, copepods belong to the class Maxillopoda (Sub-phylum Crustacea, sub-class Copepoda), which is thought to have arisen during the Paleozoic, between the Cambrian and the middle Devonian (Regier *et al.*, 2005).

Copepods represent the most abundant group of multicellular animals, and more than 14,000 species have been described to date (Humes, 1994). Their adaptive radiation is astonishing: they colonised literally every subaqueous, subaerial and subterranean environment (Huys and Boxshall, 1991; Boxshall and Halsey, 2004). The same authors suggested that all orders of copepods are derived from ancestral marine epibenthic forms, and that the invasion of the pelagic environment was accompanied by strong morphological specializations. Besides their free-living life-style, copepods have evolved symbiosis and parasitism as well (Ho, 2001). It has been hypothesised that during evolution, copepods have left the free-living habitus on at least 11 independent occasions (*e.g.*, Bayly and Boxshall, 2009). Because of their species richness and incredibly high biomass, copepods have been dubbed the 'insects of the sea' (Huys and Boxshall, 1991). In a review paper, Schminke (2007) has even emphasised this assumption, in saying that insects should be called 'the terrestrial copepods', due to the evidence that Crustacea and Insecta may form a monophyletic group called Tetraconata or Pancrustacea (Schminke, 2007). The relationship between these two sister groups has been recently validated by molecular and morphological phylogeny (Legg *et al.*, 2013). However, unlike insects, the studies on genetics and genomics of copepods is in its infancy. Here we will focus on free-living marine calanoid copepods because the ecological role they play in marine ecosystems as primary consumers is crucial for the whole food-web. Bron *et al.* (2011) have already presented the state of the art in copepod genomics in a very elegant and omni-comprehensive review. In particular, genomic resources have been used to address issues related to species identification (*e.g.*, Bucklin *et al.*, 2009; Sabia *et al.*, 2017; Di Capua *et al.*, 2017), biogeography (*e.g.*, Rubao *et al.*, 2012), cryptic species detection (*e.g.*, Schoville *et al.*, 2012; Cornils and Held, 2014), symbiosis and parasitism (*e.g.*, Fallang *et al.*, 2005; Fast *et al.*, 2007), and acute and sub-lethal responses to environmental stressors (*e.g.*, Raisuddin *et al.*, 2007; Hansen *et al.*, 2011). In this chapter, we aim to update the state of the art, paying particular attention to the techniques developed since 2011. We will emphasise recent gene expression studies on free-living keystone copepod species, and then will give a more thorough description of 'omics' studies (transcriptomics, proteomics and metabolomics), addressing specific questions in copepod biology and ecology. Finally, we will give some future perspectives for functional genomics studies in copepods.

2. TECHNIQUES

In the present section, the molecular biology techniques that will be discussed in the following sections are described.

2.1. RT-qPCR

The acronym RT-qPCR stands for Real-Time quantitative Polymerase Chain Reaction. This technique is an improvement of the conventional end-point PCR and takes advantage of fluorophores. Such fluorophores are used in two different ways: 1) they can directly bind in a stochiometric way to double stranded DNA so they emit photons when incorporated into the amplicon or 2) they are linked to a probe binding to the region which contains the DNA fragment to be amplified. The probe is linked to both the fluorophore and to a quencher which hampers fluorescence if it is in the vicinity of the fluorophore. Polymerase binds to the region to be amplified and unwinds it. Because the probe is bound the one portion of this region, polymerase removes the probe, the quencher and the fluorophore are separated, and fluorescence is released. In both cases, photons are released and detected at each PCR cycle.

The main goal of RT-qPCR is to quantify the amount of template DNA present in the sample. In the present chapter, studies based on RT-qPCR have been used to determine gene expression levels under two experimental conditions. This is achievable by amplifying complementary DNA (cDNA) samples obtained by reverse transcribing RNA from specimens under a given experimental condition. RNA is not amplifiable by PCR so it is necessary to synthesise cDNA first. To do so, a viral enzyme, the RNA-dependent DNA polymerase, colloquially called reverse transcriptase is used. This enzyme synthesizes DNA from RNA templates. When RT-qPCR is used to determine gene expression levels, the relative quantity of the template cDNA from a gene of interest (GoI) is measured in comparison to the relative quantity of a series of reference genes (RG). This procedure is needed to normalise cDNA quantity used for the reaction mix. RG's are usually house-keeping genes whose expression does not change over a broad range of experimental conditions. In order to define the suitable RG's among a set of candidates, different algorithms are used. To be valid, RT-qPCR-based works should rely on at least 3 RG's. For a wide review of RT-qPCR we suggest Valasek and Repa (2005) and Bustin *et al.* (2009).

2.2. Microarray

Microarrays, or the DNA chip technique, is based on the principle of nucleotide hybridisation. Oligomer probes (short DNA fragments) are spotted on a glass microscope slide. Oligomers can be fragments of genes or transcripts. The slide is then put in contact with nucleic acid extracts (DNA or cDNA) from two samples: one control and the other from the experimental condition. If cDNAs are hybridised to the microarray, the outcome is the gene expression level in the experimental vs the control condition. In order to obtain such a comparison, RNA samples are reverse transcribed and labelled with fluorescent dyes of a different colour. Individual fragments in template cDNAs, complementary to the probes spotted onto the chip, will anneal. The chip scanner reads the relative intensity of each dye and gives relative quantification of a given template fragment in one sample compared to the other. In the case of microarrays internal or external controls are needed to normalise the expression levels (Lippa *et al.*, 2010). In order to rule out any bias due to differential efficiency in the reverse transcription and labelling, a dye swap (*e.g.*, Hori *et al.*, 2015) is carried out. This procedure is performed by hybridising twice the chip with the same sample pair, but with the samples labelled alternately with the two different fluorescent dyes. This

constitutes a technical replicate, with cDNA that is reverse transcribed separately from the same RNA sample to label with different dyes the first cDNA strand. In addition, it is also a methodological control, possibly reducing the occurrence of false positives due to the bias of fluorescent dyes (Mary-Huard *et al.*, 2008). Microarrays can be used to measure the expression of thousands of genes simultaneously, but lack the ability to detect transcripts whose sequence is unknown. This makes such technology unsuitable to work on non-model organisms, where little or no genomic and transcriptomic information is available. Microarrays are currently applied mostly in molecular diagnostics because protocols have been standardised and validated for health and clinical investigations (*e.g.*, Yoo *et al.*, 2009; Govindarajan *et al.*, 2012).

2.3. EST and SSH

Expressed Sequence Tag libraries (EST) are sets of sequenced cDNAs from specific conditions or tissues and are used primarily for gene discovery or tissue-specific gene expression analyses (Adams *et al.*, 1991). cDNA synthesised under specific conditions or from tissues are inserted into vectors like plasmids, cosmids or fosmids and then transformed into competent bacterial cells. This procedure enables sequencing of unknown mRNA transcripts or random genomic DNA fragments. ESTs have been very useful in the field of molecular ecology to understand the population structure, and the genetic basis of phenotypic variation and adaptation of species. Through the discovery of new molecular markers, ESTs may provide a means for investigating gene family and genome evolution (Bouck and Vision, 2007).

If the problem to address is to identify genes differentially expressed between an experimental condition and that of the control (or in one tissue compared to another), one of the ways to enhance the yield of differentially expressed genes is to produce subtraction libraries by means of suppressive subtraction hybridization (SSH) (Diatchenko *et al.*, 1996). This technique enriches the library from the sample of those transcripts that are differentially expressed compared to the control. The basis of such an enrichment is the elimination of those transcripts that are present in both samples. For an extensive and exhaustive description of the method we suggest Rebrikov *et al.* (2004).

2.4. RNA-Seq and *De Novo* Assembly of Transcriptomes

In the studies reviewed in the present chapter, RNA sequencing (RNA-seq) for transcriptome production have been mainly performed using Illumina or 454 sequencing platforms. These sequencing techniques differ from the Sanger sequencing in many aspects, most importantly in that Sanger sequencing relies on capillary electrophoresis of a population of ddNTP-terminated dye labelled products that are separated by molecular weight and the dye detected. Each colour corresponds to one of the four nucleotides. Sanger sequencing is based on DNA synthesis. Illumina and 454 sequencing are based on solid-phase bridge PCR amplification, and emulsion PCR amplification, respectively. The 454 method produces longer reads compared to Illumina sequencing and with its newly released version of the platform (454 GS FLX+) can equal Sanger sequencing in read length. Although softwares

exist to assemble short reads with a very high degree of effectiveness, longer reads can help with scaffold assembly in *de novo* assemblies. Sequencing technology advances very rapidly and a complete overview of all the techniques presently available is beyond the aims of the present section. Here we will briefly describe only the two major sequencing techniques currently used in marine copepod transcriptomics analyses. The most up-to-date sequencing method is the nanopore sequencing technology (Feng *et al.*, 2015). Single molecules electrophoretically pass through a pore that separates two reservoirs, called the *cis* and the *trans* reservoirs. When the analyte passes through the pore, the voltage through it is blocked and by statistically analysing the amplitude and the time of such blockage, the nature of the molecule can be identified. This technology needs still improvements (Feng *et al.*, 2015) but will reduce costs and manipulation of samples (*e.g.*, Quick *et al.*, 2017). For a detailed description of all the Next Generation Sequencing (NGS) technologies we suggest the following papers: Branton *et al.*, 2008; Shendure and Ji, 2008; Quail *et al.*, 2012; Rhoads and Au, 2015; Wang *et al.*, 2015; Feng *et al.*, 2015; Yuan *et al.*, 2016.

Briefly, RNA-seq is based on the great technological advancement of NGS that allow obtaining billions of base pairs (Gb) and millions of short reads between 30-400 bp long per run without any cloning steps. As discussed in the above-cited papers, together with the augmentation of sequence power, the costs have dramatically decreased, enabling obtaining transcriptomes to be more widely available. An RNA-seq experiment has to be thoroughly planned beforehand with the proper questions to address. The amount of data generated by a single RNA-seq experiment is enormous and the bioinformatics analyses required are quite demanding. A transcriptome is usually produced for two main reasons; 1) to identify new genes specifically expressed in a given condition or tissue; 2) to pinpoint differentially expressed genes in different conditions or tissues or even different developmental stages. In addition to these scenarios, the only limitation to the technique is the researcher imagination. Consider the case 2. Total RNA is extracted from specimens under the experimental and control conditions. The control can be arbitrarily chosen. cDNA is synthesised as described above and libraries are constructed. The library construction depends on the sequencing technology of choice (*e.g.*, Pease and Sooknanan, 2012; Baran-Gale *et al.*, 2015). In order to be statistically robust, *i.e.*, to obtain sound results with the lowest false positive rate possible, replicates are required. Usually triplicates are accepted (Conesa *et al.*, 2016) although it has been recently demonstrated that at least six biological replicates are needed in order to be able to statistically identify differentially expressed genes (Schurch *et al.*, 2016). The output of sequencing is a variable number of variably long sequences (depending on the technique) called reads. Reads are quality filtered *in silico* in order to obtain mainly sequences from messenger RNAs, *i.e.*, expressed genes, and other RNA types like micro RNAs (miRNA), long non-coding RNA (lncRNA), etc. The assembly of short reads into full length genes is a complex bioinformatic process. Assembly can either rely on a reference genome, if available, which is usually the case for model organisms; or can be done *de novo*. In the former case, a series of algorithms (pipelines) align the reads to the reference genome sequence; in the latter, reads have to be assembled without a guide (*e.g.*, Haas *et al.*, 2013). Once the reads have been assembled into contiguous overlapping regions, referred to as contigs, which can eventually match with full length transcripts, annotation is performed. For a review on read assembly see Miller *et al.* (2010). Annotation is a bioinformatic procedure which assigns to contigs gene models (Yang and Kim, 2015; Conesa *et al.*, 2016) or other features like *e.g.*, retrotransposons or transposable elements (Criscione *et al.*, 2014; Jin *et al.*, 2015). With

appropriate normalization procedures, the number of reads can be quantified and the expression level of each transcript defined. Differential expression analyses can be now performed using adequate software (Conesa *et al.*, 2016). RNA-seq is devoid of problems derived from technical issues inherent to microarray probe performance such as cross-hybridization, non-specific hybridization and limited detection range of individual probes. Additionally, RNA-seq technology does not require species- or transcript-specific probes (Zhao *et al.*, 2014).

A recent study by Wang *et al.* (2009) compared RNA-seq, microarray and cDNA sequencing techniques in transcriptome profiling and found that RNA-seq was superior in detecting low abundance transcripts, differentiating biologically critical isoforms, and allowing the identification of genetic variants. RNA-seq also demonstrated a broader dynamic range than microarrays, which allowed for the detection of more differentially expressed genes with higher fold-change (see Table 1 in Wang *et al.*, 2009).

3. THE TARGET SPECIES

A few gene expression and transcriptomic studies have been published to date on free-living marine copepods. The most investigated genera are the calanoid *Calanus* and the harpacticoid *Tigriopus*. Calanus has been used as model organism for studies on mechanisms inducing diapause, on the life cycle in general (Tarrant *et al.*, 2008; Lenz *et al.*, 2012), and responses to microenvironmental discontinuity (Unal *et al.*, 2013). *Tigriopus* has been used for investigating temperature tolerance of different populations, ecotoxicology and for phylogenomic purposes (Raisuddin *et al.*, 2007; Schoville *et al.*, 2012). However, as it is not a planktonic species, *Tigriopus* is not dealt with in the chapter.

Only a few transcriptome studies have been performed on *Calanus* despite the extensive knowledge on its biology and ecology. The focus of these studies has been to better understand the molecular basis of their physiological responses to intrinsic and extrinsic factors. *Calanus finmarchicus* and *Calanus helgolandicus* are two of the most widely studied *Calanus* species in terms of biology (Niehoff, 2007) and distribution (Mackas *et al.*, 2012; Maud *et al.*, 2015). *C. finmarchicus* is a very abundant copepod species possessing a subarctic distribution with a latitudinal range from 40°N to 80°N (Bryant *et al.*, 1998) and inhabiting shallow to very deep waters. Its ecological importance is linked to its abundance and subsequently to the impressive amount of energy that flows through this primary consumer in most ecosystems from the primary phytoplanktonic production to economically relevant fish larvae (Prokopchuk and Sentyabov, 2006). Similarly, *C. helgolandicus* is one of the dominant zooplankton species in European waters, living in open and coastal waters of the temperate Eastern Atlantic Ocean and the Mediterranean Sea (Northern Adriatic Sea) (Bonnet *et al.*, 2005). Recent surveys showed that both species distributions are gradually shifting northward due to the increase in water temperature, with *C. finmarchicus* moving into Arctic waters and *C. helgolandicus* expanding its area towards the North Sea (Beaugrand *et al.*, 2002). As indicators of different water masses, both species are routinely identified by the Continuous Plankton Recorder (CPR) Survey, operated by the Sir Alister Hardy Foundation for Ocean Science (SAHFOS) in Plymouth (UK) (Wootton *et al.*, this volume).

Within the *Calanus* genus, it is notable that *Calanus sinicus* is a widespread cold-temperate species that inhabits the Northwest Pacific Ocean from the Yellow Sea to the Sea of Japan and the South China Sea and Taiwan Straits (Huselmann, 1994; Hwang *et al.*, 2006). *C. sinicus* has been one of the target species of the China-GLOBEC program (Sun, 2005), because it is a strong link between the anchovy and sardine fisheries and the copepod secondary production in this area (Uye, 2000; Yang *et al.*, 2014). Because of its ecological relevance, this species has recently received much attention using RT-qPCR and RNA-Seq approaches (see section 4).

4. GENE EXPRESSION APPROACH

4.1. RT-qPCR-Based Works

Gene relative expression and expression profiling can be achieved by different means, *i.e.*, RT-qPCR, DNA microarrays (section 4.2), and RNA-seq (section 4.3). The former is limited to genes already identified and for which a sequence and possibly a function are already known. This RT-qPCR approach has been extensively applied in the ecotoxicology field because stress-related genes responding to environmental stressors have been identified in copepods (*e.g.*, Hansen *et al.*, 2008 and 2013). Recently, a RT-qPCR approach was also used to explore the deleterious effects of harmful algal diets on copepod gene expression. In particular, the expression levels of genes involved in generic stress responses, defence systems and apoptosis regulation in *Calanus helgolandicus* feeding on the diatom *Skeletonema marinoi* were investigated (Lauritano *et al.*, 2011a and b). *S. marinoi* is able to produce cytotoxic metabolites called 'oxylipins' from the oxidative metabolism of fatty acids (Fontana *et al.*, 2007; Gerecht *et al.*, 2011; Di Dato *et al.*, 2017). In *C. helgolandicus*, the diatom induced the regulation of α and β tubulins, possibly revealing cellular rearrangement, and of several other genes related to stress response (heat shock protein 40), detoxification (several aldehyde dehydrogenases ALDHs) and apoptosis (cellular *apoptosis* susceptibility, CAS and inhibitor of apoptosis, IAP). These studies provided the first molecular evidence that the primary defence system that would be activated to protect copepods against toxic algae could be inhibited (Lauritano *et al.*, 2011a and b). Interestingly, this approach enabled testing for subtle differences among the responses of *C. helgolandicus* populations isolated from North Sea, Atlantic Ocean and Mediterranean Sea. Despite the only three bp difference in a 518 bp-long mitochondrial COI fragment among the three *C. helgolandicus* populations, the Mediterranean population showed stronger gene down-regulation compared to the others (Lauritano *et al.*, 2012).

Similar results were obtained with the congeneric species *Calanus sinicus* fed on *S. marinoi* for two and five days (Lauritano *et al.*, 2015). At day five, *C. sinicus* showed up-regulation of several ALDHs, catalase (CAT), and glutathione-S-transferase (GSH-S), known to enzymatically reduce free radicals and reactive species (RS).

These studies provided new insights for understanding copepod population- and species-specific responses to toxic algae and co-evolution between toxic algae and detoxification mechanisms in copepods.

The results presented above, were partially corroborated by an in situ study carried out on wild *C. helgolandicus* specimens collected at the end of the spring diatom bloom in the Northern Adriatic Sea during an oceanographic cruise (Lauritano *et al.*, 2016). Surprisingly, detoxification genes did not revel consistent regulation. This complicates our understanding of this complex phenomenon in copepods and calls for more detailed investigations. The response to a possibly noxious diet could be different if the copepods are fed with phytoplankton other than diatoms. In fact, when *C. helgolandicus* was fed on a non-toxin producing strain of the dinoflagellate *Karenia brevis* for three, five and eight days, the results were completely different (Lauritano *et al.*, 2013). β tubulin, ALDHs, CAS and HSP40 were significantly down-regulated, but only after eight days of exposure to this food. This could be due to a different effect of unknown compounds produced by the dinoflagellate. Chemical analyses of the same strain were performed but they did not reveal any harmful compound (Turner *et al.*, 2012). Such a discrepancy suggests how intricate the copepod-prey ecotoxicological landscape can be and highlights the need to more detailed functional studies.

4.2. Microarrays and EST Libraries

Microarrays or DNA chips, suppressive subtractive hybridization (SSH), expressed sequence tag (EST) libraries and RNA-seq (section 4.3) are useful techniques that have been applied to disentangle copepod transcriptional responses to different environmental stressors and different diets or to identify genes involved in reproduction and molting.

The first *C. finmarchicus* microarray was produced by Lenz *et al.* (2012). Before the target genes to spot on to the chip were defined, <11,000 ESTs were sequenced, assembled and annotated together with the other ESTs publicly available at that time for the species. Around one thousand target genes were selected according to their function. 50mers (synthetic oligonucleotides composed of 50 bp) were spotted on the chip. The microarray was first tested with *C. finmarchicus* under food stress compared to adequately fed animals. A second test was performed comparing lipid-rich and lipid-poor animals. In both cases, several genes resulted differentially expressed. This pioneering work led to the conclusion that copepods respond to food limitation similarly to other organisms and they tend to save energy.

The '*Calanus* physiological microarray' (Lenz *et al.*, 2012) was then used in an in situ investigation by Unal *et al.* (2013) who, in 2008, compared gene expression in deep and surface *C. finmarchicus* individuals. The custom 995-gene microarray was hybridised with cDNA produced from 9-12 copepod RNA extractions. Chip hybridisations involved comparison between surface and deep females, and surface females with deep juvenile stages (copepodite V, CV). No CV were detected at the surface in that sampling occasion. The differential expression patterns displayed by surface and deep individuals presented a scenario where in deep waters individuals recently emerged from diapause were completing their developmental process ready to migrate to the surface. The individuals at the surface were metabolically more dynamic with grazing and predator escaping activities (Unal *et al.*, 2013). These observations are in accordance with the bimodal vertical distribution of the species. In spring copepodites emerge from diapause and migrate to the surface to feed and reproduce. This is corroborated by the up-regulation of genes involved in development and phototransduction, as well as of some genes involved in late embryogenesis. In deep females,

the set of differentially expressed genes suggested that diapause emergence, early embryogenesis and tissue remodeling take place. Despite the absence of dye swap, the data in Unal *et al.* (2013) were statistically robust because all the possible normalization and quality controls were performed. Moreover, the work represented a novel approach.

In 2014, the first transcriptional profiling study of a copepod exposed to a harmful algal diet (the oxylipin-producing diatom *S. marinoi*) was performed by Carotenuto *et al.* (2014) on *C. helgolandicus* using a SSH technique. *Rhodomonas baltica* was used as food for control copepods. Although this method did not provide as many sequences as the RNA-seq analysis (less than 1k), it generated two reciprocal EST libraries composed of longer sequences (on average 370 bp) compared to RNA-seq. This enabled a more robust annotation of the database, with about 62% of ESTs showing a BLAST match compared to 40% reported in Lenz *et al.* (2014), and about 81% of ESTs functionally annotated into Gene Ontology (GO, http://www.geneontology.org) categories (475 out of 583) compared to 13% (5k out of 38k) (Lenz *et al.*, 2014). The annotation statistics improved when the ESTs were eventually assembled into longer contigs (on average 420 bp, and 70% with a Blast-hits). A differential expression analysis, performed on the two reciprocal ESTs libraries using the Fisher's Exact Test with Multiple Testing Correction of False Discovery Rate (FDR < 0.01), showed that several GO terms were significantly enriched in both libraries. More specifically, in *Rhodomonas*-fed *C. helgolandicus*, macromolecule biosynthetic process, ribosome biogenesis and cell cycle processes were enriched. This suggests that animals are in an active state and that they are healthy. In *Skeletonema*-fed copepods the enriched GO-terms were biological regulation, nucleotide binding, signal transduction and protein folding. This transcriptional scenario can reveal a stressing state the organisms have to cope with. The results were validated and confirmed by RT-qPCR, and led to the conclusion that in *C. helgolandicus*, the diatom diet was inducing a generalised Cellular Stress Response mechanism, characterised by over-expression of molecular chaperones aimed at re-establishing the cellular homeostasis of the copepod.

4.3. RNA-Seq

In 2013 the complete transcriptome of *C. siniucs* was sequenced using the 454 GS FLX method (Ning *et al.*, 2013). Copepodites and adult males and females were collected from the field and cDNA was obtained from total RNAs extracted from pools of CIV-CV and adult males and females. Almost 1.5×10^6 350 bp reads were obtained in total from both libraries sequenced, 7% of which did not pass quality and size control. The transcriptome was *de novo* assembled and ca. 15k genes annotated. RNA-seq data were analysed for differential expression and ca. 2k genes resulted up- and 2k down-regulated in copepodite vs. adult library. Among these genes, some were of great interest because linked to diapause regulation. In this study, RT-qPCR validation of 7 transcripts was performed on the same cDNAs used for sequencing and all of them were significantly differentially expressed validating the RNA-seq results. This validation adds robustness to the results. In the study by Ning *et al.* (2013) $>2\times10^5$ single nucleotide polymorphisms (SNPs) and $>3\times10^4$ insertions/deletions (indels) were found. The sequencing was performed on a mixture of individuals so the intrapopulation level variability could be detected. SNPs can be of great

utility for phylogenomic studies as in bacteria and coral systems (*e.g.*, Bryant *et al.*, 2016; Rosser *et al.*, 2017) or population genetics (Coates *et al.*, 2011).

Later on, the transcriptome of *C. sinicus* was also sequenced by means of Illumina technology (Yang *et al.*, 2014). The results obtained from this second study corroborated and enriched that of Ning *et al.* (2013), giving a deeper coverage of the transcriptome with nearly 6×10^6 100 bp reads sequenced. In Yang *et al.* (2014), GO term assignment was similar to that reported by their colleagues for 'molecular function' (30% vs 32%) and for 'biological process', being in both cases the most represented term. Given that Yang *et al.* (2014) obtained seven times more enzyme code (EC) assignments, *i.e.*, KEGG gene pathways annotations (KEGG: Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) with 14K vs 2.9K transcripts compared to Ning *et al.* (2013), the most represented pathways were the same: carbohydrate, amino acid and energy metabolisms. In both studies, particular importance was paid to lipid metabolisms for its involvement in diapause. Noteworthy, most transcripts identified as diapause-related, such as a long chain fatty acid elongase (ELOV), several HSPs and ferretin, were differentially expressed between copepodites and adults (Ning *et al.*, 2013). Yang *et al.* (2014) identified stress- and immune-related genes as well. This is of great interest for future studies focusing on the effect of different pollutants on marine ecosystems in general.

In 2014 and 2016 *C. finmarchicus* was the target species for three Illumina RNA sequencing projects. The first produced from different developmental stages (Lenz *et al.*, 2014), the second dealing mainly with molting (Tarrant *et al.*, 2014 and 2016) and the third showing the copepod response to a toxic dinoflagellate (Roncalli *et al.*, 2016). These studies were aimed at obtaining new resources on key copepod species. Lenz *et al.* (2014) produced the first complete transcriptome for *C. finmarchicus* and immediately after Tarrant *et al.* (2014) produced a second complete transcriptome on the same species. The former *de novo* assembly was carried out using reads from six cDNA libraries built on RNA extracts from different developmental stages of the target copepod, namely embryos, early and late nauplii (NI-NII and NV-NVI, respectively), early copepodites (CI-CII), pre-adults (CV) and adult females (CVI). Ribosomal sequences were filtered from the raw data ($>4\times10^8$ 100 bp-reads) and library-specific adaptors removed before transcriptome assembly. The number of unique contigs was 96k, of which ca. 38k presented a significant blast with protein-coding genes (about 40%). This number is in the same order of magnitude as the *Daphnia pulex* genome (ca. 31k reported genes; Colbourne *et al.*, 2011) but is twice as many putative genes as *Drosophila melanogaster*'s (ca. 18k genes; Hoskins *et al.*, 2015) and 2.5 times as many as Eriocheir sinensis crab's (14k genes; Song *et al.*, 2016). The second transcriptome by Tarrant *et al.* (2014) was carried out from pooled RNA of copepodites V individuals from two time points (three and 10 days growth). To this pool, *C. finmarchicus* RNA from wild CV's was added.

Without a complete genome sequenced, it is very difficult to extrapolate the number of genes composing a genome from RNA-seq data. On the other hand, this exercise has been done for a congeneric species, C. sinicus, and for Tigriopus californicus. In both instances, around 15k genes have been found to be expressed in these copepods' transcriptomes (Barreto *et al.*, 2011; Ning *et al.*, 2013). In comparison, the human genome has been estimated to be composed of around 19k protein coding genes (Ezkurdia *et al.*, 2014). The genome size of *C. finmarchicus* was indirectly estimated to be 6 Gb (http://www.genomesize.com), which is almost double the size of the human haploid genome.

In Lenz *et al.* (2014), the *C. finmarchicus* RNA-seq data were used to infer transcript differential expression among developmental stages. Usually, a differential expression analysis enables identification of genes that are specifically or preferentially expressed in one treatment compared to the condition set as control (*e.g.*, treated vs. non-treated). In their study, Lenz *et al.* (2014) carried out a differential expression analysis among the different developmental stages of a number of transcripts involved in lipid biosynthesis. The *C. finmarchicus* transcriptome provides a great amount of information for gene discovery. In this study at least one third of the transcripts are not expressed in any particular developmental stage. A few transcripts, though, showed pronounced stage-specificity: an acyl transferase expressed in adults and embryos; an ELOV and a Δ9 desaturase in copepodites. All these transcripts have EST support and are consistent with previous findings showing that copepodites stock fatty acids (FA) to be used in wax and the adults and embryos synthesise triacylglycerols (TAGs) suggesting a different lipid metabolism in the different developmental stages. This finding is consistent with Tarrant *et al.* (2014) as well. Voltage-gated sodium channels (Na_V) were also identified using D. melanogaster protein sequence. This protein is the target of saxitoxin poisoning and mutated Na_V proteins can be at the basis of resistant strains. Actually, different transcripts had Na_V identity. Unfortunately, in the transcriptome produced by the same group on *C. finmarchicus* fed with the toxic dinoflagellate *Alexandrium funndiense*, no Na_V genes were differentially expressed (Roncalli *et al.*, 2016). Actually, the entire detoxification machinery did not show any sign of regulation. In this second study, the authors identified cellular stress and homeostatic responses at day two and five, respectively, but no striking detoxification response as expected from comparisons with other organisms exposed to toxic compounds. Overall, this work enabled investigation of the subtle physiological response of the copepods to sub-optimal food conditions, namely the ingestion of a toxic alga, which did not reduce the copepod survival but could potentially lead to lower reproductive potential and population fitness.

Together with a previously produced database (~ 12k ESTs; Lenz *et al.*, 2012), gene model predictions and sequences can be used to design primers for specific genes involved in biological processes or environmental responses. Once an annotated transcriptome or genome becomes publicly available for a species (especially a non-model species), the research on it gets a kick onwards and thus ecologically, biologically and biotechnologically relevant questions are addressed. Moreover, the *C. finmarchicus* transcriptome will be used as reference for new RNA-seq studies, like it has been done in Roncalli *et al.* (2016).

In 2015, the transcriptome response to thermal stress of the temperate *C. finmarchicus* vs. the arctic *Calanus glacialis* was compared using an Ion Torrent RNA-seq experiment (Smolina *et al.*, 2015). Results, validated by RT-qPCR, suggest that *C. finmarchicus* responds to thermal stress activating HSPs, chaperons and proteins involved in ROS detoxification. The Arctic *C. glacialis*, on the contrary, did not show differential expression of transcripts due to the treatment. The conclusion was that the *C. glacialis* lacks the machinery to respond to thermal stress, like other organisms adapted to low temperatures (*e.g.*, Clark *et al.*, 2008). This finding does not indicate that *C. glacialis* is not stressed by temperature. On the contrary, at high temperatures the animals appeared to be dormant. Results suggest that this species is less resilient to temperature variations and hence is more vulnerable to higher temperatures. Global warming would thus endanger *C. glacialis*.

5. METABOLOMICS AND PROTEOMICS

The analyses of either endogenous or exogenous molecules like peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, that are usually below 1.5 KDa is called metabolomics. Typically, these analyses are performed by Nuclear Magnetic Resonance (NMR) or Mass Spectrometry (MS) techniques, coupled with statistical tools such as principal component analysis (PCA) and partial least squares (PLS). Metabolomics is a broad and multidisciplinary field and the description of the different techniques is beyond the aims of the present chapter. A main distinction has to be made between the non-targeted unbiased analysis of large sets of low molecular weight organic metabolites, and the targeted-metabolomic approach. The former complements genomics, transcriptomics and proteomics, and represents the closest link to phenotype; the latter is aimed at measuring defined groups of chemically characterised metabolites. For a detailed description of protocols, we suggest the book by Metz *et al.* (2011) or the review paper by Zhang *et al.* (2012).

Proteomics is a term that was first introduced in the early 90s mirroring the word 'genomics', which identified the investigation of the totality of genes present in a given genome. Actually, the word proteomics identifies a plethora of different analyses like expression proteomics, which highlights differentially expressed proteins in two conditions or samples, protein-protein interactions, proteome fingerprinting, *etc*. Techniques are mainly based on gel separation like Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE), two dimensional PAGE (2D-PAGE), or high performance liquid chromatography (HPLC) or MS.

Here we will give a short overview of recent works in targeted- and non-targeted metabolomics and proteomics on planktonic calanoids. Recently, the effect of ocean acidification (OA) and temperature variations were investigated in two planktonic calanoids, Paracalanus sp. (Garzke *et al.*, 2016) and a nearly 1:1 mixture of *Calanus helgolandicus* and *Calanus finmarchicus* (Mayor *et al.*, 2015). Targeted analysis of FA composition in adult copepods was investigated by means of Gas Chromatography, together with analysis of body size. Results suggest that in the next two centuries, warming and OA would produce a shift in copepod body size and FA composition. This would impair copepod suitability as valuable food for higher trophic levels. Changes in FA compositions in copepods would be driven by variation in the different classes of FA in phytoplankton. Remarkably, docosaexahenoic acid (DHA, 22:6 ω3) would dramatically diminish with increasing temperature and decreasing pH. This, together with the variation of other FAs, might impair the development of copepods as well (Garzke *et al.*, 2016). The reduced productivity and abundance of copepod food due to warming (Behrenfeld *et al.*, 2006; Boyce *et al.*, 2010) would lead to food stress in the future for copepods. This scenario, integrated into the context of global warming, was investigated by means of non-targeted metabolomics based on chromatography/MS techniques (Mayor *et al.*, 2015). Copepodites (CmV) of *Calanus* species were exposed to two temperatures and two pCO_2 conditions and starved for five days. The four treatment groups did not show statistically significant differences after five days of starvation, however results were different when comparing the pre-experimental to the post-experimental animals (t_0 vs t_5). Almost all polar compounds, such as amino acids, were down-regulated at t_5, while saturated and monounsaturated FA were all up-regulated. Only DHA and eicosapentaenoic acid (EPA, 20:5 ω3) were down-regulated (Mayor *et al.*, 2015). Food-deprived animals also showed

significantly higher levels of a new class of taurine-containing lipids, which the authors speculated might act as an active transporter of substrates to a catabolic site. Similar compounds were previously identified in *C. finmarchicus* and *Centropages typicus* (termed as 'copepodamides'), where they played a role as signal molecules modulating toxin production in the dinoflagellate *Alexandrium minutum* (Selander *et al.*, 2015). In conclusion, though copepods seem to be more strongly affected by starvation than by warming and OA, both influence the copepod survival by indirectly changing the phytoplankton lipid composition and productivity (Mayor *et al.*, 2015).

Non-targeted metabolomics studies in copepods are in their infancy. The pioneering environmental toxicometabolomic works by Hansen and co-authors on *C. finmarchicus* (Hansen *et al.*, 2010 and 2017) have clearly showed a relationship between copepod metabolic responses and sub-lethal xenobiotic exposure. As with transcriptomics (section 4.3), metabolomics studies as well deserve further investigation.

The first proteomics study on a planktonic calanoid, *Eurytemora affinis*, including protein sequencing, was published by Boulangé-Lecomte *et al.* (2016). The effect of two pollutants were investigated, namely phenylureic hericide commercially known as diuron (DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea) and a mixture of two alkylphenols, the 4-nonylphenol (4-NP) and the nonylphenol-ethoxy-acetic-acid (NP1EC), widely used in synthetic industry. The two pollutants produced strikingly different results, with DCMU inducing the highest protein variation. DCMU exposure induced a defense mechanism against cell damage which triggered an elevated energy request, as showed by up-regulation of ATP synthase and arginine kinase genes to cope with high ATP demand. Signal transduction and immune response machineries were regulated as well. For the latter, the 14-3-3 zeta protein contributes to oocyte mis-development, as also shown in other systems. Oxidative stress response was induced as well with GSH and superoxide dismutase proteins being up-regulated. In *E. affinis*, as in *C. helgolandicus* fed on a potentially toxic diatom (Lauritano *et al.*, 2011b), HSPs and protein-folding related proteins were up-regulated. Alkalylphenols produced an opposite result with most of the proteins down-regulated. This study, although suffering from the lack of a reference genome, shed light on copepod proteomic response to environmental concentrations of pollutants, but also posed the basis for further development of new biomarkers for water quality assessment.

6. GENOME UP-DATES

In the last few years, two genome projects for calanoids were initiated, the 'Whole genome assembly of common copepod (*Eurytemora affinis*)' as part of the Baylor College of Medicine-Human Genome Sequencing Center (BCM-HGSC) i5k Pilot Project (Accession: PRJNA203087; ID: 203087) and 'Restriction site-Associated DNA sequencing (RAD) tag study of populations of *Centropages typicus*, a marine copepod' (Accession: PRJNA265130; ID: 265130), to detect population genetic structure and connectivity among North Atlantic populations of this species (Blanco-Bercial and Bucklin 2016).

The calanoid copepod *E. affinis* is a euryhaline epibenthic species that can live both on the sediment and in the water column and is able to adapte to wide salinity gradients. For this reason, this species can disperse quite easily from estuarine areas to inland waters. Since *E.*

affinis can be a carrier of important pathogens, this ability has attracted researchers' interest to this species. In 2012 a genome sequence project of this species was started (https://www.hgsc.bcm.edu/arthropods/eurytemora-affinis-genome-project); the ~ 7K assembled contigs are available on the National Center for Biotechnology Information (NCBI) website under the ID 17731 (https://www.ncbi.nlm.nih.gov/genome/17731) where BLAST searches can be performed. A publicly accessible genome browser with automated annotation can be found at https://apollo.nal.usda.gov/euraff/jbrowse. The size of the genome was estimated to 0.6-0.7 pg DNA/cell (~587-685 Mb, assembly reaches ~ 500Mb) with a CG content of 35.8%.

This newly available resource has enabled a very elegant work on chemosensory-related gene families in arthropods, with particular focus on copepods (Eyun *et al.*, 2017). Most interesting was the discovery of gustatory receptors (GRs) in Pancrustacea and that in *E. affinis* (that contains 10 GR genes) they are poorly expressed like in insects. Also, it was demonstrated that Ionotropic Receptors (IRs) were differentially expressed in sexes. This finding could give an indication of their function. Moreover, it was found a gene family duplication of antenna IRs in males that would be a way to over-express these genes during mating.

CONCLUSION

The abovementioned studies on large-scale gene expression profiling, genomics, metabolomics and proteomics performed on planktonic calanoids have significantly improved our understanding of the mechanistic processes underlying fundamental biological and ecological issues in copepods (reproduction, molting, diapause, xenobiotic detoxification, ocean acidificiation and global warming). In the future, it is important that these analytical techniques would be integrated to better predict the species' response to changing environmental conditions, evaluate ecosystem health and environmental risk, and improve food security and nutrition. One of the main constraints limiting the full exploitation of high-throughput copepod transcriptomic studies is the lack of routine protocols for genetic transformation of copepods. These techniques will allow a better understanding of the functions of the newly sequenced genes. Such reverse-genetic tools have been already developed for the model crustacean *Daphina magna*, where exogenous RNA was injected into ovulated eggs to achieve specific gene silencing by RNA interference (RNAi) method (Kato *et al.*, 2011). Posttranscriptional gene silencing by RNAi method have been developed in *Lepeophtheirus salmonis* for identification of molecular targets of new drugs or vaccines for management of aquaculture fish parasites (Eichner *et al.*, 2014), and in *Tigriopus californicus* for testing the role of genes putatively involved in thermal adaptation (Barreto *et al.*, 2014.). In the future, we envision this approach could be also applied to other key calanoid species (*i.e.*, *Calanus*), to better elucidate the role of specific gene functions in copepod physiology and ecology, and also to generate transgenic individuals for biotechnological applications.

In conclusion, functional genomics studies in marine free-living copepods have grown in number since 2011, but the way to go is still very long. Only two genomes are currently available for planktonic calanoids and this slows down functional approaches as well as

forward and reverse genetics for those species that are not yet sequenced. Once genomic resources become available for a wider number of copepods and different molecular tools will be routinely applied, the biological, ecological, ecotoxicological and eventually biotechnological research will take major steps further. Some species can then become models for marine invertebrates.

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Chapter 72

MACROALGAE FOR FUNCTIONAL FEED DEVELOPMENT: APPLICATIONS IN AQUACULTURE, RUMINANT AND SWINE FEED INDUSTRIES

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ABSTRACT

Plant and animal derived products are the main ingredients currently used by the feed industry to produce concentrate feed. There is a need of novel feed ingredients to meet the demand of high quality products by the aquaculture, ruminant and swine production systems, together with the challenge of implementing new sustainable and environmentally friendly processes and ingredients demanded by the modern society. Macroalgae are a large and diverse group of marine organisms that are able to produce a wide range of compounds with unique biological properties. This chapter discusses the incorporation of macroalgae or macroalgal derived ingredients as a source of both macro-nutrients (i.e., proteins, polysaccharides and fatty acids) and micro-nutrients (i.e., minerals and pigments) for animal feed production. The biological health benefits of the macroalgal ingredients beyond basic nutrition for the development of functional feed in the aquaculture, the ruminant and the swine sectors are also discussed together with the industrial challenges of its application.

Keywords: macroalgae and seaweed, nutrition, feed, functional feed, aquaculture, ruminant, swine

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1. INTRODUCTION

The global human population is projected to grow to 9.6 billion individuals by 2050 and will continue to grow up to 12 billion by 2100 (Gerland et al., 2014). Within this scenario an increase of 70% in animal production and thus 235% more animal feed will be needed to sustain that growth (Herman & Schmidt, 2016). The animal feed industry faces the challenge to meet the demand of high quality products by the aquaculture, ruminant and swine production systems, and implementing new sustainable and environmentally friendly processes and ingredients demanded by the modern society. There is a need to find new alternatives to traditional animal feed ingredients that could compete in the market in terms of nutritional quality (i.e., macro and micro-nutrient composition) and environmentally sustainable (Garcia-Vaquero & Hayes, 2016).

Macroalgae are a diverse group of marine organisms with more than 10,000 different species (Collins et al. 2016). Based on their pigments marine macroalgae are classified as brown (Phaeophyta), red (Rhodophyta) and green macroalgae (Chlorophyta). Marine macroalgae are able to adapt to the changing and extreme marine environmental conditions i.e., salinity, temperature, nutrients, radiation and combination of light and oxygen concentration by producing unique secondary metabolites including proteins, polysaccharides, lipids, pigments and minerals (Collins et al., 2016). Macroalgae are known for their richness in the previously described bioactive molecules with wide variety of biological properties i.e., anti-oxidant, anti-bacterial and anti-tumour amongst others (Holdt & Kraan, 2011). Thus, macroalgae represents great potential for its use in human food and animal feed or for the extraction of biologically active compounds that could be incorporated into the animal's diet.

This chapter discusses the incorporation of macroalgae or macroalgal derived ingredients as a source of both macro-nutrients (i.e., proteins, polysaccharides and polyunsaturated fatty acids) and micro-nutrients (i.e., minerals and pigments) for animal feed production. The nutritional and biological health benefits of the incorporation of macroalgal ingredients for the development of functional feed in aquaculture, ruminant and swine nutrition were discussed together with the advances and challenges for macroalgal incorporation in animal feed.

2. MACROALGAE AS SOURCE OF MACRO-NUTRIENTS

Seaweeds have a highly variable composition, with large differences in the final content of both macro- and micro-nutrients depending on multiple factors such as macroalgae species, date of collection and environmental conditions including pollution, water temperature, light intensity and nutrient concentration in water (Mišurcová, 2011).

2.1. Macroalgal Proteins

Macroalgae species and the season of collection are the most common factors affecting both macroalgal protein content and amino acid composition (Joël Fleurence, 1999). The

protein content described in brown macroalgae is generally low in comparison with green (10-26%) and red macroalgae species (35-47%) with protein contents comparable to protein-rich foods such as soybean, cereals, eggs and fish (Garcia-Vaquero & Hayes, 2016). Seasonal variations were appreciated in red macroalgae, with higher protein concentrations in the biomass harvested during the winter season (approximately 22%) when compared to the summer period (~ 12%) (Galland-Irmouli et al., 1999).

In addition, most seaweed species are considered a rich source of essential amino acids (Garcia-Vaquero et al., 2016) and acidic amino acids such as aspartic acid and glutamic acid (Fleurence, 2004). The macroalgae contents of amino acids such as threonine, lysine, tryptophan, cysteine, methionine and histidine in macroalgal proteins are higher than those found in terrestrial plants (Fleurence, 1999).

Together with protein, macroalgae also contains large amounts of non-protein nitrogen (i.e., nitrates), resulting in an overestimation of their protein content when analyzed by traditional laboratory methods. Nitrogen to protein conversion factors of 5.38, 4.92 and 5.13 have been proposed for brown, red and green algae respectively as alternatives to the traditional factor of 6.25 (Makkar et al., 2016).

Table 1. Protein contents (% dry weight) in selected macroalgae described in the literature

Algal species	Protein content (% DW)	Reference
Phaeophyta (Brown macroalgae)		
Laminaria spp.	7	Dawczynski, Schubert, and Jahreis (2007)
	4-8	Schiener, Black, Stanley, and Green (2015)
Sargassum spp.	17	Rodrigues et al. (2015)
	12-14	Yu, Zhu, Jiang, Luo, and Hu (2014)
Undaria pinnatifida	17	Taboada, Millan, and Miguez (2013)
	20	Dawczynski et al. (2007)
Himanthalia elongata	5	Cofrades et al. (2010)
	5	Sánchez-Machado, López-Cervantes, López-Hernández, and Paseiro-Losada (2004)
Rhodophyta (Red macroalgae)		
Porphyra spp.	39	Cofrades et al. (2010)
	33	Taboada et al. (2013)
Gracilaria spp.	19	Rohani-Ghadikolaei, Abdulalian, and Ng (2012)
	10	Tabarsa, Rezaei, Ramezanpour, and Waaland (2012)
Chlorophyta (Green macroalgae)		
Ulva spp.	25	Lee, Chang, and Lee (2014)
	7	Satpati and Pal (2011)
Ulva lactula	11	Tabarsa et al. (2012)
	17	Rohani-Ghadikolaei et al. (2012)

Protein is regarded as the most expensive nutrient in animal feed (Rezaei et al., 2013). Thus, the high protein levels described in macroalgae (see *Table 1*) together with its amino acid profile could suggest its incorporation in animal feed as an alternative source of high quality protein

2.2. Macroalgal Polysaccharides

The total polysaccharide concentrations in macroalgae range from 4% to 76% of dry weight with the highest contents described in *Ascophyllum*, *Porphyra* and *Palmaria* spp., in comparison with green macroalgae (Holdt & Kraan, 2011). Differences in polysaccharide content and composition could be appreciated depending on the macroalgae species, parts of the macroalgae sampled and seasonal variations (Skriptsova et al., 2011; Kim, 2012; Men'shova et al., 2012).

Macroalgae contain a number of complex carbohydrates and polysaccharides in variable amounts depending on the macroalgae species. Brown algae contain alginates, sulphated fucose-containing polymers and laminarin; red macroalgae is a rich source of agars, carrageenans, xylans, sulphated galactans and porphyrans; and green algae contain xylans and sulphated galactans (Makkar et al., 2016).

Marine polysaccharides from seaweeds are an integral part of a globally thriving marine-bio industry. Seaweeds are the most abundant source of polysaccharides including (1) alginate, agar, agarose and carrageenan with commercial applications in the biomedical and pharmaceutical industries (Venkatesan et al., 2015) and (2) laminarin and fucoidan with promising potential in food and animal feed. Fucoidan and laminarin, showed a wide range of biological activities such as anti-inflammatory, anti-microbial, anti-coagulant, anti-adhesive, anti-oxidant, anti-viral, anti-peptic, anti-tumour, anti-apoptosis, anti-proliferative and immunostimulatory, in both *in vitro* and *in vivo* model systems (Hahn et al, 2012; Kadam et al., 2015).

2.3. Macroalgal Fatty Acids

Brown macroalgae typically have the highest total lipid content, followed by green and red macroalgae (Gosch et al., 2012). However, there is considerable variation in total lipid between species and also season. I.e., levels of 68.9 mg/g dry weight (DW) and 57.5 mg/g DW were described in different species of green macroalgae within the Bryopsidales order (Gosch et al., 2012). Also high lipid contents were described in winter and spring in *Ulva lobate*, *Egregia menziesii* and *Chondracanthus canaliculatus* (Nelson et al., 2002).

In recent years, lipid composition in marine algae has raised considerable interest due to their high content of polyunsaturated fatty acids (PUFA). PUFA contents varied from 34% of the total fatty acids content in *Porphyra* spp. to 74% in *Undaria pinnatifida* (Dawczynski et al., 2007). Macroalgal PUFA include α-linolenic (18:3 n-3), octadecatetraenoic (18:4 n-3), arachidonic (20:4 n-6) and eicosapentaenoic acids (20:5 n-3) (Kendel et al., 2015). I.e., *Ulva* spp. is unique in comparison with plant and fish derived oils due to high levels of octadecatetraenoic acid, as well as offering essential dietary eicosapentaenoic and docosahexaenoic acids, which are generally absent in terrestrial plants (McCauley et al., 2016). PUFA are considered essential nutritional components in humans and animals, playing an important role in the prevention of cardiovascular diseases, osteoarthritis and diabetes. Additional beneficial health properties include anti-microbial, anti-viral, anti-inflammatory and anti-tumour properties (Kendel et al., 2015).

Recent studies showed the future potential of the controlled cultivation of *Ulva* spp. biomass to generate an algal-based oil for human and/or animal nutrition, as part of a biorefinery process for high value products (McCauley et al., 2016).

3. MACROALGAE AS SOURCE OF MICRO-NUTRIENTS

3.1. Minerals

The capacity of macroalgae to accumulate metals depends on multiple factors, but it is mainly related to the bioavailability of the metals in the surrounding water, the uptake capacity of the macroalgae (Besada et al., 2009) and the efficient adsorption of metal/organometallic species from seawater (Besada et al., 2009; Romaris-Hortas et al., 2010).

Important essential minerals for human health, such as iodine, copper, selenium and zinc were found in high proportions in macroalgae. *Laminaria* spp. are known to be the best iodine accumulators among all living systems and the accumulation of iodine can be up to 30,000 times larger than in the surrounding environment (Holdt & Kraan, 2011). Copper, selenium and zinc levels described in macroalgae in Norway showed to be safe when applied the macroalgae as food and feed, with higher levels of zinc described in red and brown macroalgae (Duinker et al., 2016). Reported accumulation of heavy metals in seaweed includes arsenic, cadmium, chromium, nickel, vanadium, mercury, lead, cesium-137, and radium-226 (van der Spiege et al., 2013). Large amounts of arsenic in its inorganic form were found in *Sargassum* spp., while the organic arsenic contents ranged from 38 to 75% (Almela et al., 2002). However, in a recent study the health risk due to the toxic elements in seaweed was estimated and the contribution to total element intake of arsenic, cadmium and lead of macroalgae does not appear to pose any threat to the consumers, although the concentrations of these heavy metals should be controlled to protect the consumers' health (Desideri et al., 2016).

3.2. Pigments

There are three different groups of light harvesting and photoprotective pigments in macroalgae named chlorophylls, carotenoids and phycobiliproteins present in different proportions depending on the macroalgae species (Hallerud, 2014).

Chlorophylls are green lipid-soluble photosynthetic pigments found in all macroalgae species, terrestrial plants and cyanobacteria (Holdt & Kraan, 2011). Chlorophyll a is found in all photosynthetic macroalgae, while chlorophyll b and c are found in green and brown macroalgae respectively (Hallerud, 2014). The increasingly restrictive legislation concerning the origin of food preservatives (anti-oxidants and anti-microbials) and the growing demand for natural compounds, has renewed the interest in anti-oxidants such as chlorophylls from natural sources, instead of chemically synthesised molecules (Guedes et al., 2013).

Carotenoids include carotenes and xanthophylls with relative abundance variable depending on the macroalgae species. Green macroalgae species include β-carotene, lutein,

violaxanthin, neoxanthin and zeaxanthin, red macroalgae contain mainly α-and β-carotene, lutein and zeaxanthin and brown macroalgae are a rich source of β-carotene, violaxanthin and fucoxanthin (Holdt & Kraan, 2011). Due to their potential health benefits, the incorporation of carotenoids into functional foods or dietary supplements is a major interest of both consumers and the food industry (Salvia-Trujillo et al., 2013). The beneficial effects of carotenoids are thought to be due to their role as anti-oxidants. Other activities include the pro-vitamin A ability of β-carotene (Holdt & Kraan, 2011), the protective role against eye disease showed by lutein and zeaxanthin (Johnson, 2002) and the promising anti-tumour activities of fucoxanthin (Holdt & Kraan, 2011).

Phycobiliproteins include phycoerythrin, phycocyanin, allophycocyanin, and phycoerythrocyanin. Phycoerythrin levels of 12% DW in *Palmaria palmata* and 0.5% in *Gracilaria tikvahiae* (Fleurence, 2004). Phycobiliproteins showed spontaneous fluorescence, property that is used by the biomedical industries in the development of diagnostic techniques such as fluorescent immunoassays (Harnedy & FitzGerald, 2013). Also, phycobiliproteins showed multiple biological activities i.e., anti-oxidant, anti-inflammatory, anti-viral and anti-tumour (Sekar & Chandramohan, 2008) that could be used in the development of functional foods (Langellotti et al., 2013).

4. MACROALGAE AS FUNCTIONAL FEED

There is an increased interest in the scientific community to discover new functional foods or functional food ingredients. Functional foods were described as foods or dietary components that may provide a health benefit beyond basic nutrition (Wildman et al., 2016). Several functional food ingredients of different chemical nature have been reported to possess anti-oxidant, anti-bacterial, anti-hypertensive, anti-inflammatory and anti-tumour activities (Wildman et al., 2016). Functional foods could help in the prevention or reduce the progression of many chronic diseases, such as cardiovascular disease, cancer and degenerative diseases (Olaiya et al., 2016).

Animal nutrition (i.e., aquaculture, ruminant and swine) has been traditionally evaluated in terms of productive parameters such as animal weight gain or feed utilization (France et al., 2000). Recently, animal nutrition has gained attention as an effective way to produce functional food ingredients with beneficial health effects that could increase the price of animal products in the market (Siró et al., 2008). In example, the use of supplements in cattle (Rey-Crespo et al., 2014; López-Alonso et al., 2016) or swine feeds (Dierick et al., 2009) to increase the contents of essential trace elements in milk or meat.

In animal nutrition, functional feed has not a clear definition to date (see *Figure 1*). Functional feed for pet animals adopt the definition previously described for functional foods, focusing on the additional health benefits that the functional ingredients could provide to the pets such as reduction of risk of obesity, osteoporosis, colon cancer and inflammatory bowel disease (http://www.petfoodindustry.com/articles/2926-advances-in-functional-petfood-ingredients). However, functional feed in production animals such as aquaculture have been modify to incorporate the important economic benefits of the incorporation of the ingredients in animal production. In this sense, functional feeds were described as dietary ingredients that

provide growth, health, environmental and economic benefits beyond traditional feeds (Olmos Soto et al., 2015).

Due to the wide variety and biological properties of the compounds discovered in macroalgae, the incorporation of algal biomass or isolated molecules in functional feed formulation could represents a great opportunity in animal nutrition.

4.1. Aquaculture Nutrition

Aquaculture is the fastest growing sector of the food economy, increasing by more than 10% per year and currently accounts for more than 50% of all shrimp/fish consumed (Olmos Soto et al., 2015). Feeding represents 40-60% of the total production costs in shrimp/fish farming (Olmos Soto et al., 2015) thus, the development of new ingredients or novel feed formulations that could help to reduce the production cost and improved animal health represents a promising field from both scientific and industrial points of view. The use of animal protein sources, such as fish meal in aquaculture feeds is expected to be reduced or completely eliminated as a consequence of increasing economic, environmental and sanitary regulations (Olmos Soto et al., 2015). Macroalgae species with elevated protein content and production rates could be considered as potential novel feed ingredients in aquaculture (Valente et al., 2006).

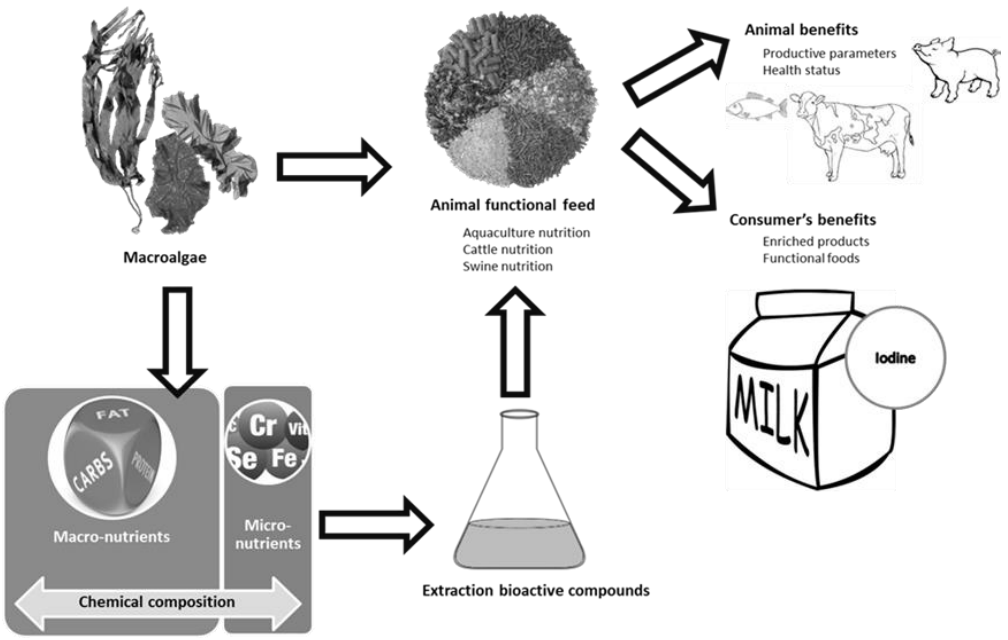

Figure 1. Scheme showing the possibilities for incorporation of macroalgae or macroalgal derived ingredients for the development of functional feeds for animals.

The partial substitution or inclusion of different percentages of macroalgae into the diet of fish showed promising results improving productive parameters in fish (i.e., growth rates), enhancing animal health (i.e., metabolic rates or response to stress) and increasing certain

beneficial compounds in derived animal products (i.e., pigmentation or iodine concentration). *Eucheuma denticulatum* can be efficiently utilized by Japanese flounder juvenile (*Paralichthys olivaceus*) and promote best growth and feed utilization at a level of 3% (Ragaza et al., 2015). The addition of 5% of *Ascophyllum nodosum*, *Porphyra yezoensis* or *Ulva pertusa* to the feed of fingerling red sea bream (*Pagrus major*) increased body weight, feed utilization and muscle protein deposition in comparison with the fish fed a normal diet (Mustafa et al., 1995). *Porphyra dioica* at levels of 10% in rainbow trout's feed showed no negative effects on the growth performance and increased the flesh pigmentation of the fish (Soler-Vila et al., 2009). The inclusion of 5% *Gracilaria* or *Alaria* spp. into the feed of meagre (*Argyrosomus regius*) modulated the metabolic rates and enzymatic responses during a bacterial infection without affecting the growth performance of the fish (Peixoto et al., 2016). Similarly dietary macroalgae supplementation (*Ulva*, *Gracilaria* and *Fucus* spp.) improved the immune and antioxidant responses in European seabass (*Dicentrarchus labrax*) without compromising growth performance of the fish (Peixoto et al., 2016). The inclusion of up to 5% of *Gracilaria vermiculophylla* in diets for rainbow trout (*Oncorhynchus mykiss*) did not affect the growth of the fish. The supplemented fish showed improved flesh quality traits (higher colour intensity and juiciness) and the flesh iodine content on the flesh doubled in comparison with the fish of the control diet (Valente et al., 2015).

Similarly, recent studies used macroalgae in the diet of important aquaculture production systems such as shrimps and molluscs. Commercial feed of marine shrimp (*Litopenaeus vannamei*) could be replaced up to 50% with *Ulva lactuca* as source of protein and lipids without negative effects on the growth performance of shrimps (Pallaoro et al., 2016). The co-culture of juvenile shrimp and green macroalgae *Ulva clathrata* showed increased growth rates, diminished lipids in shrimp carcass and also and higher body carotenoids content in comparison with the animals without co-cultured macroalgae (Cruz-Suárez et al., 2010). In mollusc culture, the culture of macroalgae *Hynea spinella*, *Hynea musciformis* and *Gracilaria cornea* in a biofiltration unit with fishpond waste water effluents was successfully used as feed in juvenile abalone (*Haliotis tuberculata coccinea R.*). The survival and growth rates of juvenile abalone were similar to those raised commercial conditions (Viera et al., 2005).

4.2. Ruminant Nutrition

There is growing interest and evidence of the benefits of using macroalgal biomass in livestock production systems, particularly for ruminants (Machado et al., 2015). The use of extracts from macroalgae *Ascophyllum nodosum* was extensively reported in feed-lot steers (Evans & Critchley, 2014). The potential benefits of the macroalgae extracts include improved carcass characteristics and meat quality (Braden et al., 2007), ruminal organic matter and total tract crude protein digestibility in cattle (Leupp et al., 2005) and color stability and extend beef shelf-life of meat products (Montgomery et al., 2001). Also, the inclusion of 2% of Ascophyllum nodosum extract in feedlot cattle diets showed a reduction in *Escherichia coli* in fecal samples (Braden et al., 2004)

In small ruminants the macroalgae *Laminaria digitata* and *Laminaria hyperborea* biomass could be used as an alternative feed source due to the high organic matter content, digestibility, and rumen dry matter degradability of these macroalgae species (Hansen et al., 2003). The addition of 1% of macroalgal meal to the forage of lambs had no significant

influence on relative growth of body components, but it influenced hot carcass weight (Al-Shorepy et al., 2001). Furthermore, the addition of *Ascophyllum nodosum* extract at 2% in the diet of goats improved the anti-oxidant status of the animals exposed to simulated pre-slaughter stress (Kannan et al., 2007).

The use of seaweed to increase mineral content in animal products is currently of interest, especially in relation to increasing the iodine content of foods (Rey-Crespo et al., 2014; López-Alonso et al., 2016). A mixture of three macroalgal species *Ulva rigida*, *Sargassum muticum* and *Sachorhiza polyschides* at 0.5% of the total daily feed intake in organic dairy cattle significantly improved the animals and milk mineral (mainly iodine and selenium) status (Rey-Crespo et al., 2014; López-Alonso et al., 2016). Similarly, the dietary inclusion of *Ascophyllum nodosum* in dairy cows led to an improvement of the iodine content in milk, and to a modification of its microbiota with a positive effect on milk hygiene and transformation (Chaves Lopez et al., 2016).

4.3. Swine Nutrition

Moderate to high amounts of brown macroalgae in the diet may be detrimental to pigs (Makkar et al., 2016) i.e., the inclusion of *Ascophyllum nodosum* at 10% in pigs diets produced weight loss in the animals after several weeks (Jones et al., 1979). However, the incorporation of macroalgal derived polysaccharides, such as laminarin and fucoidan showed promising results used as prebiotics in pigs diets to modulate the microbiota in the digestive tract and immunomodulating properties in pigs (Sweeney et al., 2012; Walsh et al., 2013). *In vivo* studies incorporating laminarin from brown macroalgae in the diet of pigs showed a down-regulation of the expression of inflammatory cytokines in the colon (Sweeney et al., 2012) and mucin gene expression in the ileum and colon (Ryan et al., 2010; Smith et al., 2010). A down-regulation of pro- and anti-inflammatory cytokines was appreciated in the colon of post-weaning pigs supplemented with laminarin and a reduction in *Enterobacteriaceae* counts was appreciated in the animals supplemented with fucoidan (Walsh et al., 2013).

As in the case of ruminants, the use of macroalgae in pigs feed has been proposed to increase iodine concentration in pig meat that could be beneficial for its consumption by deficient population. The organic iodine found in *Laminaria* or *Ascophyllum* spp. is readily metabolized and stored in the muscle, unlike inorganic iodine (Banoch et al., 2010). The addition of 2% of *Ascophyllum nodosum* to the diet of pigs increased the concentration of iodine by 2.7-6.8 in different animal derived products. The consumption of these iodine enriched products could be one safe strategy to help deficient population (Dierick et al., 2009). Similarly, the inclusion of *Enteromorpha* sp. to the diet of pigs did not affect the growth and productive parameters of the animals in comparison with animals supplemented with inorganic salts of copper and zinc. The meat of the animals supplemented with macroalgae showed higher manganese (49%), but also slight increases in iron (13%), copper (12%) and zinc (4%) when compared to the meat of the piglets supplemented with inorganic salts (Michalak et al., 2015).

5. FUTURE PROSPECTS

Crop cultivation induces a significantly high carbon debt and high water consumption, thus, terrestrial biomass seems not to be sustainable at present due to environmental as well as economic impacts (Jung et al., 2013). Macroalgae could be a good alternative as a novel ingredient in both human and animal feed. Overharvesting of wild macroalgae could lead to negative environmental impacts and problems in the sustainability of the supply in the market. Despite this, growth rates of marine macroalgae far exceed those of terrestrial biomass (Kraan, 2013). Also, widely marketed species such as *Ascophyllum nodosum* have shown quick re-generation and growth after being hand harvested in portion only on the top of the fronds allowing annual harvesting from the same beds (Ugarte et al., 2007).

Mariculture could provide a solution for sustainable supply of macroalgae. Cultivation systems include rope farms, tidal flat farms and floating cultivation systems that are currently used successfully in the production of *Laminaria* spp. (offshore), *Ulva* spp. (tidal flat farm) and *Sargassum* spp. (floating cultivation) (Kraan, 2013). Also, the combination of macroalgal culture with other production animals such as fish and molluscs could be a viable commercial alternative (Burg et al., 2013).

As previously commented, the incorporation of macroalgae biomass in animal feed as source of macro-nutrients (i.e., protein) in substitution of traditional ingredients or as a source of micro-nutrients (i.e., mineral or pigments) could be a viable alternative for the development of both functional animal feed and for the fortification of the derived animal products that could be used in the market of functional foods (see **Figure 1**). Also, the extraction of macroalgal compounds (i.e., polysaccharides) for their incorporation into animal feed has shown promising results as pre-biotics improving animal's health.

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Chapter 73

ENVIRONMENTAL IMPACTS OF SEAWEED CO-CULTURE ON COASTAL FISHERIES

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ABSTRACT

This chapter provides a brief review of seaweed co-culture and its environmental impact on coastal fisheries. First, the current situation and problems facing the coastal fisheries, and the plans to overcome these issues, are discussed. Finally, the positive and negative effects of seaweed culture, role of seaweed co-culture, and the overall environmental impact are addressed.

Although worldwide mariculture production has increased steadily, productivity has tended to decline due to continued aquaculture activity in confined areas and natural disasters, such as, typhoons and outbreaks of red tide. In particular, the nutrient loading from unconsumed feed waste results in the deterioration of the water quality and outbreaks of diseases. To overcome these problems, integrated multi-trophic aquaculture has been suggested. In the integrated culture of seaweed and fish, seaweed plays an important role as both a CO_2 sink and biofilter, greatly reducing the environmental impacts on coastal fisheries. The addition of the integrated culture of lugworms to this system could also have further benefits because of its potential for waste recycling and value as bait, which is expected to contribute towards a more sustainable and productive form of aquaculture. Furthermore, the use of cultured seaweed and their waste has been expanded based on the development of useful microbes, reducing their environmental impact.

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Keywords: coastal fisheries, integrated multi-trophic aquaculture, seaweed co-culture, environmental impact, lugworm

1. COASTAL FISHERIES

1.1. Current Situation and Problems

1.1.1. Global Situation

The growth in global aquaculture has remained strong up to present day. Annual marine aquaculture production has reached 20.1 million tons, and global food fish production has grown almost twelve times in the past three decades (FAO, 2012). By 2020, fish consumption in the developing countries is predicted to increase by 57%, and that in developed countries is predicted to increase by 4% (Delgado et al., 2003). In the developing countries, this estimate is based on the changes in supply and demand for fish protein under rapid population growth, increasing affluence, and urbanization. Total aquaculture production shows an average annual increase of 6-7%, and approximately 90% of this production comes from Asia (Halwart et al., 2007). Although cages were originally used in Asia for holding and transporting fish almost two centuries ago (Pillay and Kutty, 2005), the commercial marine cage culture was pioneered in Norway in the 1970s for salmon farming (Beveridge, 2004). In Asia, the start of cage farming in brackish and inshore waters is relatively recent, and was first adopted in Japan. Cage farming is diverse with a variety of species cultured at different intensities. With no large individual operations, the farming activities form a cluster due to the limited site availability in coastal waters (Halwart et al., 2007).

Apart from traditionally farmed species, such as, amberjacks and snappers, the inshore marine cage farming of groupers and cobia has spread in Southeast Asia. Some cage farming in Asia is still dependent on hatchery stock obtained from wild catch, especially for groupers. China is the largest finfish producer in the region, and its marine fish farming is expected to expand further in line with the rapid economic development (Halwart et al., 2007). Although the future prospects for cage farming appear relatively positive in Asia, it has been suggested that the large-scale, capital-intensive, and vertically-integrated marine cage farming seen in northern Europe and South America are unlikely to occur. Instead, clusters of small cage farming that attain a high efficacy are likely to be seen in the future. Similarly, off-shore cage farming is not expected to become widespread in Asia, due to difficulties in the availability of capital and the hydrography of the surrounding seas. Despite these limitations and constraints, cage farming in Asia will continue to contribute significantly to global aquaculture production, leading the world in total production (Halwart et al., 2007).

Because of the rapid development of aquaculture, fishmeal production cannot sustain the feed demand. In particular, lack of feedstock for fishmeal production has caused a serious problem in aquaculture. This is because the feedstock, such as, sardine, Jack mackerel, and mackerel are also used as food. Therefore, feedstock that is unused at present will have to be newly exploited to balance the demand for aquaculture feed. Due to the imbalance in supply and demand for marine products, increases in the price of marine products often occur through inflation.

1.1.2. Domestic Situation

Fisheries production in Korea has remained at 3,180-3,330 thousand tons since 2010 (Statistics Korea, 2015). Among these fisheries, the production from shallow-sea cultures has steadily increased and reached 1,662 thousand tons in 2015. However, the domestic production of fish aquaculture reached a maximum in 2009 and has decreased since then. The total number of cultured fish was approximately 353,293,000 in 2015. One of the reasons for this trend is that the increase in the production of fish, such as, flounder and rockfish, resulted in a sudden fall in fish price and, as a result, there was intervention in production at aquaculture farms.

In 2014, the total number of households managing fish aquaculture was 1,793, which was 4.0% lower than that in 2013 (Statistics Korea, 2015). This was because of the merging of low competition, small-scale fish aquaculture farms, and the shutdown of others because of red-tide damage. Recently, mass damage of fish culture farms by high water temperature has also occurred due to global climate change. Other factors causing lower production are long-term sequential culture, genetic recessiveness, and high-density culture (iPET, 2010). Particularly in cage-culture farms, self-pollution resulting from feeding is unavoidable. Furthermore, most culture farm sites located in Korea are concentrated in gently-flowing bays that are vulnerable to pollution. Therefore, lower fishery production is also caused by environmental problems (Halwart et al., 2007).

The multiple uses of coastal waters restrict further development of marine fish aquaculture, and local cage-culture industries have some difficulty in maintaining the initial production levels (Halwart et al., 2007). Another issue in domestic aquaculture is the difficulty of aquaculture farm succession, as the current population of farmers and managers ages. Furthermore, security of fishmeal as aquaculture feed in Korea is mainly dependent on imports. Recently, the imbalance in the supply and demand of fishmeal has increased due to the increase in demand by latecomers, including China, into fish farming. Therefore, it is expected that there will be difficulty in securing a stable supply of fishmeal.

1.1.3. Problems Facing Coastal Fisheries

The development of marine aquaculture has caused some negative impacts, such as, the breakdown of marine habitats, introduction of non-native species, eutrophication, and disease outbreaks. The balance between the risk of marine aquaculture and its benefits has to therefore be considered (Price and Beck-Stimpert, 2014). Another potential impact on aquaculture production is the change in climate caused by global warming, which often results in environmental disasters (De Silva and Soto, 2009). Furthermore, self-pollution from marine net cages is a serious problem confronted by mariculture, clearly showing the importance of environmental management of marine aquaculture (Halwart et al., 2007). The following is a description of factors that are causing problems in coastal fisheries.

1.1.3.1. Environmental Impact

Marine cage culture operations can have an ecological impact on the marine environment and, therefore, on sensitive habitats and biodiversity. Typically, nutrients (mainly C, N, and P) are discharged as excess feed remains or as fish waste, resulting in a series of chemical and biological reactions. In nature, organic waste settling on the ocean floor is consumed by bottom feeding animals or degraded by microbes. Organisms in the sediment take up the

nutrients, and promote the diversity of plants and animals. However, if the accumulation of organic waste becomes excessive, the chemical and biological reactions shift to an anaerobic state with little or no available oxygen. Once this phenomenon occurs, it can lead to a decrease in the diversity of the benthic community. Appropriate farm operations in well-flushed areas can help minimize nutrient accumulation, causing the least impact on the benthic community (Price and Beck-Stimpert, 2014). As a result, understanding potential site-specific ecological impacts is necessary prior to initiating farm operations.

1.1.3.2. Diseases

The continued intensification of culture practices has caused an increase in disease outbreaks in marine cage farming (Bondad-Reantaso et al., 2002). Diseases are caused principally by environmental and management effects, nutritional issues, and viral, bacterial, parasitic, and fungal pathogens (Arthur and Ogawa, 1996). The associated viruses include nodavirus, iridoviruses, lymphocystis virus, herpes virus, astro-like virus, and reovirus (Bondad-Reantaso et al., 2002). There is much concern about the increase in the intensification and clustering of marine cage farming in restricted areas. These types of culture practices may lead to outbreaks of major epizootics. In addition, there is a high possibility of trans-boundary movement of broodstock, fry, and fingerlings across cage-farming regions, especially in Asia. However, little attention has been paid to this problem. When such movement occurs, exotic diseases, pests, and invasive alien species can be spread extensively with potential impacts on biodiversity (Halwart et al., 2007).

1.1.3.3. Fish Production

The production of some fish species in cage-farming systems is dependent on the capture of wild juveniles because of the limited availability of hatchery-produced fry and fingerlings of target fish species (Halwart et al., 2007). If sufficient seed fish under high-level hatching skill is secured, this can lead to overproduction exceeding the demand (FAO, 2006; Merican, 2006; Ottolenghi et al., 2004; Rimmer, 2006). Another negative effects on cage farming are the continuous increase in working expenses, such as, feed, oil, and seed, and concerns about the hesitation of fish consumption according to an advance in fish price. The dependency of cage farming systems on feed has increased because feedstock, such as, fishmeal, fish oil, and low-value ‘trash fish’ species, are not sufficiently available as feed (Halwart et al., 2007). If fish feed is not sufficient, it leads to low production that cannot meet the consumer demands. As a substitute for raw fish feed, formulated feed mixture has been developed to provide a stable supply.

1.1.3.4. Social Concerns

Community concerns about the use of shared coastal water bodies for cage farming systems may increase due to problems relating to pollution and the possible displacement by other uses, resulting in the need for consultation with all stakeholders (FAO, 2006). Furthermore, the long-term environmental and ecological sustainability of rearing fish in cage-based farming systems is also of public concern (Goodland, 1997). Therefore, there is an increased need for governmental control of the development of the farming sector under environmental monitoring and implementation of good on-farm management practice (Alston et al., 2006; Boyd, 2005; FAO, 2006).

1.1.3.5. Geographical Effects

The size and strength of cages, and how they respond under in-situ conditions have an effect on fish production in cage-based farming systems (Halwart et al., 2007). For example, when large and robust cages, such as those of Norwegian design, were introduced in Langkawi Island, Malaysia, they were less successful because they were unable to function at full capacity due to the lack of support facilities for such large cages. The regions that have seasonal severe typhoons are not geographically appropriate for cage-farming. Locations, such as the South China Sea, are relatively shallow, and have strong surface and bottom currents but less wave height. Under these geographical features, open-ocean cages must be built to reduce drag rather than to withstand the wave height (Halwart et al., 2007).

1.2. Development Schemes

1.2.1. Existing Schemes

1.2.1.1. Environmental Monitoring

Sediment monitoring protocols should include the early detection of excessive nutrient loads, anoxia, and heavy metals, making the implementation of adaptive management possible. Prior to restocking, site planning should be established that includes cage rotation in the event that there is a measurable impact on the sediment. This practice usually takes less than two years, and can provide chemical and biological recovery (Price and Beck-Stimpert, 2014). A way to reduce metabolic waste is the use of formulated feed under good feed management practice. Today some farms install underwater cameras to reduce nutrient discharge to the water column by monitoring feeding and fish activity (Price and Beck-Stimpert, 2014).

1.2.1.2. Feed Management

The use of formulated feed under good feed management practice can help reduce the amount of excess feed discharge. This results in a reduction in the release of heavy metals into the environment because they are less soluble in water compared with fish feed (Price and Beck-Stimpert, 2014). To date, raw fish feed has been used more. In Asian aquaculture, the total amount of trash fish used as feed for marine cage farming was estimated at approximately 53,301 kilotons in 2010 (FAO, 2014). The main reasons for the continued use of trash fish as feed for cage farming are: the farmer's perception of better performance by trash fish, low price of trash fish compared with formulated feed, the ease of availability, the shortage of suitable formulated feed for all life cycle stages of culture species, and social restraints with the existing livelihood strategies of many coastal fish farmers compared with more organized lot farming (Halwart et al., 2007). However, farmers should be encouraged to use formulated feed to remove the stress of negative environmental impacts by the use of raw fish feed. To reduce the nutrient load on the sediment, high energy feeds with high digestibility should be formulated and used (Halwart et al., 2007).

1.2.1.3. Limitations

Marine cage farms must be placed away from sensitive ecosystems including corals, seagrass, and mangrove habitats (Price and Beck-Stimpert, 2014). In some countries, liquid fisheries waste is disposed of through the municipal sewage system or discharged directly into a waterbody. The receiving waterbody must be able to degrade the biological and chemical constituents present in the waste to have no detrimental effect on the aquatic fauna and flora (Naidoo and Olaniran, 2014; UNICEF and WHO, 2012). Furthermore, the damage on submerged habitats by farm installation must be considered, because they may be sensitive to shading from net pens, cages, and other moored structures. In some cases, many surrounding species of marine organisms can be influenced by the slow leaching of toxicants from copper-based antifoulant coating on nets or cages. Furthermore, the use of chemicals to remove fouling should be avoided. Instead, mechanical scrubbing by divers, or lifting cages or nets out of the water to desiccate fouling organisms is recommended (Price and Beck-Stimpert, 2014). Onsite cleaning may cause an increase in the organic load on the benthos.

1.2.1.4. Farming Site Management

Reducing waste and debris can contribute to the maintenance of water quality at cage farming sites. The timely removal of dead fish will avoid fouling of the water column, impede proliferation of predators, and prevent the spread of disease. During harvest and slaughter, waste can be reduced through careful treatment (Price and Beck-Stimpert, 2014). To prevent disease outbreak, enhanced means for disease resistance or vaccines must be developed (Halwart et al., 2007).

1.2.2. Integrated Multitrophic Aquaculture

The use of integrated multitrophic aquaculture (IMTA) can reduce the impact of nutrient loading on water quality. IMTA is the integrated culture of low-level trophic organisms (shellfish and seaweed) and high-level trophic organisms (fish and prawn). For example, it is common practice in finfish culture to combine production with shellfish (mussels and oysters) and seaweed that filter waste particulates. Lobsters and sea urchins have also been used with some success (Price and Beck-Stimpert, 2014). Therefore, this approach has some potential to mitigate environmental impacts and it simultaneously expands the economic base of the farming operation. As a result, IMTA is an experimentally sustainable marine aquaculture, and in some cases can obtain social acceptance (Price and Beck-Stimpert, 2014). In Figure 1, the simple IMTA model is shown where fish, as the high-level trophic organism, and seaweed, as low-level trophic organism, are cultured together.

1.2.3. Use of Lugworm

Global legal controls exist to protect the marine environment, and this consecutive effort has made aquaculture farms environmentally-friendly. Polychaete worms have the potential to mitigate environmental impact (Brown et al., 2011). The *Nereis* species can efficiently use solid waste (unconsumed feed and fecal material) collected from a marine recirculating system, and is an excellent candidate for integrated aquaculture and waste recycling (Bischoff et al., 2009; García-Alonso et al., 2008; Honda and Kikuchi, 2002). The use of aquatic worms also has great potential in bioremediation of waste deposited on net pens or in fish ponds (Kinoshita et al., 2008; Riise and Roos, 1997). Therefore, their role in the decomposition and

mineralization of organics can be critical for recovery in impacted coastal aquaculture sites (Heilskov et al., 2006). Furthermore, their availability at these sites can be used as an indicator of the level of environmental impact by coastal aquaculture operations (Tomasseti and Porrello, 2005).

Lugworm is an important marine resource because of its role in water purification and simultaneous commercial value, mainly as bait (Cho, 2011). The market price of lugworm is twice as much as the prime cost from its culture (Jung, 2014). Therefore, the benefits of lugworm can be applied to maximize the potential of IMTA in terms of productivity and to reduce the level of nutrient loading release into the marine environment. The IMTA models primarily represent co-culture of fish with invertebrates, such as, sea cucumbers and sea urchins. This IMTA model can be modified by considering the main parameters to simulate the growth and nutrient uptake of different species under various environments. Lugworms settle beneath the farm site and perform effectively in recycling the larger organic particles that are produced from the other (feed or non-feed) components in the IMTA system (Aquaculture in Canada, 2013).

2. SEAWEEDS

2.1. Diversity

Seaweeds are a group of photosynthetic plant-like organisms, or macroalgae, that are macroscopic and multicellular. They are classified into three major groups based on their dominant pigmentation: red (*Rhodophyta*), brown (*Phaeophyta*), and green (*Chlorophyta*). Seaweeds have been traditionally used as food in East Asia. For this reason, about 33 genera of seaweed, mainly red and brown, are commercially harvested and cultivated (McHugh, 2003). Furthermore, close to 500 species in about 100 genera are used locally (Mouritsen, 2013).

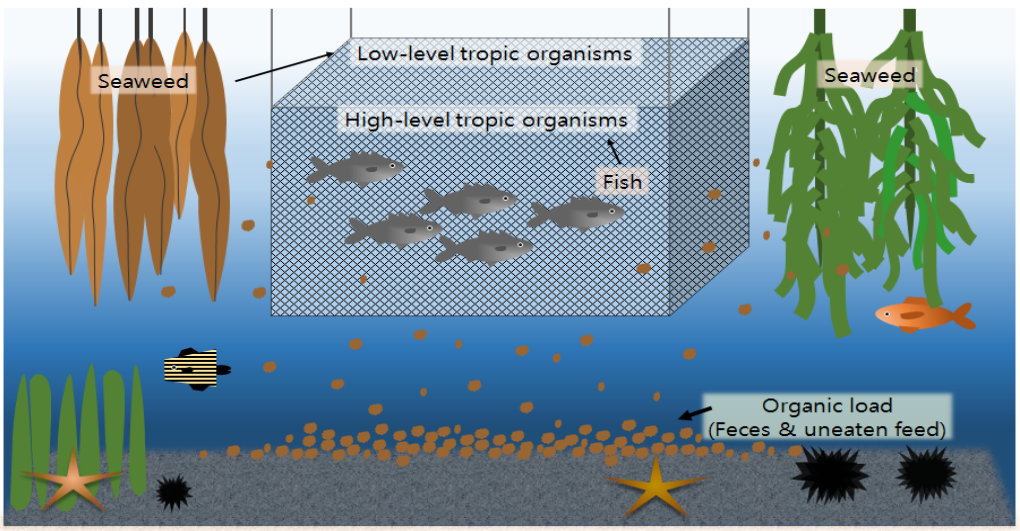

Figure 1. Simple IMTA model showing co-culture of fish and seaweed.

The representative red seaweed include *Porphyra tenera*, *Gelidium amansii*, and *Gracilaria verrucosa*, and their carbohydrate content based on dry weight ranges between 40-75% (Suo et al., 1986). Brown seaweed, including *Undaria pinnatifida*, *Laminaria japonica*, *Sargassum fulvellum*, and *Hizikia fusiforme*, contain about 36-60% carbohydrate, while green seaweed, including *Enteromorpha compressa*, *Ulva lactuca*, *Monostroma nitidum*, *Codium fragile*, and *Capsosiphon fulvescens*, contain about 41-53% carbohydrate. The main carbohydrates contained in each seaweed are different: agar, carrageenan, cellulose, mannan and xylan in red seaweed; alginate, laminaran, fucoidan, cellulose, and mannitol in brown seaweed; and cellulose, mannose, xylan, and starch in green seaweed. The content based on dry weight of proteins and lipids are 2-39% and 0-2% (red seaweed), 6-20% and 1-3% (brown seaweed), and 17-23% and 0-1% (green seaweed), respectively.

2.1.1. Current State in Korea

Brown and red seaweed are a good match for the oceanic climate of Korea. The seaweed culture sites in Korea are mainly located on the western side of the south coast, accounting for almost 90% of total seaweed cultivation. The total production of seaweed in 2015 was 1,105,498 metric tons at a value of USD 421,754,469 (Ministry of Oceans and Fisheries, 2015). More specifically, the production of *Pyropia*/*Porphyra*, *Saccharina*/*Laminaria*, *Undaria*, and others were 419,024; 372,311; 283,714; and 30,449 metric tons at a value of USD 269,447,654; USD 67,515,494; USD 58,614,306; and USD 25,177,014, respectively. The production of *Pyropia*/*Porphyra* has increased since the 1980s due to artificial seeding, development of the float culture system, transplantation of new species, and expansion of the culture sites. Its high market price is the result of continuous consumer demand for high-quality products (NOAA, 2015).

2.1.2. Domestic Uses

Koreans have traditionally used raw or dried seaweed for food, and industries have manufactured diverse seaweed products since the 1980s. Recently, fast food-type and diversely packed products have also been developed. Dried laver is the most valuable marine product obtained from a single fishery. Another important product of processed seaweed is salted *Undaria* that is manufactured mainly on the southwest coast of Korea. The annual production of *Undaria* has increased steadily due to the increase in the demand for healthy food (NOAA, 2015). At present, about 80% of the total seaweed production is directly consumed by humans. The remaining 20% is used as a source of the phycocolloids used in food, industrial, cosmetic, and medical industries (Browdy et al., 2012; Critchley et al., 2006; Lahaye, 2001; McHugh, 2003; Mouritsen, 2013; Ohno and Critchley, 1993), and as an animal feed additive, fertilizer, water purifier, and probiotic in aquaculture (Abreu et al., 2011; Chopin, 2012; Chopin et al, 2001; Chopin et al, 2012; Fleurence et al., 2012; Kim et al., 2014; Neori et al., 2004; Pereira and Yarish, 2008; Pereira and Yarish, 2010; Rose et al., 2010).

2.2. Seaweed Culture

Seaweed culture is practiced using various culture methods and each method interacts in some way with the environment (Phillips, 1990). Therefore, the nature of the interaction and

environmental impact are dependent on the culture method, surface area of the farm, and farming site. Seaweed culture is significantly influenced by environmental factors, including, turbidity, levels of organics and heavy metals, phytoplankton blooms, and temperature and salinity fluctuations (Trono, 1986).

2.2.1. Positive Effects

2.2.1.1. Physical Aspects

There is some potential for large-scale seaweed farms to increase sedimentation of organic matter and to influence coastal water movement, demonstrated by the large areas covered by the *Laminaria japonica* culture in China (Phillips, 1990). Seaweed farms can protect coastal areas from erosion. The large-scale seaweed farms may also provide shelter for other sensitive culture species and systems. For example, mussel or scallop culture species and systems have been protected under *Laminaria japonica* culture zones in China. Furthermore, facilities installed in seaweed culture, such as, rafts, ropes, and anchors, may enhance the production of other marine organisms due to an increase in the substrate surface area. Seaweed culture may also be effectively used to rehabilitate degraded coastal areas.

2.2.1.2. Ecological Aspects

Seaweed culture is an extensive system that depends mainly on a natural nutrient supply. Therefore, seaweed culture has the potential to deplete nutrients present in coastal waters. Lower nutrient levels have been detected in *Laminaria japonica* culture areas (UNDP/FAO, 1989), implying a good correlation between nutrient concentrations and seaweed production (Chung, 1986). The use of supplementary feed in intensive aquaculture systems causes an increase in nutrient levels, and it has a positive effect on seaweed production (NCC, 1989). In some areas placed in a nutrient-depleted state, fertilization is needed for seaweed growth (UNDP/FAO, 1989). The benthic area under seaweed cultures has been used for production of other aquatic animals, such as, abalone and sea cucumber, therefore making the best use of the allotted area (Phillips, 1990). Facilities for seaweed culture also have a significant influence on the productivity of coastal invertebrate and vertebrate populations due to the increased availability of shelter and food (Suo et al., 1986). Furthermore, seaweed culture has been grown in abandoned shrimp ponds, thus making use of wasted resources (Phillips, 1990).

2.2.2. Negative Effects

2.2.2.1. Physical Aspects

The main physical impact of seaweed culture systems is likely the large surface area required. Other factors include site preparation, routine management, and culture facilities. For some species, site preparation involves the removal of rocks or other obstructions, including competitive grasses and predators (Juanich, 1988). Such operations may cause some damage to coastal ecosystems, and in some cases, the loss of species of conservation interest, such as, seagrasses (Pullin, 1989). The routine management of seaweed culture in shallow waters may also cause damage through trampling and accidents. Some areas of seaweed culture are shaded by the culture facilities, and this physical shading may result in

changes to the benthic communities and primary production in the water column. Although the influence of seaweed culture on benthic communities has not been studied extensively, a shading problem in large-scale seaweed farming can potentially reduce benthic productivity, especially in shallow inshore areas.

2.2.2.2. Ecological Aspects

Extensive aquaculture as a result of overstocking causes an eventual decrease in aquaculture production because the 'carrying capacity' of the environment has been exceeded (Beveridge, 1984). In some locations, over-production has resulted in outbreaks of disease (ICES, 1989), which may be linked to nutrient decline (UNDP/FAO, 1989). To overcome these problems, the balance between the ecological requirements for seaweed culture and the carrying capacity of the environment must be carefully considered. Another concern that has arisen in intensive and semi-intensive aquaculture is the potential impact of chemicals used for the control of disease, predators, and fouling organisms (Santelices and Doty, 1989; North, 1987). Furthermore, the attractive characteristics of seaweed can foster prey-predator interaction between the seaweed, invertebrates, and finfish (North, 1987).

2.3. Seaweed Co-Culture

Seaweed production accounts for approximately 35% of the global mariculture production, but its harvest value represents only 7% of the total value (FAO, 2010). To increase space efficiency and yields, co-culture with alternative or valuable aquaculture species in existing seaweed-culture areas may be necessary. Therefore, co-culture is more efficient than monoculture, especially in limited coastal areas (Beltran-Gutierrez, 2016). Fish cage culture relies on external food supplies, which results in a negative impact on water quality (Fang et al., 2016). Conversely, seaweed culture can reduce nutrient loading from fish aquaculture. Therefore, co-culture of seaweed with other species could provide more profit, and concurrently have ecological benefits (Fang et al., 2016).

2.3.1. Role of IMTA

The implementation of IMTA can be beneficial to an ecosystem. Fish cage culture always produces unconsumed feed waste. This causes nutrient loading on the seabed and results in the release of CO_2 as a greenhouse gas. In this situation, co-culture of seaweed with fish can turn the system into a CO_2 sink through photosynthesis by uptake of nutrients (Tang et al., 2011). In some IMTA systems, co-culture of seaweed with abalones, clams, and sea cucumbers is possible (Tang et al., 2013): seaweeds and phytoplankton proliferate by the uptake of CO_2 and NH_4 from the nutrients present in water. Abalones and clams then grow feeding on the seaweed and phytoplankton, respectively, and produce some detritus. Sea cucumbers finally consume these detritus for their growth. These co-cultured organisms at different levels of the food chain simulate the functioning of natural ecosystems, and this type of a balanced system with recycling nutrients would ultimately provide healthier waters (Aquaculture in Canada, 2013).

Seaweed, including kelp, is able to extract dissolved inorganic nutrients, such as, nitrogen and phosphorus, helping reduce levels of dissolved inorganic nutrients generated in the IMTA

system. Therefore, seaweed plays an important role as a bio-filter (Hayash et al., 2008; Neori et al., 2007). To date, *Sacchaarina japonica*, *Gelidium amansii*, and *Codium fragile* can effectively use the nutrients in an IMTA system (Kim et al., 2014). Co-culture of seaweed *Ulva* sp. with Atlantic salmon (*Salmo salar*) and sea urchin (*Paracentrotus lividus*) has also been shown in another IMTA model that considered the environmental conditions on the west coast of Scotland (Lamprianidou et al., 2015). The model simulated the growth, uptake, and release of nitrogen by these organisms to study the nitrogen bioremediation potential of low-level trophic organisms (Lamprianidou et al., 2015). Overall, seaweeds as primary coastal producers can provide nurseries, habitats, and food for aquatic fauna (Ohno, 1993; Watanuki and Yamamoto, 1990).

2.3.2. Association with Lugworm

As a candidate for integrated aquaculture and waste recycling, lugworms can be affiliated with the IMTA system (Bischoff et al., 2009; García-Alonso et al., 2008; Honda and Kikuchi, 2002). They settle beneath the farm site and play an important role to effectively reduce the amount of organic particles produced from the other components in the IMTA system (Aquaculture in Canada, 2013). Therefore, their role is important to remedy impacted coastal aquaculture sites (Heilskov et al., 2006). As a result, they contribute towards a more sustainable and productive form of aquaculture (Aquaculture in Canada, 2013).

For the practical use of lugworms in the IMTA system, at least 2-month old, grown, juvenile lugworms must be used because of high mortality during the larval stage. The normal feed for worms is decaying organic material, such as, seaweed, microalgae, dead animals, and some bacteria (Alyakrinskaya, 2003). In lugworm culture, the early larval stage was fed with marine *Chlorella* and green laver powders, and then feed for adult breeding was fed after the juvenile stage in which the grown larvae infiltrated into sediment (Choi, 2006). Seaweed can then be used as a supplementary feed for lugworms (Harris, 2010). The lugworm species, *Tubifex*, was cultured using a biofloc system to enhance their survival and growth, because the biofloc system could provide good water-quality by the decomposition of waste loading, including ammonia (Wahyuni et al., 2016).

2.4. Schemes for Diverse Uses

Seaweed is cultivated on the waste resulting from fish culture in IMTA systems, while they are used as ingredients for fish feed, such as, immune-stimulating, feces-binding, and fishmeal-replacing additives (Palstra et al., 2015). The reuse of the seaweed waste generated during their processing has recently gained interest, as the number of people seeking seaweed as a health food increases. Therefore, the security of useful microorganisms for the reuse of seaweed waste is important to reduce the environmental impact of the waste.

2.4.1. Acquisition of Useful Microbes from the Lugworm viscera

Benthos inhabiting in intertidal zones have been used in the removal of organics and in water purification (Campos et al., 2002; Davidson et al., 2008; Palacios and Timmons, 2001; Vigneswaran et al., 1999). In particular, lugworms, including the Perinereis sp., have been used in shrimp culture farms (Fujioka et al., 2007). Suspension feeders, including lugworms,

have been studied extensively to understand the nutritional function of suspended bacteria (Gili and Coma, 1998; Orejas et al., 2000; Prieur et al., 1990). Lugworm Sabella spallanzanii was able to efficiently accumulate and enrich bacteria from the surroundings (Stabili et al., 2006). This is because the lugworm's viscera can provide an optimum environment for microbial growth (Kathrin et al., 1997). In earthworm viscera, Brevibacillus agri, Bacillus cereus, Bacillus licheniformis, Bacillus licheniformis, and Brevibacillus parabrevis were found to be indigenous microbes showing proteolytic and lipolytic activities (Kim et al., 2010). The Bacillus species found in lugworm viscera have also synthesized various enzymes, including protease, amylase, and cellulase to degrade diverse organics (Ziaei-Nejad et al., 2006; Verschuere et al., 2000; Ghosh et al., 2002). Furthermore, some strains isolated from lugworms showed antimicrobial activity, indicating that they could be used as a potential source to research bioactive molecules, drugs, and antifouling compounds (Shankar et al., 2015). Among the bacteria, Bacillus species found in the marine environment are used as probiotics, because they are able to stimulate the alimentary and immune systems (Gatesoupe, 1999; Ziaei-Nejad et al., 2006). B. subtilis is used as live feed by many organisms (Ziaei-Nejad et al., 2006; Verschuere et al., 2000), and some Bacillus species have been suggested as alternative antibiotics in shrimp culture farms (Phillips, 1990). In addition, B. subtilis (Vaseeharan and Ramasamy, 2003), B. licheniformis (Arena et al., 2006), and B. pumilus (Ghosh et al., 2002; Sugita et al., 1998) are able to produce antibiotics, antiviralagent, and digestion-related enzymes, respectively.

2.4.2. Biodegradation of Seaweeds

As a favorite food, various types of seaweed products are sold in Asian countries, in particular. The increase in seaweed consumption results in large generation of seaweed waste. Seaweed waste is composed of carbohydrates, proteins, and lipids (NFRDI, 2009). Through biodegradation, these components can be converted to useful compounds, such as, bioactive substances. To date, many studies have been performed on diverse microbial strains: cellulolytic *Acremonium strictum* (Goldbeck et al., 2013), alginate- and laminarin-degrading *Microbacterium oxydans* (Kim et al., 2013), agar- and carrageen-degrading *Bacillus alcalophilus* (Kang and Kim, 2014), proteolytic *Bacillus pseudofirmus* (Raval et al., 2014), and lipolytic *Aneurinibacillus thermoaerophilus* HZ (Masomian et al., 2013). The biodegraded seaweed waste can also be reused as liquid fertilizer by mixed microbes (Kim et al., 2007; Kim et al, 2010). As useful resources, they can often be applied as fertilizer, fungicides, herbicides, and phycocolloids, such as, alginate, carrageenan, and agar.

2.4.3. Others

Seaweed can be a sink for anthropogenic CO_2 emissions via photosynthesis in the coastal waters of some countries. Therefore, CO_2 removal belts formed by the seaweed culture in coastal waters are expected to be developed together with sustainable seafood production. Furthermore, seaweed is a good resource for the production of reducing sugars, and studies on the use of the produced biomass for biofuel are currently in progress. Seaweed culture can therefore contribute, to some extent, in meeting the global food, feed, and pharmaceutical requirements (Israel et al., 2010).

CONCLUSION

The development of marine aquaculture has caused some negative impacts, such as, the breakdown of marine habitats, self-pollution, disease outbreaks, and environmental disasters, clearly requiring environmental management. To reduce the water-quality impact of nutrient loads sinking to the seabed, an IMTA system has been suggested. Seaweed culture is considered to be an indispensable component of this system because of its role as both CO_2 sink and biofilter. In addition, the introduction of lugworm culture into the established IMTA system is expected to result in more sustainable and productive form of aquaculture. To reduce the environmental impact caused by the seaweed waste, the diversity of microbes associated with lugworms has been studied. This effort for the development of useful microbes has resulted in diverse uses of cultured seaweed and their waste.

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Chapter 74

SUSTAINABLE PRODUCTION OF SEAWEED IN MALAYSIA: A REVIEW OF POLICIES AND FUTURE PROSPECTS

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ABSTRACT

Malaysia has the potential to be a key seaweed production player in the region if proper management and possible interventions take place. The seaweed industry in Malaysia has its own historical background as the first seaweed cultivation activity took place in 1978 in Sabah, East Malaysia. The importance of the seaweed industry has been realised when seaweed was found as an important commodity to be exported to various countries. This brings economic benefits to the country and the development of human resource in this sector. The rural people play an important role in the human resource aspect that produce and cultivate the seaweed within their respective areas. In Malaysia, Sabah is the main state for seaweed cultivation activity because of its natural abundance resources and good climate. Department of Fisheries (DOF) Sabah indicated that seaweed production in Sabah has been increased every year amounting to 32 percent from 2008-2012. This is a good opportunity for seaweed production in Sabah where it has the potential to be developed on a bigger scale. The government of Malaysia has introduced seaweed transformation initiatives through Mini Estate System (MES) and Cluster System (CS) in year 2012 in order to enhance the seaweed industry in adhering to the scientific approach and management. These capacity building programmes' objective is to transform the conventional seaweed cultivation techniques to modern seaweed cultivation techniques by applying scientific approach. In terms of community livelihood activity, the MES and CS systems provide the seaweed cultivators with knowledge and skills in order to transform their conventional seaweed cultivation techniques to new technological approach. This MES and CS systems are still new in terms of practical

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implications. Therefore, a study to identify the effectiveness of these programmes is needed. Thus, this study is only focused on the development of both programmes by using available secondary data sources and most importantly through the documentation of these programmes. It will benefit the future researchers that are interested in this seaweed studies in Malaysia as well as in other developing countries.

Keywords: seaweed industry, government-led programmes, development, Sabah

1. INTRODUCTION

It is evident that seaweed industry has brought significant economic revenues to the country as well as improves the standard of living of the coastal communities, for instance, the case of Zanzibar (Msuya, 2006). Seaweed has been considered as important commodity which brings economic benefits to the developing countries that produce seaweed on a large scale such as Indonesia, Malaysia and Philippines. In Malaysia, seaweed industry has been paid attention by the government with intention that it could be able to increase the country revenue through exports. Despite focus on economic benefit alone, seaweed cultivation also has potential to provide side incomes to the fishers those reside in coastal areas of Sabah, Malaysia (Hussin, Yasir, Kunjuraman & Hossin, 2015). Realising on the potential of seaweed industry, the government of Malaysia has been introduced with some possible interventions to scaling up the seaweed industry to ensure the benefits to be shared by all stakeholders. Sabah is the only state in Malaysia that is commercially producing seaweed with focus in four districts on large scale such as Semporna, Lahad Datu, Kudat and Kunak. The coral triangle are of Philippines, Indonesia and Brunei are the best locations and most appropriate for seaweed farming. With this speciality, seaweed cultivation activities are mainly being focused in Sabah and few small scale in west Malaysia.

However, the seaweed industry in Malaysia needs a transformation in terms of scientific management approach in order to achieve the country's aim. The transformation we meant was the conventional cultivation approach to scientific cultivation approach which considered reliable and efficient for this new era. Since 1970s when seaweed was introduced in Sabah, the conventional approach played a role in its growth and being practised by coastal communities in Sabah. Since then, there were no any initiatives and good methods introduced by any parties in Malaysia. Therefore, this is the right time to consider a new scientific approach in seaweed cultivation activities. There was an argument claimed by Yasir and Ali@Ally (2012) that conventional seaweed cultivation activities brought less productivity and low quality as well as over-reliant towards labour intensive based industry. Thus, the authors urged that new scientific approach in seaweed cultivation is vital in order to boost the growth and production of seaweed which could benefit the country as well as local community. Therefore, several questions arise. First, what is the new scientific approach which could be considered good for seaweed cultivation compared to the conventional methods? Who are the parties involved in developing new scientific approach? Is this new scientific approach fix into people or local community visions despite in fully focus on economic benefits? Is this scientific approach will be proven to increase the seaweed production compared to previous experience? What are the implications of introducing new scientific approach in seaweed cultivation? In the light of the above, the paper proposes that

future researchers who are interested in seaweed and development studies would consider the insights of this paper as the main reference for them to carry out the related studies of seaweed in near future.

1.1. Seaweed Cultivation in Malaysia: An Overview

Seaweed cultivation was introduced in Malaysia since year 1978 and cultivated in the island of Semporna called *Karindingan* Island (Yasir & Ali@Ally, 2012). It was a first cultivation farm in Sabah and has been spread to the other island of Semporna as well as few states in west Malaysia. For seaweed cultivation, the East Coast Sabah waters have been identified as an Aquaculture Seaweed Industry Zone due to its resources. What is seaweed? Seaweed refers to a kind of floating vegetables on top of the sea and cultivated mainly by the coastal communities especially local ethnic groups called 'Bajau'. In native language, seaweed has been called as '*sayur hijau'* or green vegetables. Mohamad, Ahmad, Noh & Saari, (2013) described seaweed as macro-algae which is growing near to the sea and has been classified into three major groups based on its pigmentation of brown (*Phaeophyceae*), red (*Rhodophycea*) or green (*Chloropphyceae*). In year 1989, seaweed species like *Kappaphycus alvarezii* has been commercialised in Sabah after Philippines (1973) and Indonesia (1986). As mentioned earlier, seaweed cultivation activities have been concentrated in four places of Sabah such as Semporna, Kudat, Kunak and Lahad Datu. This is because such areas are suitable for seaweed farm and climate as well as sufficient manpower for cultivation activities. In Asian Pacific region especially in Sabah, seaweed species such as *Euchema Cottonii* and *Kappayachus Alvarezii* are farming as a natural flora. These types of seaweeds grow along the Seaside of Sulu until Celebes Sea, which is located between of Semporna until Zamboanga Island in Philippines (Yasir, 2012). Yasir & Ali@Ally, (2012) stated that Malaysia has became a significant contributor in producing red seaweed species namely *Euchema Cottonii* and *Kappayachus Alvarezii* amounting to 20,000 metric ton of dried seaweed. The revenue are MYR60 million in year 2010. In addition, through this activity, the local community who resides in coastal areas of Sabah enjoyed the economic benefits and 9,000 hectares of seaweed farm is allocated for farming. Other than that, seaweed cultivation activities are mostly being carried out in Kerindingan Island with 15,000 hectares, Bum Bum Island with 3,500 hectares, Sebangkat Island with 7,000 hectares, Sibuan Island with 5,000 hectares and 10,000 hectares in Omadal Island. Due to these characteristics, Malaysia has the potential to be a major seaweed manufacturer and exporter in the region in terms of infrastructure development, manpower, product quality, transfer of technology, industrial support and marketing issues (Kaur & Ang, 2009).

2. THE INTERVENTIONS OF MALAYSIAN GOVERNMENT AGENCIES IN SEAWEED PRODUCTION

Realising the seaweed industry has a potential to boost country's economy, the Malaysian government has proactively introduced a few of intervention programmes to enhance the country's seaweed production at par with other producing countries. In Malaysia, seaweed

has been considered as one of the new sources of aquaculture commodity since 2006. A government agency called Ministry of Agriculture and Agro-Based Industry of Malaysia have been given the responsibility to deal with seaweed related issues. Seaweed was introduced as important commodity in the Malaysian National Agro-Food Policy (2011-2020) (NAP4) and assumed it could be gain economic benefits to the country. In addition, under the above-mentioned policy, seaweed was identified as one of the high-value commodities under the program of Entry Point Project 3 or EPP3 (Venturing into Commercial Scale Seaweed Farming in Sabah) which was linked with the themes of 'Capitalizing on Malaysia's Competitive Advantage' (Safari, 2015). To ensure the future sustainability of the seaweed industry in Malaysia, the government has introduced various action plans such as establishing Malaysian Seaweed Industrial Development Committee, Strategic Reform Initiatives for establishing quality and standard protocol as well as enhancing research and development activities. Such government-led interventions indicate that seaweed has a big potential to be promoted nationwide as well as considered an important commodity for export purposes. Since seaweed has a potential to boost the country's economic value, the government has realised that without the partnership from the private sectors, the mission could not be achieved. Under the current policy, the government has initiated the partnership from the private sectors in order to increase the productivity of the seaweed industry. Seaweed industrial zone namely Semporna, Kudat, Kunak and Lahad Datu have been targeted to improve the current productivity of seaweed yields and enhance the coordination of quality production of seaweed. For this reason, the private companies have been given the responsible for this matter. On the other hand, these private companies also responsible to monitor and coordinate the seaweed cultivation, improve the quality of seaweeds, manpower arrangement, marketing of seaweeds and the processing of seaweeds into the high-value commodity to be exported. These stakeholders' participation in the seaweed industry in Malaysia could be beneficial for the parties who involved directly as well as the local community who produces or cultivates the seaweeds. In addition, the quality of seaweeds always be an important issue to be tackled by the government. Thus, Research and Development (R&D) is a most important element that should be included in the seaweed related interventions in order to increase the productivity of the seaweeds and safe for consumption. A few public universities also included to undertake the research on related projects of seaweed cultivation and the suitable methods for cultivation activities. Under the EPP's project, the public universities such as Universiti Malaysia Sabah (UMS), Universiti Kebangsaan Malaysia (UKM), and Universiti Sains Malaysia (USM) are in the midst of commercialising eight seaweed products which developed based on R&D initiative. In fact, Partnership with government and research institutions has been carried out to develop a Standard Operating Procedure (SOP) for the seaweed cultivation practises (Safari, 2015). Partnership with the multiple organisations are welcomed in seaweed industry and to ensure the sustainability of the industry in the long run. Safari (2015), shared that at present the seaweed production has achieved one metric ton per day and its mission to produce 10 metric tons per day by 2020. Moreover, in order to equip such big amount of seaweed production, Sabah State Government has planned to gazette another 3,000 ha of land for seaweed production. The government mission seems ambitious but with the systematic approach played by the relevant parties are important to achieve this mission.

Number	Responsible agency	Type of agency
1	Universiti Malaysia Sabah (UMS)	Government
2	Universiti Sains Malaysia (USM)	Government
3	Universiti Kebangsaan Malaysia (UKM)	Government
4	Universiti Malaya (UM)	Government
5	Department of Fisheries (DOF)	Government
6	Avanova Group Sdn Bhd	Private
7	Perdana Seaweed Farm	Private
8	*Pertubuhan Peladang Kawasan Semporna*	Semi-government
9	SALS Agriculture	Private
10	NIZH Goodwill Sdn Bhd	Private
11	VC United Sdn Bhd	Private
12	Madesjaya	Private
13	Sebangkat Reef Eco-Plant	Private
14	Ko-Nelayan Negeri Sabah	Semi-government
15	*Kluster Look Butun*	Semi-government
16	*Kluster Gellam-Gellam*	Semi-government
17	*Kluster Koperasi Bajau Laut*	Semi-government
18	*Kluster Kuala Merotai*	Semi-government
19	Permata Sekitar Sdn Bhd	Private
20	Department of Fisheries (DOF) Sabah	Government

Source: Pemandu, 2016.

Figure 1. Partners of seaweed in Malaysia.

2.1. Mini Estate System (MES) and Cluster System (CS) as a New Approach in Seaweed Cultivation in Sabah, East Malaysia

Realising the potential of seaweed as one of the important commodity which could generate economy of the country, the Malaysian government has initiated and introduced new transformation programme called as Seaweed Mini Estate Development. Under the Economic Transformation Programme (ETP), the Malaysian government has identified seaweed as one of the National Key Economic Areas (NKEA). It was placed in one of the 16 Entry Point Project (EPP). Such initiatives have been acknowledged that seaweed was the most important commodity exported by the country. A total of 46 million was allocated to boost the seaweed industry in Malaysia by the government in 2011 (Utusan Borneo, 2011). The seaweed industry in Malaysia especially in Sabah had gained the transformation status (EPP3) when Mini Estate System (MES) has been introduced with the aim to transform the conventional seaweed cultivation to a new scientific approach. The conventional seaweed cultivation refers to those old practices such as tie seaweed with nylon string, and over exposure of seaweed drying under sunlight made the seaweed not healthy and less productive. Such conventional methods of seaweed cultivation need a transformation with the new scientific knowledge like MES. In order to manage the MES programme systematically, Universiti Malaysia Sabah (UMS), a local public university in Sabah has been given the responsibility. Since 2010, the MES programme managed by the UMS and Dr Suhaimi Md. Yasir was the director and leader of this project. The two phases of MES started in 2010 and run concurrently by the UMS. The first phase (2011-2010) focused on the initial development of MES infrastructure as well as appointment of leading companies who have the stake in seaweed cultivation. On the other hand, identification of seaweed cultivation farms also being focused in this phase.

Semporna district was identified as suitable place for seaweed production. The second phase (2013-2020) focused on the product development based on seaweeds and marketing issues. It was aimed that MES programme could increase the productivity of seaweed and could produce 150, 000 metric tons of high quality processed seaweeds worth to MYR1.45 billion by the year 2020 (New Strait Times, 2013).

Fully funded by the government, MES using the approach of 'community-based, commercial approach'. MES is a fine concept which is implemented in the seaweed cultivation programme in Semporna, Sabah. There are a few characteristics and definitions of MES. A manual book entitled '*Sistem Mini Estet Industri Rumpai Laut Negara'* was published by the Department of Fisheries Malaysia clearly mentioned about the background of MES and its significance to the seaweed industry in Malaysia. According to the manual book, MES refers to a better and new approach to the seaweed cultivation. There have been a few important factors considered in the early process of seaweed cultivation activity, namely (1) Holistic management transformation-'poor-man industry to lucrative industry', (2) Science and Technology based mechanism, (3) Reduce labour intensive production, (4) Friendly and sustainable environmental management, (5) seaweed as a new commodity, (6) 'community based, commercial approach', and (7) High quality yields and seaweed related products. There are elements in the MES that could provide the local community and industry manufacturers with the new knowledge and skills in order to embark on the new seaweed cultivation production in Malaysia.

Through the MES, an important part of the seaweed cultivation process is the availability of platform facilities. The platform facilities and accommodation for the seaweed cultivators would enable them to be involved seriously. Previously, a high level of poverty among the fishermen in both islands (Selakan and Bum Bum Islands) has led and forced them to carry out seaweed cultivation activities in a conventional way. Hence, the new technological-based approach have been introduced to assist these people to adapt themselves with the new process of seaweed cultivation. It is hoping for them to enhance their livelihoods. In MES, other facilities that have been introduced including: (1) holistic transformation (an enhancement of physically socio-economic image), (2) management system (previously 80% work on the sea and 20% on seaweed platform, but now 80% work on seaweed platform and 20% on the sea), (3) an integrative management complex, (4) estate management, (5) block management, and (6) a model of entrepreneur development (Yasir, 2012). As a result, the MES has transformed the conventional seaweed cultivation to a new and modern seaweed cultivation management.

According to Yasir (2012), the MES has its own speciality where it had many improvements over the conventional seaweed cultivation methods. For instance, the MES eliminates the use of nylon string to tie seaweeds which has been practised for decades and replaced with the UMS designed eco-friendly Tie-Tie, which is seaweed-based rope. In addition, the MES also introduced organic seaweed fertilizers for high yields and seedling pin tables or commonly known as Casino Tables as well as anchor for systematic and hygiene seaweed cultivation management.

The CS was implemented after the MES. It is considered as a branch of MES because it also focused on the production of a commercial basis rather than the domestic used. However, CS are managed by the Sabah Fisheries Department. Under the CS programme, the community that already engaged in seaweed cultivation activities are considered as

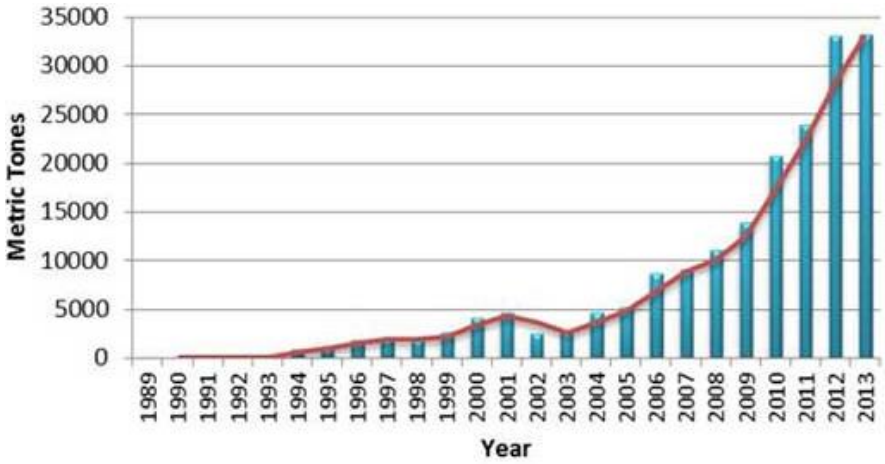
participant. The areas selected for cultivation are Bum-Bum Island, Silungan Island and Merotai Island located in the district of Semporna, Sabah, Malaysia.

3. SEAWEED SOCIO-ECONOMIC BENEFITS TO MALAYSIA'S DEVELOPMENT

In Malaysia, the seaweed production has taken place in Semporna, Sabah in year 1978 and continuously growing until to date. In the initial phases of seaweed cultivation, the seaweed primarily used for coastal communities' daily meals. For coastal communities of Semporna, seaweed cultivation has been the secondary livelihood activity after the fishing activity. Since then, the seaweed cultivation became one of the significant economic activity for the coastal communities in Sabah. In terms of economic benefits, the production of seaweed has been growing gradually after 1989. The seaweed cultivation then started to grow with big scales and spread to the other potential areas of Sabah. According to the Sabah Annual Fisheries Statistic in 2013 showed that the total production of dried seaweed recorded were gradually increased from 1989 to 2001. This is an initial phase of production where it always needs some period of time to increase the efficiency of seaweed cultivation production since year 2001. In year 2002 and 2003, there were a drastic decline in seaweed production. This situation has raised attention for relevant parties to take the necessary actions. After the declination of seaweed production in year 2002 and 2003, the production of seaweed has steadily increased since 2004 until 2013 (refer Figure 2). In terms of value, in 2013, the seaweed production from Sabah amounted to 28 per cent by volume (33, 210 mt) and 3 per cent (MYR198.93 million). This value is based on the total marine aquaculture production. In fact, the seaweed production from Sabah has been recorded to be increased slightly by volume in 2013 to 110.0 metric tons compared to 2012 (Safari, 2015). This is a good revenue where the seaweed production from Sabah has high potential to be developed if serious proactive measures and interventions are taken to enhance this industry run systematically.

Figure 3 shows the statistics of Malaysian exports and imports of dried seaweeds from 2009 to 2012. The total amount of Malaysian dried seaweeds exported in the past four years were recorded a slight fluctuation with the volume increasing from 236.94 metric tons in 2009 to 656.63 metric tons in 2010. This shows a steady increasing of quantity of seaweeds exported to other countries. Table 2 also depicts that in 2011, there was a further increment of exported seaweeds amounting to 1,320.70 metric tons with revenue of 3,101,858 US dollars. However, in 2012 the amount of exported seaweeds has dropped to 502.45 metric tons. Obviously, this trend has reflected the value of seaweeds exportation showed in the parallel trend. Table 2 also indicates the statistics of dried seaweeds imported from 2009 to 2012. The amount of dried seaweeds imported have been decreased from 1,058.8 metric tons in 2009 to 668.9 metric tons in 2012. However, the value of imports where it fluctuated with a comparable average of 25 per cent from US$ 5.4 million in 2009 increasing to US$ 6.0 million in 2010 and US$ 7.9 million in 2011 (Safari, 2015). This trend significantly reflected that seaweed industry in Malaysia is fragile in terms of export and import. This trend could be sustained if the stakeholders aware about the marketing strategy and increment of seaweed production. The usage of seaweed for commercial products has raised economic benefits to the country where an element of carrageenan extraction plays an important role for the

production. Thus, carrageenan from the seaweeds is valuable and potentially sustained for the seaweed industry in near future.



Source: Safari, 2015.

Figure 2. Total production of seaweeds in Malaysia (1989-2013).

Year	Export		Import	
	Volume (mt)	Value (US$)	Volume (mt)	Value (US$)
2009	236.94	1,235,767	1,058.8	5,446,238
2010	856.63	1,969,601	917.9	6,033,002
2011	1,320.70	3,101,858	582.6	7,890,801
2012	502.45	931,814	668.9	5,958,578

Source: Safari, 2015.

Figure 3. Exports and Imports of Malaysian dried seaweeds (2009-2012).

4. THE POTENTIAL USAGE OF SEAWEED IN BUSINESS VENTURES OR COMMERCIALISATION

At present, the world demand for seaweed production especially the carrageenan. The demand was started during the Second World War where it could be the substitute for animal fat, the most popular colloid for food processing. Generally, the carrageenan was extracted from red seaweeds. In addition, the usage of seaweed specifically the carrageenan was applied in multiple commercial products such as in the food processing, pharmaceutical, animal feed, fertilizers and cosmetic industries (Phang, 2006; Valderrama, Cai, Hishamunda & Ridler, 2013). There are two types of seaweeds in Sabah such as *Euchema Cottonii* and *Kappayachus Alvarezii* were the major sources of carrageenan. Currently, there are three types of carrageenan extraction available for production of materials such as Highly-Refined Carrageenan, Refined Carrageenan and Semi-Refined Carrageenan (Safari, 2015). Such categorisation of carrageenan is widely used by manufacturers to produce products that have commercial value (see Figure 4). In order to produce semi-refined carrageenan, there are two major factories located in Sabah since year 2000 such as Tacara in Morotai, Tawau and Omi-Gel in *Jalan Kemiri* (Kemiri Road), Semporna (Phang, Yeong, Lim, Nor & Gan, 2010).

Carrageenan products	Alkali-treated cottonii (ATC) chips	Semi-refined carrageenan (SRC)		Refined carrageenan (RC)	
		Non-food-grade SRC	Food-grade SRC (PES)	RC (gel-press)	RC (alcohol)
Major raw materials used (Panlibuton, Porse and Nadela, 2007)	Cottonii			Cottonii or *Gigartina*	Any kind of carrageenan seaweed: cottonii, *spinosum, Gigartina* or *Chondrus*
Processing method (McHugh, 2003)	Seaweed treated in hot alkaline solution of potassium hydroxide to remove water-soluble contents; dried; then:			Carrageenan first extracted into an aqueous solution; then recovered by:	
	chopped into pieces	milled	bleached, milled, and sterilized	gel-press method	alcohol-precipitation method
Main contents (McHugh, 2003)	Kappa + cellulose			Kappa	Any type of carrageenan: kappa/ iota/lambda
Main uses (McHugh, 2003; Panlibuton, Porse and Nadela, 2007; Bixler and Porse, 2011).	Further processed into SRC or RC	Pet food	Low-end product: meat (ham, pre-cooked poultry, fat replacement), dairy (cheese, chocolate milk, etc.), water-based food (water-gel, salad dressing, etc.)		High-end product: toothpaste, cold-soluble dairy products, pharmaceutical products (capsules, etc.)
Major producing regions/countries (Neish, 2008a; Bixler and Porse, 2011).	China, Indonesia and the Philippines			Europe, Americas, China and the Philippines	
Share of total carrageenan production worldwide in 2009 (%), based on the estimation in Bixler and Porse (2011)		10	41	26	23

Source: Adapted from Cai, Hishamunda & Ridler, 2013: 20.

Figure 4. Summary of products based on carrageenan extraction.

For the past decades, coastal communities of Sabah used seaweed as daily meals whether as raw or blanched as salads (Phang, 2006). The seaweeds rich with vitamins such as A, C and E and good for health. In addition, it has been also considered as side dish for most of the coastal communities around the Semporna that cultivated seaweed as their full-time or part-time employment. The use of seaweeds as food are well-documented in the literature. There are different types of seaweeds have been used. Types of seaweeds used for food namely Rhodophytes *Gracilaria changii*, *Gracilaria tenuispitata*, *Eucheuma* species, Chlorophytes *Caulerpa lentillifera* and *Caulerpa racemose* are popular (Phang, 2006). Seaweeds are popular among the Chinese communities in Malaysia where they perceived it has traditional medicine value and healthy for consumption. Since seaweeds are largely cultivated in Sabah, the local community especially *Sabahans* are favour for seaweeds to include in their meals. Such popularity of seaweeds as food could sustain the seaweed industry in Sabah. Furthermore, it could become an alternative mechanism for people who prefer healthy lifestyles when it comes to food consumption. Seaweeds are not only famous among the local Malaysian but also among Japanese people. Restaurants and street food courts are now serving seaweeds as food and cook seaweeds in different methods. Among the popular species of seaweeds used in Japanese and Chinese restaurants are the temperate species of *Porphyra* (nori), *Undaria* (wakame), *Laminaria* (kombu) and *Nostoc* (fa' tsoi). In terms of daily food consumption, people can buy dried *Sargassum* and *Turbinaria* in most Chinese medicine shops in Malaysia (Phang, 2006).

5. PROSPECT OF SEAWEED INDUSTRY AND WAY FORWARD

Carrageenan powder is the most important source for used in many commercial products. Therefore, it can boost the country's economy where more innovative products could be developed if the carrageenan production is sustained. The demand of carrageenan was estimated value of 40, 000 metric tons in 2006. It is projected to increase at a rate of 10-15 per cent per annum with an estimated value of US$3.3 billion. With regards to Malaysian development perspectives, the Malaysia's Ministry of Agriculture and Agro-Based Industry targeted for wet seaweed production is expected to grow by 19.7 per cent from 365, 000 metric tons in 2015, tripling to about 900, 000 metric tons in 2020 (Safari, 2015). With this projection, the production of carrageenan are also expected to increase in line with this target. The importance of carrageenan production can be realised through government's interventions. The present government's interventions are seriously focus on the sustainability of the seaweed industry as well as the carrageenan production. It also assumed that the export value of seaweed could be tripled by 2020, from MYR376 million in 2015 to MYR1.4 billion in 2020. In context of food and pharmaceutical industries, the uses of carrageenan are important for the development of various commercial products of related industries. In Malaysia, it is hoped that the production of carrageenan could be enough to supply and fulfil the current demands. In fact, it was estimated that the world population of 8 billion in 2030, thus the overall food consumption is definitely going to increase. In line with this fact, the total production of carrageenan is also expected to increase in near future.

CONCLUSION

Based on the review of Malaysian government policies related to seaweed industry, the study found that the Malaysian government is seriously committed in introducing new interventions in seaweed cultivation in order to enhance the seaweed industry in the country. This is reflected in the form of policy where seaweed production and commercialisation has become an important industry in Malaysia. The government has highlighted seaweed as one of the high-value commodity under the Malaysian National Agro Policy for the year 2011-2020. This is proven that Malaysian seaweed industry has high potential to be developed. It is also could bring economic benefits for the country as well as socio-economic development. Kaur and Ang (2006) believed that Malaysia has the potential to be a major seaweed manufacturer in Southeast Asia region. This vision however, could not be achieved if the proper management and systematic approach take place by the relevant stakeholders. To some extent, such interventions are already in place. But it is too ambitious to claim that the seaweed industry will grow faster and sustain in near future. This is because the seaweed industry is consider as a 'fragile industry' and unpredictable in terms of its implications. Therefore, continuous monitoring efforts from stakeholders are always needed where future proactive measures are still relevant to consider. The sustainability of the seaweed industry is rely on the people hands (in this context is the local people who cultivating seaweed) and industry manufacturers in order to stabilize the production value and the volume of seaweed production. Thus, more efforts especially R&D, prevalent technologies and scientific approach are needed to boost the seaweed industry in the future in Malaysia.

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BIOGRAPHICAL SKETCHES

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Velan Kunjuraman, Rosazman Hussin & Abdul Rahman Ahmad. 2015. International tourist satisfaction towards tourism activities and facilities: A case study in Mamutik Island, Kota Kinabalu, Sabah, East Malaysia. *Journal of Tourism, Hospitality and Culinary Arts,* 7, (1): 76-92. ISSN: 1985-8914.

Rosazman Hussin, Suhaimi Md. Yasir, Velan Kunjuraman & Aisah Hossin. 2015. Enhancing Capacity Building in Seaweed Cultivation System among the poor fishermen: A case study in Sabah, East Malaysia. *Asian Social Science,* 11, (18): 1-9. ISSN: 1911-2017 & E-ISSN: 1911-2015.

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Chapter 75

THE IDENTIFICATION OF MACROALGAE AND THE ASSESSMENT OF INTERTIDAL ROCKY SHORES' ECOLOGICAL STATUSES IN THE CENTRAL WESTERN COAST OF CONTINENTAL PORTUGAL

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ABSTRACT

This work studied the marine macroalgae thriving at intertidal rocky shores on the west coast of Portugal (Peniche, Central Portugal). Four seashores (Baleal-Norte, Gamboa, Portinho da Areia do Norte and Consolação) located near the borderline between two adjacent types of the Portuguese coast were surveyed, the first 3 inside the northern exposed coast (type A5) and the last shore from the moderately exposed coast at the southwest coast of continental Portugal (type A6).

The aim of the study was to contribute to improve knowledge on the marine macroalgal flora of this transition zone, where the combined influence of cold waters (from North Atlantic Ocean) and warmer waters (from subtropical Atlantic Ocean and the Mediterranean Sea) may favour the development of unique macroalgal communities (different arrangement of species). The collected material was housed at herbaria and photographed (records inserted in MACOI – Portuguese Seaweeds Website), and data were compared with historical information, aiming to update the local taxonomic list of marine macroalgae, and to infer about the species evolution along a 50 years' period. Data were also used to assess the ecological quality status of the four sampled sites by applying the methodology MarMAT (Marine Macroalgae Assessment Tool).

A total of 87 macroalgal species were identified in the four localities, from which 16% have been not registered 50 years ago in local surveys. Meanwhile, more *taxa* were

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identified after the initial campaign so no new species were recorded in the present study. Taxonomic composition showed similarities for all sites but sufficient differences were still present and Consolação (A6 typology) appeared separate from the northern shores in the MDS analysis. From the application of MarMAT to present registers and old data, resulted a similar ecological quality status. Although all sites assessed in this study resulted on High quality, MarMAT reported equally high Ecological Quality Ratio for old and some recent data, indicating that sites preserved their excellent quality along the last decades.

Keywords: western coast of Portugal, Rocky shores, intertidal, marine macroalgae, MarMAT, ecological quality status

INTRODUCTION

The first phycological studies of the Portuguese coast were published by Vandelli in 1788 [in 1] and Correa da Serra in 1796 [2]. In the following years, other studies were carried out by Welwitsch [3], Hauck [4], Palminha [5-8], Mesquita Rodrigues [9, 10], Póvoa dos Reis [11]. However, the most complete study on the Portuguese phycological flora was carried out by Ardré [12-14]. Actually, according to Sousa-Pinto [15], the Portuguese phycological flora did not suffer a significant change in terms of the number of species registered since the study made by Ardré in 1970 [13] in the late sixties, of the last century. Ardré [13] studied, identified and described 404 macroalgae species, from which 246 were Rhodophyta (red algae), 98 Ochrophyta (brown algae) and 60 Chlorophyta (green algae). In 2009, Araújo et al. [16] based on literature references, new records and herbarium data, obtained an updated checklist of the benthic marine algae of the northern coast of Portugal. This checklist includes 346 species, namely 200 Rhodophyta, 70 Ochrophyta, 50 Chlorophyta, and 26 Cyanobacteria. Of these, 33 species were recorded for the first time in this region and 21 were new records for the Portuguese coast. These new records were probably mainly related to the sampling effort, undertaken recently across more localities than in previous works, with a consequent increase of diversity on the habitats surveyed [16]. Recent new floristic records and taxonomic data were published meanwhile by other authors [17-24].

The Portuguese coast is located in a transition region, between the cold waters of the North Atlantic Ocean and warmer waters of subtropical Atlantic Ocean and the Mediterranean Sea, favourable to the development of a unique combination of species that compose the Portuguese macroalgal communities [16, 17]. Southwards there is an increasing number of Rhodophyta species and a simultaneous decrease in Ochrophyta, which enables the division into two groups: algae of the northern zone (between the mouth of the Minho river and the mouth of the Tagus river) and algae of the southern zone (between the mouth of the Tagus river and the Algarve) [25, 26]. Although the intertidal macroalgae in northern Portugal tend to be more similar to those of the central European coast, Brittany and South of British Isles, and the macroalgae of southern Portugal to get included several Mediterranean and African species [15], the global composition of macroalgal communities represents well this transition situation. This is evident when Feldmann [27] and Cheney [28] ratios are compared by geographical region, varying from 2.53 in Britain and Ireland to 3.71 in Moroccan coast, with a higher value registered for the Iberian coasts (3.57 to north Portugal, 4.09 to Basque Country) [16].

Located in the central region of the country, the Baleal-Norte, Gamboa, Portinho da Areia do Norte and Consolação coastal areas also have a great diversity in terms of phycological flora in their mediolittoral rocky shores. The first phycological records of the Baleal-Norte rocky shore were made in 1961 and 1970 by Ardré [12, 13]. Later, inserted in the project MACOI-Portuguese Seaweeds Website [29] (1998 to present), new *taxa* were identified and added to the previously described by Ardré. From a total of 131 species recorded, 20 were Chlorophyta, 29 Ochrophyta and 82 Rhodophyta. The first inventory of Gamboa beach was done by Ardré in 1966 [30] and later in 1970 [13] recording a total of 148 species (21 Chlorophyta, 33 Ochrophyta and 94 Rhodophyta). In Portinho da Areia do Norte and Consolação sites the first records appeared with the project MACOI-Portuguese Seaweeds Website [29], accounting 71 species for the first location (9 Chlorophyta, 18 Ochrophyta and 44 Rhodophyta) and 24 species for the second (5 Chlorophyta, 5 Ochrophyta and 14 Rhodophyta) [12, 13, 29, 30].

Taxonomic composition of macroalgae is important for the structure and functions characterizing marine coastal communities and, consequently, it has influence on the dynamics and changes occurred in coastal environments [31, 32]. Conversely, it is also known that species richness, taxonomic composition and the abundance of certain macroalgal species at intertidal rocky shore communities (proportion of opportunists' coverage in relation to total coverage) may reflect changes resulting from alterations in the water quality. These characteristics allowed the Water Framework Directive (WFD, 2000/60/EC), an European environmental legislation piece, to require the use of marine macroalgae as one of the biological quality elements (BQE) integrated in evaluation schemes developed by European Member States (MS) [31] to detect any significant ecological degradation resulting from anthropogenic activities. The implementation of the international environmental legislation's court forced to a concerted effort between the administration of various MS, regarding the development of environmental and ecological indicators [33]. From this need it was developed in Portugal the Marine Macroalgae Assessment Tool (MarMAT), a methodology created to evaluate the quality of coastal waters based on data collected from intertidal macroalgae communities [34].

Mostly, assessment methods require either a correct identification of macroalgal species present and a deep knowledge of taxonomic lists naturally characterizing the coastal zone under evaluation (i.e., type specific reference conditions) [34, 35]. These aspects are eventually more important in transition zones of the coast, such as the study area here presented, where assessment methods may experience changes in boundary values or in the reference conditions they have to use to compare with but where the availability of data is also low. Due to this, the increase on quality and quantity of information from those sites earns extreme importance for a robust assessment of ecological conditions of rocky shores. In this sense, the present chapter aims to improve the knowhow about the behaviour of intertidal macroalgal communities from a coastal transition zone, namely: (1) to register and update the intertidal phycological flora of four rocky shores from the western coast of Portugal (diversity and photographic records); (2) to analyse the macroalgal community structure, its similarity between study sites, and to compare historical and present taxonomic data; and (3) to assess the Ecological Quality Status (EQS) of coastal water bodies for present and first sampling situations, at the same time that MarMAT robustness is validated for sites located at the borderline of transition zones.

SEASHORES OF THE WESTERN COAST OF PORTUGAL (PENICHE)

Located in the North-East Atlantic (NEA) region of the European coast, the four study sites belong to typology NEA 1/26 of EU coastal waters' category [36]. In terms of national classification, the sites (Baleal-Norte, Gamboa, Portinho da Areia do Norte and Consolação) are displaced on the borderline between two adjacent coastal water types, which are type A5 (exposed coasts, includes the first three sites) and type A6 (moderately exposed coasts, includes the last shore). The classification of Portuguese categories includes also for open coastal waters the sheltered type (A7) on the south part of the country (Algarve), and types A1 to A4 representing transition waters (estuaries) and sheltered coastal waters (coastal lagoons) along all territory [37]. The coast is influenced by a semidiurnal tide, mesotidal (2-4 m amplitude) with prevailing winds from the North/Northwest in the summer months. The sea surface temperature varies between 15 °C and 17 °C and the air between 18 °C and 25 °C during the summer season [38-40].

Inserted on the central region of the west coast of Portugal, the Baleal-Norte, Gamboa, Portinho da Areia do Norte and Consolação shores have characteristics in common, including the fact that they possess mixed substrates, more or less extensive, composed by thin, gold sands and rocky outcrops (Figure 1). However, every location presents a particular physical feature of the coast, given by the arrangement and interactions between the water and these substrates or platforms. Resulting from distinctive abiotic and biotic characteristics, the side by side presence of exposed coasts (A5 coastal waters Portuguese typology: Baleal-Norte, Gamboa and Portinho da Areia do Norte) and moderately exposed ones (A6 coastal waters Portuguese typology: Consolação), promotes the emergence of many species of macroalgae and the consequent enhancement of general diversity in the area.

Figure 1. Location of the studied areas on the Portuguese coast: A) Baleal-Norte (39°22'24.21''N - 9°20'11.36''W); B) Gamboa (39°21'51.19''N - 9°22'19.55''W); C) Portinho da Areia do Norte (39°22'7.46''N - 9°22'42.28''W); D) Consolação (39°19'30.57''N - 9°21'37.86''W).

SURVEY OF THE PHYCOLOGICAL FLORA

Seaweed samples were collected between July 2012 and June 2013; maximum possible information was obtained from several visits to sites. The methodology used for harvesting macroalgae in the intertidal level was equal to all sites. The collection of specimens began before the low tide hour (information found at the Hydrographic Institute website) [41] and lasted until after that, making possible the visualization of the lower zone of the intertidal level. The macroalgal species richness was assessed through a random path in the rocks and tide pools area, from the lowest tide level to the highest level in the intertidal. All species found along this course were photographed in place, harvested and stored in labelled plastic bags. In this study was chosen a destructive method, proceeding to the collection of specimens through the uprooting in the case of larger algae and in the case of smaller algae by scraping the surface of the rocks.

The collected material was separated into two groups, the larger algae were conserved in herbarium and the small sized species were transferred into labelled vials and preserved in a solution of formaldehyde at 4% in seawater. The largest specimens were identified into the main taxonomy groups of red, brown and green algae in the laboratory, except when the accurate identification was possible in the field (Cyanobacteria were not included in this study). The screening of small sized algae was done in the laboratory under a Stereo Microscope (Lan binocular optics with digital camera, DCMC 130, 1.3M pixels) and a Light Microscope (Motic BA 310 with camera incorporated) (see Annex I). All collected species were identified and data inserted into MACOI - Portuguese Seaweeds Website [26, 29].

MACROALGAL DIVERSITY AND COMMUNITY STRUCTURE

Along the four intertidal areas were identified in total 87 species of macroalgae. Of these 87 species, 10 belonged to Chlorophyta, 20 to Ochrophyta and 57 to Rhodophyta. Carrier of the highest species richness, the Baleal-Norte beach contained 55 species, than the beaches of Consolação with 53, Portinho da Areia do Norte with 50 and Gamboa with 36 species of macroalgae (Table 1). Of the 55 species of macroalgae referring to the Baleal-Norte beach, 7 belong to Chlorophyta, 12 to Ochrophyta and 36 to Rhodophyta. Of the 53 species from the Consolação beach, 7 belong to Chlorophyta, 13 to Ochrophyta and 33 to Rhodophyta. Of the 50 species from the Portinho da Areia do Norte beach, 7 belong to Chlorophyta, 10 to Ochrophyta and 33 to Rhodophyta. And of the 36 species of Gamboa beach, 5 belonged to Chlorophyta, 14 to Ochrophyta and 17 to Rhodophyta.

In all shores, the number of species belonging to Rhodophyta was the highest, followed by Ochrophyta and Chlorophyta, which permanently had the lowest number of species. Regarding the number of Orders, it was noted that in all sites, the Bryopsidales and Ulvales had the highest number of species from Chlorophyta. On Ochrophyta the Orders that stood out on the Baleal-Norte shore were Dictyotales and Fucales, on Gamboa and Portinho da Areia do Norte were Dictyotales, Fucales and Sphacelariales, and from Consolação were Fucales, Ectocarpales and Sphacelariales. In Rhodophyta, Ceramiales was more abundant in all sites, followed by Corallinales. Apart from these two Orders, on the Portinho da Areia do Norte and Consolação also Gigartinales showed a significantly high number of species. In

contrast it was found that Nemaliales, Peyssonneliales, Plocamiales and Rhodymeniales had only a single species each. Similarly, to what was verified individually for seashores, also throughout the species registered on sites was found that Bryopsidales and Ulvales Orders were the most representative of Chlorophyta with 3 and 5 species, respectively. On Ochrophyta, Dictyotales, Ectocarpales and Fucales were the most evident with 5 species each. In Rhodophyta, Ceramiales was the most expressive with 36 species, followed by Gigartinales with 7 species (Figure 2 and 3).

Table 1. List of species identified for the study sites and indication of photograph number

Species	ESG	Opport.	Study sites				RTL
			Baleal-Norte	Gamboa	Portinho da Areia do Norte	Consolação	
Phyllum Chlorophyta							
Order Bryopsidales							
Codium adhaerens (Figure 5)	II			x	X	X	1
Codium tomentosum (Figure 6)	II		x	x	X	X	1
Derbesia tenuissima (Figure 7)			x	x	X		-
Order Cladophorales							
Cladophora prolifera (Figure 8)	II	Yes	x				2
Valonia utricularis (Figure 9)					X	X	-
Order Ulvales							
Ulva clathrata (Figure 12)	II	Yes	x		X	X	3
Ulva compressa (Figure 10)	II	Yes			X	X	3
Ulva rigida (Figure 11A, C)	II	Yes	x	x	X	X	4
Ulva rigida var. *fimbriata* (Figure 11B, D)	II	Yes	x				4
Ulvaria obscura (Figure 13)	II	Yes				X	4
Phyllum Ochrophyta							
Order Dictyotales							
Dictyota dichotoma (Figure 14)	II		x	x	X	X	5
Dictyota spiralis (Figure 15)	II		X				5
Dictyopteris polypodioides (Figure 16)	II		x	x	X		6
Padina pavonica (Figure 17)			x	x	X		-
Taonia atomaria (Figure 18)			x	x	X		-
Order Ectocarpales							
*Colpomenia peregrine** (Figure 19A)	II		x	x		X	7
Colpomenia sinuosa (Figure 19B)	II				X		7
Ectocarpus fasciculatus (Figure 20)	II	Yes		x		X	8
Hincksia granulosa (Figure 21A)						x	8
Hincksia hincksiae (Figure 21B)						x	8
Order Fucales							
Bifurcaria bifurcata (Figure 22)	I		x	x		x	9
Cystoseira baccata (Figure 23)	I		x	x			10
Cystoseira tamariscifolia (Figure 24)	I		x	x	X	x	10
Fucus spiralis (Figure 25)	I					x	11
Sargassum vulgare (Figure 26)			x	x	X		-
Order Laminariales							
Laminaria ochroleuca (Figure 27)						x	12
Order Sphacelariales							
Cladostephus spongiosus (Figure 28)	I		x	x	X	x	13
Halopteris scoparia (Figure 29)	II		x	x	X	x	14
Sphacelaria rigidula (Figure 30)	II	Yes		x		x	8

Species	ESG	Opport.	Study sites				RTL
			Baleal-Norte	Gamboa	Portinho da Areia do Norte	Consolação	
Order Tilopteridales							
Saccorhiza polyschides (Figure 31)					X	x	15
Phylum Rhodophyta							
Order Bonnemaisoniales							
*Asparagopsis armata** (Figure 32B-D)	II		x	x	X	x	16
Falkenbergia rufolanosa (Figure 32A)	II		x			x	16
Order Ceramiales							
Acrosorium ciliolatum (Figure 33)	II		x	x	X		17
Aglaothamnion pseudobyssoides (Figure 34A, B)	II	Yes				x	18
Aglaothamnion sepositum (Figure 34C, D)	II	Yes	x			x	18
Anotrichium furcellatum (Figure 35)			x				-
Antithamnion densum (Figure 36)	II	Yes			X		18
Apoglossum ruscifolium (Figure 37)	II				X		19
Boergeseniella fruticulosa (Figure 38A, C)	II	Yes	x	x	X	x	18
Boergeseniella thuyoides (Figure 38B, D)	II	Yes	x			x	18
Bornetia secundiflora (Figure 39)	II		x		X	x	20
Callithamnion tetragonum (Figure 40)	II	Yes				x	18
Ceramium ciliatum (Figure 41)	II	Yes	x	x	X	x	18
Ceramium echionotum (Figure 42)	II	Yes			X		18
Gayliella flacida (Figure 43)	II	Yes				x	18
Ceramium pallidum (Figure 44)	II	Yes		x		x	18
Ceramium tenuicorne (Figure 45)	II	Yes	X				18
Ceramium virgatum (Figure 46)	II	Yes			X	x	18
Chondria coerulescens (Figure 47)	II		x		X	x	21
Compsothamnion thuyoides (Figure 48)	II	Yes	x			x	18
Cryptopleura ramose (Figure 49)	II					x	17
Crouania attenuata (Figure 50)					X		-
Erythroglossum laciniatum (Figure 51)			x	x			-
Halurus equisetifolius (Figure 52)	II		x				22
Herposiphonia tenella (Figure 53)	II	Yes			X		18
Hypoglossum hypoglossoides (Figure 54)	II		x		X	x	19
Laurencia pyramidalis (Figure 55)	II		x	x	X		23
Leptosiphonia schousboei (Figure 56)	II	Yes	x				18
Lophosiphonia reptabunda (Figure 57)	II	Yes		x	X		18
Nitophyllum punctatum (Figure 58)	II		x			x	24
Ophidocladus simpliciusculus (Figure 59)	II	Yes	x				18
Osmundea pinnatifida (Figure 60)	II					x	23
Pleonosporium borreri (Figure 61)	II	Yes				x	18
Polysiphonia denudata (Figure 62)	II	Yes	x	x	X	x	18
Polysiphonia fucoides (Figure 63)	II	Yes	x			x	18
Xiphosiphonia ardreana (Figure 64)	II	Yes	x				18
Pterosiphonia complanata (Figure 65)	II		x	x	X	x	25
Xiphosiphonia pennata (Figure 66)	II	Yes	x		X		18
Order Corallinales							
Ellisolandia elongata (Figure 67A)	I		x		X		26
Corallina officinalis (Figure 67B)	I			x	X	x	26
Jania rubens (Figure 68)	I		x	x	X	x	26
Lithophyllum incrustans (Figure 69A)	I		x	x	X	x	27
Lithophyllum byssoides (Figure 69B)	I		x		X	x	27
Mesophyllum lichenoides (Figure 70)	I				X		27
Order Gelidiales							
Gelidium corneum (Figure 71A, B)	I		x				28
Gelidium spinosum (Figure 71C, D)	I		x				28

Table 1. (Continued)

Species	ESG	Opport.	Study sites				RTL
			Baleal -Norte	Gamboa	Portinho da Areia do Norte	Consolação	
Order Gigartinales							
Ahnfeltiopsis devoniensis (Figure 72)	II		x		X	x	29
Caulacanthus ustulatus (Figure 73)	II				X	x	30
Chondracanthus acicularis (Figure 74A)	II		x		X	x	31
Chondracanthus teedei (Figure 74B)	II		x		X		32
Dilsea carnosa (Figure 75)	II					x	33
Gigartina pistillata (Figure 76)	II					x	34
Sphaerococcus coronopifolius (Figure 77)	I		x	x	X		35
Order Nemaliales							
Liagora viscida (Figure 78)				x	X		-
Order Peyssonneliales							
Peyssonnelia coriacea (Figure 79)	I		X				36
Order Plocamiales							
Plocamium cartilagineum (Figure 80)	I		x	x	X	x	35
Order Rhodymeniales							
Champia parvula (Figure 81)	II				X	x	37

ESG - ecological status groups (I - perennial species; II - annual species); Opport. – opportunistic species; X - species recorded; RTL - different entries in the reduced taxa list (RTL).

The numbers indicate different entry present in the reduced list of species (RTL), since there are several species grouped in some single entry. Thus, the following entries consider more than one possible taxa in RTL (1) *Codium* spp.; (2) *Cladophora* spp.; (3) *Ulva* spp. 'tubular-type'/*Blidingia* spp.; (4) *Ulva* spp. 'sheet-type'/*Ulvaria obscura*/*Prasiola stipitata*; (5) *Dictyota* spp.; (7) *Colpomenia* spp./*Leathesia marina*; (8) filamentous Phaeophyceae; (10) *Cystoseira* spp.; (11) *Fucus* spp.; (12) *Laminaria* spp.; (14) *Halopteris filicina*/*Halopteris scoparia*; (16) *Asparagopsis armata*/ *Falkenbergia rufolanosa*; (17) *Acrosorium ciliolatum*/*Callophyllis laciniata*/*Cryptopleura ramosa*; (18) other filamentous Rhodophyta; (19) *Apoglossum ruscifolium*/*Hypoglossum hypoglossoides*; (20) *Bornetia* spp./*Griffithsia* spp.; (21) *Chondria* spp.; (23) *Laurencia* spp./*Osmundea* spp.; (26) erect calcareous species; (27) encrusting calcareous species; (28) Gelidiales; (29) *Ahnfeltiopsis* spp./*Gymnogongrus* spp.; (30) *Catenella caespitosa*/ *Caulacanthus ustulatus*; (33) *Dilsea carnosa*/*Schizymenia dubyi*; (35) *Plocamium cartilagineum*/ *Sphaerococcus coronopifolius*; (36) *Peyssonnelia* spp.; (37) Champiaceae.

*Exotic species (non-native).

From the above mentioned distribution of taxa was possible to estimate the Cheney ratio - (R + C)/P [28] for all sites. The highest values were achieved by Portinho da Areia do Norte with 4.0, Baleal-Norte was next with 3.58, Consolação follow it with 3.08, and Gamboa was the last with 1.57. Except for the last site, all the others fall inside the ranged by other studies for the European coast [16], which varied from 2.53 to Britain and Ireland, 4.09 to the Basque Coast, 3.57 to North Coast of Portugal, and 3.71 to the Atlantic Coast of Morocco. Concerning the Feldmann ratio - R/P [27], a similar situation was observed, with the highest values registered for Portinho da Areia do Norte with 3.3, next was Baleal-Norte with 3.0, Consolação achieved 2.54, and Gamboa was 1.21. The tendency here (except for Gamboa site) was to be next the values obtained in other studies for the European coasts [16], which varied from 1.87 to Britain and Ireland, 3.31 to the Basque Coast, 2.86 to North Coast of Portugal, and 2.93 to the Atlantic Coast of Morocco.

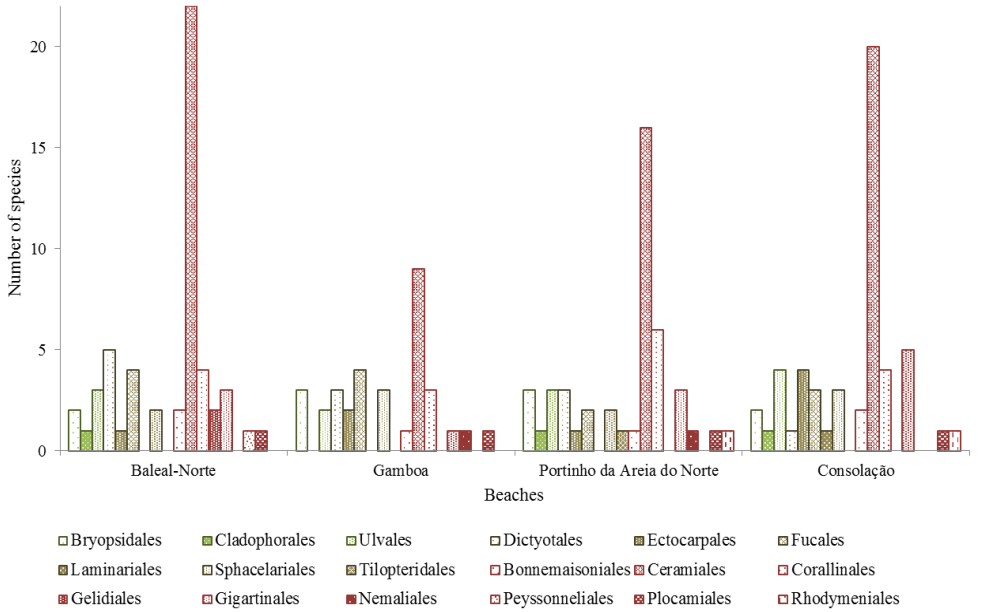


Figure 2. Total number of species per order on each beach: Baleal-Norte; Gamboa; Portinho da Areia do Norte; and Consolação.

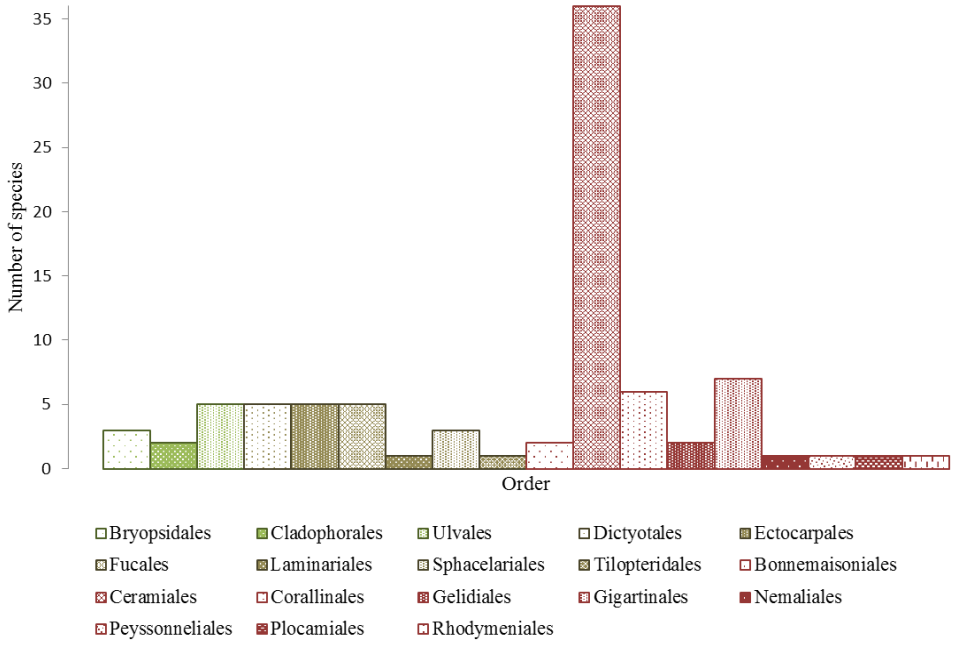


Figure 3. Total number of species per order in the four beaches of the municipality of Peniche.

Despite the geographical proximity of study sites, the macroalgal communities were relatively diverse along the sampled space. This was pretty much evident when the community was analysed based on the taxonomic composition present at different sites. Macroalgae presence/absence data were analysed through a non-metric Multidimensional Scaling (n-MDS), with Bray Curtis index as similarity measure (PRIMER 6 + PERMANOVA© software) [42, 43], and produced an ordination of sites through a virtual space where larger distances meant also more differences between pairs of shores. Ordination results showed that, independently of the variation found between the different taxonomic groups, the taxonomic composition present was more similar between Gamboa, Portinho da Areia do Norte and Baleal-Norte, then in comparison to Consolação (Figure 4). The last site was not grouped together with the other three due to the slightly lower affinity of the fourth site existing on taxonomic compositions. Although the number of species registered on Gamboa site was considerably lower, 36 against more than 50 for the other sites, this was not sufficient to displace it from the northern shores' group. Consolação presented the second highest diversity but its list of species may be on the basis of the separation from the rest, with 8 species present in all other three sites except here (*Derbesia tenuissima*, *Dictyopteris polypodioides*, *Padina pavonica*, *Taonia atomaria*, *Sargassum vulgare*, *Acrosorium ciliolatum*, *Laurencia pyramidalis*, *Sphaerococcus coronopifolius*) and, in the opposite, 13 taxa being exclusive from this site (*Ulvaria obscura*, *Hincksia granulosa*, *H. hincksiae*, *Fucus spiralis*, *Laminaria ochroleuca*, *Aglaothamnion pseudobyssoides*, *Callithamnion tetragonum*, *Gayliella flacida*, *Cryptopleura ramosa*, *Osmundea pinnatifida*, *Pleonosporium borreri*, *Dilsea carnosa*, *Gigartina pistillata*).

From taxa absent from Consolação, 3 species (*Dictyopteris polypodioides*, *Laurencia pyramidalis*, *Sphaerococcus coronopifolius*) may be considered as Northern-cold species [16] or were not registered in southern locations during the first surveys [12, 13, 30], and from taxa exclusive form this site, eight species (*Ulvaria obscura*, *Fucus spiralis*, *Laminaria ochroleuca*, *Callithamnion tetragonum*, *Cryptopleura ramosa*, *Gigartina pistillata*, *Osmundea pinnatifida*, *Pleonosporium borreri*) are considered Southern-warm species [16] or were not registered for Portuguese northern coast in the first surveys made on the area [12, 13, 30].

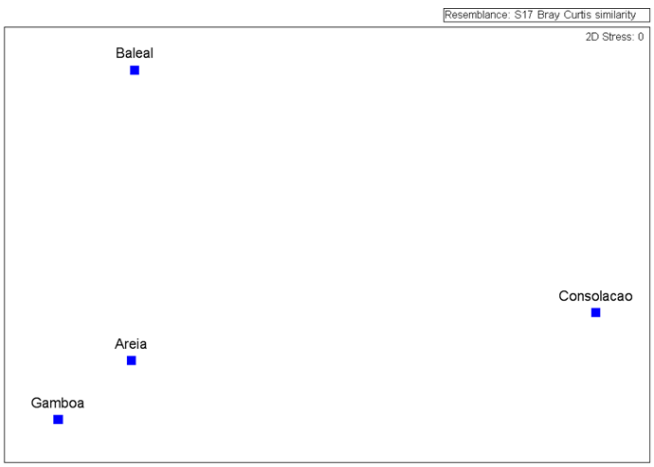


Figure 4. Plot of the four studied rocky shores. Non-metric Multidimensional Scaling (n-MDS), with Bray Curtis index as similarity measure, based on macroalgae presence/absence data.

Comparing the current data, collected in the present survey, with those historically reported in the literature it was found that 84% of the species recorded (73 species) have been already identified 50 years ago by Ardré [12, 13, 16, 30]. The species not identified at that time constitute mainly new species, described more recently, or small sized species that could be easily not seen in the field. These were 14 species (16%) distributed between Ochrophyta and Rhodophyta, respectively with 3 and 11 species. All species from Chlorophyta have been indicated before as present at the surveyed area. From those 14 species, 3 have been registered for northern and southern areas by the Ardré at that time. From the 14 species not present in historical records, 3 species (*Dictyota spiralis, Padina pavonica, Erythroglossum laciniatum*) have been observed by Ardré only at southern sites from the study area, and one species (*Aglaothamnion sepositum*) for northern areas. Additionally, from the 14 species not detected by Ardré but registered in the present survey, these have been meanwhile reported for the local flora listed by recent studies [16, 44].

ASSESSMENT OF THE ECOLOGICAL STATUS OF COASTAL WATER BODIES

The methodology developed in Portugal to assess the ecological quality of rocky shores fulfilling the WFD requirements is known as the Marine Macroalgae Assessment Tool (MarMAT). This tool includes seven different metrics: species richness, proportion of Chlorophyta, number of Rhodophyta, number of opportunists/ESG I (ratio), proportion of opportunists, shore description and coverage of opportunists [34]. The taxonomic composition data (presence/absence of species) registered on site is compared to a Reduced Taxa List (RTL), created for each different water typology based on macroalgae species present under different pressure levels (from naturally undisturbed to heavily impacted conditions). All MarMAT metrics, except the 'Proportion of Opportunists,' are calculated based on RTL structure.

More in detail, the first step is the *in situ* identification of *taxa* and its assignment (or not) into one of the entries in RTL. *Taxa* not assigned are not included in further calculations. Still in the field, is to do the shore description, filing in specific information into an appropriate classification table, and, at last, to calculate the percentage coverage of opportunistic macroalgae in relation to the total species coverage [34]. Species richness, proportion of Chlorophyta, number of Rhodophyta, proportion of opportunists and the ratio of the number of opportunists/Ecological State Group (ESG) I are calculated based on *taxa* in the RTL. The coverage of opportunists is usually estimated from 1-meter sq. photographed quadrats, having in mind the species considered as opportunists in the RTL [34]. The RTL considered in this study was developed by Gaspar et al. [35] for the A5 coastal waters Portuguese typology. The EQS is calculated by attributing scores to each of the MarMAT metrics (Table 2), according to the established in Neto et al. [34]. The metric 'Shore Description' functions as a correcting factor, aiming to reduce the influence of different rocky shores' morphology on the composition and abundance of macroalgae.

Table 2. Quality class scores attributed to different metrics integrated in MarMAT and the translation of EQR values into Ecological Quality Status (EQS)

Metrics	Bad	Poor	Moderate	Good	High
Species Richness[a]	0-6	7-13	14-20	21-27	28-54
Proportion of Chlorophyta	0.32-1	0.27-0.31	0.21-0.26	0.15-0.20	0-0.14
Number of Rhodophyta	0-3	4-8	9-12	13-17	18-33
Number of opportunists/ESG I	≥1.23	1.01-1.22	0.80-1.00	0.58-0.79	<0.58
Proportion of opportunists	0.59-1	0.47-0.58	0.35-0.46	0.23-0.34	0-0.22
Coverage of opportunists (%)[a]	72-100	59-71	4-58	33-45	0-32
Shore Descriptions	-	15-18	12-14	8-11	1-7
Corresponding Score to Metrics class	0	1	2	3	4
Sum of Scores	0-7	8-14	15-21	22-28	29-36
EQR	0-0.20	0.21-0.40	0.41-0.60	0.61-0.80	0.81-1
EQS	Bad	Poor	Moderate	Good	High

[a]factor of 2, counts twice in the metrics sum of scores calculation.

Applying the methodology MarMAT to macroalgae data it was possible to assess the Ecological Quality Status (EQS) of the four coastal rocky shores. MarMAT was applied to data collected in the end of June 2013 and using the RTL developed for the Portuguese typology A5. In Table 1 are shown species collected in this survey and the assignments into RTL. The site with the highest species richness was Baleal-Norte with 55 species, however, for the calculation methodology (after assignment to RTL) were recorded 29 *taxa*. The following site with higher number of species registered was Consolação with 53 species and 31 RTL entries, then Portinho da Areia do Norte with 42 species and 27 entries. The Gamboa site was the one recording the lowest species richness with only 36 species and 19 RTL entries. In all monitoring events, the number of Rhodophyta species was always higher than the number of Ochrophyta and Chlorophyta species. The last registered the lowest number of species for all study sites.

In Table 3 are discriminated the results and respective scores of different MarMAT metrics, as well as the final quality assessment of studied rocky shores. For the metric shore description Baleal-Norte station presented the highest results, with 11, Portinho da Areia do Norte beach had a score of 10 and Gamboa and Consolação had 9. Regarding the coverage of opportunistic algae, Baleal-Norte showed the lowest value with 21.1%, followed by Consolação and Gamboa stations with 26.3% and 32.6%, respectively. With 45.2% the Portinho da Areia do Norte beach was the one with the highest percentage cover of opportunistic algae. The ecological quality ratios (EQR) of Baleal-Norte, Gamboa, Portinho da Areia do Norte and Consolação were 0.97, 0.81, 0.83 and 0.97, respectively. Despite the EQR differences, results rated all sites with 'High' EQS, which confirms the general elevated quality of sites. Having in mind the macroalgal species registered 50 years ago, during the first intertidal surveys, and assuming that cover of opportunistic macroalgae should have been not worst then nowadays, the calculation of the EQS for 1970 achieved also High status (EQR = 0.97) for the area. From these results, similar to the today's best, it's possible to say that quality of the study area remained in High status since then.

Although Consolação site belongs to A6 the RTL for A5 was the one used. This was due to the geographical proximity of all sites, being also possible to validate the applicability of specific RTLs to sites located near the boundary limits but already outside the typology area.

Table 3. Assessment (MarMAT) results for studied sites (Peniche)

Metrics/Beaches		Baleal-Norte	Gamboa	Portinho da Areia do Norte	Consolação
Results	Shore Description	11	9	10	9
	Species Richness	29	19	27	31
	Number of opportunists/ESG I	0.4	0.4	0.4	0.4
	Proportion of Chlorophyta	0.14	0.11	0.11	0.10
	Number of Rhodophyta	18	9	17	18
	Proportion of opportunists	0.14	0.16	0.11	0.13
	Coverage of the opportunists (%)	21.1	32.6	45.2	26.3
Scores	Shore Description	3	3	3	3
	Species Richness (a)	4 (8)	2 (4)	3 (6)	4 (8)
	Number of opportunists/ESG I	4	4	4	4
	Proportion of Chlorophyta	4	4	4	4
	Number of Rhodophyta	4	2	3	4
	Proportion of opportunists	4	4	4	4
	Coverage of the opportunists (%)[a]	4 (8)	4 (8)	3 (6)	4 (8)
	Sum of the scores	35	29	30	35
	EQR	0.97	0.81	0.83	0.97
	EQS	High	High	High	High

[a] counted twice in the final sum.

CONCLUSION

In this survey, a total of 87 species of macroalgae were identified, from which 10 were Chlorophyta, 20 Ochrophyta and 57 Rhodophyta. The studied seashore with the greatest biodiversity was Baleal-Norte with 55macroalgal species, followed by Consolação with 53 Portinho da Areia do Norte with 50 and Gamboa with 36 species. Among the four sites, it was observed that the biodiversity in terms of taxonomic composition was not too different since a high number of repeated species was found among stations, in particular the large sized ones. Despite that, the taxonomic composition presented was sufficiently different to separate Consolação from the other three sites, which were grouped together in a compact way. The presence of *taxa* simultaneously in all sites constituting the larger group, but not in Consolação, and, by opposition, the exclusive presence of *taxa* only in Consolação, dictated the mentioned relative distribution of sites. The distribution of species through the macroalgae Phyla also places the study sites inside the expected variation for the European coast. The achieved results (except for Gamboa) were perfectly in line with results obtained from studies where the sampling effort was higher [16]. This indicates the survey was balanced and performed in a trustful way, which is important when delicate issues and conclusions are extracted from the collected data (e.g., WFD Ecological Quality Assessment).

Comparing the current data with those historically reported in the literature it is found that about 84% of the species recorded have been already identified 50 years ago by Ardré [12, 13, 16, 30], and all the remain species were meanwhile identified by other researchers so no species were added as a new register by the present survey.

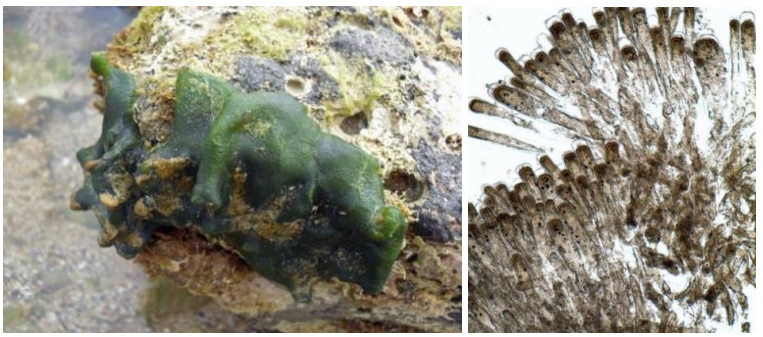
The ecological quality status of Baleal-Norte, Gamboa, Portinho da Areia do Norte and Consolação reached ‘High,’ thus confirming MarMAT’s sensitivity in the assessment of ecological quality of coastal waters. The quality assessment reporting to data collected in 1970 allowed to confirm the high quality of this coastal area, where the High status was achieved for the former survey as well as for the present one.

ACKNOWLEDGMENTS

This work had the support of Fundação para a Ciência e Tecnologia (FCT), through the strategic project UID/MAR/04292/2013 granted to MARE. This work had also the support from the European Union through EASME Blue Labs project AMALIA - Algae-to-MArket Lab IdeAs (EASME/EMFF/2016/1.2.1.4/03/SI2.750419). RG was financed by the programs POPH (Portuguese Operational Human Potential Program) and QREN (Portuguese National Strategic Reference Framework) (FSE and national funds of MEC) through a PhD grant (SFRH/BD/82014/2011). The authors also thank Ignacio Bárbara for his valuable help in the identification of some species of macroalgae and for reviewing this chapter.

ANNEX I. MACROALGAE BIODIVERSITY IN IMAGES AND THEIR CHARACTERISTICS

Chlorophyta – Green Algae



(A) (B)

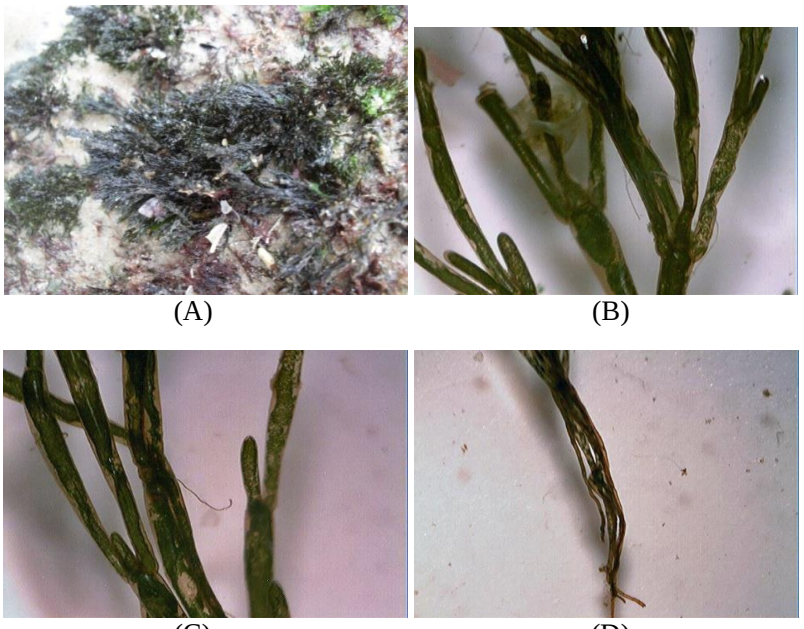
Figure 5. *Codium adhaerens*: (A) Spongy thallus, green light, prostrate, irregularly shaped and presenting with the appearance of a plane carpet firmly fixed to the substrate; (B) cross section of the thallus, showing the narrow and elongated utricles (L.M.100X).

(A) (B)

Figure 6. *Codium tomentosum*: (A) A small green alga (up to 30 cm long) with a dichotomously branched, cylindrical frond; the frond is solid and spongy with a felt-like touch; (B) cross section of the thallus, showing the non-mucronate utricles (L.M. 400X).

(A) (B)

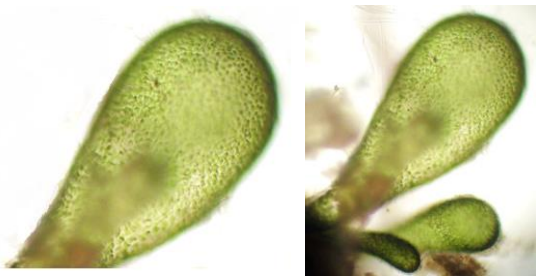
Figure 7. *Derbesia tenuissima*: (A) Thalli bright green, 10-60 mm tall, on rock or on other plants, threadlike, sparsely branched, transverse septa absent; (B) specimens in its natural habitat.



(A) (B)

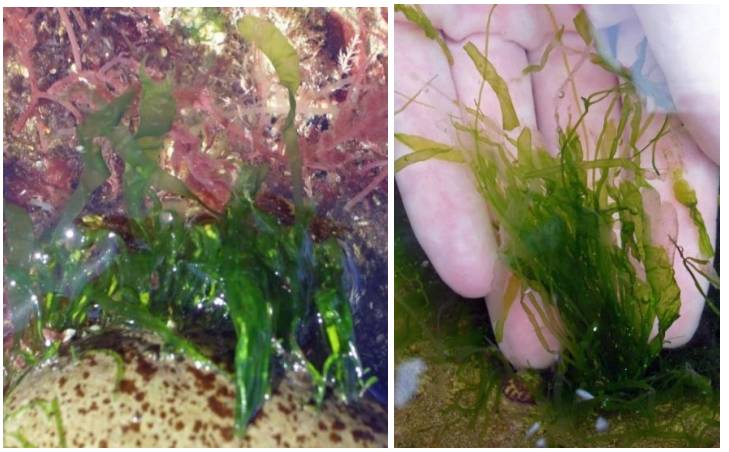
(C) (D)

Figure 8. *Cladophora prolifera*: (A) Unattached or basally attached coarse filaments that are usually less than 0.5 mm wide and 3-5 cm long; (B) the filaments are formed of a single row of often swollen cells; if attached then by a discoid base or by rhizoidal outgrowths (S.M.15X); (C) ramifications with rounded apexes (S.M.15X); (D) basal part with rhizoids (S.M.15X).



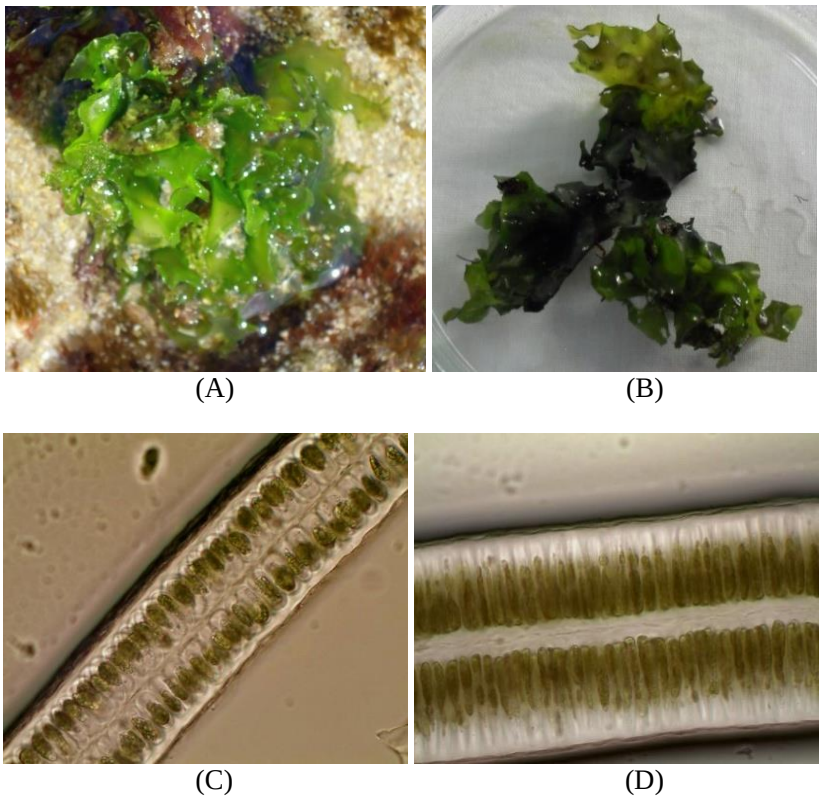
(A) (B)

Figure 9. *Valonia utricularis*: (A) Thallus, translucent light- to dark-green, primarily consisting of a large (up to 5 mm thick and 20 mm long) bladder- or cluc- to hose-like "cell," branching at the base rhizoidally. Later due to outgrowths of this cell cylindrical-clavate branches, often contorted and almost gapless densely packed, thus forming intertwined erect stands [32] (L.M. 400X); (B) vesicles forming tufts (L.M. 400X).



(A) (B)

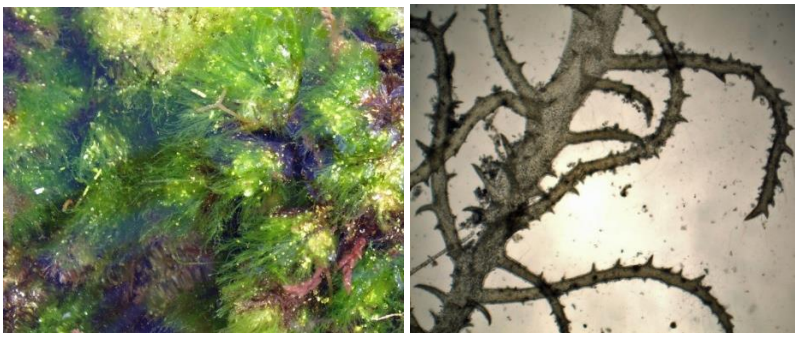
Figure 10. *Ulva compressa*: (A-B) This species can have one of two different growth forms: the first is a flat, narrow sheet with ruffled edges; the second form (often referred to as *Enteromorpha compressa*) is a hollow tube of tissue, rounded at the top. In both forms the sheets of tissue are very thin, in fact they are exactly one cell thick; several blades or tubes arise from a common attachment point and can grow up to 200 mm long.



(A) (B)

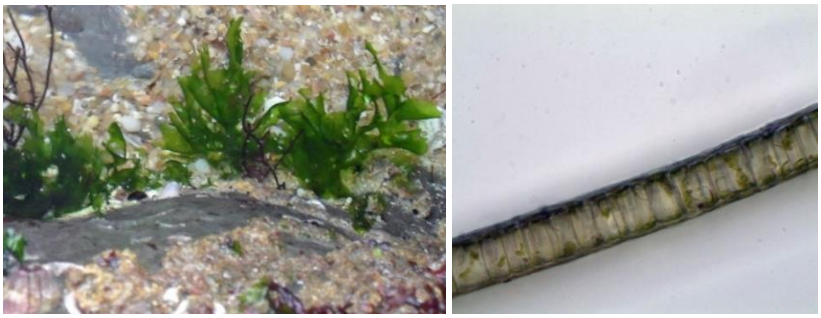
(C) (D)

Figure 11. (A) General appearance of *Ulva rigida* thallus; (B) general appearance of *Ulva rigida* var. *fimbriata* thallus; (C) cross section of *U. rigida*'s thallus, in which it is possible to see the rectangular shape of the cells (L.M. 100X); (D) cross section of *U. rigida* var. *fimbriata* thallus, in which it is possible to see the conical shape of the cells (L.M. 400X).



(A) (B)

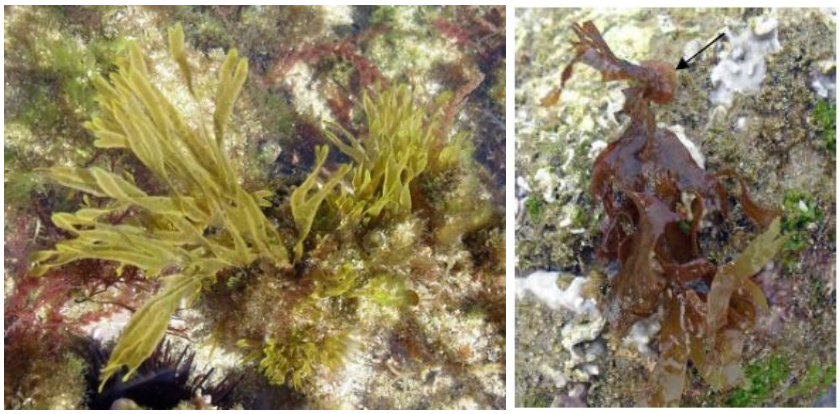
Figure 12. *Ulva clathrata*: (A) This species forms tufts, bright green, composed of branched axes, which can reach several centimeters long (20-30 cm); the main axis and branches are covered with a very characteristic (B) conical branchlets (L.M. 100X).



(A) (B)

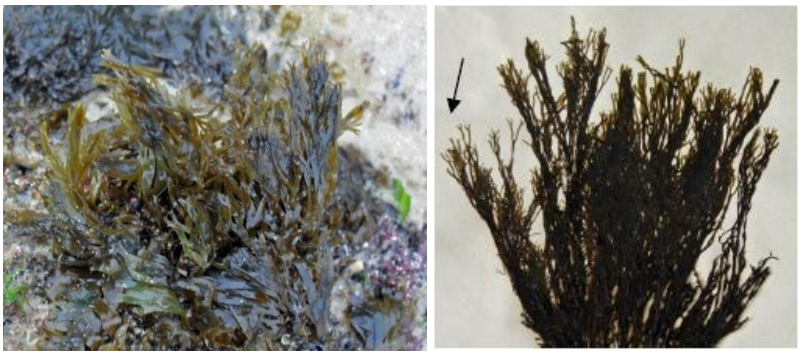
Figure 13. *Ulvaria obscura*: (A) Thallus widely-bladed, monostromatic (B), similar to that of laminar *Ulva*, turning brown on dying (L.M. 100X).

Ochrophyta (Phaeophyceae) – Brown Algae



(A) (B)

Figure 14. *Dictyota dichotoma*: (A) Thallus flat, homogenous yellow-brown to darker brown, with fairly regular dichotomous branches with parallel sides to 30 cm long, the tips usually bifid; branches 3 to 12 mm wide, membranous, without a mid-rib; (B) fixing disk (arrow).



(A) (B)

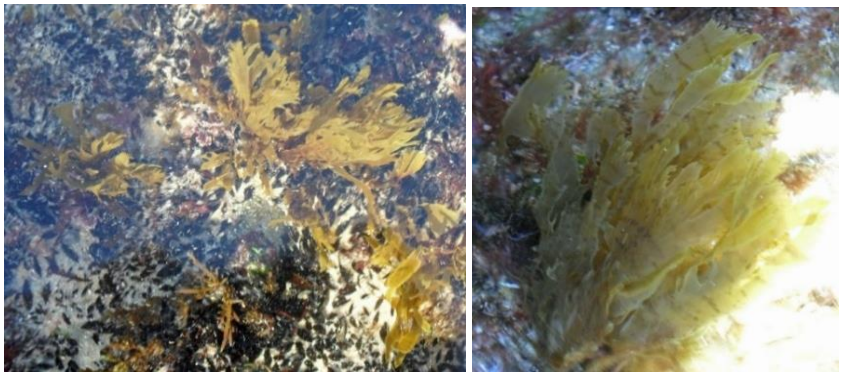
Figure 15. *Dictyota spiralis*: (A) Thallus leathery, 5-15 cm high, reddish-brown, attached by decumbent stolons; branching dichotomous, often irregular; some segments cease to ramify, others grow closely by 3-4 in tuft; segments near-linear, slightly wider at nodes, basal parts often narrowed; (B) apices broad and blunt (arrow) [30].

(A) (B)

Figure 16. *Dictyopteris polypodioides*: (A) Thallus flat and leaf-like, to 300 mm long and 20-30 mm broad; fronds olive to yellow-brown, translucent, and ± regularly dichotomously forked with a prominent midrib extending to the apices; margins sometimes split to the midrib (B) initially with an unpleasant smell shortly after collection, and degenerating quickly [31].

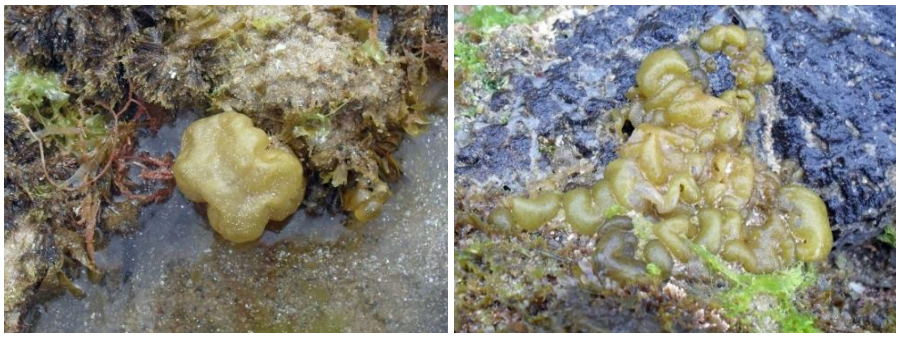
(A) (B)

Figure 17. *Padina pavonica*: (A) The frond is thin and leafy, flattish and entire when young, but often concave, or almost funnel shaped in mature specimens, with a laciniate or irregularly lobed margin; (B) the inner (or upper) surface is covered in a thin coating of slime, and the outer (or lower) surface is banded with zones of light brown, dark brown and olive green.



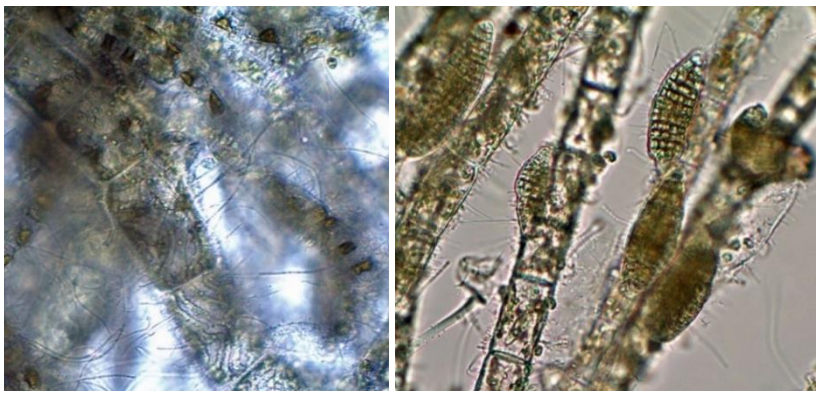
(A) (B)

Figure 18. *Taonia atomaria*: (A) Thalli erect, attached by matted, branched rhizoids, up to 30 cm long, complanate, flabellate or lacerate with many elongate, cuneate branches, 0.5-6 cm broad, often tapering to the apex; (B) thallus 2 cells thick near apex increasing to 5-7 cells thick towards the thallus base, not arranged in rows in transverse section.



(A) (B)

Figure 19. *Colpomenia peregrina* (exotic species - non-native): (A) Sometimes regularly spherical (B) or more or less irregular outline (*Colpomenia sinuosa*), yellowish-brown color, fixed to the substrate by filamentous rhizoids.



(A) (B)

Figure 20. *Ectocarpus fasciculatus*: (A) The thallus consists of profusely branched uniseriate filaments; the cells of the main filaments are taller than broad and have tape-shaped plast (L.M. 400X); (B) lateral branches containing cylindrical plurilocular structures (L.M. 400X).

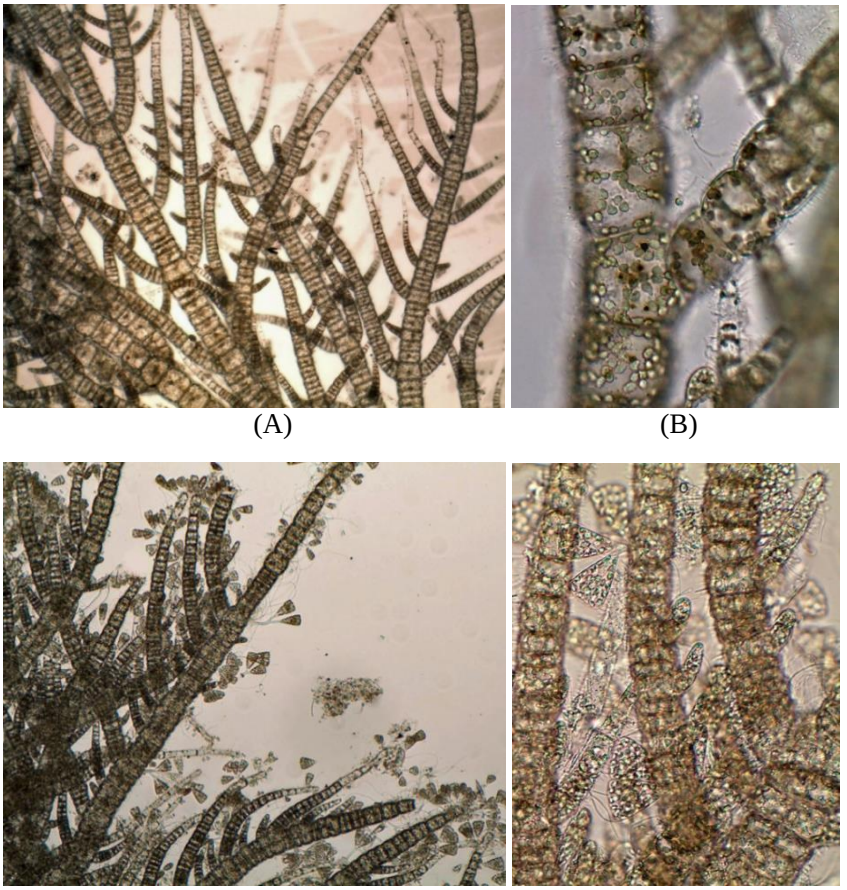


Figure 21. *Hincksia granulosa*: (A) Uniseriate filamentous thalli, erect and with opposing branches that originating several long lateral branches (L.M. 100X); (B) the cells are longer than large, with numerous discoid plasts (L.M. 400X); *Hincksia hincksiae*: (C) The main axes branch out, unilaterally, and support several plurilocular sporangia (L.M. 100X); (D) the cells have many discoid plasts (L.M. 400X).

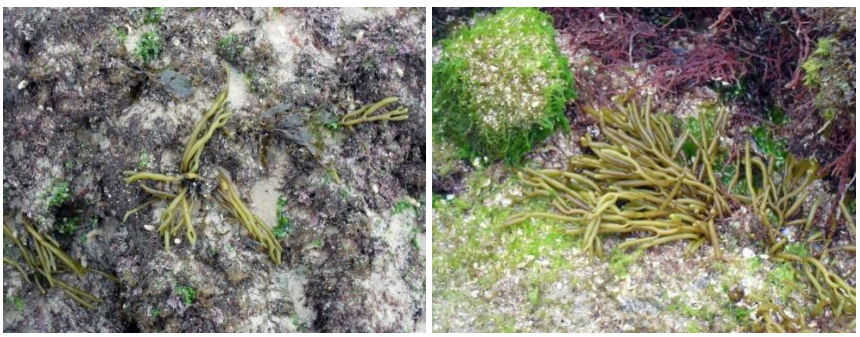


Figure 22. *Bifurcaria bifurcata*: (A-B) Up to 30 cm in length; olive-yellow in color, but much darker when dry; holdfast expanded and knobbly; frond cylindrical, unbranched near base then branching dichotomously; elongate reproductive bodies present at ends of branches; rounded air bladders sometimes present.

(A) (B)

Figure 23. *Cystoseira baccata*: (A) Plants usually solitary, 1 m or more in length, attached by a thick, conical attaching disk; axis simple or branched, up to 1 m in length, flattened, about 1 x 0.4 cm in transverse section; apex smooth and surrounded during periods of active growth by incurred young laterals.; lateral branch systems distichous, alternate, radially symmetrical, profusely branched in a repeatedly pinnate fashion and bearing sparse, filiform, occasionally bifurcate appendages on the branches of higher orders [32]; *Cystoseira nodicaulis:* (B) in water, the thallus has a yellowish-brown coloration.

(A) (B)

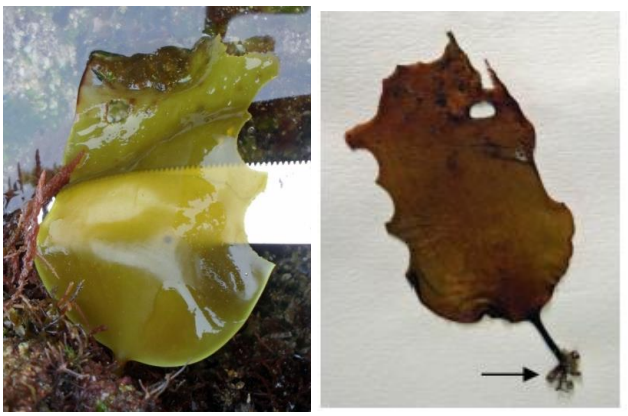
Figure 24. *Cystoseira tamariscifolia*: (A-B) bushy seaweed, up to 60 cm in length but usually 30-45 cm; it has a cylindrical frond with irregularly branches; olive green in color, almost black when dry; when the plant is seen underwater it has a blue-green iridescence.

(A) (B)

Figure 25. *Fucus spiralis*: (A) The fronds are usually easily recognizable by the flattened, twisted, dichotomously branched thallus, lacking bladders, and the large, oval receptacles at the frond tips, each receptacle being surrounded by a narrow rim of vegetative frond. Nevertheless, younger plants are not always so easy to identify, and even mature plants can be confused with other *Fucus* species; (B) the thallus is composed of a blade with midrib and receptacles in the apical zone.

(A) (B)

Figure 26. *Sargassum vulgare*: (A-B) Much branched, bushy plants that grow to 50 cm tall and are attached by a discoid holdfast; thalli pseudo-parenchymatous; primary and secondary branches are cylindrical and bear lanceolate foliar branches (4 cm long, 3 mm wide) with serrate margins; bladders are formed on short pedicels [33].



(A) (B)

Figure 27. *Laminaria ochroleuca*: (A-B) Young specimen; glossy, yellow-brown kelp that is prevalent along the intertidal zones; this kelp is quite conspicuous as it grows quite large under the right conditions; the maximum length recorded is 4 m long, but this length is rarely attained and occurs only in specific areas; under normal conditions *L. ochroleuca* is more likely to reach a maximum length of about 2 m; *L. ochroleuca* has a large heavy holdfast made up of thick haptera or rhizoids (up to 18 cm in diam.) that supports the plant and anchors (arrow) it to rocks.

(A) (B)

Figure 28. *Cladostephus spongiosus*: (A-B) Fairly stiffly branched fronds growing from a crust-like discoid holdfast (arrow), covered with small branchlets arranged in whorls; maximum length usually about 15 cm.

(A) (B)

Figure 29. *Halopteris scoparia*: (A) Dark brown algae that forms beautiful fluffy clumps in shallow rocky-bottomed water; growing only up to 15 cm in length, *H. scoparia* has a main axis with alternate plumed (B), S.M. 20X) branches which are more or less fan-shaped when flat, though when buoyed up by water they form inverted cone-shaped tufts with a very delicate appearance due to the many filamentous branches.

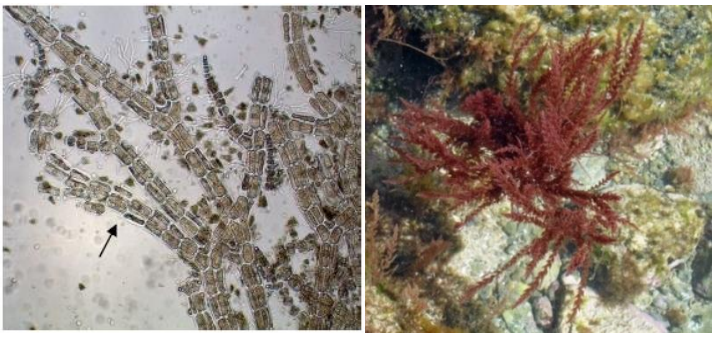
(A) (B)

Figure 30. *Sphacelaria rigidula*: (A) Filamentous cushion-like tufts, also occurring as scattered filaments in mixed turf communities, 5-10 mm high, medium to dark brown; branching irregular, sparse (L.M. 40X); (B) the filaments are cylindrical and emerge laterally the propagules, which are also cylindrical and trifurcated (L.M. 100X).

(A) (B)

Figure 31. *Saccorhiza polyschides*: (A) Species with a distinctive large warty holdfast and a flattened stipe with a frilly margin (in adult specimens); the stipe is twisted at the base and widens to form a large flat lamina, which is divided into ribbon-like sections; (B) presence of a large bulbous holdfast with warty appearance.

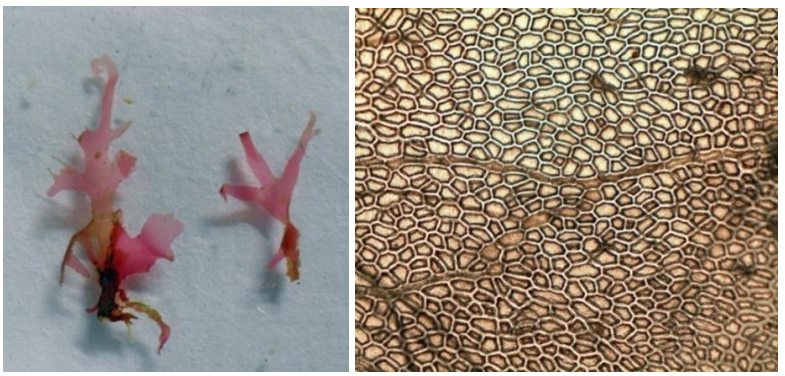
Rodophyta – Red Algae



(A) (B)

(C) (D)

Figure 32. (A) Morphology of the *Falkenbergia rufolanosa* (tetrasporoporic phase of *Asparagopsis armata*) (exotic species – non-native) thallus, where it can be seen that it is composed of slightly branched filamentous axes and the filaments composed of an axial cell and three periaxial cells (L.M. 400X); (B) general appearance of the feathery thallus of *A. armata*; (C-D) spiny, harpoon-shaped (arrow) branches some cm in length.



(A) (B)

Figure 33. *Acrosorium ciliolatum*: (A) Flattened, membranous, deep-red fronds, 30-200 mm long; frond deeply divided into linear-lanceolate, irregularly branched segments, often terminating in hooks; (B) the frond is traversed by network of microscopic veins, but macroscopic veins are absent (L.M. 100X).

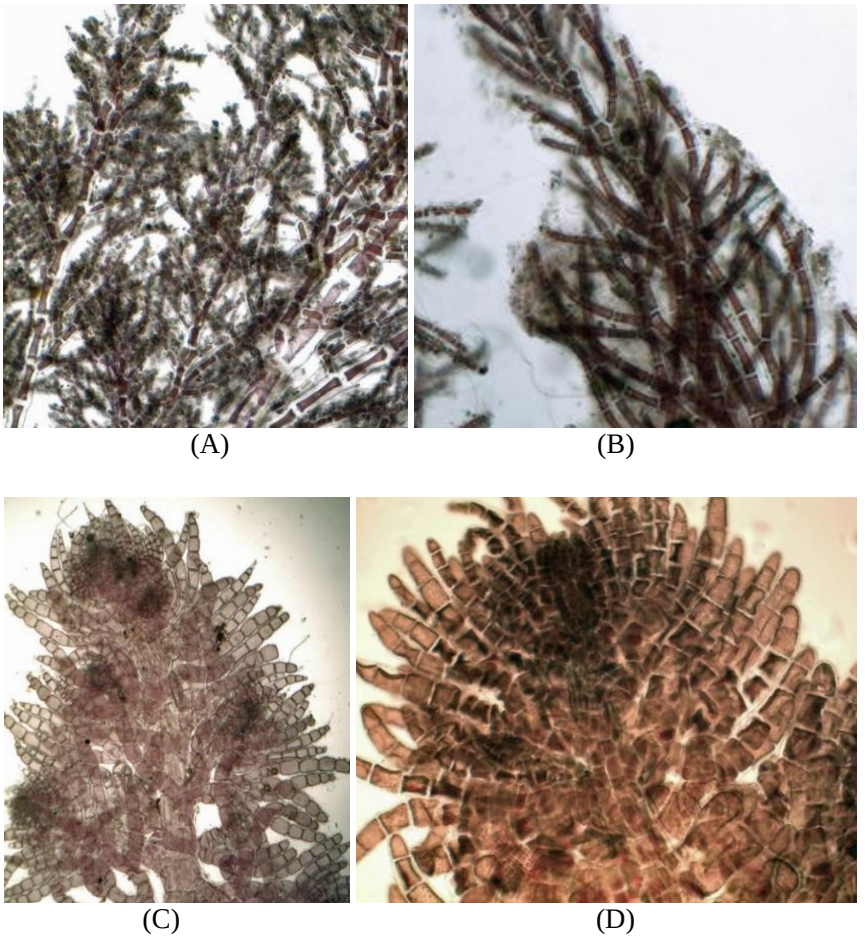


Figure 34. *Aglaothamnion pseudobyssoides*: (A) Uniseriate filaments, uncropped and with alternate ramifications (L.M. 100X); (B) the apical cells of the lateral branches have the rounded ends (L.M. 400X); *Aglaothamnion sepositum*: (C) Thick, corticated, filamentous filament with alternate ramifications (L.M. 40X); (D) the apical cells of the lateral branches assume a false conical shape (L.M. 100X).

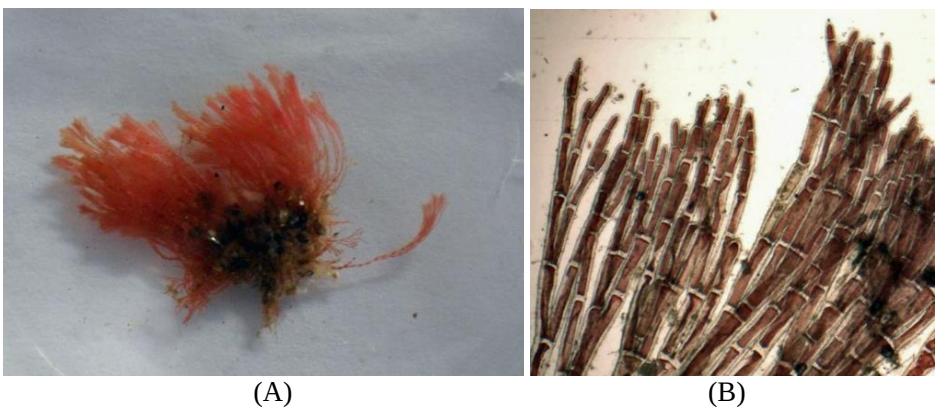
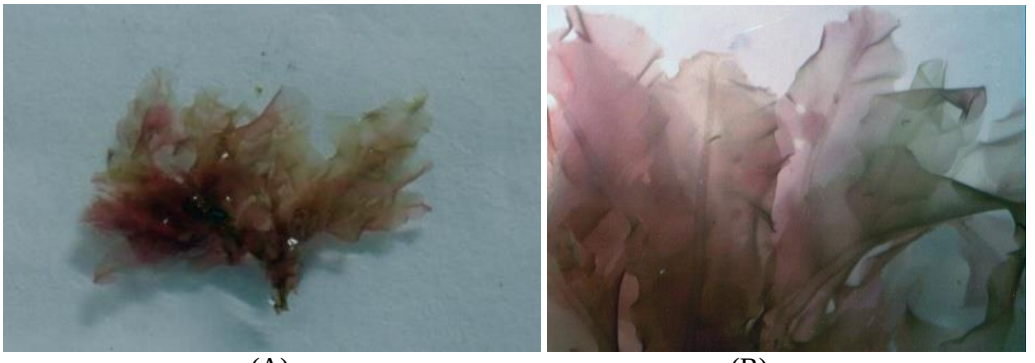


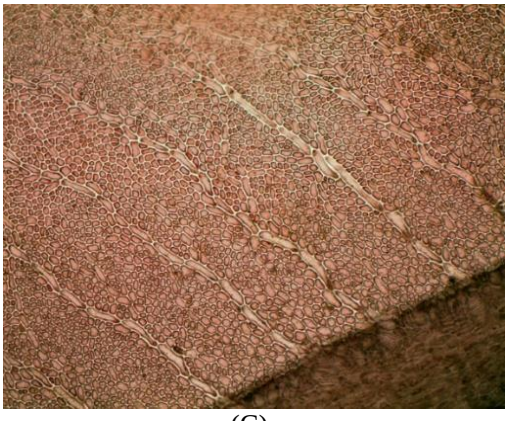
Figure 35. *Anotrichium furcellatum*: (A) Small reddish-pink filamentous tuft; (B) Thin filaments, uniseriate, erect and composed of apical cells with a conical shape (M.O. 40X).

(A) (B)

Figure 36. *Antithamnion densum*: A) Uniseriate filamentous thallus with opposing branches (L.M. 100X); (B) the plumed clusters are mostly arranged at the same side (L.M. 100X).

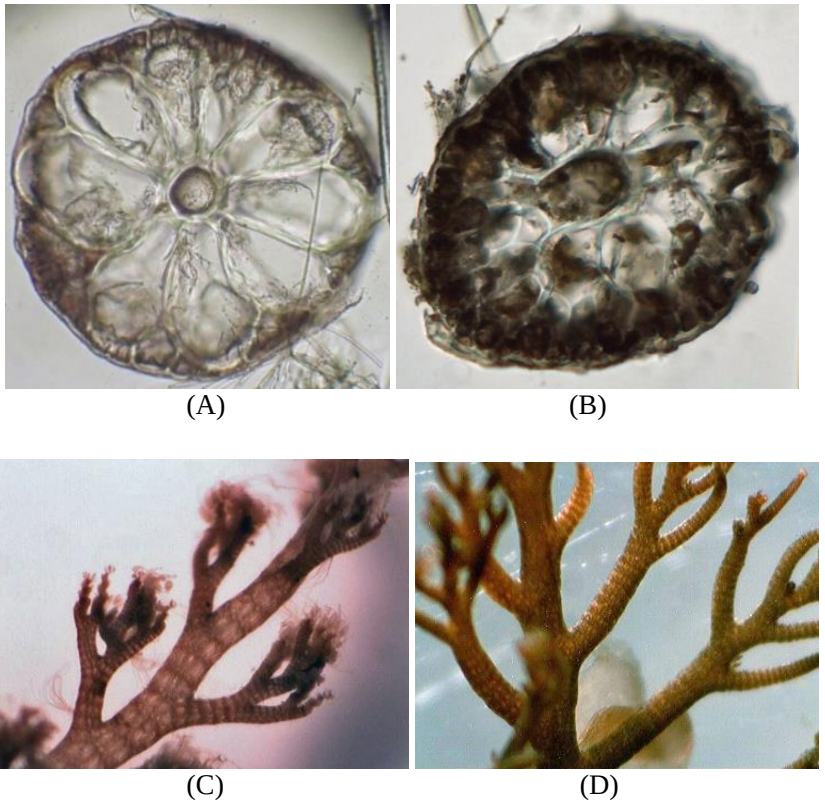


(A) (B)



(C)

Figure 37. *Apoglossum ruscifolium*: (A) Membranous, tufted, bright red fronds, to 100 mm long; (B) frond with conspicuous midrib and wavy margin, to 6 mm wide, repeatedly branched from midrib, apices blunt (S.M.20X); (C) numerous microscopic veins, at wide angle from midrib (L.M. 100X).



(A) (B)

(C) (D)

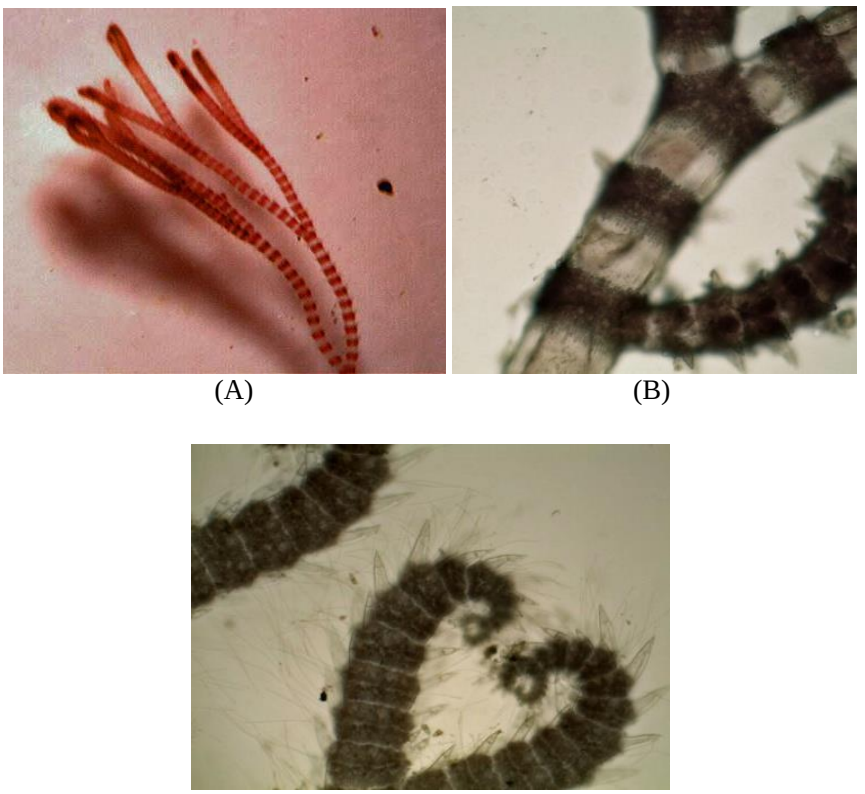
Figure 38. (A) Transverse section of the main axis of *Boergeseniella fruticulosa*, formed by nine periaxial cells, which are surrounded by a reduced cortical cell layer (L.M. 100X); (B) Transverse section of the main axis of *Boergeseniella thuyoides*, formed by ten periaxial cells, which are coated by several layers of cortical cells (L.M. 100X); (C) *Boergeseniella fruticulosa* is composed of thin transverse bands of cortical cells (S.M.35X); (D) *Boergeseniella thuyoides* has transverse bands of thicker cortical cells (S.M.35X).

(A) (B)

Figure 39. *Bornetia secundiflora*: (A) Dark red in color, firm and rigid when fresh; the thallus is 5-20 cm high when erect, fan-shaped with blunt tips (apices), much branched and tufted, with branches often curved over; the plant has a jelly-like texture; branches are sparse at the base becoming denser towards the apices; (B) each filament consists of a single row of cells that are arranged end-to-end (S.M.20X).

(A) (B)

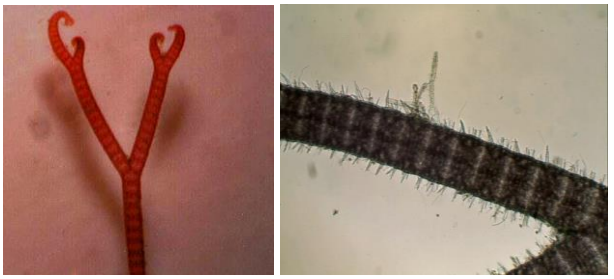
Figure 40. *Callithamnion tetragonum*: (A) Brownish-red fronds, to 50 mm long; uniseriate filaments, corticated below, repeatedly branched with simple, alternate branches (L.M. 40X); (B) ultimate branchlets densely clothed with tufts of alternate ramuli, corymbosely, incurved, attenuate at base and apex (L.M. 100X).



(A) (B)

(C)

Figure 41. *Ceramium ciliatum*: (A) Uniaxial, cylindrical, erect thalli, corticated in transverse bands, in a discontinuous way (S.M.45X); (B) the filaments at the base are comprised of cells wider than tall (between nodes) that are surrounded by transverse bands of cortical cells (nodes) (L.M. 100X); (C) the branches apexes are bifurcated and curved (L.M. 100X).



(A) (B)

(C)

Figure 42. *Ceramium echionotum*: (A) Rough, densely tufted, purplish red fronds, to 150 mm long, repeatedly dichotomously branched, axils wide, apices strongly hooked inwards filaments of almost uniform diameter throughout.(S.M.45X); (B) Articulations corticated at nodes, 3-4 times as long as broad in lower parts, very short distally, with numerous, irregularly distributed, colorless, unicellular, needle-like spines on corticating bands (L.M. 100X); (C) the apexes are wrapped in fork and transverse bands of cortical cells emerge the single-celled spicules (L.M. 100X).

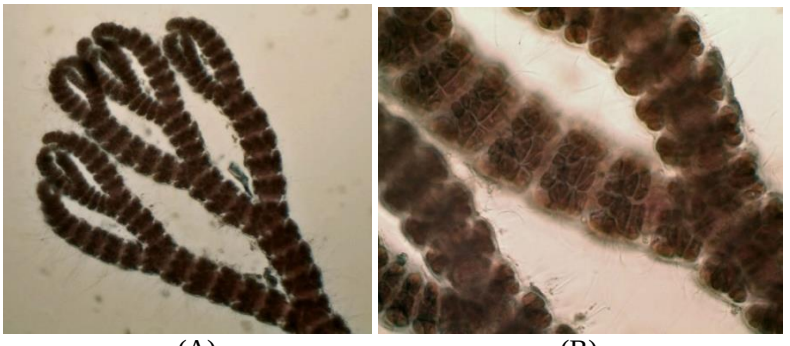
(A) (B)

(C)

Figure 43. *Ceramium pallidum*: (A) Uniaxial, cylindrical, erect thalli, corticated in transverse bands, in a discontinuous way (S.M.40X); (B) the filaments have an alternating branching and are constituted by transverse bands of cortical cells that are intercalated by transparent cells (S.M.35X); (C) bifurcated and slightly curved apexes (S.M.45X).

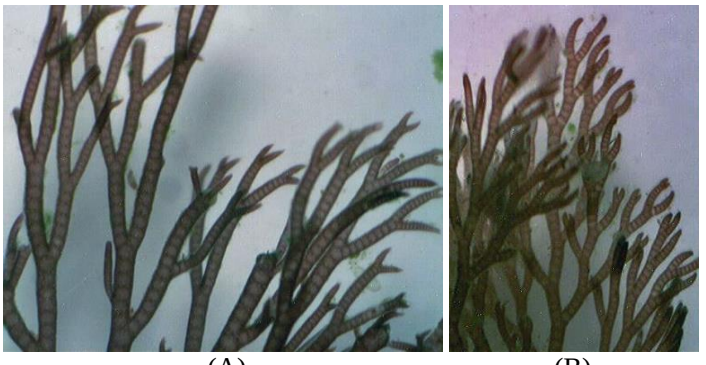
(A) (B)

Figure 44. *Ceramium pallidum*: (A) The filaments are composed by transparent cells which assume various shapes and sizes and which are arranged alternately, or not, between the dense transverse bands of cortical cells (L.M. 100X); (B) the branches apexes are bifurcated and curved (L.M. 100X).



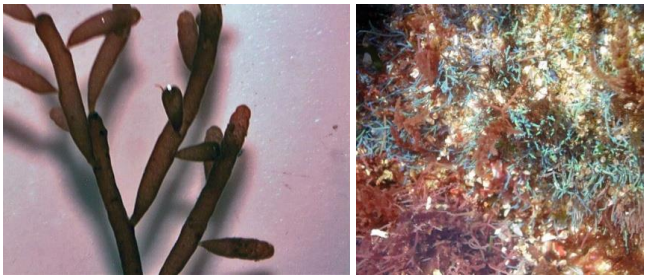
(A) (B)

Figure 45. *Ceramium tenuicorne*: (A) Uniaxial, cylindrical, erect and dark red thallus, in which the branches apexes are bifurcated and curved (L.M. 100X); (B) the filaments are composed of transverse bands of low and thick cortical cells (nodes), which are interspersed by small nodes (L.M. 400X).



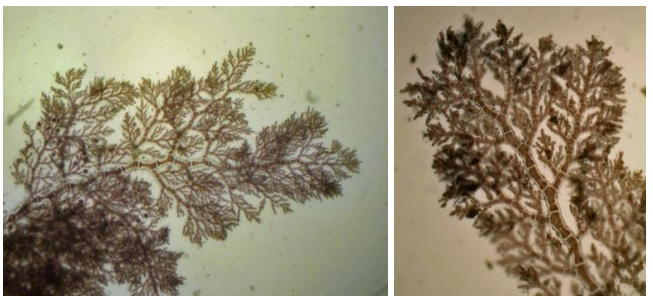
(A) (B)

Figure 46. *Ceramium virgatum*: (A) Small red seaweed growing up to 30 cm tall; it has a filamentous frond that is irregularly and dichotomously branched, (S.M.45X); (B) with the branches narrowing towards pincer-like tips (S.M.40X); these little seaweeds are often found growing epiphytically in association with *Fucus* spp., such as *Fucus vesiculosus*.



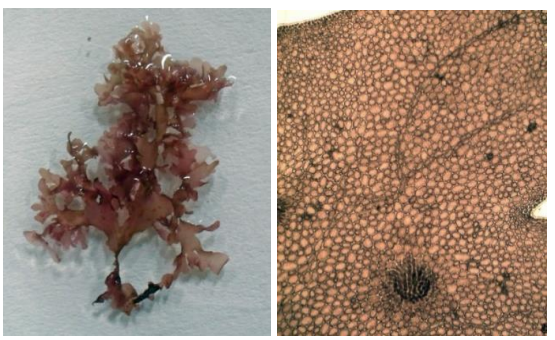
(A) (B)

Figure 47. *Chondria coerulescens*: (A) The fronds are flexible and cartilaginous in texture, turning black when dry (S.M.30X); (B) in water this seaweed has bluish or yellowish fronds with blue iridescence.



(A) (B)

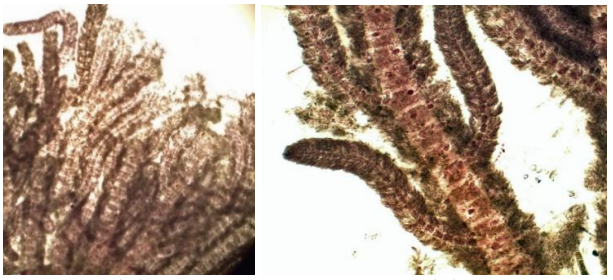
Figure 48. *Compsothamnion thuyoides*: (A) Uniseriate thallus, erect and with alternate ramifications (L.M. 40X); (B) the lateral branches are, in turn, branched alternately giving rise to branches of higher order which are also branched (L.M. 100X).



(A) (B)

(C)

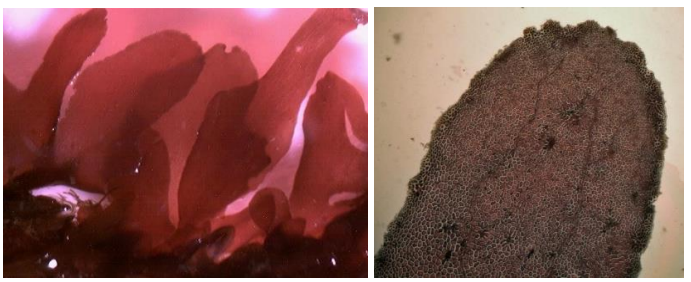
Figure 49. *Cryptopleura ramosa*: (A) Thin, membranous, brownish red fronds, to 200 mm long; frond ± dichotomously divided, becoming irregular in upper parts; (B) the blades have microscopic veins that bifurcate and recombine at various points (L.M. 100X); (C) surface view of cystocarp (L.M. 400X).



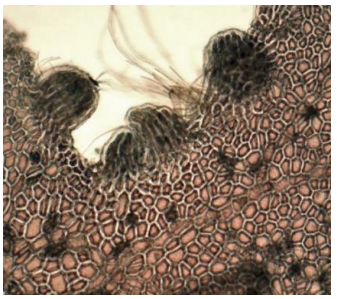
(A) (B)

(C)

Figure 50. *Crouania attenuata*: (A) Small pompous tufts of reddish-pink color (L.M. 40X); (B) The tufts are constituted by protruding main axes that ramify of irregular and alternated form, giving lateral branches (L.M. 100X); (C) the lateral branches are formed by quadratic cells that are covered by curved and mucronate ramuli, vertically displaced (L.M. 400X).

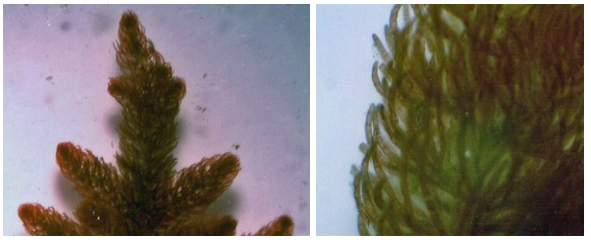


(A) (B)



(C)

Figure 51. *Erythroglossum laciniatum*: (A) Thallus a short stipe, erect, consisting of one or more blades to 16 cm long and 15 or 20 cm wide, fan-shaped and divided into lobes with pointed apices (S.M.15X); (B) veins macroscopic, also fine veins (M.O. 40X); (C) lateral view of cystocarp on blade edges (L.M. 100X).



(A) (B)

Figure 52. *Halurus equisetifolius*: (A) Erect plants consisting of up to 7 irregularly branched main axes growing 6 to 22.5 cm high and 2 to 3 mm wide, resembling a horsetail fern (S.M.15X); (B) the main axes branch to 4 orders and are clothed (either sparsely or densely) in whorls of between 5 and 8 short incurved branchlets which divide di- to trichotomously; the branchlets are 1.4 to 2.4 mm long and consist of 4 to 7 cells of which the terminal one is mucronate (L.B. 30X).

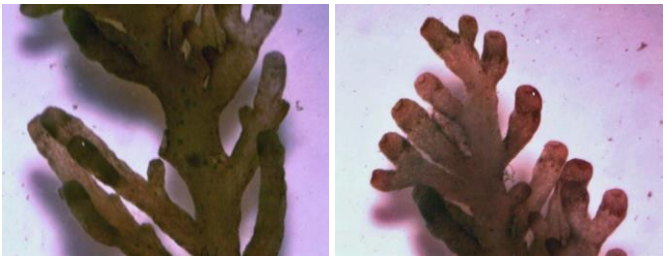
(A) (B)

Figure 53. *Herposiphonia tenella*: (A) Plants coarse and brownish red; thalli terete, having a branching pattern of three determinate axes followed by one lateral indeterminate (d/d/d/i pattern); prostate axes 120-150 µm diam., segments 0.8-1.3 diam. long., 7-9 pericentral cells (L.M. 100X); (B) erect determinate axes up to 1 mm in height, 70-80 µm diam., 8-13 segments with 0.81.0 diam. long, 7-8 pericentral cells; apex blunt with an inconspicuous apical cell. Vegetative trichoblasts rudimentary, 1-3 at the tips of determinate axes in successive segments, pseudo-dichotomously divided 24 times (L.M. 400X) [34].

(A) (B)

(C)

Figure 54. *Hypoglossum hypoglossoides*: (A) Membranous, rose-pink fronds, 20-300 mm long, arising from a discoid base; frond linear-lanceolate, with well-marked midrib and thin membranous margins, 1-8 mm wide, repeatedly branched irregularly from midrib (M.O. 40X); (B-C) fronds with pointed apices, margins without microscopic veins, monostromatic except in midribs (M.O. 400X).

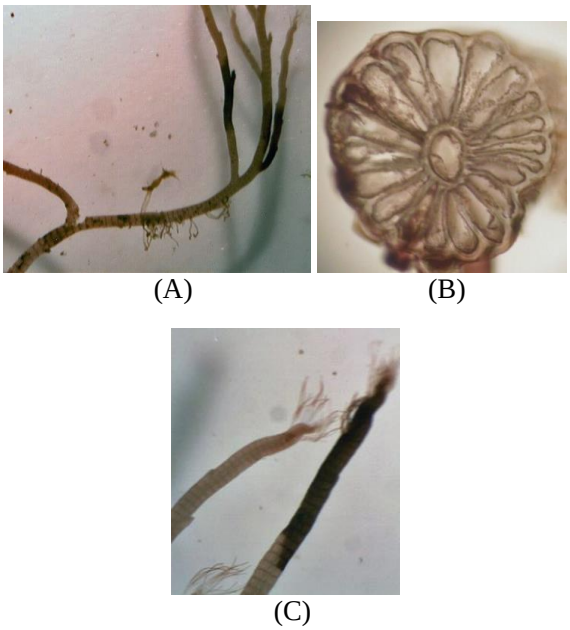


(A) (B)

Figure 55. *Laurencia pyramidalis*: (A) Globose tufts of brittle, cartilaginous, narrow, cylindrical, reddish brown to yellowish red fronds, 150 mm long, from small discoid base (S.M.15X); (B) axis simple, branches patent, often opposite, spirally arranged, shorter towards apex giving regular pyramidal outline; bifurcated tips and a central hole (S.M.15X).

(A) (B) (C)

Figure 56. *Leptosiphonia schousboei*: (A) Thallus composed by uniaxial axes, corticated and prostrate at the base, attached to the substrate through rhizoids (L.M. 100X); (B) secondary erect axes, polysifonate and presenting apical trichoblasts (L.M. 100X); (C) cross section of the thallus with 14 periaxial cells (L.M. 400X).



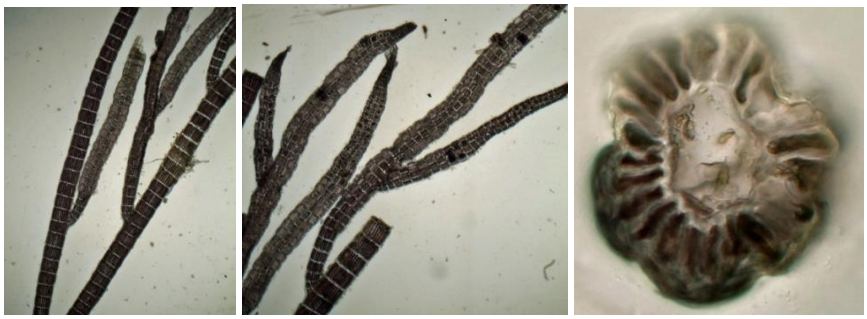
(A) (B)

(C)

Figure 57. *Lophosiphonia reptabunda*: (A) Thallus consisting of prostrated axes, from which depart secondary axes which have a curvature up to the apex (S.M.35X); (B) cross section of the thallus with 19 periaxial cells (L.M. 100X); C) secondary axes with tricoblasts arranged helically (S.M.45X).

(A) (B)

Figure 58. *Nitophyllum punctatum*: (A) Delicately membranous, rose-pink fronds with an elongate fan-shaped outline, margins distinctly frilly, to 40 mm or, exceptionally, to 1 m, sessile or shortly stipitate (<2 mm long); (B) frond veinless, undivided or deeply sub-dichotomously divided to the base; apices blunt or rounded, often ribbon-like; gametophyte plants form rounded spots to 5 mm in diameter whilst tetrasporphyte plants form characteristic elongated spots.

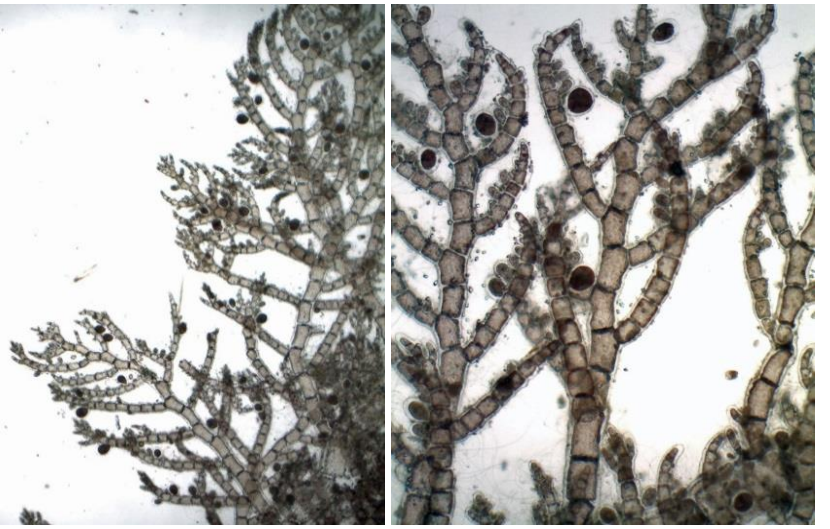


(A) (B) (C)

Figure 59. *Ophidocladus simpliciusculus*: (A) Branches erect simple or branched 1-2 times, with 16-23 periaxial cells (L.M. 40X); (B) from the branches arise tetrasporic secondary axes (L.M. 40X); (C) cross section of the thallus with 23 small periaxial cells surrounding a larger axial cell (M.O. 100X).

(A) (B)

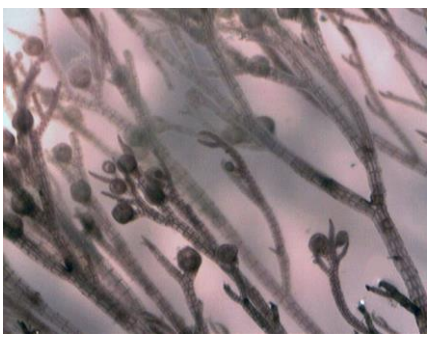
Figure 60. *Osmundea pinnatifida*: (A) Cartilaginous, usually markedly compressed, dark purple to pale yellow fronds, to 100 mm or more long, from discoid base; very variable in size and form; main axis usually simple; (B) branching alternate distichous, repeatedly pinnate; ultimate ramuli short, blunt (S.M.15X).



(A) (B)

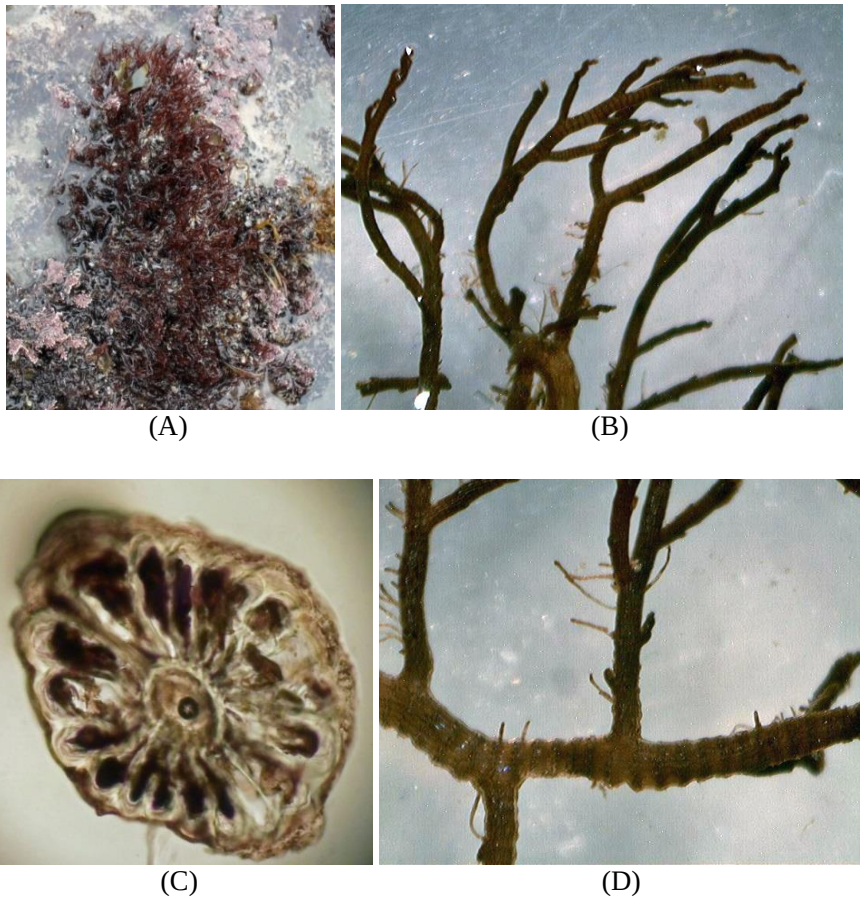
Figure 61. *Pleonosporium borreri*: (A) Erect thallus, filamentous, uncropped and with alternate ramifications (L.M. 40X); (B) the lateral branches also divide alternately giving rise to the cystocarps, which consists of small quadratic cells and rounded apices (L.M. 100X).

(A) (B)



(C)

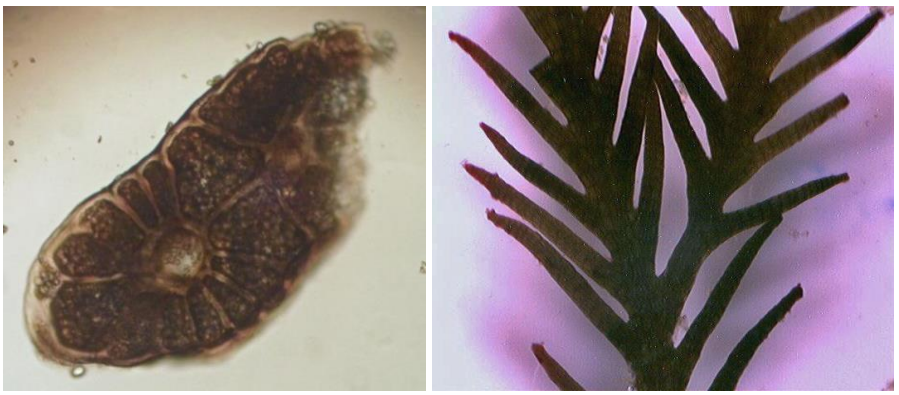
Figure 62. *Polysiphonia denudata*: (A) Small fronds reddish-brown color; (B) transverse section of the thallus with 6 periaxial cells (L.M. 100X); (C) composed thallus of cylindrical, uncrossed axes that branch in dichotomous form giving rise to secondary branches bearing cystocarps (S.M.45X).



(A) (B)

(C) (D)

Figure 63. *Polysiphonia fucoides*: (A) Cartilaginous, cylindrical, tufted, brownish purple fronds, to 300 mm long (more usually about 70 mm long), from branched rhizoidal holdfast; (B) branching ± alternate, tripinnate, ramuli with terminal tufts of colorless dichotomous fibrils (L.B. 40X); (C) cross section of the thallus with 19 periaxial cells (L.M. 100X); (D) corticated only at base (S.M.45X).



(A) (B)

Figure 64. *Xiphosiphonia ardreana* (formerly *Pterosiphonia ardreana*): (A) Sub-cylindrical thallus composed by 12 periaxial cells (L.M. 100X); (B) the branches are arranged alternately in an almost continuous way and are long and thin (S.M.20X).

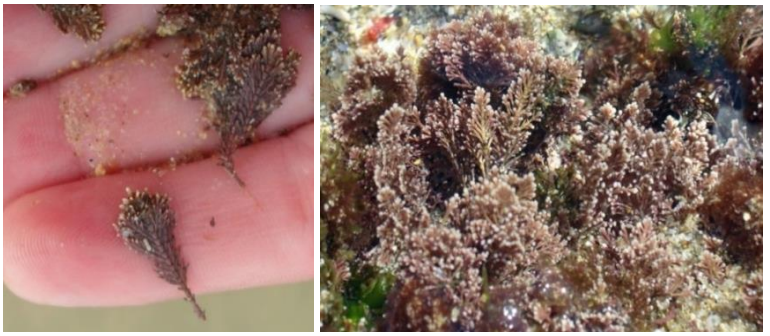
(A) (B)

(C)

Figure 65. *Pterosiphonia complanata*: (A) Thallus with prostate and erect axes, the erect ones flat-compressed and with distichous alternate pinnate branching; (B) brownish-red color, flexible and cartilaginous texture; attached by rhizoids which form discoid adhesive structures; veining visible with a magnifying glass (arrow) (S.M.25X); (C) cross section of main axis with multiple periaxial cells (L.M. 100X).

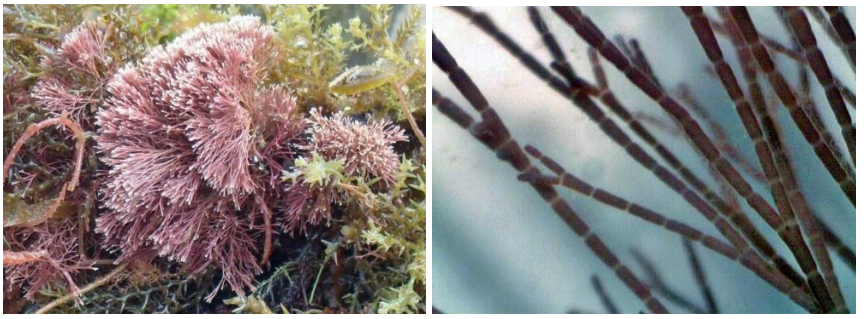
(A) (B)

Figure 66. *Xiphosiphonia pennata* (formerly *Pterosiphonia pennata*): (A) Small brownish-red seaweed up to 8 cm high, with a rigid texture; transverse section of the main axis with 9 periaxial cells (L.M. 100X); (B) from the main axis emerge thin and long branches, which are arranged alternately (L.M. 40X).



(A) (B)

Figure 67. *Ellisolandia elongata* (formerly *Corallina elongata*): (A) Whitish pink to reddish lilac, calcified, articulated fronds, fish-bone-like arrangement, to 50 mm high, axis compressed, repeatedly pinnate from discoid base; *Corallina officinalis*: (B) whitish-pink to lilac, calcified, articulated fronds, 60-120 mm high, axis cylindrical to compressed, repeatedly pinnate from and expanded discoid base, branching often irregular.

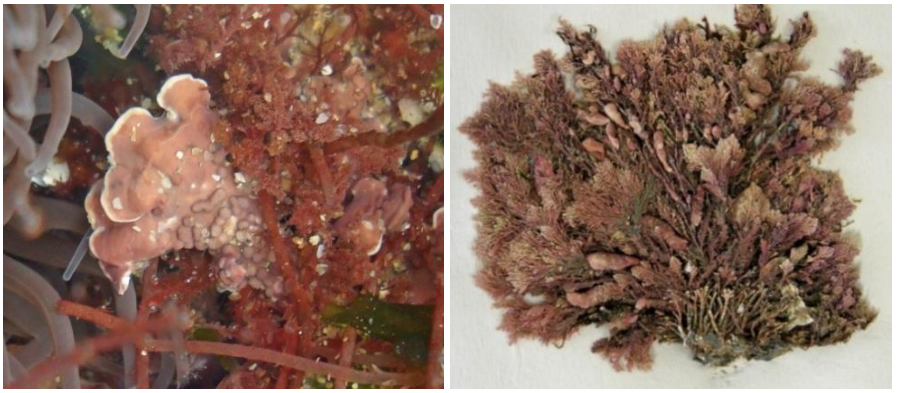


(A) (B)

Figure 68. *Jania rubens*: (A) Slender, rose-pink, articulated, calcified fronds, to 50 mm high; repeatedly dichotomously branched, luxuriant specimens secondarily pinnate; fixed by small conical disc, but spreading vegetatively by developing attachment discs from branches in contact with solid substratum; (B) segments cylindrical, to 120 µm diam., those bearing branches somewhat compressed, to 180 µm diam (S.M.20X).

(A) (B)

Figure 69. *Lithophyllum incrustans*: (A) Thick, dull chalky, yellowish, pink or lavender calcareous crusts forming irregular concretions, to 40 mm thick, margins ridged where crusts meet; *Lithophyllum byssoides*: (B) calcified and rigid encrusting thallus, strongly adhering to the bedrock; small hemispherical cushions 1 to 2 cm high, white to dark pink; crust covered with upright lamellae 10 mm high and 800 µm wide, anastomosed to each other.



(A) (B)

Figure 70. *Mesophyllum lichenoides*: A) Pale to dark purple thin, brittle, leafy calcified fronds, attached at base, margins free, lobed; fronds semicircular, concentrically banded; reproduction takes place in winter and spring in small, wart-like conceptacles; B) Small specimens often epiphytic on *Ellisolandia elongata.*

(A) (B)

(C) (D)

Figure 71. *Gelidium corneum*: (A) Dark red, hard consistency, cartilaginous, and may reach 30 cm in length thalli; (B) branches with obtuse apex and attenuated at the base; this species typically forms dense stands of clumped fronds, often under a Kelp canopy; *Gelidium spinosum*: (C) dark red, hard consistency, cartilaginous, and may reach 30 cm in length thalli. Small alga, cartilaginous, crimson to purplish red, 20-60 mm long; *Pterocladiella capilacea:* (D) main axes distinctly flattened, often narrower at base, ultimate branches short, often opposite, spine-like or spatulate.

(A) (B)

Figure 72. *Ahnfeltiopsis devoniensis*: (A) Small red marine alga growing to only several cm in length from a disc-like holdfast; (B) it forms a medium-sized flattened frond with regular dichotomous branching; the branches have parallel sides; the reproductive structures (cystocarps) are internal.

(A) (B)

Figure 73. *Caulacanthus ustulatus*: (A) Thalli forming small dense entangled tufts of up to 5 cm, reddish-brown color, which blackens by desiccation, with rough touch; (B) ramifications with acute apices and behaving small triangular-shaped spines (S.M.25X).

(A) (B)

Figure 74. *Chondracanthus acicularis*: (A) Dark-red or blackish, sometimes bleached yellow, cartilaginous, cylindrical, erect or re-curved or prostate, branched thalli, with 7 cm long; branches are irregular, curved and sharply pointed; *Chondracanthus teedei*: (B) upper thallus-branches cylindrical, lower ones flattened, dark crimson to black-red; branching repeatedly irregular-pinnate, cartilaginous-firm, lateral branches distant, pointed, the yougest terminal sections thorn-like; discoid base.

Figure 75. *Dilsea carnosa* (drift specimen): Dark red, frequently becoming yellow above, thickest of the foliose red algae in the NE Atlantic, flattened cartilaginous fronds, arising in groups of small, medium and large from a thick, discoid holdfast, obtuse, ovate with tapered base, to 500 mm long, 250 mm broad.

Figure 76. *Gigartina pistillata*: thalli are erect, up to 20 cm tall, dark-red or red-brown, cartilaginous, elastic, dichotomously branched, attached to the substrate through a small disk; presence of external, rounded cystocarps in female gametophyte fronds.

(A) (B)

Figure 77. *Sphaerococcus coronopifolius*: (A) Narrow, compressed, two-edged, cartilaginous, scarlet fronds, main axes dark brownish-red, to 300 mm long; (B) branching abundant, distichous, sub-dichotomous or alternate, terminal branchlets acute, fringed with short marginal proliferations.

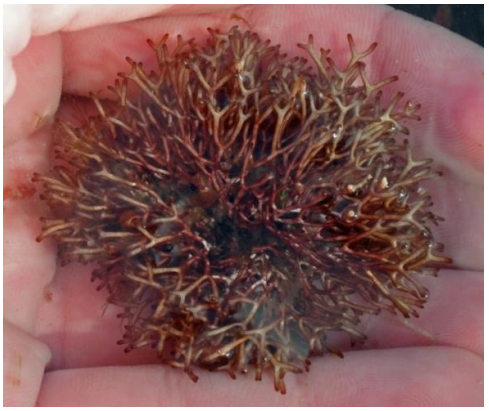
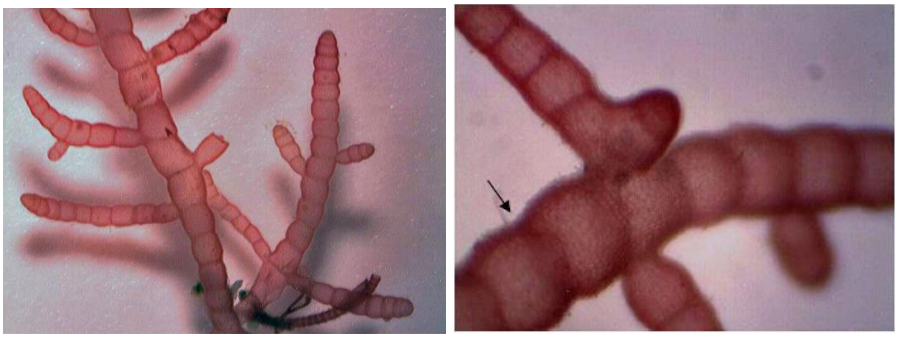


Figure 78. *Liagora viscida*: Thallus tufted, grey-purple to greenish-white or pink, repeated dense branching at almost the same length, branches terete, tapering towards the top; terminal branches usually spreading as wide-angled bifurcations, moderately calcified, texture flexible-firm [32].

Figure 79. *Peyssonnelia coriacea*: Laminar algae with slightly calcified flat thallus, with wavy margins and subject to the fund by the back with concentric grooves in the margins; its diameter is about 3 cm; color can vary from red to yellow with greenish hues.

(A) (B)

Figure 80. *Plocamium cartilagineum*: (A) Narrow, compressed, cartilaginous, bright scarlet fronds, to 300 mm long, tufted, much divided; (B) branching irregularly alternate, pinnules alternately second in twos to fives (arrow), with acute apices, lowest of each set a simple spur, others increasingly strongly pectinate (arrow) (S.M.25X).



(A) (B)

Legend: L.M. - Light Microscope; S.M. - Stereo Microscope.

Figure 81. *Champia parvula*: (A) Soft, gelatinous, pinkish red, much-branched fronds, densely matted, with blunt apices, to 100 mm high (S.M.25X); (B) axes segmented, with nodal diaphragms (arrow), segments about as broad as long, filled with a watery mucilage (S.M.45X).

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Chapter 76

POTENTIAL APPLICATIONS OF *ULVA RIGIDA* FOR BIOFUEL AND BIOCHEMICAL PRODUCTION

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ABSTRACT

Problems of environmental deterioration and energy demand could be alleviated by the paradigm shift from fossil to biofuels. Innovative strategies, such as the use of microwave irradiation, sonochemical treatment and solar irradiation were recently developed for the exploitation of biomass for biofuel production. The concept of biomass itself can be understood in an unconventional sense. Apart from terrestrial plant resources, nowadays, seaweed, industrial emissions such as CO_2, and organic remains such as glycogen are being explored as new feedstock for biofuels/chemicals production. Our research group in Israel is working on converting biomass (terrestrial and marine) to biofuels (bioethanol) and biochemicals (levulinic acid, furfural, formic acid) using microwave, sonochemical, and hydrothermal methods. Among several types of biomass, marine algae are a promising choice due to several advantages. Marine algae form an abundant and rich source of biomass. Bioethanol production process based on marine algae could be sustainable. A mild sonication-assisted simultaneous saccharification and fermentation (SSF) process for the conversion of *Ulva rigida* to bioethanol in a single step is developed in the current study. Bioethanol is a potential biofuel owing to the similarity of its energy density value (23 MJ/L) to that of gasoline (35 MJ/L). Bioethanol could also be a feedstock for the production of C_2 hydrocarbons. Any progress in the direction of development of a marine-algae-based bioethanol process would open up a new avenue towards sustainable biorefinery. *Ulva rigida*, comprising 37 wt%

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carbohydrate was used as a feedstock for the SSF process. Initially, saccharification process of *Ulva rigida* (with amylases and cellulases) was carried out under mild sonication conditions (40 kHz, 37°C); 3.1 times higher glucose yield was obtained using sonication of *Ulva rigi*da relative to conventional incubation. The hydrolysate was found to contain glucose exclusively. Subsequently, the SSF process for converting the algae (*Ulva rigida*) to bioethanol in a single step was also accelerated using sonication. The improvement was observed in the total carbohydrate content of the algae using multi-tropic aqua culture. 27-41 times higher specific growth rates were achieved using this approach. Under specific optimal conditions of growth, a starch amount as high as 32 wt% was accumulated. The high-carbohydrate algae were subjected to the sonication-based SSF process. Under optimal process conditions, an ethanol yield as high as 16 wt% was achieved. A unique solar-energy-based continuous flow process for the direct conversion of *Ulva rigida* to bioethanol is outlined. The conversion of macroalgae to the strategically significant chemical, levulinic acid is discussed. In an acid-catalysed hydrothermal process, 12.8 wt% levulinic acid was produced from *Ulva rigida*. We therefore elaborate in this chapter on the unconventional strategies developed for the farming as well as conversion of *Ulva rigida* to biofuels and biochemicals.

Keywords: seaweeds, macroalgae, *Ulva rigida*, bioethanol, SSF, fermentation, sonication, solar energy, levulinic acid, biofuels, biochemicals

INTRODUCTION

Growing population and increasing standards of living demand additional resources. The emphasis of this chapter is on two major sectors of human well-fare, namely, energy needs and the environment. Petroleum alternatives are an additional resource that would improve the standard of living of human societies. Biomass encompasses a renewable and cost-effective source of petroleum alternatives for fuel and chemical use. The US Department of Energy has listed twelve major chemicals that can be produced from biomass which would act as building blocks for producing a variety of other fuels and chemicals that are used for transportation and materials applications. The term biomass should not be restricted to conventional terrestrial lignocellulosic and algal resources but should be extended to abundant natural resources such as CO_2, another potential carbon feedstock for biorefinery. However, for the sake of brevity, the present compilation is restricted to the exploitation of seaweeds (macroalgae) for the production of biofuels and biochemicals. Oceans make up 70% (2/3rd) of the earth's surface and offer a wide unused space. Seaweeds possess bioenergy-production potential similar to that of land plants. Thus, cultivating and harvesting huge quantities of seaweed as an energy crop and devising energy-efficient strategies for its conversion to biofuels generate a new alternative energy source that could replace fossil fuels and in turn substantially reduce the CO_2 emissions that lead to global warming and environmental deterioration [1].

Energy insecurity and environmental pollution are the major problems facing mankind in the 21st century. Reduced dependence on fossil-based fuels is the need of the hour. Problems of environmental deterioration and energy demands could be alleviated by the paradigm shift from fossil fuels to biofuels. Innovative methods of activation, such as the use of microwave irradiation, sonochemical treatment and solar irradiation were recently developed for the exploitation of seaweeds for biofuels production. Among several types of biomass, seaweeds

(marine algae) are a promising choice owing to several advantages such as (i) absence of lignin which is a hindrance to hydrolysis and fermentation, (ii) absence of food vs fuel conflict, (iii) absence of the requirement of land for growing the biomass and (iv) availability of vast sea shores (2/3 of Earth's surface is ocean). Marine algae forms an abundant and rich source of biomass and biofuels (especially, bioethanol) so that production processes based on marine algae could be sustainable.

Algal biomass is classified into two main groups, namely, microalgae (bluegreen algae, dinoflagellates, bacilloriophyta) and macroalgae (seaweeds like green, brown and red algae). Red algae (*Gelidum, Palmaria, Poryphyra*) capture light with the reddish protein pigments (phycoerythrins) and as a result have the characteristic reddish colour. Green algae (*Ulva, Codium*) have chlorophyll as the light-absorbing pigments. Brown algae (*Laminaria, Fucus, Sargassum*) contain the carotenoid, fucoxanthin, as the dominant light-capturing pigment. Brown and red algae can grow deeper than the green algae. Most seaweeds grow bound to the substrate (rocks near-shore or artificial surfaces) except certain species like *Sargassum* that float freely on the surface of water. Microalgae are a rich source of highly bioactive compounds found in marine resources. Red algal seaweeds are an active metabolite. The conventional use of seaweeds includes human food resources and as a source of gum (phycocollides such as agar agar, alginic acid and carrageenam) [2]. Unconventional applications being explored include biofuels and biochemicals.

Seaweeds are multicellular, macroscopic marine algae classified as non-vascular plants. Global production of seaweeds is more than a million tons (dry weight matter) relative to no more than 20,000 tons of microalgae production. As a result, the cost of macroalgae is nearly ten times lower than that of microalgae. Macroalgae have higher carbohydrate and lower protein and lipid contents compared to microalgae and so the macroalgal seaweeds are an ideal candidate for bioethanol and biochemicals production via the conversion of the carbohydrate component. The carbohydrate content in macroalgae can be up to 80% organic matter (ash-free dry weight) with about 20% protein and 15% lipids. Depending on the method of analysis, growth conditions of the seaweeds and the species under study, the composition varies [3].

Seaweeds as Biofuel Feedstock

The macroscopic nature of the seaweeds allows simple and low-cost harvesting. Methane, bioethanol and biobutanol are the three common fuels that can be derived from macroalgae. *Ulva species* have relatively high carbohydrate content and can be readily digested to methane gas and subjected to simultaneous scharrification and fermentation (SSF) processes for bioethanol production. As early as 1960’s, Howard Wicox suggested the production and exploitation of macroalgae as a solution to the energy crisis and global warming [4]. During the period from 1970-1980, extensive research efforts were devoted in the US to developing open-ocean macroalgae farms as a substitute to natural gas. Despite nearly half a century of research in the area of macroalgae conversion to biofuels, the R&D is still at the initial stage with several practical challenges to be surmounted. The major challenge pertains to the economically competitive sustainable production of the macroalgae. The extension of cultivation systems to deeper, less protected ocean areas without anchoring facilities is a challenge. Even in the cultivation systems near the shore, the production costs are high and

major breakthroughs in the cultivation of algal seaweeds are awaited. In spite of these hurdles, beyond doubt, seaweed holds the promise of a sustainable feedstock for food, feed, fuels and chemical production. Motivated by the challenges and rewards, during the last decade, there has been renewed interest in the exploitation of seaweeds for biofuel production with the growing energy demands.

Japan's efforts (Ocean Sunrise Project) were focused on the harvesting of *Sargassum fulvellum* and its conversion to bioethanol in unused maritime areas around Japan [1]. A farming technology was used for *Laminaria* and *Undaria pinnatifida* in both coastal and off-shore areas. The aim of the project is to produce seaweed bioethanol by farming and harvesting *Sargassum horneri*, utilizing the world's sixth largest (4.47 million km^2) areas of the exclusive economic zone (EEZ) and maritime belts of Japan. The research was conducted by Tokyo Fisheries Promotion Foundation. Japan's long-term plan is to proudce 6 million kL of biofuel by 2030. The key to achieving the target lies in farming large quantities of seaweed at low cost.

The UK and Ireland's BioMara joint project aims at the production of sustainable fuels (methane and ethanol) from marine biomass by studying the process feasibility and economics. The objectives of the project comprise of chemical characterization of selected seaweeds (*Laminaria digitata* and byperborea, *Alaria esculenta*, *Saccharina latissima*, *Palmaria palmata* and *Ulva lactuca*), pretreatment (thermal/enzymatic), and fermentation. Utilization of the alginate component of the seaweed by employing alginate lyase for breaking down the alginate matrix exposes the polysaccharide. The action of alginate lyase is similar to hemicellulase. 20% enhancement in ethanol yield was observed upon pretreatment of *Laminaria digitata* with alginate lyase. Successful utilization of alginate component of seaweeds for bioethanol production is projected to enhance the bioethanol yield as high as ~ 400%. German researchers have developed an offshore ring system for farming *Laminaria Saccharina* for food and fuel applications The methodology comprises growing the macroalgae sporophytes in the lab to the appropriate length and then placing them in the ring structures. The ring structural design was found to be stable for offshore farming [5]. China has successfully demonstrated the sustained commercial cultivation of *Laminaria japonica* with yields of ~ 25 t/ha [6]. Similar attempts were made in Korea for the conversion of *Gelidium amansii* to bioethanol [7]; in India for the conversion of *Kappaphycus alvarezii* to bioethanol; in Denmark for the conversion of *Ulva lactuca* to bioethanol [8]. In Israel, Gedanken's group has developed innovative strategies for the farming of carbohydate-rich marine macroalgae *Ulva rigida* and has designed unconventional pathways, based on sonication and solar energy for the single-step conversion of the *Ulva rigida* to bioethanol, which will be descibed in the following sections [9-13].

Unconventional Strategies for the Farming and Conversion of *Ulva rigida* to Bioethanol

Bioethanol has relevance to major sectors of human activity such as energy, chemicals, materials and the environment. Bioethanol is a potential biofuel owing to the similarity of its energy density value (23 MJ/L) to that of gasoline (35 MJ/L). Bioethanol production is important not only for transportation applications, but also for its use as feedstock for the production of C_2 hydrocarbons [13]. Fermentation is one of the intrinsic and crucial reactions

involved in the conversion of biomass carbohydrate fraction to bioethanol and is usually slow. Glucose fermentation reaction carried out under mild sonication (40 kHz) at 30°C using baker's yeast (*Saccharomyces cerevisiae*) was accelerated by a factor of 2.3. The acceleration of the fermentation process was observed for glucose concentrations as high as 40 wt%. The kinetics of the glucose fermentation reaction was monitered by ^{13}C NMR spectroscopy as well as by measuring the weight loss of the fermentation broth during the course of the reaction. Theoretically, 1 mol of D-glucose yields 2 mol of ethanol and 2 mol of CO_2 during the fermentation process by yeast. The evolution of CO_2 is reflected in the weight decrease which can be correlated to the amount of ethanol produced. However, this method of evaluation is not very accurate as the substrate glucose is not only converted to ethanol and CO_2 but also to the inevitable secondary metabolite glycerol. So another method based on the use of ^{13}C NMR spectroscopy was developed for the evaluation of the kinetics of glucose fermentation. A fairly good correlation between the two methods for the measurement of glucose conversion values and calculation of reaction rate constants of glucose fermentation was observed as depicted in Figure 1.

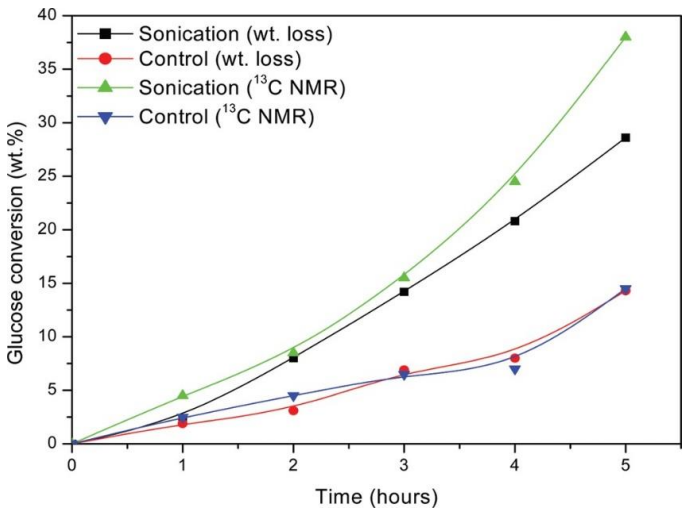
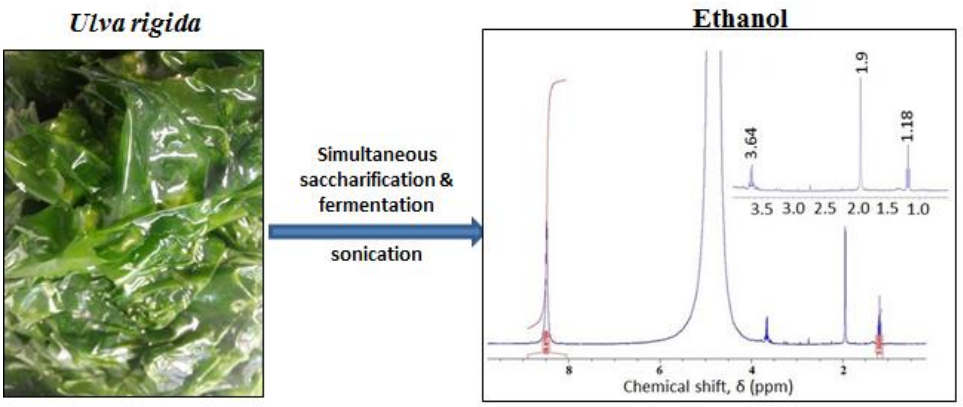


Figure 1. Effect of ultrasound (40 kHz) irradiation on the kinetics of glucose fermentation at 30°C. [Indra Neel Pulidindi, Aharon Gedanken, Rakefet Schwarz and Eleonora Sendersky (2012) Mild sonication accelerates ethanol production by yeast fermentation, *Energy and Fuels*, 26, 2352-2356]. Adopted by permission of the American Chemical Society.

The glucose conversion values in the fermentation process under mild sonication and conventional stirring at 30°C, measured by the weight decrease of the fermentation broth and by ^{13}C NMR spectra, are shown in Figure 1. The mean rate constant values deduced from weight-loss measurements (13.4×10^{-6} s^{-1}) and ^{13}C NMR analysis (17.3×10^{-6} s^{-1}) are in good agreement. The average of these values is considered as the reaction rate constant for the fermentation of glucose under sonication at 30°C (15.35×10^{-6} s^{-1}) while the corresponding value under conventional stirring at 30°C is 6.67×10^{-6} s^{-1}. The ratio between these values (2.3) is the enhancement factor in the kinetics of glucose fermentation achieved by carrying out the fermentation reaction under sonication.

Extending this methodology for the acceleration of the fermentation process to real biomass (*Ulva rigida*) is a challenge and was attempted successfully [14]. *Ulva rigida* is a

common seaweed with the potential for biofuel and biochemical production. Other potential seaweeds reported in literature for bioethanol production include *Gelidium amansi* [15], *Laminaria japonica* [16], *Codium fragile* [17] and *Nizimuddinia zanardini* [18]. The option of seaweeds as feedstock offers several advantages including rapid growth rates of the biomass relative to terrestrial plants: large sea area (70% of earth surface available in principle even though off-shore cultivation is challenging), higher carbohydrate content, higher theoretical ethanol yields, low concentration of crystalline components (like lignin that hinder the action of enzymes and yeast in the hydrolysis and fermentation reactions respectively), no competition with food crops and cultivable land area, serves as a bioremediation crop by lowering eutrophication impact on in-shore waters [19], no requirement of fresh water supply or nutrients [20, 21]. In spite of these advantages, inefficient methods of harvesting, pretreatment, hydrolysis and fermentation processes have resulted in lower ethanol yields from seaweeds, thereby offering wide scope for further research and development. Any progress in the direction of development of a marine algae based bioethanol process would open up a new avenue towards sustainable biorefinery. In view of these aspects Gedanken *et al.* developed a mild sonication-assisted simultaneous saccharification and fermentation (SSF) process for the conversion of *Ulva rigida* to bioethanol [9, 10]. A schematic representation of the sonication based SSF process for the conversion of *Ulva rigida* to bioethanol is shown in Scheme 1.



Scheme 1. Schematic depiction of the conversion of *Ulva rigida* to bioethanol [9].

Ulva rigida, comprising 37 wt% carbohydrate (23.8 wt% cellulose, 7.6 wt% starch, and other components such as ulvan and xylan) was used as a feedstock for the SSF process. Initially, the saccharification process (in the presence of enzymes, amylases and cellulases) of *Ulva rigida* alone was carried out under mild sonication conditions (MRC Clean-01 Ultrasonic cleaner, 40 kHz ultrasound frequency, 120 W ultrasonic power, 37°C) without any prior pretreatment. As a control study, the reaction was studied under identical conditions in an incubator under shaking. The progress of the reaction is monitored by estimating the amount of glucose formed with time (Figure 2). The difference in the glucose yields in the sonication-assisted and control process is more pronounced in the initial period (30 min) of the reaction. A higher (by 3.1 times) yield of glucose is obtained in the sonication-assisted hydrolysis process. The advantage of obtaining higher glucose yield in the hydrolysis process

aided by sonication, at any given time, relative to a conventional incubator process under identical conditions is evident from Figure 2. A maximum of 19.6±0.02 wt% glucose is produced in a sonication-based process in 3 h relative to a yield of 16.7±0.27 wt% in an incubation process in 24 h. The accelerated release and improved yield of glucose from *Ulva rigida* upon sonication is due to mechanical and thermal effects.

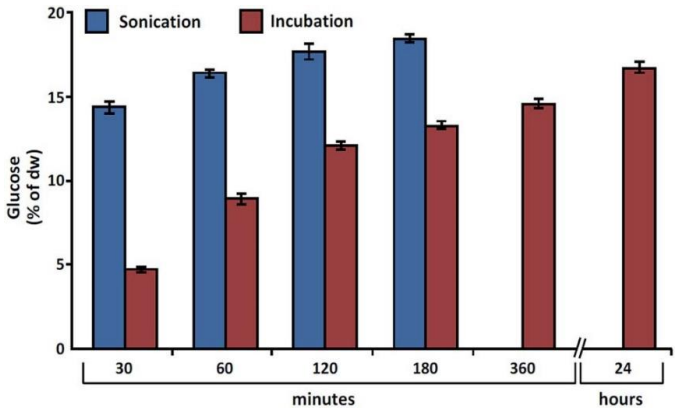


Figure 2. Glucose yields as a function of time in the enzymatic saccharification of *Ulva rigida* under sonication vs incubation at (37°C) [Leor Korzen, Indra Neel Pulidindi, Alvaro Israel, Avigdor Abelson and Aharon Gedanken (2015) Single step production of bioethanol from the seaweed *Ulva rigida* using sonication, *RSC Advances*, 5, 16223-16229]. Adopted with permission from the Royal Society of Chemistry.

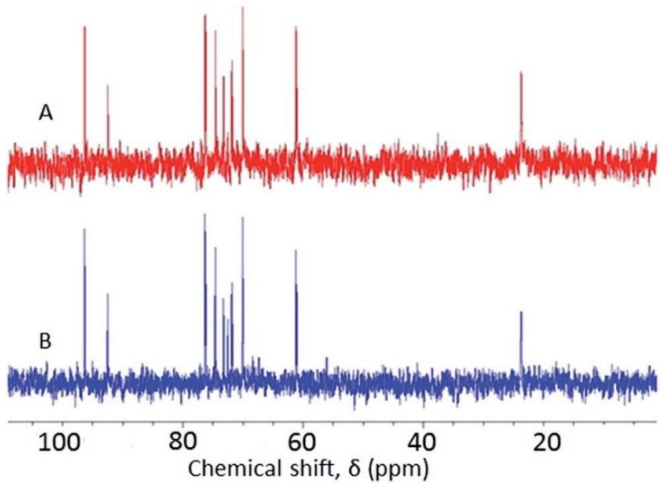


Figure 3. ^{13}C NMR spectra of aliquots from the hydrolysate of *Ulva rigida* produced under sonication (A) and incubation (B) at 37°C at 120 min. [Leor Korzen, Indra Neel Pulidindi, Alvaro Israel, Avigdor Abelson and Aharon Gedanken (2015) Single step production of bioethanol from the seaweed *Ulva rigida* using sonication, *RSC Advances*, 5, 16223-16229]. Adopted with permission from the Royal Society of Chemistry.

The structural rigidity of components of algal biomass is reduced due to sonication. Effective mixing, phase transfer, diffusion of enzymes across the algal cell membranes results in the acceleration of saccharification of *Ulva rigida* during the sonication process [9].

Irrespective of the method of hydrolysis, glucose is produced exclusively as the fermentable sugar. The ^{13}C NMR spectra of the aliquots of the hydrolysate under bath sonication (Figure 3a) and incubation (Figure 3b) at 120 min are shown in Figure 3. Well-resolved intense signals in the range of 60-100 ppm (60.9 (C6), 69.9 (C4), 71.8 (C2β), 73.1 (C2β), 74.5 (C3), 76.2 (C5), 92.4 (C1β) and 96.2 (C1β)) are typical of the α and β isomers of D-glucose. An additional signal at 23.6 ppm is attributed to the carbon nuclei of CH_3COONa used as buffer for the enzymatic hydrolysis of algae.

Further studies were carried out on the single step conversion of *Ulva rigida* (SSF) to bioethanol under sonication. In addition to the algae and enzymes (amylogucosidase, α-amylase, and cellulase), baker's yeast was also added to the reaction medium in glass media bottles with cap (Fisher brand, 100 mL). The progress of the SSF process, under sonication and in an incubator, was monitored by evaluating the amount of ethanol formed at regular intervals of time. The ethanol amount in the analytes was quantified using ^1H NMR. The SSF process was also found to be faster in a sonication driven process as shown in Figure 4 [9].

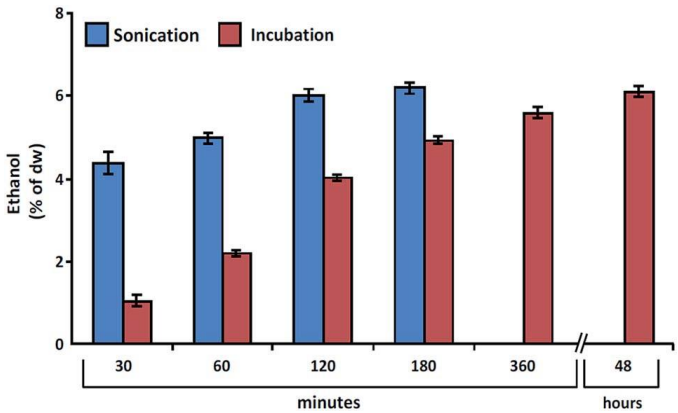


Figure 4. Ethanol yield as a function of time in the enzymatic hydrolysis of *Ulva rigida* under sonication vs incubation at 37°C (replicate no. n = 3; error bars indicate standard deviation, SD). [Leor Korzen, Indra Neel Pulidindi, Alvaro Israel, Avigdor Abelson and Aharon Gedanken (2015) Single step production of bioethanol from the seaweed *Ulva rigida* using sonication, *RSC Advances*, 5, 16223-16229]. Adopted with permission from the Royal Society of Chemistry.

Higher ethanol yields (4.3 ± 0.26 wt%) were observed in a sonication-aided process relative to a process under incubation (1.0 ± 0.13 wt%) in a short duration of 30 min, reaching a maximum value of 6.2 ± 0.13 wt% in 3 h. The 3H (t, 1.18 ppm) and 2H (q, 3.64 ppm) confirm the formation of ethanol from *Ulva rigida* in the SSF product (Figure 5). The signal, 3H (s, 1.9 ppm) corresponds to the buffer (sodium acetate) used. The peak, 1H, s, at 8.5 ppm, is characteristic of the internal standard (HCOOH) used for the quantification of ethanol. Thus an unconventional sonication-based SSF process for the single step conversion of *Ulva rigida* to bioethanol is developed. Compared to a conventional incubation process (4.9 wt% ethanol in 48 h), the current process is faster and the ethanol yield is higher (6.2 wt% ethanol in 3 h) [9].

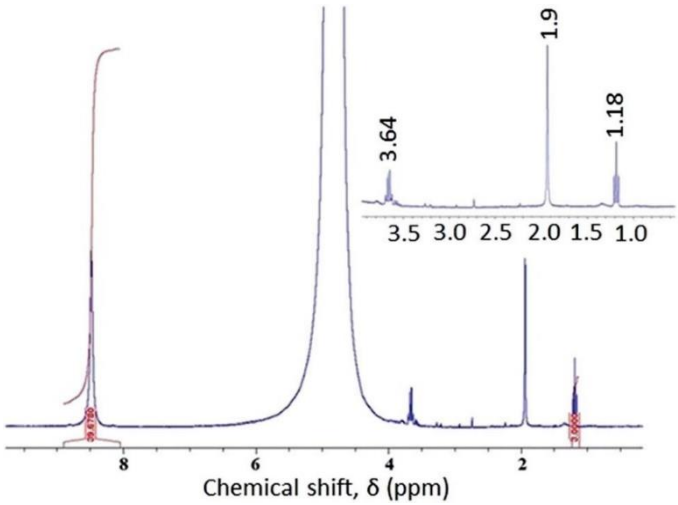


Figure 5. ^{1}H NMR spectrum of the aliquot of sample collected from the fermentation (SSF) broth under mild sonication at 120 min. [Leor Korzen, Indra Neel Pulidindi, Alvaro Israel, Avigdor Abelson and Aharon Gedanken (2015) Single Step Production of Bioethanol from the Seaweed *Ulva rigida* using Sonication, *RSC Advances*, 5, 16223-16229]. Adopted with permission from the Royal Society of Chemistry.

Table 1. Process efficiency of SSF of *Ulva rigida* in a sonication vs incubation process*[9]

SSF process	Glucose yield (gg^{-1} biomass)	Ethanol yield (gg^{-1} glucose)	Process efficiency (%)
Sonication (t=3 h)	0.196	0.33	64.7
Incubation (t=48 h)	0.173	0.34	66.6

*Reaction conditions: biomass (dry weight) = 1.68 g; distilled water = 40 mL; cellulase = 0.1 g (0.3 units per mg); α-amylase = 40 µL (250 units per mL); amyloglucosidase = 100 mL (300 units per mL); sodium acetate buffer = 40 mL.

Although the SSF process of algae is accelerated upon sonication, the process efficiency (64.7%) remained similar to that of the conventional incubation process (66.6%) (Table 1). Moreover, the bioethanol production process is still a batch process rather than a continuous-flow process with large scope for further improvement.

Typical challenges in the exploitation of seaweed include production in large quantities and also the lower fermentable sugar content available currently. For the commercial-scale production of bioethanol, it is imperative to develop farming strategies resulting in carbohydrate-rich as well as fast growing biomass. Towards attaining this target Gedanken *et al.* developed integrated multi-tropic aquaculture (IMTA) as an alternative farming strategy to the conventional algal mono-culture. The concept of IMTA involves reuse and recycling of internal feed within a culture system minimizing the wastage of resources (nutrients, water and energy). Integrating seaweed farming with aquaculture operations has several advantages. Seaweed turns waste into productive resources and reduces the impact of wastes on the ecosystem. Integration of seaweed cultivation with fed mariculture facilitates recapturing of waste nutrients, leading to increased growth rate, improved fermentable sugar contents in the *Ulva rigida* farmed downstream to fish-culture net pens. So far, the methodology of

integrated culture of fish and algae in marine open-waters is focused towards economic and environmental aspects but not towards energy (bioethanol) and chemical (levulinic acid) production which has been the focus of the study of Gedanken's group [10].

The seaweed *Ulva rigida* was co-cultured with fed-fish culture (*Sparus aurata*) in an off-shore fish cage aquaculture complex with the objective of obtaining high-yield carbohydrate-rich biomass. *Ulva rigida* cultivation is carried out in an open sea fish farm (Lev-Yam aqua-culture Ltd) located off the Michmoret coast, Israel. The specific location is shown in the map (Figure 6a).

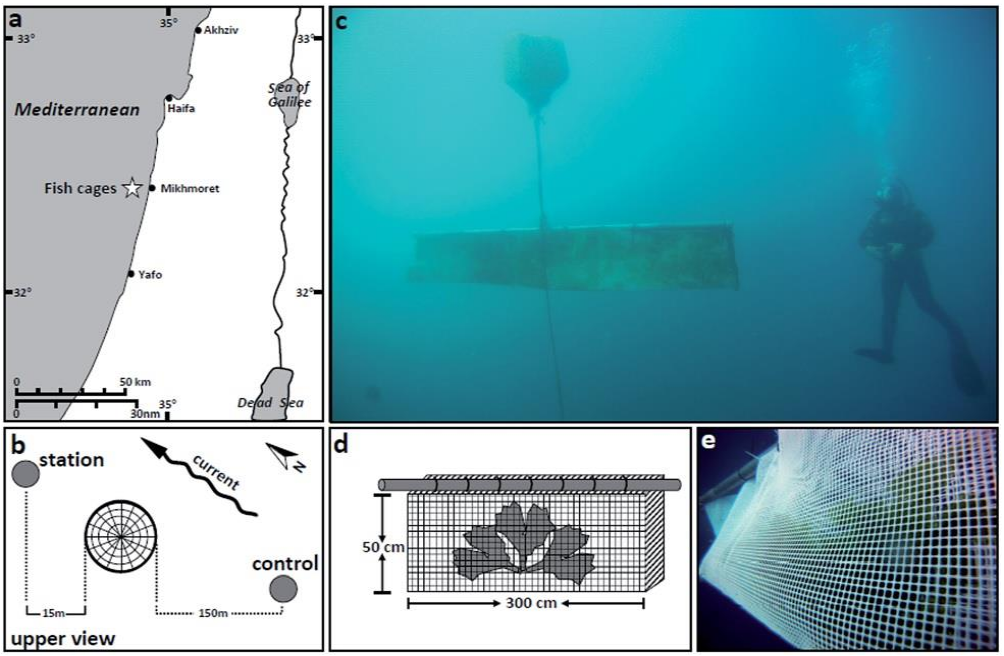


Figure 6. Schematic (a) map of the study area showing the fish farm, (b) scheme of the fish cage and the algal culture cages, (c) algal culture cage suspended at 3 m depth, (d) scheme of an algal culture cage and (e) *Ulva rigida* in culture cage. [Leor Korzen, Indra Neel Pulidindi, Alvaro Israel, Avigdor Abelson and Aharon Gedanken (2015) Marine integrated culture of carbohydrate rich *Ulva rigida* for enhanced production of bioethanol, *RSC Adv* 5, 59251-59256]. Adopted with permission from the Royal Society of Chemistry.

About 500 g of *Ulva rigida* were placed in nylon net cages (Figure 6e). The cages were attached to buoys and placed at two positions. One site is located within the fish cage surroundings, 15 m downstream to the cages' main water current direction and the control site is located 150 m upstream to the fish cages (Figure 6b). Algal culture cages were placed at a depth of 3 m (Figure 6c). Specific growth rates and the starch content of *Ulva rigida* cultured during Sept/Oct. 2013 (Figure 7 a & c) and during Nov. 2013 (Figure 7 b & d) were shown pictorially in Figure 7. The initial culture or grow-out period is for 14 days and an additional 5 days in a low-nutrient site. Remarkable enhancement in the growth rate of *Ulva rigida* grown under nutrient-rich conditions downstream from the fish cages was observed (Figure 7a & b). The enhancement in the specific growth rates is by a factor of 27 and 41 for the Sept. (Figure 7a) and Nov. (Figure 7b) trials respectively. The availability of inorganic nutrients is the vital parameter facilitating the growth and productivity of seaweed *Ulva rigida*.

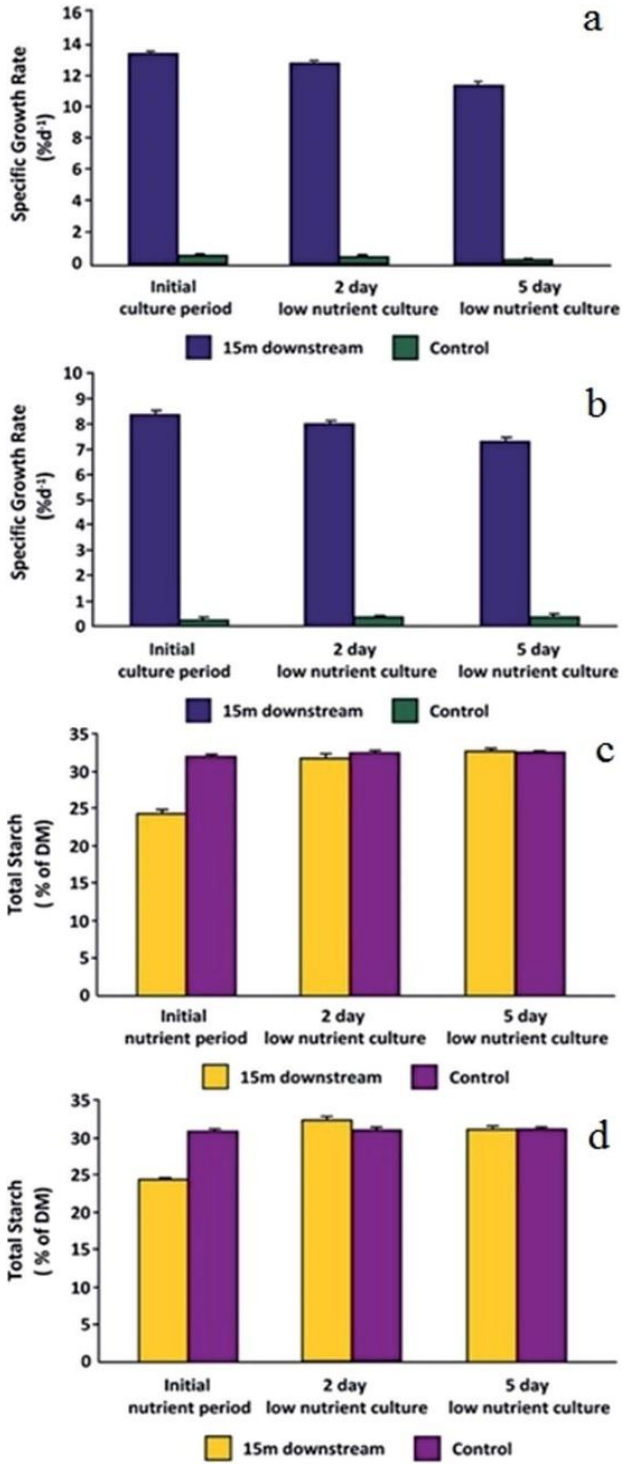


Figure 7. *Ulva rigida* culture and starch content. [Leor Korzen, Indra Neel Pulidindi, Alvaro Israel, Avigdor Abelson and Aharon Gedanken (2015) Marine integrated culture of carbohydrate rich *Ulva rigida* for enhanced production of bioethanol, *RSC Adv* 5, 59251-59256]. Adopted with permission from the Royal Society of Chemistry.

In both trials (Sept. Figure 7c and Oct. Figure 7d), the carbohydrate (starch) content was higher at the control site (31.5 wt% of dry weight, Sept. trial) compared to the nutrient- rich site downstream to the cages (24 wt%). This observation is in inverse proportion to the ambient seawater nutrient concentrations. However, the starch contents bounced up and levelled with values similar to those at the control site on culture manipulation for two days at the low-nutrient site. High nutrient concentrations altered the proximate composition in seaweeds and caused a shift to lower levels of starch. Thus, the developed strategy based on IMTA resulted in the accelerated production of *Ulva rigida* with high starch content (31 wt%). The high-carbohydrate *Ulva rigida* was subsequently converted to bioethanol in a sonication-based SSF process.

Under optimized process parameters (enzyme loading, 1 wt%; algal consistency in the broth, 15 wt%, sonication time, 4 h) a high bioethanol yield of 16 wt% is produced (Figure 8).

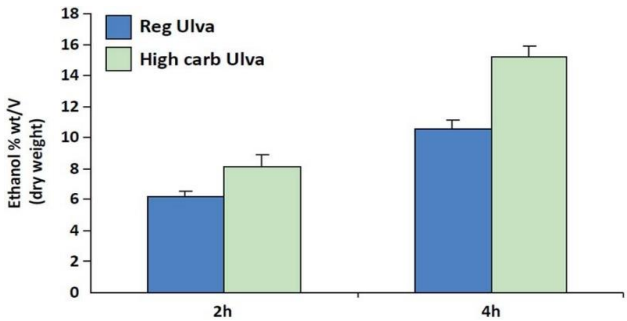


Figure 8. Effect of tailoring the carbohydrate content of *Ulva rigida* on the ethanol yield in the sonication-based SSF process with 15 wt% solid consistency and 1 wt% enzyme loading (replicate no. n = 3; error bars indicate standard deviation, SD). [Leor Korzen, Indra Neel Pulidindi, Alvaro Israel, Avigdor Abelson and Aharon Gedanken (2015) Marine integrated culture of carbohydrate rich *Ulva rigida* for enhanced production of bioethanol, *RSC Adv* 5, 59251-59256]. Adopted with permission from the Royal Society of Chemistry.

Formation of bioethanol from *Ulva rigida* is confirmed by the two characteristic signals at 17.5 and 58.1 ppm typical of ethanol (Figure 9).

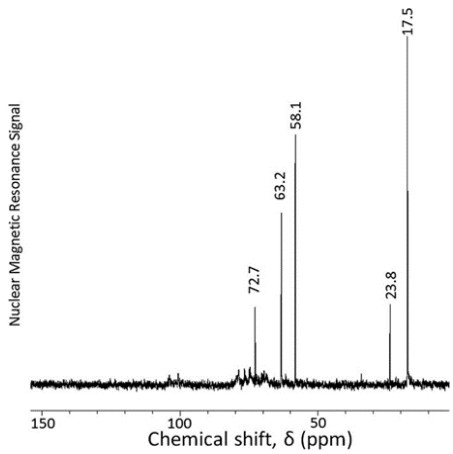


Figure 9. ^{13}C NMR spectrum of aliquot of sample collected from the fermentation (SSF) broth under optimal reaction conditions. [Leor Korzen, Indra Neel Pulidindi, Alvaro Israel, Avigdor Abelson and Aharon Gedanken (2015) Marine integrated culture of carbohydrate rich *Ulva rigida* for enhanced production of bioethanol, *RSC Adv* 5, 59251-59256]. Adopted with permission from the Royal Society of Chemistry.

Scheme 2. Strategy adopted for the production of starch-rich *Ulva rigida* and its conversion to high-concentration bioethanol [10].

Additional signals at 63.2 and 72.7 ppm correspond to the inevitable secondary metabolite glycerol and the signal at 23.8 ppm corresponds to the buffer (CH_3COONa) used in the SSF medium. The absence of signals in the range 60-100 ppm indicates the complete metabolism of glucose by yeast and the effectiveness of the SSF process under sonication. The whole process of farming the high-carbohydrate *Ulva rigida* by the integrated multi-tropic aquaculture (IMTA) and its subsequent conversion to high-concentration bioethanol in a single-step sonication-assisted SSF process is represented in Scheme 2.

Continuous-Flow Solar-Energy-Driven SSF Process for the Conversion of *Ulva rigida* to Bioethanol

The exploitation of solar energy, which is abundant, renewable and environmentally friendly, for the production of bioethanol is an innovative idea. Successful utilization of solar energy for bioethanol production from biomass has the potential to solve the fuel-shortage problem. Utilization of solar energy for the production of bioethanol has never been tested experimentally so far. Gedanken's group was the first to envisage the prospects as well as potential benefits in using solar radiation for biofuel production [22]. Using solar energy for bioethanol production has economic, energy and environmental benefits. The product, bioethanol, has diverse applications apart from being a potential transportation fuel. Bioethanol could act as an economic driver for the upcoming biorefinery industry as it could be a feedstock for alkanes as well as ethers. Moreover, the solar-energy-driven bioethanol process is sustainable owing to the abundance of solar radiation and also because of the wide range of biomass that could serve as feedstock for carbohydrates that are converted to bioethanol in the process of SSF. The feasibility of using the bioethanol produced in a solar-energy-driven process (from glucose and starch as feedstock) was demonstrated for generating electricity from fuel cells, and the performance of the fuel cells was on a par with commercial ethanol of similar concentrations. Such an application is the first of its kind in the literature and can be regarded as a breakthrough and prelude to several other applications (in the field of transportation sector and chemical industries) which are currently unanticipated [12, 13]. The use of this alternate green renewable resource may provide a solution to meet the growing energy demands. The utilization of solar thermal energy for biofuel production has a significant impact on the overall energetics (energy return on energy invested, EROEI) of the process. The exploitation of solar energy for the direct conversion of biomass to bioethanol has not been attempted before and hence the invention has potential for easy adaptation by industry. Moreover, the unique design of the solar reactor (Figure 10) not only

accelerates the simultaneous saccharification and fermentation (SSF) process for the conversion of biomass to bioethanol but also facilitates the *in situ* separation of the ethanol formed in the broth by an evaporation-condensation process [11].

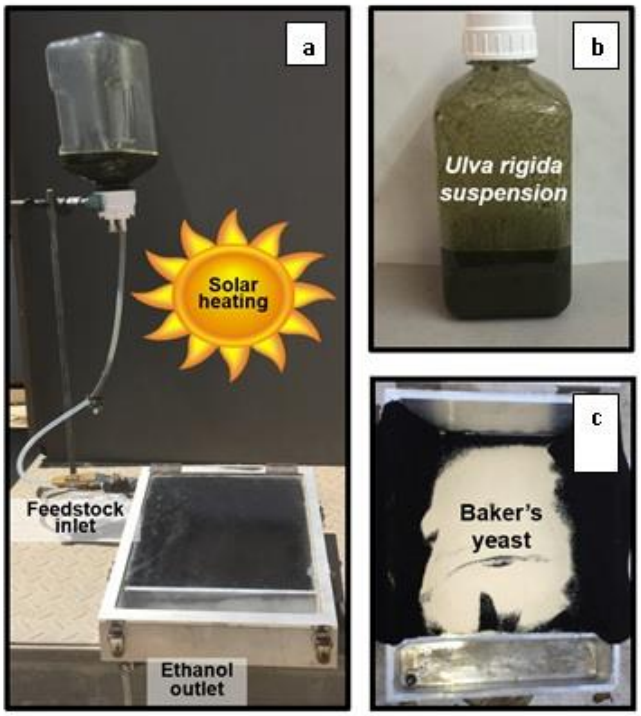


Figure 10. Continuous-flow solar reactor for the single-step conversion of marine macroalgae *Ulva rigida* to bioethanol; (a) complete experimental setup, (b) stable 5 wt% aqueous *Ulva rigida* suspension with enzymes, and (c) fermentation chamber loaded with baker's yeast on activated carbon cloth [11].

A typical continuous-flow process for the solar-aided conversion of an aqueous suspension of *Ulva rigida* to bioethanol consists of feeding the algal solution (500 mL, 5 wt%, prepared by ultrasonication) mixed with specific amounts of enzymes (α-amylase, amyloglucosidase, endo-cellulase, exo-cellulase, and β-glucosidase) into the fermentation chamber of the solar reactor (at a flow rate of 3.9 mL/h) loaded with instant baker's yeast (*Saccharomyces cerevisiae*) covered with activated carbon cloth. In this solar-energy-driven continuous-flow SSF process, the enzymes led to the hydrolysis of starch and cellulose components of the algae to glucose which was further fermented by the yeast and converted to ethanol. When 500 mL *Ulva rigida* feedstock was completely fed to the reactor, DDW was flown through the reactor (with the same flow rate, 3.9 ml/h) in order to convert the residual carbohydrates of the algae to bioethanol. When the DDW was completely fed, additional 500 mL of the feedstock was fed to the reactor to test the reusability of the enzymes and the yeast bed as well as the continuous operability of the system. Again, when the feedstock was completely fed to the reactor, DDW was allowed to flow in order to convert the residual carbohydrate content of the algae to bioethanol. It is important to note that similar bioethanol results were observed when the same experiment was repeated by loading the enzymes and the baker's yeast on activated carbon prior to SSF process.

Time-on-Stream Studies of Solar-Energy-Driven Bioethanol Production from Continuous-Flow SSF of Ulva rigida

The continuous-flow SSF process of 5 wt% *Ulva rigida* was monitored for 37 days in the solar reactor (with the same enzymes and the yeast) at 31/24°C average day/night temperature. The fermentation was continuous; most of the evaporation occurred during the day and only a negligible amount occurred at night. The aliquots of products were collected at regular time intervals and quantified for ethanol using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and high performance liquid chromatography (HPLC). The ethanol yield (deduced from ^{1}H NMR analysis) as a function of time is depicted in Figure 11.

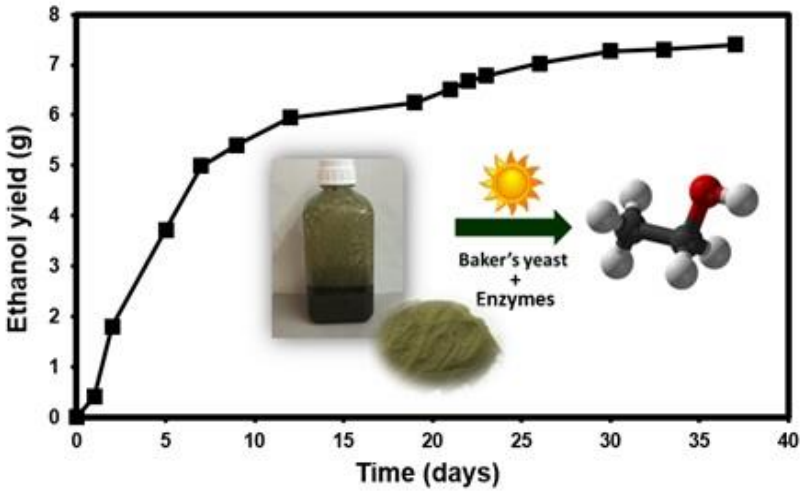


Figure 11. Time-on-stream studies of solar-energy-driven bioethanol production in a continuous-flow SSF process of *Ulva rigida* (5 wt%) ($T_{average}$ day/night: 31/24°C) [11].

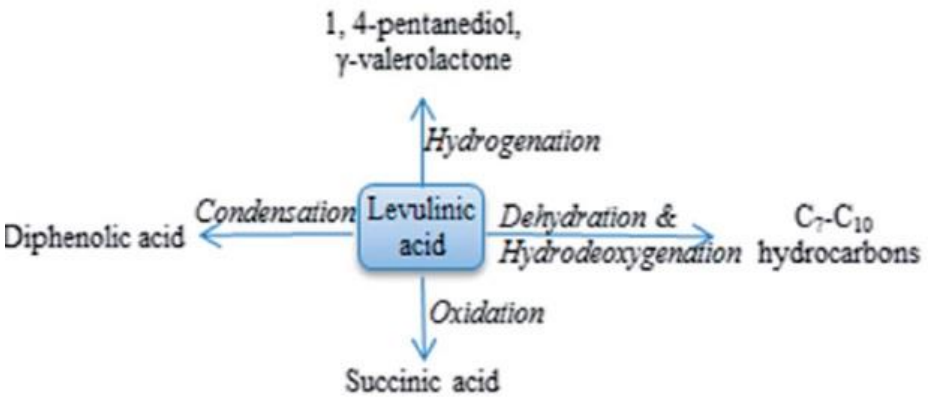
High ethanol yields (84.2 wt% of the theoretical ethanol yield) were observed throughout the SSF process of *Ulva rigida* (7.4 g ethanol in 37 days, 2.64 g ethanol/day/m^2), based on the fermentable sugar content of the biomass. Thus the solar-aided conversion of *Ulva rigida* to bioethanol is highly energy- and atom-efficient.

Can Seaweeds Be a Sustainable Feedstock for the Production of Levulinic Acid, a Key Economic Driver for the Biorefinery?

Levulinic acid is a chemical of strategic significance. In fact, production of levulinic acid from cellulose would be more advantageous than converting cellulose to bioethanol. The theoretical yields of levulinic acid and bioethanol from cellulose are 64 [23] and 51 wt% [14] respectively. Not only is the atom efficiency of levulinic acid production process higher than that of the bioethanol production process, but also owing to the multifunctionality of levulinic acid, it could be a building block for several other fuel-grade chemicals such as γ-valerolactone (Scheme 3).

Although the production of levulinic acid has been attempted from a variety of carbohydrate feedstock including direct conversion of widely available agricultural wastes

such as *cicer arietinum*, cotton, *Pinus radiata*, sugar cane bagasse and rice straw [23, 24], very few attempts have been made for the exploitation of seaweeds as feedstcok for levulinic acid production [25, 26]. Gedanken's group made preliminary studies towards the utilization of the seaweed, *Ulva rigida*, for the production of levulinic acid. An acid (HCl, 3 M) catalyzed hydrothermal process (150°C, 3 h) yielded 12.8 wt% levulinic acid from *Ulva rigida*. However, the process needs to be optimized further with respect to reaction temperature, reaction time, and acid concentration to obtain improved yields of levulinic acid. Using marine algae for levulinic acid production is advantageous and makes the process sustainable. In addition to conventional mineral acid, alternate solid acid catalysts such as (polyoxometallates) need to be developed for the conversion of *Ulva rigida* to levulinic acid.



Scheme 3. Reactivity of levulinic acid making it a key strategic chemical [Amudhavalli Victor, Indra Neel Pulidindi, Aharon Gedanken (2014) Levulinic acid production from *Cicer arietinum*, Cotton, *Pinus radiata* and Sugar cane bagasse, *RSC Advances*, 4, 44706-4471]. Adopted with permission from the Royal Society of Chemistry.

The design of diverse process pathways for product diversification makes the biorefinery sustainable. Thus strategies should be developed for converting the carbohydate component of seaweeds to various products apart from levulinic acid and bioethanol by devising innovative strategies for improving the yields and reducing the process severity. For instance, in principle, the theoretical limit on the yield of bioethanol from cellulose can be raised from 51 wt% by capturing and reusing the CO_2 evolved in the fermentation process by charging the fermentation broth with cyanobacteria that could be metabolizing CO_2 and synthesizing glycogen which could be further converted to bioethanol. Such efforts need to be made in near future. Likewise, the current commercial levulinic-acid production processes use conventional mineral acid as catalyst. However, the process could be environmentally benign as well as profitable if a reusable solid acid catalyst like activated-carbon-supported heteropoly acids is employed [24]. Development of such pathways requires further research. In addition, the inevitable reaction byproduct of the bioethanol production process, glycerol, could be a valuable feedstock for the production of propane diols which are nearly 20 times more costly and value added [27]. Conversion of glycerol to valuable chemicals is another avenue in biofuel production from seaweed.

CONCLUSION

Typical challenges in the farming and conversion of seaweeds to biofuels and biochemicals are outlined. Solutions, such as the adoption of integrated multi-tropic aquaculture for the effective utilization of resources and production of high carbohydrate (32 wt%) *Ulva rigida* with accelerated growth rates (by a factor of 41), were offered. In addition, unconventional methods, such as ultrasound irradiation and solar energy for the simultaneous saccharification and fermentation (SSF) of seaweeds to bioethanol were demonstrated successfully. The marine seaweed, *Ulva rigida*, is a potential feedstock for the high-yield production of bioethanol (16 wt%) and levulinic acid (12.8 wt%). Solar-energy-driven continuous-flow process for the SSF of *Ulva rigida* to bioethanol is appealing for industrial adaptation owing to its atom and energy efficiency.

ACKNOWLEDGMENTS

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BIOGRAPHICAL SKETCHES

Dr. Indra Neel Pulidindi

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Education: Dr. Pulidindi received his primary and secondary education from APPMills Model High School, Rajahmundry, India from 1985 to 1995. He received his MSc from Andhra University, India in 2002. He received his PhD from IIT Madras, India in 2010 with the doctoral thesis entitled "Development and exploitation of carbon materials from plant sources."

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Professional Appointments:

Dr. Pulidindi was given an opportunity to work in the laboratory of Professor Tae Hyun Kim at Kongju National University, Korea (1.11.20116 to 28.2.2017) and at Hanuyang University, Ansan campus, Korea (1.3.2017 to till date) as a visiting scientist. He was a

postdoctoral researcher under the supervision of Prof. Aharon Gedanken in Department of Chemistry, Bar-Ilan University, Israel for six year (2.9.2010 to 30.10.2016).

Research and Professional Experience:

Dr. Pulidindi's research interests include nuclear chemistry, catalysis, materials science, renewable energy, and biofuels. He has published 33 peer-reviewed manuscripts, two book chapters and has applied for 5 patents. He has an h-index of 10 and i10-index of 10 with 428 citations. He is passionate about identifying solutions for societal problems such as energy crisis or environmental pollution by means of utilizing natural resources such as solar energy, CO_2, and biomass. Energy crisis and environmental deterioration are the two major problems human society is currently facing. His research focuses on developing alternate renewable energy sources. Towards realization of this goal, it is imperative that one should utilize the solar energy which is abundantly available and also devise strategies to utilize CO_2 as an organic raw material for the production of fuels and chemicals. Dr. Pulidindi is currently working exclusively on the conversion of both terrestrial lignocellulosic and algal biomass to biofuels and biochemicals using solar energy for fuels production and lignin valorization. Mankind need to learn and mimic nature for sustainability and wellbeing; therefore, he focuses on understanding and mimicking nature by utilizing natural resources and trying to solve societal problems.

Honors:

Recipient of advanced scientific-brain fellowship of the Korean Federation of Science and Technology Societies, 2016-2017. Editorial board member of Journal of Catalyst and Catalysis, Editorial board member of Emerging trends in Chemical Engineering, Prof. M. N. Sastry Shastaibdpoorthi Celebration Gold Medal (2002, for 1st ranking in M. Sc., 2nd year), Prof. B. S. V. Raghava Rao Memorial Prize (2001, for 1st ranking in M. Sc., 1st year), Prof. R. Sambasiva Rao Prize (2001, for 1st ranking in M. Sc., 1st year), Gold medalist (Best outgoing student in X^{std}, 1995, APPMills Model High School, Rajahmundry).

Publications in the Last Three Years:

(1) Alex Tangy, Indra Neel Pulidindi and Aharon Gedanken (2017) Continuous flow biodiesel production from waste cooked oil using microwave irradiation and supported SrO catalyst, *Bioresource Technology*, 224, 333-341.

(2) Alex Tangy, Vijay Bhooshan Kumar, Indra Neel Pulidindi, Yael Kinel-Tahan, Yaron Yehoshua and Aharon Gedanken (2016) In situ transesterification of *Chlorella vulgaris* using carbon dot functionalized strontium oxide as heterogeneous catalyst under microwave irradiation, *Energy & Fuels*, 30, 10602-10610.

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Patents:

(1) A. Gedanken, P. Indra Neel, T. Betina, Solar-aided conversion of marine algae and Biomass to bioethanol, US Provisional Patent Application No 62450107, 25th January 2017.

Book Chapters:

(1) Betina Tabah, Indra Neel Pulidindi, Venkateswara Rao Chitturi, Leela Mohana Reddy Arava, Aharon Gedanken (2017) Solar-energy driven bioethanol production from carbohydrates for transportation applications, in *Solar Energy: Systems, Performance and Recent Developments*, Book chapter, Nova Science Publishers, Inc.
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Research and Professional Experience:

Leor's research interests include finding sustainable solutions to global problems, namely aquaculture of marine biomass and the conversion and production into value-added products.

Publications Last Three Years:

(1) Korzen, Leor, Avigdor Abelson, and Alvaro Israel. "Growth, protein and carbohydrate contents in *Ulva rigida* and Gracilaria bursa-pastoris integrated with an offshore fish farm." *Journal of Applied Phycology* (2015): 1-11.
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(4) Korzen, Leor, et al. "An economic analysis of bioethanol production from the marine macroalga Ulva (Chlorophyta)." *Technology* (2015): 114-118

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Professional Appointments:

PhD candidate under the supervision of Prof. Aharon Gedanken in Department of Chemistry and Institute for Nanotechnology and Advanced Materials at Bar-Ilan University.

Research and Professional Experience:

Betina's PhD thesis focuses on novel methods for the conversion of biomass to bioethanol. She is passionate about finding green solutions to global problems. Her research interests include potential alternative energy sources, biofuels and renewable energy studies, utilizing different biomass for energy applications, developing biofuel production systems and up-scaling to industrial applications, and conversion of wastes and plant residues into value-added products.

Honors:

Betina was selected as a 2014 Rieger-JNF Fellow and 2015 Marshall Tulin Fellow in Environmental Studies by Rieger Foundation, USA. She has received many awards including Jewish National Fund-Keren Kayemeth LeIsrael prize for excellence in research (2011), The Robert Equey prize for excellence in desert studies (2011), Selim and Rachel Benin award of merit (2013), David Brekovsky award for academic excellence (2014), Turkish Union in Israel award of merit (2014), The Salti Foundation award of merit (2015), and Cyd Hessner prize for academic excellence and contribution to the community through volunteer work (2015). Her recent manuscript published in *ChemSusChem* was selected as a Key Scientific Article by Renewable Energy Global Innovations (Canada) for contributing to the excellence in energy research (2016). Her poster presentation entitled "Solar-energy driven solid-state fermentation for continuous flow bioethanol production" has won the "Best Poster Award" at NanoIsrael 2016 conference.

Publications in the Last Three Years:

(1) Tabah, B., Pulidindi, I. N., Chitturi, V. R., Arava, L. M. R., Gedanken, A. Solar-energy driven bioethanol production from carbohydrates for transportation applications. In *Solar Energy and Solar Panels: Systems, Performance and Recent Developments*; Joel G. Carter, Ed.; Energy Science, Engineering and Technology Series; Nova Science Publishers, Inc.: New York, 2017; pp 1-66.

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Amudhavalli Victor

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Education: Mrs. Amudhavalli received her BSc and MSc degrees in Pharmacy from the Tamil Nadu Dr M G R Medical University. Mrs. Amudhavalli carried out her doctoral studies in the field of renewable energy under the supervision of Prof. Aharon Gedanken and submitted her doctoral thesis titled "Can biomass be a sustainable feedstock for biorefinery?" to the Department of Chemistry, Bar Ilan University, Israel on 22/09/2016.

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Professional Appointments:

Mrs. Amudavalli was a doctoral researcher at the department of Chemistry, Bar Ilan University from 01/03/2013 to 22/09/2016. She has six years (2006-2012) of teaching experience as a lecturer at the SRM college of Pharmacy, SRM university, Chennai, India.

Research and Professional Experience:

Environmental deterioration and energy insecurity are the vital challenges society is facing currently. Mrs. Amudhavalli's research emphasis is on developing alternate chemical and electrochemical energy sources that are renewable and sustainable. Utilization of natural and abundant resources (biomass, agricultural and forest wastes, food wastes, kitchen wastes, municipal wastes, CO_2 and solar energy) for attaining this objective is one of the strategies that she has adopted. Currently, she is developing microwave, sonochemical and solar energy based strategies for the production of biofuels and biochemical, especially from lignin. Also her interest is centered around activation of CO_2 via chemical and photochemical means. Another area of her research focus is to develop cost effective and selective sensors for biomolecules like dopamine that have relevance to health sector. She has 3 peer-reviewed publications to her credit during the last four years of research in the area of renewable energy.

Publications in the Last Three Years:

(1) Amudhavalli Victor, Indra Neel Pulidindi, Tae Hyun Kim, Aharon Gedanken, (2016) Design of a selective solid acid catalyst for the optimization of glucose production from Oryza sativa straw, *RSC Adv*., 6, 31.

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After his post-doctoral research at UCLA, US, he joined the Israel Oceanographic Institute in Haifa, Israel in 1992 where he is currently a senior scientist and Head of Department of Marine Biology. He contributed to the understanding of photosynthesis in seaweeds in response to environmental factors particularly those related to climate change. He lead several national and international projects in marine macroalgae cultivation and biotechnology including development of land- and sea-based cultivation systems. Israel's research interests include carbon fixation and ecology of marine algae, taxonomy of seaweeds applied phycology and renewable energy (biomass conversion to biofuels and biochemicals). Israel has published over 60 papers in peer-reviewed journals.

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Research and Professional Experience:

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(2) Ecological processes and principles of benthic marine environments, with emphasis on human impact on marine ecosystems
(3) Artificial Reefs: Planning, design and implementation
(4) Settlement and recruitment of benthic organisms
(5) Development and implementation of bio-monitoring methods (from molecular to community levels).
(6) Marine Protected Areas (MPAs) and community-based management
(7) FADs (Fish Aggregating Devices) and Fishery Management
(8) Sustainable aquaculture - Integrated Multi-Trophic Aquaculture (IMTA)

(9) Coral reef ecology
(10) Marine bioinvasion – Invasion and introduction of exotic marine organisms and their environmental impact on marine communities

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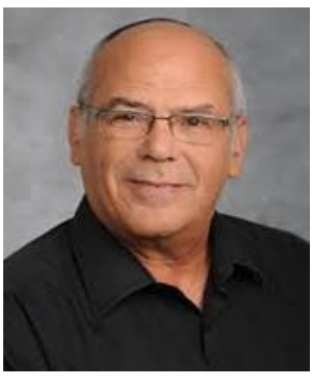

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Research and Professional Experience:

After his post-doctoral research at USC, USA, Prof. (Em.) Gedanken returned to Bar-Ilan University in 1975 as a senior faculty in Chemistry Department. He was a visiting scientist at AT&T Bell Laboratories several times in 1980-1988, and at NIDDK, NIH, USA in the summers of 1989-1991. **In 1999-2001, he was the** chairman of national committee for strategic studies in advanced materials and chemical technologies. In the EC program FP7, he was the Israeli representative to the Nanotechnology, Materials, and Processes (NMP) committee. He was also a partner in 12 EC projects, in which two of them were coordinated by him. From 2012 to 2016, he was a visiting chair professor in Department of Materials Science and Engineering, NCKU, Taiwan. Gedanken's research interests include sonochemistry, surface coating, synthesis of nanomaterials, microwave superheating, synthesis reactions under autogenic pressure at elevated temperatures, fuel cells, renewable energy (biomass conversion to biofuels), carbon materials, sensors, medicinal chemistry, and polymers. Gedanken has published over 725 papers in peer-reviewed journals, has 38 patent applications, and his h-index is 87.

Honors:

He is the recipient of 2009 Israel Vacuum Society and 2012 Israel Chemical Society awards of excellence in research.

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Chapter 77

ALUMINIUM IMPACT ON THE GROWTH OF BENTHIC DIATOM

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ABSTRACT

A laboratory experiment was performed, in tidal artificial conditions, with natural defaunated sediments that were artificially contaminated with aluminium salts. Benthic diatoms were cultivated on these contaminated sediments in a tidal mesocosm with natural seawater, in order to assess the aluminium impact on the growth of benthic diatom. The sediments were also leached by HCl (1M) to estimate the available/mobile part of the aluminium in the sediment.

This experiment highlighted that Al-contamination sediments had a strong effect on diatom communities growing at sediments surface. Drastic negative effect is perceptible beyond 10 mg of aluminium salts added by kg of natural sediment (which is a low contamination stress with regard to the Al initial content). The 1M HCl-extraction, widely used in the literature for metals mobility evaluation in marine sediments seems to underestimate aluminium availabilities for phytobenthic communities.

Keywords: marine sediment, HCl extraction, aluminium, phytobenthos

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1. INTRODUCTION

Heavy metals contamination in sediments is often assessed by the determination of their total contents and comparison with either established national guidelines or some background reference values (Covelli and Fantolan, 1997; Giancoli Barreto et al., 2004; Leleyter and Baraud, 2005; Dubrulle et al., 2007; Meybeck et al., 2007; Chand and Prasad, 2013). However metal concentrations derived from these total digests do not necessarily provide biologically meaningful data. The alternative is to use a partial extraction that only targets the labile mineral phases, as these are most likely to exert an influence on biota (Tam et al., 1989; Scouller et al., 2006). Thus, other studies focused on the potential mobility of these heavy metals in the sediments, estimated by either simple or sequential extraction procedures (Tessier et al., 1979; Leleyter and Probst, 1999; Sutherland, 2002; Rousseau et al., 2009; Devesa-Rey et al., 2010; Leleyter et al., 2012; Roussiez et al., 2013; El Azzi et al., 2013; Alvarenga et al., 2014; Islam et al., 2015; Hamdoun et al., 2015a and b).

For marine sediments, HCl (1M) extraction is recommended by many authors (Doherty et al., 2000; Snape et al., 2004; Burton et al., 2005; Scouler et al., 2006; Larner et al., 2008; Leleyter et al., 2012; Pena-Icart et al., 2014; Hamdoun et al., 2015a and b), as HCl is assumed to extract heavy metals thanks to its acidic properties combined with the chelatant property of Cl^-. For a comprehensive environmental risk assessment, both approaches (total and labile contents) are generally complementary in order to fully characterize the degree of sediments contamination.

Natural aluminium (Al) is the third most abundant element in the earth crust (around 8%, Allègre and Michard, 1973). Aluminium is known as a refractory element, typically not diagenetically labile. Moreover, anthropological activities (aluminium industry, water treatments, steel protection with aluminium sacrificial anodes, ...) release aluminium in the environment. As aluminum is more stable in solid than in aqueous phase, the anthropological aluminum poured in marine waters tends to be sorbed onto surrounding sediments (Pineau et al., 2008; Gabelle et al., 2012), which could act as a sink of aluminium to biota. Only a small part of Al in sediments is available (i.e non-silicate-bound), involving reactions such as solubilization, polymerization, complexation, crystallization, which are strongly controlled by the pH conditions (Tam et al., 1989; Gunkel et al., 2011). Many studies have proved that Al toxicity is an important factor affecting the growth of plant and aquatic biota in acidic ecosystems (Meiri et al., 1993; Platt et al., 2001; Lu et al., 2011). However, detecting anthropogenic aluminium contamination could be especially difficult. Indeed, the usual trace metals contaminations studies (using background references comparisons) could not be easily transposed into a major element study (such as aluminum), because anthropological quantities are often negligible with regard to the natural amount. Partial extractions seem to provide more sensitive indication of aluminium contamination, as the first tests on natural sediments (Gabelle et al., 2012; Leleyter et al., 2012) highlighted some differences in the mineralogical partitioning of natural or anthropological aluminium.

The processes controlling the fate of dissolved aluminium are still not well understood (Wang et al., 2015) and up to now, the complex biogeochemical cycle of this element has been poorly understood (Gunkel et al., 2011). Benthic diatoms are an important component of phytobenthos inhabiting the intertidal mudflats by constituting the first primary producer in muddy estuaries (Underwood and Kronkamp, 1999) by biofilms at the sediment surface

(Decho, 1990; Wingender et al., 1999). Benthic diatoms can be used as bioindicators of metallic pollution (Gold et al., 2003a; Lai et al., 2003; Cunningham et al., 2005; Morin et al., 2007), because of their high level of primary productivity in coastal sediments (Underwood and Kronkamp, 1999), their sensitivity to changes in water quality (Dixit et al., 1992; Stevenson and Pan, 1999), and their fundamental role in the food webs (Lefebvre et al., 2009). *In situ* studies conducted at sites exhibiting a high level of metals and microcosm experiments have demonstrated a decrease in productivity, diversity and changes in species composition of diatom communities (Takamura et al., 1989; Hill et al., 1997; Sabater, 2000). As reported by Gold et al. (2003b) and Duong et al. (2010), metal contamination had a strong effect on the density of diatom communities, possibly corresponding to a reduction in the rate of cell division of diatom species as demonstrated by Rivkin (1979), cessation or interruption of cell division (Dickman, 1998), the development of teratogenic forms (Arini et al., 2013), and also physiological impairments regarding photosynthesis, mitochondrial metabolism (Arini et al., 2012) and gene expression (Kim Tiam et al., 2012). Thus changes in benthic diatoms have been revealed to be a good estimator to assess heavy metal contamination (such as Cd, Cu, Zn, …) in freshwater ecosystems (Say and Whitton, 1980; Foster, 1982; Medley and Clements, 1998; Sabater, 2000). However, benthic diatoms in rivers can handle high concentrations of metal pollution such as cadmium, and biomonitoring surveys show the bioremediation process exerted by benthic diatoms (Arini et al., 2012) while reverse potential of teratogenic forms can show cadmium decontamination after experimental contamination (Arini et al., 2013). The behaviour of Al at the water-sediment interface is important for controlling dissolved concentrations in bottom waters via resuspension processes. The major processes removing dissolved Al from the water column include active biological uptake by diatoms (Wang et al., 2015), as benthic diatoms are ecosystem engineers capable of bioremediation of metallic pollution (Arini et al., 2012).

A mesocosm experiment was performed, with natural defaunated sediments that are contaminated with aluminium salts. Benthic diatoms are cultivated on these contaminated sediments in a tidal Mesocosm (Ubertini et al., 2015), in order to evaluate the aluminium impact on the growth of benthic diatom. The sediments are also leached by HCl (1M) to estimate the available/mobile part of the aluminium in the sediment. The aim of the present work is to study the potential uptake by benthic diatoms of non-natural Al in marine sediments, levels of toxicity for benthic diatoms and the capacity of the aluminium geochemical characterization procedures (total and labile) to be used in the evaluation of aluminum environmental risk.

2. MATERIAL AND METHODS

2.1. Sediments

A stock of fine sediment was collected in the Orne estuary (WGS84, 49.28°N, -0.24°W) from 10 to 20 cm below the surface and brought back to the lab. After 1 month in darkness, this sediment was sieved on a <1 mm mesh to remove the macro-fauna, then crushed and homogenized in an agate mortar and sieved at 80 µm. Control experiment consisted of Al natural sediment (T). Some aliquots of T-sediment were mixed thoroughly with variable

quantities of aluminium sulphate ($Al_2(SO_4)_318,H_2O$, from Riedel-Haën, puriss quality, > 99%), in order to obtain five Al-contaminated sediments, designed hereafter as C1 to C5 with added content of Al from 1 to 1000 $mg.kg^{-1}$ (Table 1). Then, contaminated sediments (C1 to C5) were sprayed with ultra-pure water and manually mixed. After drying (40°C), the C-sediments were crushed and homogenized in an agate mortar.

Table 1. Total Al contents measured in the sediments after Al contamination, values are expressed per kg of dry sediment; SD: Standard Deviation (3 replicates); Normalisation ratio with [Cx] and [T] = total concentration in sediment Cx and T respectively

	Added Al *mg.kg^{-1}*	Measured total content *g.kg^{-1}*	*SD*	Normalisation ratio: [Cx]/[T]
T	0	21.7	*0.9*	1.00
C1	1	22.2	*0.1*	1.02
C2	10	22.2	*0.1*	1.02
C3	100	21.9	*0.1*	1.01
C4	500	22.7	*0.3*	1.05
C5	1000	22.8	*0.2*	1.05

Al total contents in all the sediments were obtained after solubilisation of the solid matrix by an alkaline fusion procedure (NF ISO 14869-2, AFNOR 2002): 0.2g of dried sediment was thoroughly mixed with 0.8g of lithium metaborate and 0.2g of lithium tetraborate in a Pt crucible. The mixture was heated at 1000°C for 45 min and then the fusion product was immediately put in 60 mL of 1 $mol.L^{-1}$ HNO_3 solution until total dissolution of the occurred residue. The recovered solutions were made up to a volume of 100 mL with 1 $mol.L^{-1}$ HNO_3 solution.

The aluminium concentrations were measured by Inductively Coupled Plasma- Atomic Emission Spectrometry (ICP-AES, Varian, Vista MPX). The analytical quality of the chemical data was controlled using the standard certified material HR-1 (Canada Center for Inland Waters National Laboratory for Environmental) and the standard marine sediment (PACS2) (Gabelle et al., 2012; Hamdoun et al., 2015a).

Single extraction with 1 mol. L^{-1} HCl was also applied to all the sediments (T, C1 to C5). 1g of dry sediment was mixed with 10 mL of 1 $mol.L^{-1}$ HCl solution under agitation (200tr/min) at room temperature for 1h. The resulting mixture was centrifuged (3000 rpm; 2min), filtered at 0.45 µm (syringe filter, Millex - SLHN033N). The obtained leachates were analyzed by ICP-AES.

2.2. Sediment Preparation and Benthic Diatom Cultivation Experiment in Response to Contamination by Aluminium

24 PVC cores (diameter: 15 cm, height: 20 cm) were filled with 2 layers: bottom layer with natural sediment and a superficial mud layer (1st cm of each core) that was treated with different levels of contamination (T and C1 to C5 sediments; 4 replicates) before being inoculated with fresh benthic diatoms. The first upper cm was then homogenized with an epipelic MPB inoculum collected from a mudflat located in the Orne estuary (WGS84,

49.28°N, -0.24°W) in Basse-Normandie during April 2011. It was collected by scratching the sediment surface. The biofilm was mainly composed of pennate diatoms including small *Navicula sp.* (length ~17µm, >95% of total MPB), *Amphora sp*., *Pleurosigma sp*., *Niztschia sp.* and *Cylindrotheca closterium*. The cores were positioned in trays (1.20 m x 1 m x1m, 12 cores capacity) (Figure 1). The first tray (called series I) contained T, C1 and C2 sediments, whereas the other tray (called series II) was performed with C3 to C5 sediments cores. The surface of the sediment was gently smoothed and cores were cultivated in a tidal mesocosm able to simulate a high/low tide alternation every 6 hours in order to simulate immersion and emersion phases.

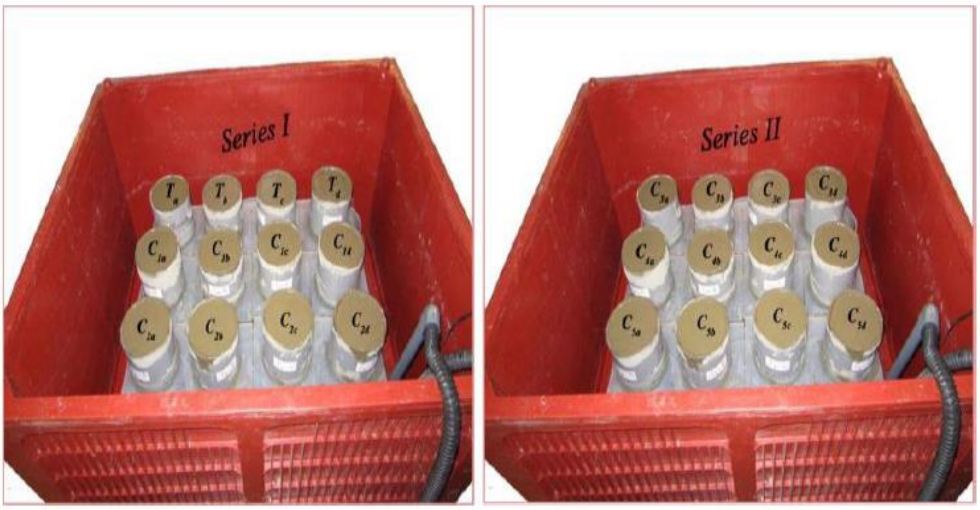

Figure 1. Unit (series I and II) with the 12 cores each.

The experimental mesocosm consists of two trays placed one on the top of the other (Figure 2). The lower unit served as seawater reservoir, to produce high and low tide, thanks to submersible pumps. The upper unit is used for the implementation of sediment cores (series I or II) and is covered with a roof equipped with a light intensity of 1600 µmol photons $m^2.s^{-1}$ (LUMINUX, 36W Osram), to reproduce day (during low tide) and night cycles. The experimental design was carried out according to Orvain et al. (2003) with 6h light: 18h dark regime. Natural seawater filtered over filters (1µM) was used for this experiment.

Water samples were collected at 0, 5 and 9 days and filtered through a 0.45 µm (Millipore) in polyethylene flask. The filtrates (14 mL) obtained were then acidified with HNO_3 (suprapur: 60%) and stored at 4°C for Al and trace metal analysis by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES, Varian, Vista MPX).

Phytobenthic biomass was assessed by measuring the chlorophyll *a* (Chl *a*) content. Sediments samples were taken from the experimental cores at 5 and 9 days at the beginning of diurnal emersion periods in order to access respectively the growth and stationary phases of the biofilm (Orvain et al., 2003). The first upper cm of the sediment (2 replicates) was sampled with a 2-cm diameter cut syringe. The uppermost first centimeter was cut and mixed in 50 mL Falcon tubes. All frozen samples were lyophylised. Chl *a* extraction was applied on a given amount of dry sediment, with approximately 150 mg of sediment that was accurately

weighed. Phytobenthic biomass was estimated by quantifying fluorometrically the chloropigments after extraction in 90% acetone and in the dark overnight under constant agitation at 4°C and centrifugation (4°C, 5 minutes, 3500 rpm). The chlorophyll extracts were measured on a Turner Designs TD 700 fluorometer (USA) following the method of Welschmeyer (1994). In order to avoid the dewatering over the emersion period (Perkins, 2003), water content and the bulk density of sediments could be used to express the Chl*a* as a content per m^{-2}.

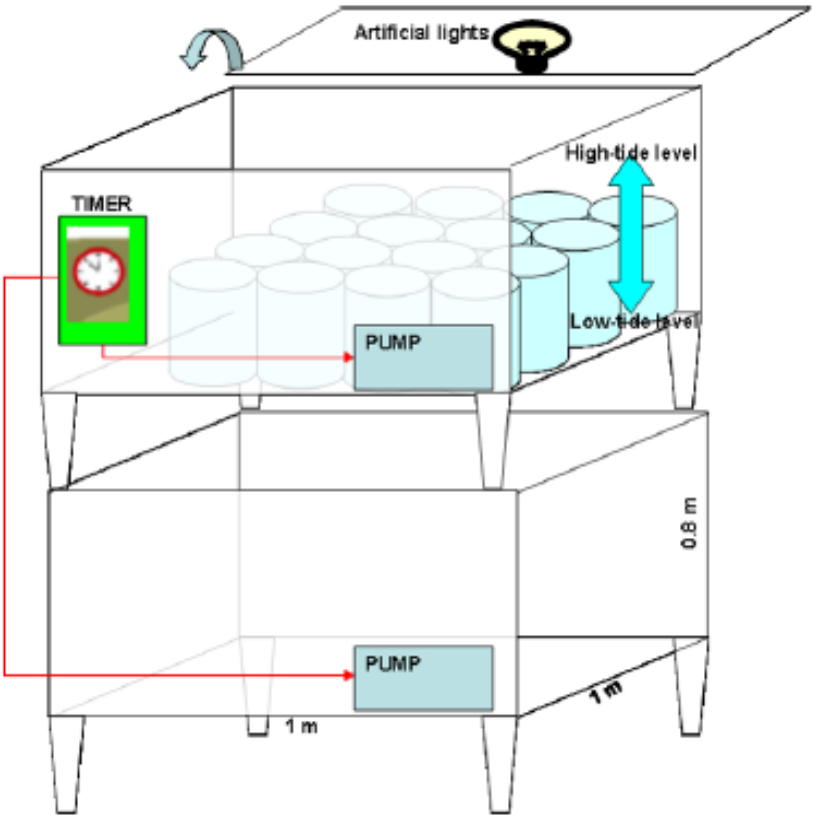

Figure 2. Tidal mesocosm reproducing the alternation of diurnal daytime low-tide period lasting 6 hours (8 :00AM – 2 : 00 PM), and nocturnal high-tide period lasting 18 hours (2:00 pm – 8:00).

3. Results and Discussion

3.1. Sediments Contamination

The measured quantities (Table 1) for all the contaminated sediments indicated that the contamination procedure was correct, without any loss or excessive contaminations. We noticed no meaningful difference (<5%, Table 1) between the Al total content in the background (T sediment) and the Al total content in contaminated sediments (low contamination with regard to the Al initial content which is naturally a major element in sediments).

Mobile metals concentrations are measured (3 replicates) in the tested sediments (T, C1 to C5, see Table 2) in the leachates after the HCl extraction and the mobile part is expressed as the percentage of the total Al content initial measured in the corresponding sediment. The quantities of labile Al remain quite stable (521 to 564 $mg.kg^{-1}$) for the low contamination rates (0 to 100 $mg.kg^{-1}$). On the opposite, the extracted quantities increased for the highest contamination rates (500 and 1000 $mg.kg^{-1}$), respectively 873 and 1311 $mg.kg^{-1}$, suggesting that an important part of the added aluminium is scavenged in the labile fraction of the sediment.

The resulting percentage of aluminium extracted by HCl 1M in contaminated sediments (C4 and C4, i.e., 500 or 1000 $mg.kg^{-1}$ of sediment) increased (respectively 4 and 6%) and thus doubled the quite stable percentage (2 to 3%) in sediments with low contamination rate (T and C1 to C3, from 0 to 100 $mg.kg^{-1}$ of added Al).

Various authors (Agamian and Chau, 1976; Sutherland et al., 2001; Matus, 2007) reported that dilute HCl mainly extracted (from soils and sediments) Al species connected with anthropogenic contamination of the environment. Gabelle et al. (2012) also discriminated marine sediments contaminated by Al sacrificial from uncontaminated sediments thanks to similar results. They determined that Al mobilised by 1M HCl represented 2 ± 1% of the total Al present in the uncontaminated, whereas 5% to 11± 1% of the total Al was extracted by HCl from the sediments at the vicinity of Al sacrificial anodes.

Table 2. Quantities (Q_{HCl}) and percentages (% $_{HCl}$) of Al extracted by HCl extraction, SD: standard deviation (3 replicates)

	(Q_{HCl}) $mg.kg^{-1}$	SD	(% $_{HCl}$)
T	564	*3*	3
C1	536	*2*	2
C2	521	*6*	2
C3	547	*19*	3
C4	873	*9*	4
C5	1311	*6*	6

3.2. Water Contamination

The average concentrations of the total Al determined in water throughout the 9 days of experimentation range from 0.04 to 0.07 $mg.L^{-1}$ (Figure 3); these values are similar to concentrations previously reported for estuarine and marine waters. For example, concentrations from 9 to 70 µg/L, 5 to 50 µg/L, 10 to 60 µg/L, and 1 to 8 µg/L are reported for the GPMH (Seaport of Le Havre, France, Gabelle et al., 2012), Les Flamands (Seaport of Cherbourg, France, Mao et al., 2011) some Chinese estuaries (Zhang et al., 1999) and English Channel (Chou and Wollast, 1993) respectively.

The initial Al concentrations are similar for the two sets of experiment series (I and II). After 5 days, the concentrations increase in both cases, followed by a decrease for the next 4 days. The decrease is more pronounced for the low contamination set (0 to 10 $mg.kg^{-1}$ of added Al) with Al concentrations in water lower than the initial ones, whereas the final

concentrations remain higher than the initial ones for the second set (100 to 1000 mg.kg^{-1} of added Al).

The increase noticed after 5 days is probably due to the partial solubilisation of the added aluminum salts. The decrease then observed after 9 days could result from various processes, such as re-adsorption by the sediments, or uptake by the diatoms. Indeed, diatom can use a significant quantity of aluminium for their physiological requirements, but phytobenthic diatoms are above all capable of bioremediating pollutants out of their cells, by producing anionic polymers in the microenvironment (Wingender et al., 1999). These Extracellular Polymeric Substances (EPS) are very efficient to sequester cationic pollutants such as heavy metals, due to the anionic nature of these compounds (Decho, 2000). In case of physiological and environmental stress (contamination by heavy metals, pesticides, sediment evaporation, dehydration, saline stress, nutrient absence), a wide quantity of the photoassimilates of the benthic diatoms are secreted at the water/sediment interface (Orvain et al., 2003; McKew et al., 2011) to preserve cells against pollutants.

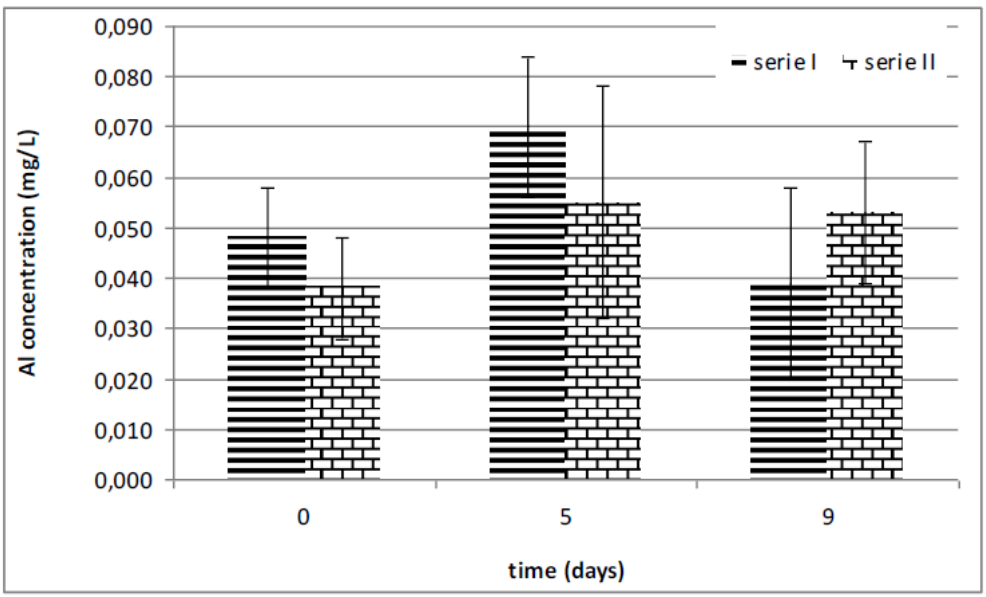

Figure 3. Al concentrations in the water throughout the experiment, 5 replicates.

3.3. Impact of Aluminium Contamination on Benthic Diatom Growth

The chl*a* content measured on the control cores (non contaminated T-sediment) remains stable from 5 to 9 days at 14 µg.g^{-1} (dry weight sediment, Figure 4) (or 120 mg.m^{-2}) because benthic diatoms show a rapid growth phase in 5 days (or less). The Chl*a* measured after 5 days are similar to the control for the two lowest contaminated samples (C1 and C2, with 1 and 10 mg.kg^{-1} of added Al). However, after 9 days, the values for C1 and C2 differ from the control: the higher the Al content is, the higher the Chl*a* is. This reflects the stimulation of the growth of benthic diatoms when there is moderately contamination in aluminium. Chl*a* content increases between T and C2 after 9 days of culture.

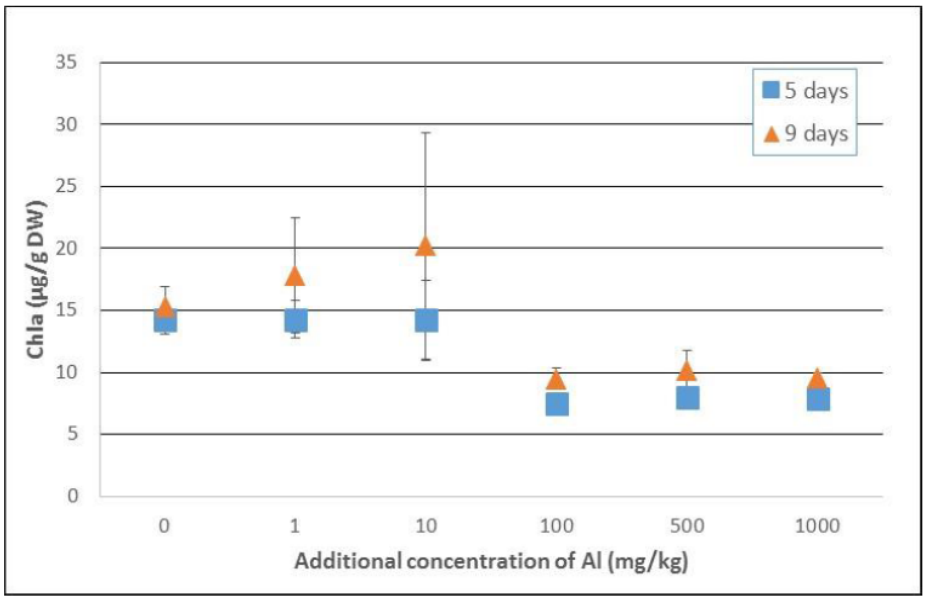

Figure 4. Influence of increasing stress of additional aluminium on Chlorophyll *a* (Chl *a*) contents in sediments.

In case of a moderate Al contamination (≤10 mg.kg^{-1}), benthic diatoms should resist to the presence of heavy metal under an apparent threshold of 10 mg.kg^{-1}. In case of physiological stress and pollution, benthic diatoms are known to secrete EPS in their micro-environment; the stimulation of this process can eventually lead to a better growth of the biofilm. We can hypothesize that benthic diatoms must enhance their photosynthesis activity, productivity and EPS secretion, to exert a phytoremediation process, that is mandatory to resist to the heavy metal contamination. Thus a moderate contamination can have a positive effect on the net growth of benthic diatoms in the epipelic biofilm: these preliminary results must be further investigated to refine the bioremediating strategy of these microalgae In contrast to the algal growth at low contamination rate (T, C1 and C2, ie, 0 to10 mg.kg^{-1} of added Al), our results indicate a lack of development of microalgae at higher contamination level (≥ 100 mg.kg^{-1} of added Al, sediments designed as C3, C4 and C5). In agreement with our results, various authors reported the same observations on the inhibitory effects of metals on algal growth capacity (Prasad and Prasad, 1982; Lasheen et al., 1989; Payne and Price, 1999; Nayar et al., 2003; Duong et al., 2010). Benthic diatoms do not grow and Chl*a* concentration remains very low, without any difference between days 5 and 9, revealing that no resilience can occur, even after some days after a strong contamination by heavy metals. The negative effects are so drastic, that this suggests a mass mortality of benthic diatoms, in response to high levels of Al contamination over 100 mg.kg^{-1}. Resilience strategies in response to Al contamination are efficient up to the threshold value of 10 mg.kg^{-1}, and this process surprisingly stimulates the growth and probably also the physiological activities in the biofilm.

CONCLUSION

This experiment has proved that the Al-contamination of sediments has a strong effect on diatom communities growing at the muddy sediments surface. The negative effect can be detected beyond 10 mg of aluminium salts added by kg of natural sediment. Indeed, benthic diatoms show a lethal threshold of aluminium contamination of 10 $mg.kg^{-1}$ of sediment. However, at lowest concentrations of added aluminium, phytobenthic are capable of resilience and a stimulation of the growth is observed probably in relation to a stimulation of photosynthetic activity, to produce and excrete more EPS in the biofilm that can absorb aluminium in excess. Further studies must be performed to better refine physiological mechanisms of benthic diatoms to exert a bioremediation of contaminating heavy metals and especially aluminium.

HCl-extraction showed that at high level of Al-salts contamination (500 mg or 1000 mg per kg of sediment), the aluminum mobility increases, respectively of 4 and 6%, compared to the initial values in natural conditions (3%). The Al mobility remained unchanged for the lowest contamination rates (1, 10 or 100 mg of salt per kg of sediment). The 1M HCl-extraction, widely used in the literature for metals mobility evaluation in marine sediments, underestimates aluminium availability for phytobenthic communities, in such case. Indeed, the % HCl for the C2 sediment (100 $mg.kg^{-1}$ of added Al), is similar to the ones obtained for lower contamination levels, whereas drastic effects on the diatoms are only observed for this specific concentration rate. On the opposite the increase of the % HCl for the highest contamination level (100 $mg.kg^{-1}$ of added Al) can be considered as a good prediction of the negative impact of Al on the diatoms that is actually observed.

Finally, in case of aluminium contamination, HCl extraction can be helpful for risk assessment only for significant contamination levels, that induces HCl mobility superior to 4%. When lower % HCl is measured complementary investigations might be necessary. Thus, an abnormal result (over 3%) for Al lability (after 1M HCl extraction) allows classifying the studied sediment as Al contaminated with important negative effects for benthic diatom. However a more 'classic' result (≤3%), does not allow the discrimination of contaminated or uncontaminated sediments.

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Chapter 78

EVALUATION OF MICRO-FABRIC NETWORK WITHIN MARINE SEDIMENTS BASED ON A ROCK MAGNETIC TECHNIQUE

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ABSTRACT

Magnetic techniques that use anisotropy of magnetic susceptibility (AMS) act as a proxy of preferred permeable orientation in basin-filling sediments, when it is applied on samples impregnated with a magnetic suspension. The unique method for quantifying heterogeneity in rocks is reviewed and its value for reconstruction of the preferred direction of pore fluid flow is reassessed critically. The authors also present results of their experiments, which dealt with secondary fracture networks developed in tight sandstones burying a foreland basin on an arc-arc collision zone. Directional analysis of AMS ellipsoid implies tectonic control on rupture development under strong trans-compressive regime. Micro-focus three-dimensional density imaging of test pieces has shown a substantial variation in pore fabric reflecting inhomogeneous impregnation of magnetic fluid within rocks.

1. INTRODUCTION

Micro-fabric of marine sediments is a versatile petrophysical information reflecting clast alignment for the reconstruction of sediment transport mechanism, direction of paleoflow and preferred permeable orientation of formation fluids since basins remote from hinterlands are filled with fine deposits lacking in visible manifestation of sedimentary structure. Hence

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many studies have explored standardized procedures for acquisition of grain fabric through conventional microscopy, which was summarized as an extensive review (Baas et al., 2007).

Baas et al., (2007) also evaluated a brand-new method utilizing magnetic techniques based on anisotropy of magnetic susceptibility (AMS) as a proxy for grain orientation, which benefits greatly from being able to measure a large number of grains in a three-dimensional space, in a short amount of time and with a lower sensitivity to user bias. The magnetic technique applied on samples impregnated with liquid containing suspension of ferromagnetic powder was originally developed for the purpose of oil exploration by Hailwood and his colleague (e.g., Hailwood et al., 1999) on the theoretical background of Pfleiderer and Halls (1994).

Because the rock magnetic method was first applied to continental setting where high porous sediments are widely distributed, previous studies tend to concentrate on evaluation of fabric of intact detrital grains and their interstitial pore geometry, omitting tectonic control on fracture generation and enhancement of effective permeability. The authors' preliminary study (Itoh et al., 2014a) on collision-related turbidite samples showed wider variety in micro-fabric reflecting secondary fracture network under strong tectonic stress. In this paper, we aim at verification of AMS usability as a textural indicator of rocks, with special emphasis on origin of fluid pathway within tight turbidite sandstones burying a foreland basin.

2. METHODOLOGY: A REVIEW

2.1. Anisotropy of Magnetic Susceptibility

To measure the initial magnetic susceptibility, a rock sample is placed in a low-intensity magnetic field of strength H, and the intensity of the induced magnetization (J) is measured for different orientations of the field. The induced magnetization is related to the field strength through the magnetic susceptibility (*K*) of the sample, where:

$$J=KH$$

The magnetic susceptibility of a sample is the summation of the susceptibilities of all mineral species within rock samples, and magnetic susceptibility is rarely identical in all directions of measurement; it more or less is an anisotropic parameter. The degree of anisotropy of magnetic susceptibility (AMS) is dependent primarily on mineral type, but even for a single type of mineral species the magnitude and anisotropy of magnetic susceptibility may considerably vary with many physicochemical properties (Tarling and Hrouda, 1993). Figure 1 presents significant parameters of AMS with typical types of susceptibility ellipsoids.

2.2. Conventional Method

As for common sedimentary rocks with low content of ferromagnetic minerals, AMS fabric generally reflects alignment of iron-rich silicates such as biotite and amphibole, of

which orientations are bound to sedimentary structure. Based on azimuth of untilted AMS principal axes of fine sediments, Itoh et al., (2006) argued that the AMS is controlled by shape anisotropy of minerals laid on bedding plane.

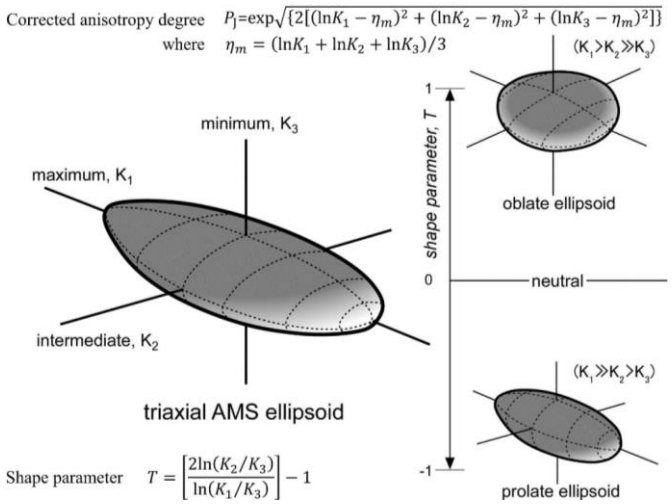

Figure 1. The susceptibility ellipsoids. Significant parameters of anisotropy of magnetic susceptibility (AMS), such as P_J and T, are calculated based on orthogonal principal susceptibilities (K_1, K_2, K_3).

Most igneous rocks are magnetically isotropic in primary state. Secondary structure may, however, cause detectable AMS trend. Based on intensive rock magnetic experiments, Itoh and Amano (2004) found that an enhanced AMS trend near a major fault cutting a granitoid pluton is originated from fine-grained authigenic magnetite grains precipitated on fracture surface.

Although such conventional methods are effective in case the possible AMS-carrying minerals are rather restricted, anisotropy of individual particles is brought about by the magnetocrystalline anisotropy or by the shape anisotropy, of which contributions vary with mineral species. Therefore more direct analytical method is necessary for quantitative description of micro-fabric of rocks.

2.3. Ferrofluid Method

Figure 2 shows a generalized scheme of analytical method utilizing liquid containing suspension of magnetic powder (hereafter referred to as ferrofluid). Since ultrafine ferromagnetic minerals in ferrofluid (mainly magnetite) are under superparamagnetic limit, coercive force (H_C) is actually negligible. As a result, maximum axis of AMS (K_1) of a rock sample impregnated with ferrofluid reflects elongate azimuth of pore network and most permeable direction.

Since the pioneering work of Hailwood, many researchers have applied the attractive method to various research areas. Nabawy et al., (2009) evaluated permeability and magnetic pore fabrics of an aquifer, and stated that pore fabrics of most formations in the Tushka Basin, Egypt are closely linked to paleocurrent directions with minor exception showing structural control (affinity for the main fault trends around the study area).

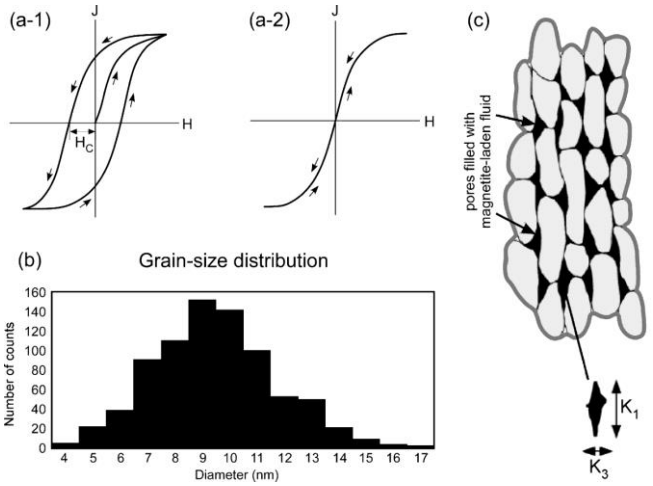

Figure 2. Generalized basis of analytical method utilizing ferrofluid. (a) Comparison of magnetic hysteresis between conventional ferromagnetic body (a-1) and ferrofluid (a-2). Ultrafine ferromagnetic minerals (mainly magnetite) are under superparamagnetic limit, and Hc is negligible. (b) Grain size distribution of a ferrofluid (provided by FerroTec Co., Ltd.). (c) Schematic view of a rock sample impregnated with ferrofluid (darkened parts).

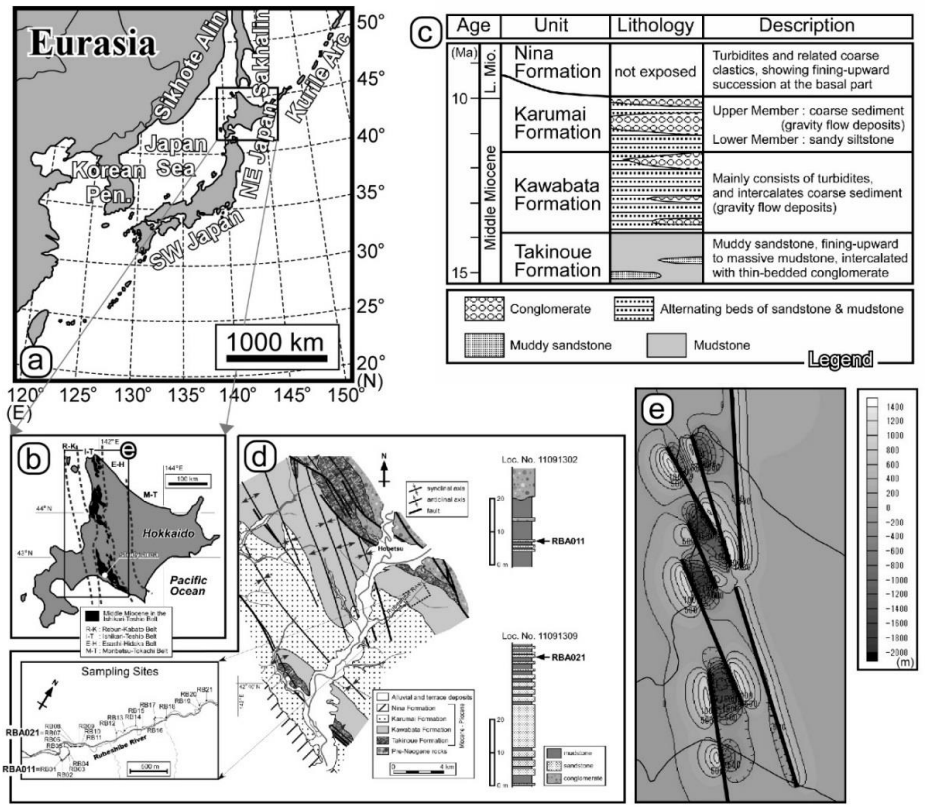

Figure 3. Geological background of the case study area. (a) Regional index. (b) Cenozoic tectonic context of central Hokkaido. (c) Neogene stratigraphy of the study area. (d) Geology of the study area (simplified from Kawakami et al., 1999), and locations of rock magnetic samples (modified from Itoh et al., 2014a). (e) Dislocation modeling of the Kawabata sedimentary basin (modified from Itoh et al., 2014b). Mapped area is shown by an envelope in (b).

Almqvist et al. (2011) utilized AMS-derived pore shape geometry for prediction of elastic properties for porous and anisotropic synthetic aggregates. It is noted that they adopted X-ray micro-tomography density contrast imaging, where attenuation of X-rays is related to the density contrast of the material, of dry and ferrofluid-processed specimens, and visually evaluated completeness of fluid saturation. At the end of this paper, we attempt to visualize various patterns of impregnation using similar apparatus.

Reflecting highly porous nature of samples in continental setting, the above studies used a vacuum chamber to impregnate ferrofluid. Although Baas et al., (2007) made a comment on high pressure treatment for complete saturation with ferrofluid, detailed experimental condition was not shown.

3. CASE STUDY

3.1. Geological Background

Sedimentary basins on convergent margins are rapidly filled by voluminous clastics, part of which is volcaniclastic reflecting active volcanism, then deformed and exhumed under compressive stress provoked by various tectonic events. On such condition, tight sedimentary rocks suffering diagenesis are less porous and instead studded by numerous secondary fractures. One presumable theory is that micro-fabrics of rocks are different from those in stable continental setting.

Our focus is set on a turbidite sequence, Kawabata Formation, widespread in central Hokkaido, which deposited during a middle Miocene arc-arc collision event. Figure 3 presents geological background of the case study area. Hokkaido is located on the northeastern margin of Eurasia, and has suffered various tectonic events through the Cenozoic. Reflecting backarc spreading and subsequent collision events, Neogene sequence of the study area shows a transgression-regression cycle (see Figure 3c). Recently a numerical modeling was executed on the Kawabata sedimentary basin (Itoh et al., 2014b) and clarified that transpressional regime followed by strong compression on the NNW-SSE regional fault zone is essential to restore the basin configuration (Figure 3e).

3.2. Previous Studies

The authors executed preliminary rock magnetic studies on the Kawabata Formation (Itoh et al., 2013, 2014a). Rock magnetic samples were taken along the Rubeshibe river in Hobetsu district in central Hokkaido, where Kawakami et al., (1999) reported detailed stratigraphic and structural data (see Figure 3d).

Itoh et al., (2013) collected samples of the Kawabata Formation with a battery-powered electric drill at 21 sites along the Rubeshibe route. The bedding attitudes were measured on outcrops to compensate for tectonic tilting later. Between seven and sixteen independently oriented cores 25 mm in diameter were obtained at each site using a magnetic compass. Cylindrical specimens 22 mm in length were cut from each core and the natural remanent magnetization (NRM) of each specimen was measured using a cryogenic magnetometer

(model 760-R SRM, 2-G Enterprises). Low-field magnetic susceptibility was measured on a Bartington MS2 susceptibility meter, and the AMS was measured using an AGICO KappaBridge KLY-3 S magnetic susceptibility meter.

Their sedimentological results are shown in Figure 4. AMS fabrics of most raw samples of the Kawabata Formation are highly oblate, as shown by positive *T* parameters near unity. This fabric is essentially confined to the bedding plane under gravitational force, and the authors considered the fabric as being governed simply by the shape anisotropy of paramagnetic minerals, i.e., alignments of elongate or platy grains such as amphibole or mica based on hysteresis study indicative of a negligible amount of ferromagnetic material.

Notably, the imbrication of the oblate AMS fabric matches visible sedimentary structures (Figure 4a), suggesting that AMS data can serve to indicate paleocurrents after the contributors to the magnetic fabric have been identified. It is also indicated that K_1 of prolate samples (with negative *T* parameters) tend to align perpendicular to the paleocurrent direction, implying that elongate grains roll on the sediment surface.

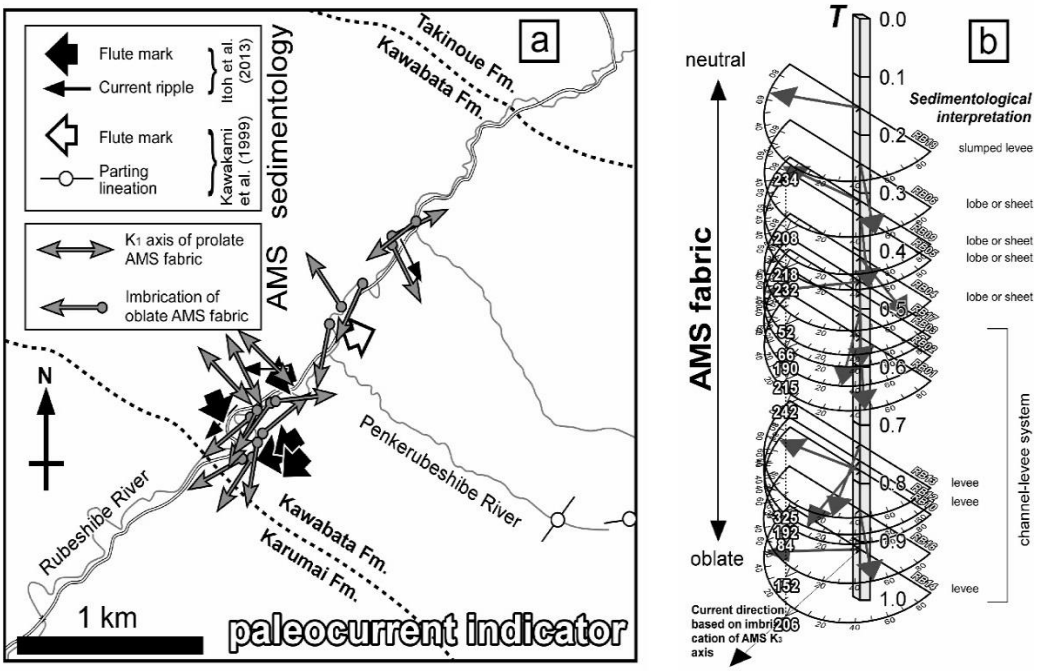

Figure 4. Summary of previous sedimentological analyses modified from Itoh et al., (2013). (a) Paleocurrent map of the Kawabata Formation around the Rubeshibe River route. Formation boundaries are after Kawakami et al., (1999). (b) AMS paleocurrent indicators of the Kawabata Formation. Directions of K_1 (gray arrows) are shown as acute angles from the dotted baseline of K_3 axis imbrication. Downcurrent orientations based on imbrication data are depicted as outlined numbers on the baseline. Vertical positions of the data represent degree of AMS oblateness shown by the *T* parameter. Samples with negative *T* values are excluded from the diagram because such cases have a large scatter in the K_3 directions.

Figure 4b shows a series of paleocurrent indicators identified in the Kawabata Formation as a function of the AMS shape parameter (*T*). The intensity of alignment forcing inferred from AMS data is closely related to sedimentary facies (shown on the right in the figure) determined by field observation. For example, weak hydrodynamic forcing corresponds to

fine rhythmically alternating facies in channel-levee systems. Thus, the sedimentological context of muddy sediments' AMS fabric can be interpreted in the light of sandy sediments' facies analysis.

Based on the sedimentological discussion above, Itoh et al., (2014a) executed ferrofluid experiments on selected Kawabata samples in the Rubeshibe route. Hand samples oriented using a magnetic compass were taken from two sites, RBA011 (muddy channel and levee turbidite) from RB01 and RBA021 (sandy sheet turbidite) from RB07. They were cut into cubic specimens with an approximate volume of 4 cm^3. Their permeability measured by a Pressure Decayed Profile Permeameter ranges 0.014~0.030 md for RBA011 and 0.053~0.151 md for RBA021, respectively. After evacuation for one day, all the samples were soaked in water-based ferrofluid (MSG W10 with saturation magnetization of 185 Gauss; provided by FerroTec Co., Ltd.) contained in a pressure vessel and impregnated under 5 MPa for 30 days.

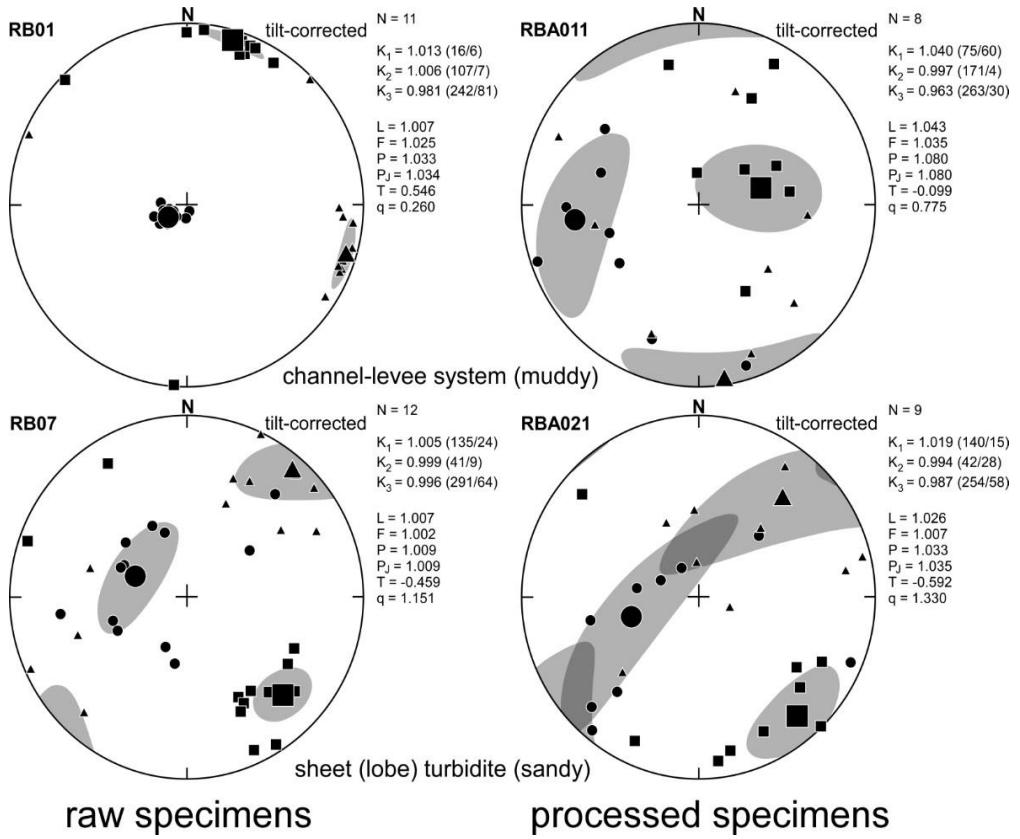

Figure 5. Tilt-corrected AMS fabric for the Kawabata Formation samples in raw (left) and ferrofluid-soaked (right) states. All the data are plotted on the lower hemisphere. Square, triangular and circular symbols represent orthogonal maximum (K_1), intermediate (K_2), and minimum (K_3) AMS principal axes, respectively, and larger symbols show their mean directions. Shaded areas are 95 % confidence limits of Bingham statistics. Anisotropy parameters posted on the equal-area diagrams are calculated based on Tarling and Hrouda (1993). Generally, ferrofluid impregnation results in enhanced anisotropy degree. In site RB01 (upper), processed specimen (RBA011) shows prolate fabric and quite different spatial arrangement of principal axes from the raw data, whereas site RB07 (lower) is characterized by similar AMS trend after ferrofluid treatment (RBA021).

Impregnated samples were washed with purified water, dried and then contained in plastic capsules. Their AMS data were measured using an AGICO KappaBridge KLY-3 S magnetic susceptibility meter, and much more than one digit larger bulk susceptibility indicates successful impregnation of ferrofluid. Measured AMS parameters were compared with those for raw samples in the same sites reported by Itoh et al., (2013). Figure 5 presents tilt-corrected AMS fabrics for the Kawabata Formation samples before and after ferrofluid treatment. Generally speaking, ferrofluid impregnation results in enhanced anisotropy degree (P_J). In site RB01, processed specimen (RBA011) shows prolate fabric and quite different spatial arrangement of principal axes from the raw data, whereas site RB07 is characterized by similar AMS trend after ferrofluid treatment (RBA021). They attributed such a variety in magnetic fabric to difference in microscopic sedimentary structure.

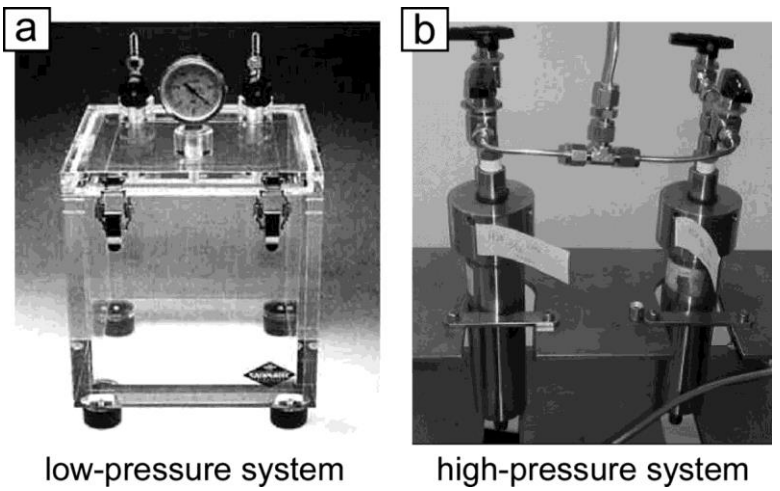

Figure 6. Experimental apparatuses to impregnate ferrofluid. (a) Vacuum chamber for low-pressure treatment. (b) Pressure vessel for high-pressure treatment.

3.3. Experimental Scheme

In the present study, the authors prepared two specimens (4 cm^3 oriented cubes cut from 25 mm diameter cores originally taken by Itoh et al., 2013) from 13 sites of the Kawabata Formation to observe lithofacies effect on the AMS fabrics. Using the water-based ferrofluid (MSG W10), one was impregnated in a vacuum chamber (Figure 6a) for 30 days, the other was impregnated in a pressure vessel (Figure 6b) under 5 MPa for 30 days. They were washed with purified water, dried and then contained in plastic capsules. Their AMS data were measured using an AGICO KappaBridge KLY-3 S magnetic susceptibility meter.

4. RESULTS

Bulk susceptibility of the processed samples were one to two digits larger than raw state indicating successful impregnation of ferrofluid. As shown in Figure 7, magnitudes of magnetic fabrics in ferrofluid-impregnated samples (solid symbol) are much greater than raw

samples (open symbol) for 13 sites of the Kawabata Formation. Low-pressure treatment did not so much affect shape parameter *T*, whereas some specimens show clear prolate fabric after high-pressure treatment. Thus we investigate a directional trend of AMS axes in the next section.

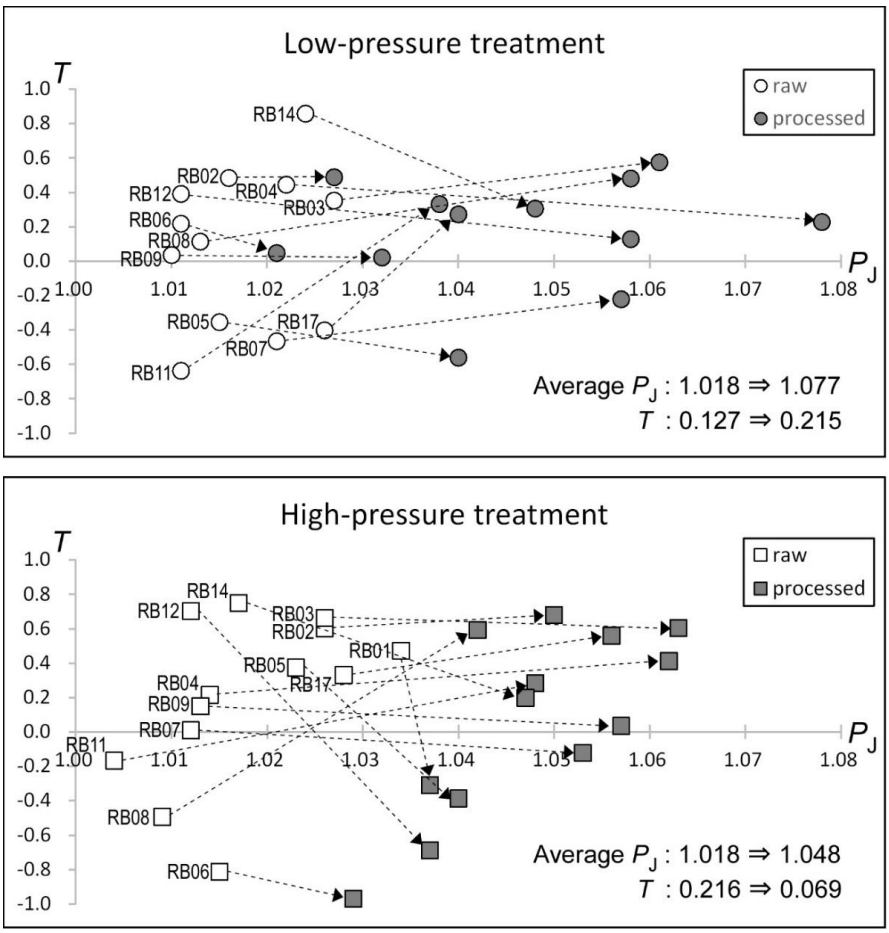

Figure 7. Magnitudes of magnetic fabrics in raw samples (open symbol) and ferrofluid-impregnated samples (solid symbol) for 13 sites of the Kawabata Formation. As for the low-pressure treatment, site RB01 is omitted because its processed P_J (1.448) is out of the range of transverse axis. Average P_J and T are arithmetic means of specimen data.

5. DISCUSSION

5.1. AMS Fabric

Figure 8 delineates tilt-corrected AMS axes for 13 sites of the Kawabata Formation samples in raw (left) and ferrofluid-soaked (right) states. Generally, ferrofluid impregnation results in enhanced anisotropy degree. In case of low-pressure treatment (upper), AMS trend is more or less similar before and after ferrofluid experiment, whereas high-pressure treatment (lower) results in considerable decrease in T parameter although spatial

arrangement of principal axes remained unchanged. This implies that pressurized ferrofluid was impregnated via a pathway which is impermeable under atmospheric pressure.

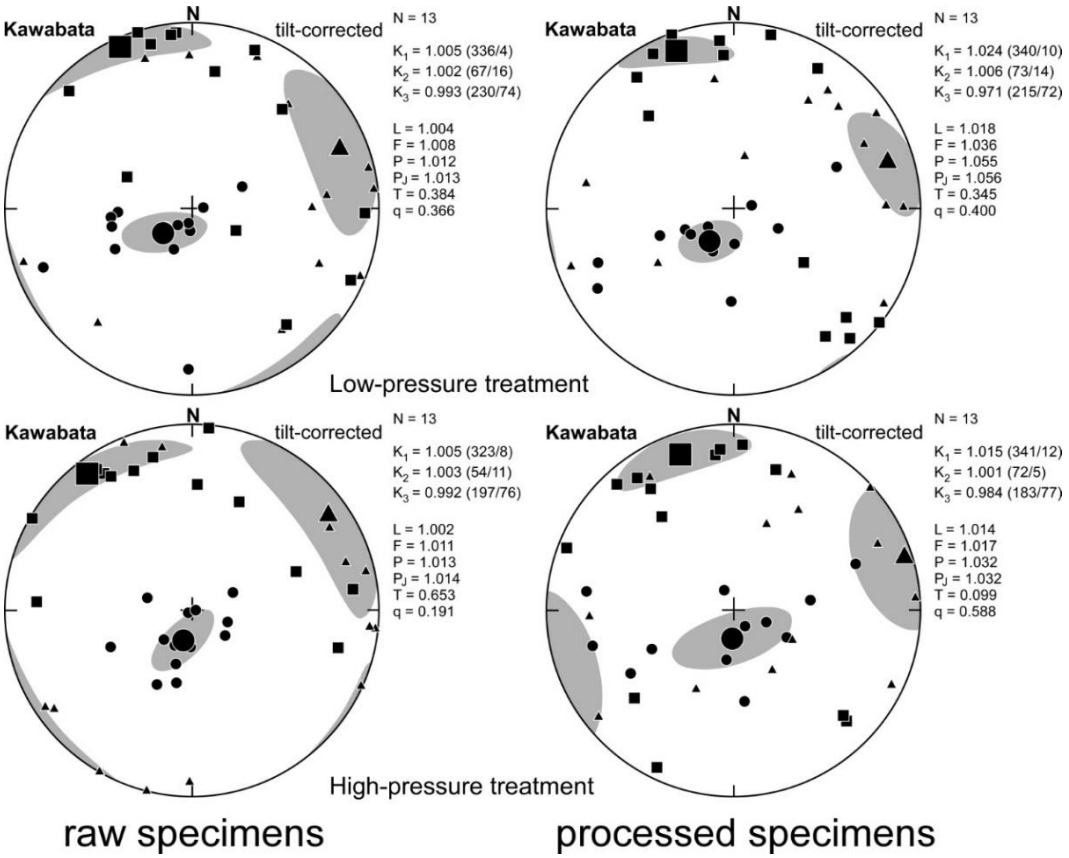

Figure 8. Tilt-corrected AMS fabric for 13 sites of the Kawabata Formation samples in raw (left) and ferrofluid-soaked (right) states. All the data are plotted on the lower hemisphere. Square, triangular and circular symbols represent orthogonal maximum (K_1), intermediate (K_2), and minimum (K_3) AMS principal axes, respectively, and larger symbols show their mean directions. Shaded areas are 95 % confidence limits of Bingham statistics. Anisotropy parameters posted on the equal-area diagrams are calculated based on Tarling and Hrouda (1993). Generally, ferrofluid impregnation results in enhanced anisotropy degree. In case of low-pressure treatment (upper), AMS trend is more or less similar before and after ferrofluid experiment, whereas high-pressure treatment (lower) results in considerable decrease in T parameter. It is noted that azimuth of the K_1 axis, which is the most permeable direction, is coincident with regional fault system in central Hokkaido (see Figure 3).

5.2. Tectonic Implication

It is noted that azimuth of the K_1 axis after the high-pressure treatment, which is the most permeable direction in subsurface condition, is coincident with regional fault system in central Hokkaido (see Figure 3). Not only in the intensive collision event during the Kawabata stage, the NNW-SSE fault system has been intermittently activated with dextral slips throughout the Cenozoic era (Kusumoto et al., 2013). Thus the AMS data with ferrofluid treatment may delineate an invisible weakness in rocks in quantitative way.

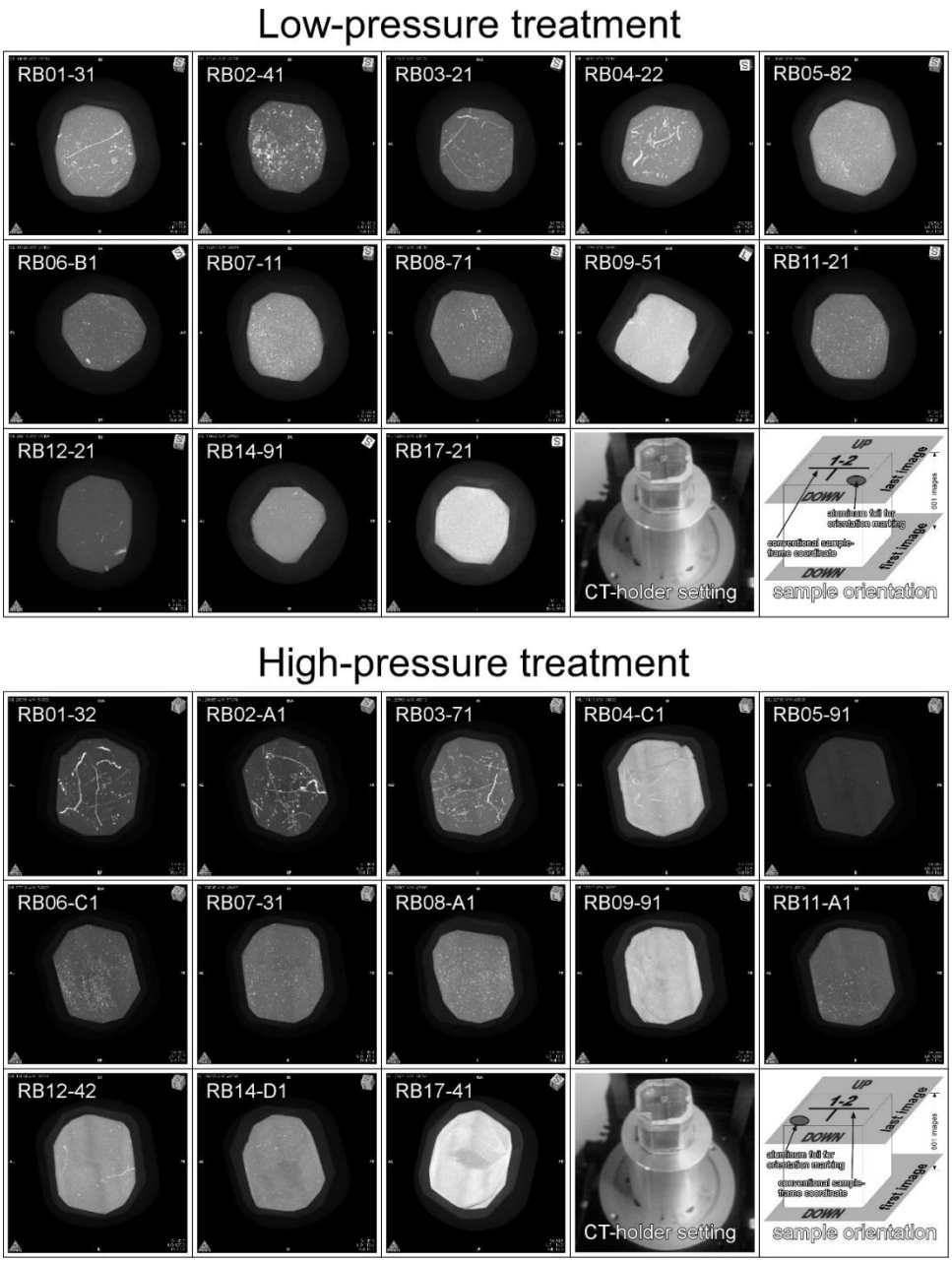

Figure 9. Three-dimensional maximum intensity projection (MIP) images of ferrofluid-processed specimens generated by OsiriX MD. Original image sequences (601 images per sample) were acquired using HMX225-ACTIS+3 Micro-Focus X-Ray CT Scanner at Center for Advanced Marine Core Research, Kochi University with 30 μm spatial resolution.

CONCLUSION

Well-organized magnetic experiments utilizing ferrofluid revealed wide variety in microscopic fabric of sedimentary rocks. Visualized spatial distribution of permeable pore

spaces in rocks shows considerable diversity reflecting experimental methods (low or high pressure) and variety in lithologic facies. Figure 9 presents three-dimensional maximum intensity projection (MIP) images of ferrofluid-processed specimens generated by OsiriX MD. It is obvious that efficiency of impregnation differs even in the same site reflecting small-scale structural disturbance, some amount of which should be owing to bioturbation that is delineated by sinuous pathway of the dense fluid. A series of movies (13 files in .mov format each for low- and high-pressure treatments) of micro-focus X-ray CT scanning images (601 per sample at 30 μm spatial resolution) are available at OPERA:Osaka Prefecture University Education and Research Archives (http://hdl.handle.net/10466/14732).

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Chapter 79

ACTINOBACTERIA FROM MARINE SEDIMENTS: DIVERSITY AND SECONDARY METABOLITES

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ABSTRACT

The widely distributed component of marine sediments in many areas such as in USA, Mexico, Bahamas, Papu New Guinea, the Republic of Palau, Guam, Fiji, Chile, Antarctica, Norway Mediterranean, Australia, India, China, Republic of Korea, Japan and Thailand, associated microbial communities represented different taxonomic groups at the phylum level including Acidobacteria, Planctomycetes, Actinobacteria, Gamma-, Alpha -and Delta-proteobacteria based on the 16S rRNA gene sequences and phylogenetic analyses .This divergence was mainly triggered by the dominance of Acidobacteria and Actinobacteria .A majority of Actinobacteria in sediment samples contained predominantly the genera *Streptomyces*, *Micromonospora*, *Actinocorallia*, *Actinomadura*, *Knoellia*, *Glycomyces*, *Nocardia*, *Nocardiopsis*, *Nonomuraea*, *Pseudonocardia*, *Rhodococcus*, *Saccharomonospora*, *Streptosporangium*, *Salinispora*, and *Sciscionella.* Actinomycetes in marine sediments produced a wide variety of secondary metabolites with diverse biological activities. *Streptomyces* strains produced various anti-cancer and anti-tumor bioactive compounds such as actinoranone, chlorizidine, galvaquinones, glucopiericidin, grincamycins, lobophorins, marfuraquinocins, nitropyrrolins,spiroindimicins,strepnonesides and sungsanpin while the rare actinomycetes in genera *Actinoalloteichus*, *Actinomadura*, *Marinactinospora*, *Marinispora*, *Micromonospora*, *Nocardiopsis*, *Saccharomonospora*, *Salinispora*, and *Verrucosispora* produced cyanogrisides, fijiolides, fluostatins, marthiapeptide, marinactinones and nocardiopsins. At present, bioactive metabolite of obligate marine actinomycete, *Salinispora tropica*, salinosporamide A is a potent proteasome inhibitor being used as the anticancer agent that currently being evaluated in phase I clinical trials as monotherapy. Additionally, many compounds firstly reported from marine sediment actinomycetes may be guides for drug discovery, for example, abyssomicin C from

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Verrucosispora, showed *in vivo* activity against *Mycobacterium tuberculosis*, and marinomycin A from *Marinispora* is potent antitumor agent with substantial activities against selected human tumor cells and drug-resistant pathogenic bacteria. In this chapter, the isolation, taxonomic approach, diversity, secondary metabolites and activities of actinobacteria are described.

1. INTRODUCTION

The component of marine sediments associated microbial communities represented different taxonomic groups mainly by the dominance of Acidobacteria and Actinobacteria (Polymenakou et al. 2009). The class *Actinobacteria* (Stackebrandt et al. 1997) Gram-positive bacteria with a high G + C content are a well-known source of secondary metabolites and the marine counterparts are proving to be a rich source of novel bioactive compounds (Bérdy, 2005; Fiedler et al. 2005; Blunt et al. 2007). The diversity of actinobacteria in marine habitats is little known based on the description of the first sea-water dependent actinobacterial genus *Salinispora* (Maldonado et al. 2005a) that supports the view of marine ecosystem, an untapped source of microorganisms of economical importance .It is only more recently that marine-derived actinomycetes have become recognized as a source of novel antibiotics and anti-cancer agents with unusual structures and properties (Bredholt et al., 2008). Some of the unusual structures and properties of compounds isolated from marine sources and the fact that 58 %of the isolated actinomycetes from marine sediments collected around Guam in the Pacific ocean required sea water for growth (Jensen et al., 2005) implies that one may find microorganisms adapted to the marine environment and producing compounds not found among microorganisms adapted to the terrestrial sources . Actinomycetes are best known as soil bacteria and were generally believed to occur in the ocean largely as dormant spores that were washed into the sea (Goodfellow and Haynes, 1984).

The distributions and ecological roles of actinomycetes in the marine environment, and the extent to which obligate marine species occur, have remained an unresolved issue in marine microbiology .Recently, Jensen et al. (2005) reported the cultivation from marine sediments of a major new group of marine actinomycetes (originally called MAR1, *Micromonosporaceae*) for which the generic epithet '*Salinospora*' was proposed (Mincer et al., 2002) and later wasrevised to be *Salinispora* gen. nov., which comprised only three species *Salinispora aenicola*, *Salinispora tropica* and *Salinispora pacifica* (Maldonado et al., 2005a; Ahmed et al., 2013). All *Salinispora* strains required seawater and more specifically, sodium for growth indicating a high level of marine adaptation.This new taxon is the source of novel secondary metabolites including salinosporamide A, a potent anticancer agent specifically targeting the 20S subunit of the mammalian proteasome (Feling et al., 2003). The discovery of this taxon provides clear evidence for the existence of autochthonous populations of marine actinomycetes, and the compounds being discovered from them indicate that cultured strains are an important resource for novel secondary metabolites. In addition, the taxon has proven to be a productive source of structurally unique and biologically active secondary metabolites (Feling et al., 2003; Jensen et al., 2005). To enhance the chance of recovering the novel actinobacteria from the sea by improving the selective isolation strategies, sample pretreatments, selective isolation media and the

dereplication of isolate collections can still help us to recover putative novel species from one of the most important biotechnologically microbial groups. Thus, there is evidence that marine actinobacteria represent an autochthonous yet little understood component of the sediment microbial community as well as a useful resource for pharmaceutical discovery.

2. SAMPLES COLLECTION, ISOLATION AND CULTIVATION OF ACTINOBACTERIA

2.1. Samples Collection

Samples for the selective isolation procedures were taken from various places such as the samples were obtained from the first 1 to 5 cm of sediment by scuba collection at depths of 10 to 40 m, collected at the Bismarck Sea, in Madang Province, the northern coast of Papua New Guinea, and to the Solomon Sea, in Milne Bay Province, and placed in sterile 50-ml conical tubes and the samples were kept at room temperature during the expedition and at 4°C upon return to the laboratory (Magarvey et al., 2004) while the marine sediments from a depth of 300 m was kept under liquid nitrogen until processing (Maldonado et al. 2009). Each wet sediment sample (approximately 50 mg) was used to inoculate on NaST21Cx agar plate supplemented with 25 μg of cycloheximide/ml. (Magarvey et al., 2004) as shown in Table 1. Whatman no.1 sterile filter paper disks (precut to fit the agar surface) were placed on the agar, and the sediment material was dispersed evenly on the surfaces of the cellulose disks. The plate was incubated in a humidified chamber at 30°C for 30 to 90 days. Following incubation, selected colonies were streaked on ISP-2 medium (BD Diagnostics, Sparks, MD.) prepared with artificial seawater (ISP-2/ASW) and containing cycloheximide (25 μg/ml) and nalidixic acid (25 μg/ml). Mincer et al. (2002) collected the top 1 cm of sediment samples in sterile 50-ml plastic Whirl-Pak bags (NASCO, Modesto, Calif.) by divers using scuba gear. Sediment sample depth ranges, numbers, locations, and dates were as follows: 0 to 30 m, Sea of Cortez, Mexico. The samples were processed as soon as possible after collection (generally, 4 h) using the selective methods of (i) stamping and (ii) dilution and heat shock or both (as described below) and were inoculated onto isolation media (M1 to M5). The dilution-and-heat-shock method was carried out as follows: 1 ml of wet sediment was added to 4 ml of sterile seawater, heated for 6 min at 55°C, vigorously shaken, and further diluted (1:4) in sterile seawater, and 50 l of each dilution was inoculated by spreading with a sterile glass rod onto agar-based isolation media. The stamping method was carried out as follows: 10 ml of wet sediment was aseptically placed into a sterile aluminum dish, dried (ca. 24 h) in a laminar flow hood, ground lightly with a pestle, pressed into a sterile foam plug (14 mm in diameter), and inoculated onto agar media by stamping eight or nine times in a circular fashion, giving a serial dilution effect. All media were prepared with 100% filtered natural seawater. Bredholdt et al., (2007) collected the marine sediment samples by scuba divers close to shore outside the Trondheim Biological station, Norway, 63 ′27 ‘N and 10 ′21-′E.) Only the upper 5 cm of the sediments were collected. The samples including, sample 2: Depth 28 m, fine mud and clay, no vegetation, sample 5: Depth 27 m, fine mud and sand, no vegetation, sample 7: depth 6 m, clay and stones, vegetation, brown layer from sedimented algae and sample 10: depth 4.5 m, fine mud with small stones, upper layer covered with diatom and bacteria, some vegetation.

Sediments from 4.5, 6.0, 27 and 28 m depths were collected by scuba divers, while sediments from 450 m depth were sampled with a box-corer .The upper 5 cm of the sediments were collected in zip-lock bags (scuba) or with a sterile spade (box–corer) and transferred to 1 liter sterile plastic containers (Bredholdt et al., 2008).

2.2. Selective Isolation Procedure

The wet sample from marine sediment (1 g) of each Gulf, was transferred to a 15 ml centrifuge tube which contained 9 ml of seawater solution (3.5%; Instant Ocean, USA) and 1 g of sterile glass beads (710 – 1.180 lm; Sigma, USA). Each centrifuge tube was then shaken for 1 h in a blood tube rotator model SB2 at fixed speed (Fischer Scientific, USA). A second and third series of dilutions (1 ml into 9 ml of sea water) were then prepared for each sample (10^{-2} and 10^{-3}). The three dilutions were then employed to inoculate (100 µl) a set of selective isolation plates by duplicate (Maldonado et at. 2009). Bredholdt et al. (2007) prepared the samples by aseptically diluting the wet sediment (1 ml each in 9 ml sterile water). The actinobacteria selective treatments were performed on dried sediments (Speed vac. 30°C, 16 h (including dry heat) 120°C, 60 min), phenol (1.5%, 30 min at 30°C), dry heat and phenol, dry heat and benzethonium chloride (0.02%, 30 min at 30°C), as well as pollen baiting as previous reported (Hayakawa et at. 1991; Palleroni, 1980). Ten microlitres of the dilution were spread over the surface of isolation media in Petri dishes. Isolation plates were incubated at 20 – 28°C for 2 – 6 weeks (Bredholdt et al. 2008; Maldonado et at. 2009). Actinomycetes generally appeared after 2 to 6 weeks of incubation and were considered to be any colony with a tough leathery texture, dry or folded appearance, and branching filaments with or without aerial mycelia. They were detected by eye and by using a binocular microscope (Olympus Optical Co., Ltd, Tokyo, Japan) fitted with a long working distance objective.

Table 1. The isolation media for actinomycetes from marine sediments

Medium	Medium Components	References
M3	K_2HPO_4, 0.466 g; Na_2HPO_4, 0.732 g; KNO_3, 0.1 g; NaCl, 0.29 g; $MgSO_4{:}7H_2O$, 0.10 g; $CaCO_3$, 0.02 g; Na-propionate, 0.20 g; $FeSO_4{:}7H_2O$, 200 µg; $ZnSO_4{:}7H_2O$, 180 µg;$MnSO_4{:}4H_2O$, 20 µg; thiamine-HCl, 4.0 mg; agar, 18 g in 1 l of natural seawater	Maldonado et al., 2005
M4	Chitin, 2 g; agar 18 g in 1 l of natural seawater	Mincer et al., 2002
M5	Agar, 18 g in 1 l of natural seawater	Mincer et al., 2002
Medium 1 (AMM)	Agar, 8 g; starch, 10 g; yeast extract, 4 g; peptone, 2 g in 1 l of natural seawater	Jensen et al., 2005
Medium 2 (NPS)	Noble agar, 8 g; NPS (nutrient poor sediment) extract, 100 ml in 1 l of natural seawater. NPS extract was prepared by washing (extracting) 900 ml (wet volume) of sand collected from a high-energy beach with 500 ml of sea water	Jensen et al., 2005
Medium 3 (NRS)	Noble agar, 8 g; NRS (nutrient rich sediment) extract, 100 ml in 1 l of natural seawater. NRS extract was prepared as above using 300 ml (wet volume) of sediment collected at low tide from a mangrove channel	Jensen et al., 2005
Medium 5 (SMC)	Noble agar, 8 g; mannitol, 500 mg; casamino acids, 100 mg; nystatin (50 µg/ml) in 1 l of natural seawater	Jensen et al., 2005
Medium 4 (SHG)	Noble agar, 8 g; humic acid sodium salt, 100 mg; galactose, 500 mg; nystatin (50 µg/ml)	Jensen et al., 2005

Medium	Medium Components	References
Medium 6 (SMP)	Noble agar, 8 g; mannitol, 500 mg; peptone 100 mg; rifampicin (5 µg/ml) in 1 l of natural seawater	Jensen et al., 2005
Medium 7 (SNC)	Agar, 8 g; novobiocin (25µg/ml) in 1 l of natural seawater	Jensen et al., 2005
Medium 8 (SPC)	Agar, 8 g; polymixin B sulfate (5 µg/ml) in 1 l of natural seawater	Jensen et al., 2005
Medium 9 (SRC)	Agar, 8 g; rifampicin (5 µg/ml) in 1 l of natural seawater	Jensen et al., 2005
NaST21Cx agar	Solution A (750 ml of artificial seawater containing K_2HPO_4, 1.0 g; and Bacto agar, 10 g) and solution B (250 ml of artificial seawater containing KNO_3, 1.0 g; $MgSO_4$, 1.0 g; $CaCl_2.2H_2O$, 1.0 g; $FeCl_3$, 0.2 g and $MnSO_4:7H_2O$, 0.1 g). Solutions A and B are autoclaved separately and mixed, supplemented with 1 ml of trace element solution	Magarvey et al., 2004
Raffinose histidine agar (RH)	Raffinose, 10 g; *L*-histidine, 1.0 g; K_2HPO_4, 1.0 g; $MgSO_4:5H_2O$, 0.5 g; $FeSO_4$, 0.01 g; agar, 15 g in 1 l of natural seawater	Maldonodo et al., 2009
Soil agar-sea salt	Agar 20 g; sea salt, 30 g in 1 l of soil extract solution. 1 ml of vitamin complex solution (calcium pantothenate, 10.0 mg; nicotinic acid, 10.0 mg; thiamine chloride, 1.0 mg; biotin, 1.0 mg in 20 ml of tap water. Soil extract was prepared by mixing 200 g soil in 1 l water, followed by boiling the mixture for 30 min.	Bredholdt et al., 2007

2.3. Media for Selective Isolation

Mincer et al. (2002) isolated actinomycetes using various media (M1 to M5): M1, 10 g starch, 4 g yeast extract, 2 g peptone, 18 g agar, and 1 liter of natural seawater; M2,6 ml glycerol, 1 g arginine, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 18 g agar, and 1 liter of natural seawater; M3, 6 g glucose, 2 g chitin (United States Biochemical, Cleveland, Ohio), 18 g agar, and 1 liter of natural seawater; M42 g chitin, 18 g agar, and 1 liter of natural seawater; and M5, 18 g agar and 1 liter of natural seawater. All isolation media were amended with filtered (0.2-m pore size) cycloheximide (100 g/ml) and rifampin (5 g/ml), after autoclaving. Mincer et al. (2005) collected marine sediment samples around the islands of the Bahamas .Sediment samples ranged from fine carbonate muds to coral rubble and were collected using SCUBA or a modified, surface deployed sampler. All sediment samples were processed in the field as soon as possible after collection by using desiccation and heat shock as selective cultivation methods. These methods were designed to reduce the numbers of Gram-negative bacteria and to enrich for slow-growing, spore-forming actinomycetes. Treated samples were then inoculated onto medium M1 or M5 (Table 1) and incubated for 4 to 8 weeks at room temperature. Novobiocin (10 μg/ml) or rifampin (5 μ/ml) was added to reduce the number of unicellular bacteria. The antifungal agent cycloheximide (100 µg/ml) was added to all isolation media. Enrichment cultures were prepared in 20-ml vials by adding 1.5 g of wet sediment (homogenized and previously frozen) to either 10 ml of seawater enriched with crude chitin (0.1% w/v), 10 ml of sediment extract [SE; autoclaved supernatant from a 0.5% (w/v) sediment-seawater solution], or 10 ml of medium M1 low (0.2% starch, 0.08% yeast extract, 0.04% peptone, seawater). Each of the three enrichment conditions was supplemented with either 5 μg of rifampin/ml or 25 μg of novobiocin/ml (final concentrations). Similar enrichments were prepared in the field with 0.5 g sediment and one of the following antibiotics (final concentrations): kanamycin (20 μg/ml), novobiocin (10 μg/ml), vancomycin (5 μg/ml), gentamicin (2 μg/ml), or tetracycline (4 μg/ml). All enrichment cultures were incubated for 4 to 15 weeks at room temperature and observed at x10 to x64 magnification using a stereomicroscope. Bacteria that formed visible mycelia were harvested directly from

the enrichment cultures by using a sterile pipette, serially washed three times in 10 ml of sterile seawater, and plated on medium M5.

Jensen et at. (2005) collected shallow sediments from the depths of 1–20 m, Guam Island while the remaining sediments were collected using a modified, surface-deployed sediment sampler to depths of 570 m. All samples were processed within a few hours of collection using a variety of techniques designed to reduce the numbers of Gram-negative bacteria and to enrich for slow-growing, spore-forming actinomycetes. Samples were processed and inoculated onto various agar media [medium 1 to medium 12, [Medium 1 (AMM), Medium 2 (NPS), Medium 3 (NRS), Medium 4 (SHG), Medium 5 (SMC), Medium 6 (SMP), Medium 7 (SNC), Medium 8 (SPC), Medium 8 (SPC), Medium 9 (SRC), Medium 10 (SSC), Medium 11 (STC), Medium 12 (SMY)] (Table 1) using one, or in some cases (especially for the deeper sediments) as many as three, of the eight methods described below. All algal samples were processed using method 1 (with grinding) while all sponges were processed using method 3. Method 1 (dry/stamp). Sediment was dried overnight in alaminar flow hood and, when clumping occurred, ground lightly with an alcohol-sterilized mortar and pestle. An autoclaved foam plug (2 cm in diameter) was pressed onto the sediment and then repeatedly onto the surface of an agar plate in a clockwise direction creating a serial dilution effect. Method 2 (dry/scrape). This method was used for small rocks that had been dried overnight in a laminar flow hood and then scraped with a sterile spatula generating a powder that was processed as per method 1. In some cases, the powder was collected with a wet cotton-tipped applicator or the rock was rubbed directly with the applicator which was then used to inoculate the surface of an agar plate. Method 3 (dry/dilute). Dried sediment (*c* .0.5 g) was diluted with 5 ml of sterile (autoclaved) seawater (SSW). The diluted sample was vortex mixed, allowed to settle for a few minutes, and 50 ml of the resulting solution inoculated onto the surface of an agar plate and spread with an alcohol-sterilized glassrod. Method 4 (dilute/heat). Dried sediment was volumetrically added to 3 ml of SSW (dilutions 1:3 or 1:6), heated to 55°C for 6 min, and 50 – 75 ml of the resulting suspension inoculated onto an agar plate as per method 3. Method 5 (dilute/heat/2). Dried sediment was treated as per method 4 (dilution 1:6) with the addition of a second heat treatment at 60°C for 10 min. Method 6 (dry/stamp+dilute/heat). The surface of an agar medium was inoculated using a sample treated as per method 1. The dried sediment was then processed using method 4 and the same agar plate inoculated a second time with the heat-treated samples. Method 7 (freeze/dilute). Wet sediment was frozen at -20°C for at least 24 h, thawed, volumetrically diluted in SSW (1:3–1:120 depending on particle size), and 50 ml of the resulting suspension inoculated onto the surface of an agar plate asper method 3. Method 8 (freeze/dilute/2). Wet sediment was treated as per method 7 except that the thawed and diluted sample was incubated at room temperature for 48 h before inoculation onto the surface of an agar plate. Processed samples were inoculated as described above onto the surface of from one to eight of the following agar media. All media were prepared with 1 l of natural seawater and contained the anti-fungal agents cycloheximide (100 mg/ml) and, when listed, nystatin (50 mg/ml). Inoculated Petri dishes were incubated at room temperature (*c*. 28°C) and monitored periodically over 3 months for actinomycete growth . Actinomycetes were quantified on each plate by eye and with the aid of a Leica MZ6 stereomicroscope (x10 –x64).

Bredholdt et at. (2007) isolated actinobacteria using the following media: *soil agar*: 1 l of filtered soil extract, 20 g agar, pH 7.0 (soil extract was prepared by mixing 200 g soil in 1 l water, followed by boiling the mixture for 30 min); 1 ml of vitamin complex solution (10.0

mg of calcium pantothenate, 10.0 mg of nicotinic acid, 1.0 mg of thiamin chloride, 1.0 mg of biotin, 20 ml of tap water) was added into soil agar. *Soil agar* with 3% sea salt added. *Modified organic agar 2 Gause:* 5.0 g of peptone, 3.0 g of tryptone, 10 g of glucose, 5.0 g of NaCl, 1 l of tap water. *Modified organic agar 2 Gause* with 3% sea salt added. *Modified organic agar 2 Gause* supplemented with tobramycin (10 mg/ml) (Terekhova et al., 1991). *Modified organic agar 2* Gause supplemented with rubomycin (5 mg/ml) (Lavrova et al., 1972). *Mineral agar 1 Gause*: 20.0 g of starch-soluble, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4$, 1.0 g of KNO_3, 0.5 g of NaCl, 0.01 g of FeSO4, 20.0 g of agar, 1 l of tap water. *Soy-bean meal agar*: 3.0 g of soy-bean meal, 0.2 g of KNO_3, 0.5 g of K_2HPO_4, 0.4 g of $MgSO_4$, 20.0 g of agar, 1 l of distilled water. *Soy-bean meal agar* with 3% sea salt added. *Yeast-corn-starch agar*: 10.0 g of starch-soluble, 10 g of yeast extract, 10.0 g of corn meal extract, 2.0 g NaCl, 20 g of agar, 1 l of water. *Pea-meal agar*: 10.0 g of pea meal, 10.0 g of glucose, 5.0 g of NaCl, 1.0 g of $CaCO_3$, 20 g of agar, 1.0 l of tap water. The pH of all media was adjusted to 7.0 – 7.5. Nalidixic acid (10 mg/ml) and nystatin (50 mg/ml) were supplemented to all media to inhibit the growth of Gram-negative bacteria and fungi. For isolation, a variety of methods was used, including the pretreatment of sediment samples with physical factors: UV irradiation (Galatenko and Terekhova, 1990), super high frequency (SHF) radiation (Bulina et al., 1997, 1998), extremely high frequency (EHF) radiation (Li et al., 2002), cold-shock by freezing sediment samples at -18°C. UV-irradiation of the wet sediment suspension (5 ml) was performed in open Petri dishes for 30 s. with the use of UV lamp at emission wavelength of 254 nm and power of 15 W. The distance from the irradiation source was 20 cm. SHF radiation treatment of the suspension (2.5 ml) placed into sterile Eppendorf tubes was carried out in a microwave oven at a frequency of 2460 MHz and power of 80 W for 45 s. EHF-radiation treatment of the suspension (5 ml) was carried out in Petri dishes from the bottom. Emitted radiation had a non-thermal intensity and was amplitude-modulated at a frequency of 1 kHz within wavelength band of 8 – 11.5 mm using industrial generator (Russia). In addition, the two-layer soil agar with the following transfer of the upper layer to organic agar 2 Gause was used (Galatenko et al., 2004). Before inoculation agar media in Petri dishes were dried for 30 min to prevent water condensation on agar surface. The isolation plates were incubated at 28°C for 2 weeks. Colonies showing the characteristic appearance of filamentous actinomycetes were selected for isolation and the colonies growing on agar plates were examined under the microscope *in situ* using the long working distance condenser and objectives. Actinomycete colonies were picked up and inoculated onto oatmeal agar slants (40 g oatmeal, 20 g agar, 1 l tap water, pH 7.2.) which were incubated at 28°C for 2 – 3 weeks.

Bredholdt et al. (2008) reported to use isolation media consisted of the following: IM5 (humic acid agar, Hsu and Lockwood, 1975), with sea water), humic acid (1 g), K_2HPO_4 (0.5 g), $FeSO_4.7H_2O$ (1 mg), agar (20 g), vitamin B solution (1ml), natural sea water (0.5 1) and distilled water (0.5 1); IM6 glycerol (0.5 g), starch (0.5 g), sodium propionate (0.5 g), KNO_3 (0.1 g), asparagine (0.1 g), casein (0.3 g), K_2HPO_4 (0.5 g), $FeSO_4.7H_2O$ (1 mg), agar (20 g), vitamin B solution (1 ml), natural sea water (0.5 l) and distilled water (0.5 1); IM7 (chitin agar, with sea water) chitin (Sigma), K_2HPO_4 (0.5 g), $FeSO_4.7H_2O$ (1 mg), agar (20 g), vitamin B solution (1 ml), natural sea water (0.7 l) and distilled water (0.3 l); IM8, malt extract (1 g), glycerol (1 g), glucose (1 g), peptone (1 g), yeast extract (1 g), agar (20 g), natural sea water (0.5 l) and distilled water (0.5 l). The pH of the isolation media was adjusted to pH 8.2. Vitamin B solution consisted of the following: thiamine-HCl (50 mg), riboflavin

(50 mg), niacin (50 mg), pyridoxine-HCl (50 mg), inositol (50mg), Ca-pantothenate (50 mg), p- aminobenzoic acid (50 mg), biotin (25 mg) and distilled water (100 ml). All isolation media were amended with filtered) 0.2-µm pore size (cycloheximide) (50 μg/ml), nystatin (75 μg/ml) and nalidixic acid (30 μg/ml). Seventeen media were used according to well known recipes or from known suppliers (Maldonado et at. 2009) and they were supplemented with nystatin (antifungal; 50 mg/ml) and rifampicin (5 mg/ml). Some media were also supplemented with nalidixic acid (10 mg/ml to diminish the growth of marine bacteria using both distilled and sea water (35 g/1 l; Instant Ocean, USA).

3. IDENTIFICATION AND DIVERSITY OF ACTINOBACTERIA

3.1. Identification Techniques

Actinomycetes strains were taxonomic studied by employing the polyphasic approach. The isolates were subcultured onto two media, glucose-yeast-malt extract agar (ISP 2 medium; Shirling and Gottlieb 1966), and glucose-yeast extract agar (GYEA; Gordon and Mihm 1962) prepared with normal and sea water, incubated for up to 3 weeks at 28 °C and then checked for purity by a light microscope (Maldonado et al., 2009). The suspensions of hyphal fragments or spores of isolates were preserved in 20% glycerol (w/v) at -20°C and -80°C for long term maintenance. Morphological grouping assigned the isolates from each sample on the basis of the properties of the colonies (i.e., colour, pigment formation, etc.) when they grew on the solid media. If aerial mycelium or spores were evident, the microorganisms were then also prepared for scanning electron microscopy (SEM) by examining gold-coated, dehydrated material, prepared from up to 28-days-old cultures.

The phenotypic characteristics of isolates compared with the type strains including morphological, cultural, physiological and biochemical characteristics such as carbon utilization, the ability to produce acid from sugars, the temperature tests and the pH tolerance test were determined by following the standard protocol of the International Streptomyces Project (ISP) (Shirling and Gottlieb 1966; Arai, 1975; Williams and Cross, 1971; Gordon et al. (1974).The chemotaxonomic characteristics including the determination of menaquinone (Collins 1977), polar lipid composition of isolate (Minnikin et al. 1984), mycolic acids (Minnikin et al. 1980), the DNA G+C mol% content (Gonzalez and Saiz-Jimenez, 2002) and DNA-DNA hybridization in microdilution-well plates, as reported by Ezaki et al. (1989) were conducted. 16S rRNA gene sequencing analyses was determined using the method of Kim et al. (1999) for which the chromosomal DNA, PCR amplification and direct sequencing of the PCR products of isolates were carried out. The 16S rRNA gene sequence was multiple-aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases by using CLUSTAL W version 1.81 (Thompson et al., 1997). Phylogenetic trees were reconstructed by using the neighbour-joining (Saitou and Nei, 1987), maximum-parsimony (Kluge and Farris, 1969) and maximum-likelihood (Felsenstein, 1981) methods in the program MEGA5.0 (Tamura et al., 2011). The confidence values of nodes were evaluated by using the bootstrap resampling method with 1000 replicates (Felsenstein, 1985).

3.2. Diversity of Actinobacteria

The novel actinomycetes from marine sediments are proposed including *Verrucosispora gifhornensis* HR1-2^T, a new genus, isolated from a peat bog near Gifhorn, Lower Saxony, Germany (Rheims et al., 1998); *Pseudonocardia antarctica* DVS 5a1, isolated from McMurdo Dry Valleys, Antarctica (Prabahar et al., 2003); *Sciscionella marina* SCSIO 00231^T, gen. nov., sp. nov., isolated from a sediment in the northern South China Sea (Tian et al., 2009); *Verrucosispora sediminis* MS426^T, from a deep-sea sediment sample of the South China Sea, a cyclodipeptide-producing actinomycete from deep-sea sediment (Dai et al., 2010); *Streptomyces glycovorans* YIM M 10366^T, *Streptomyces xishensis* YIM M 10378^T and *Streptomyces abyssalis* YIM M 10400^T from marine sediments collected from the Xisha Islands in the South China Sea. (Xu et al., 2012); *Spinactinospora alkalitolerans* CXB654^T gen. nov. in the family *Nocardiopsaceae*, isolated from marine sediment collected at a depth of 17.5 m near the Yellow Sea Cold Water Mass, China (Chang et al., 2011); *Verrucosispora maris* AB-18-032^T a novel deep-sea actinomycete isolated from a marine sediment which produced abyssomicins (Goodfellow et al., 2012); *Streptomyces pharmamarensis* PM267^T isolated from a marine sediment, sediment sample in the Mediterranean Sea (Carro et al., 2012); *Micromonospora sediminicola* SH2-13^T, isolated from a marine sediment sample collected from the Andaman Sea of Thailand (Supong et al., 2013); *Verrucosispora fiedleri* MG-37, an actinomycete isolated from a fjord sediment which synthesized proximicins (Goodfellow et al., 2013); *Verrucosispora qiuiae*, isolated from mangrove swamp sediment (Xi et al., 2012); *Streptomyces chumphonensis* isolated from marine sediments in Thailand (Phongsopitanun et al., 2014) and *Micromonospora fluostatini* PWB-003^T, which produced fluostatins B and C antibiotics, isolated from nearshore sediment collected from Panwa Cape, Phuket Province, Thailand (Phongsopitanun et al., 2015).

The distribution of obligate marine bacteria from ocean sediments within the order *Actinomycetales* designated MAR 1 has been reported (Mincer et al., 2002). Actinobacterial populations designed as MAR 1 for *Micromonosporaceae* (*Micromonospora* and *Salinispora*); MAR2 and MAR3 and MAR4 for *Streptomycetaceae* (*Streptomyces* and *Kitasatospora*); MAR5 and MAR6 for *Thermomonosporaceae (Actinomadura*) were described by Jensen et al. (2005) based on the small subunit rRNA (*SSU rRNA) gene sequences* data analysis. Stach et al., 2003 investigated bacterial diversity in a deep-sea sediment, Atlantic ocean deep-sea sediment collected from the edge of the Saharan debris flow near the Canary Islands (27º02.39'N 18º29.02'W) at a depth of 3,814 m using a piston corer during a scientific cruise aboard the RRS Charles Darwin by constructing actinobacterium-specific 16S ribosomal DNA (rDNA) clone libraries from sediment sections taken 5 to 12, 15 to 18, and 43 to 46 cm below the sea floor at a depth of 3,814 m. Clones were placed into operational taxonomic unit (OTU) groups with >99 %16S rDNA sequence similarity; the cutoff value for an OTU was derived by comparing 16S rRNA homology with DNA-DNA reassociation values for members of the class Actinobacteria. The 5- to 12-cm sediment actinobacterium community was dominated by bacteria most closely related to *Streptomyces* species (45%), though OTUs from this sediment section were distributed throughout the phylogenetic tree. OTUs from the 15- to 18-cm sediment section were dominated by bacteria most closely related to *Rhodococcus* species (56%), with only one OTU (M16) being most closely related to a *Streptomyces* species. The 43- to 46-cm

community was also dominated by *Rhodococcus* species (62%), with no *Streptomyces* species present.

Magarvey et al. (2004) reported 102 actinomycetes isolated from subtidal marine sediments collected from the Bismarck Sea and the Solomon Sea off the coast of Papua New Guinea. A combination of physiological parameters, chemotaxonomic characteristics, distinguishing 16S rRNA gene sequences, and phylogenetic analysis based on 16S rRNA genes provided strong evidence for the two new genera (represented by strains of the PNG1 clade and strain UMM518) within the family *Micromonosporaceae* including *Micromonospora* and *Verrucosispora*. Actinobacteria isolated from marine environments have been dominated by *Micromonospora*, *Rhodococcus* and *Streptomyces* species (Maldonado et al., 2005). The dominant actinomycete recovered from marine samples collected around the island of Guam belonged to the seawater-requiring marine taxon *Salinispora*, a new genus within the family *Micromonosporaceae* and two major new clades related to *Streptomyces* spp., tentatively called MAR2 and MAR3, including the new genera within the *Streptomycetaceae* and five new marine phylotypes, including two within the *Thermomonosporaceae* (Jensen et al., 2005). Phylogenetic analysis of 189 representative isolates from sediments collected in the Republic of Palau from the intertidal zone to depths of 500 m., based on 16S rRNA gene sequence data, indicated that 124 (65.6%) belonged to

Table 2. Distribution of actinobacteria in marine-sediments

Species	Sources/Locations	References
Actinomadura sp.CNU-125	Marine sediments from Kahlisco, El Cajon, CA	Gontang et al., 2007
Amycolatopsis sp. GY109, *Frankia* sp., *Kitasatospora* sp.	Deep sea sediment (3800 m)	Stach et al., 2003
Microbacterium sp. strains CNJ-797, CnJ-743, CNJ-930	Marine sediments from Kahlisco, El Cajon, CA	Gontang et al., 2007
Micromonospora sp. strains CNB394 and CNB512	Marine sediments from Bahamas	Mincer et al., 2002
M. rhodorangea, *M. halophytica*	Marine sediment from Papu new Guinea	Magarvey et al., 2004
M .sediminicola SH2-13^T	The gulf of Thailand	Supong et al., 2013
Marinactinospora thermotolerans	Marine sediment in the northern South China Sea	Tian et al., 2009a
Nocardia sp. CnS-044	Marine sediments from Kahlisco, El Cajon, CA	Gontang et al., 2007
Pseudonocardia alaminiphila DVS 5a1^T, *P. aurantiaca*, *P. aalnii*, *P. antarctica*	Deep sea sediment (3800 m) McMurdo Dry Valleys region of Antarctica	Maldonado et al., 2005b; Stach et al., 2003; Prabahar et al., 2004
Salinispora aenicola CNS-051, *S. tropica* CNS-055	Marine sediments from Bahamas	Maldonado et al., 2005a
	Marine sediments from Kahlisco, El Cajon, CA	Gontang et al., 2007
Sciscionella marina SCSIO 00231^T	Marine sediments in the northern South China Sea	Tian et al., 2009b
Spinactinospora alkalitolerans CXB654^T	Marine sediment from the Yellow Sea Cold Water Mass, China	Chang et al., 2011
Streptomyces glycovorans YIM M10366^T, *S. xishensis* YIM M 10378^T, *S. abyssalis* YIM M 10400^T	Marine sediment from the Xisha Island, China	Xu et al., 2012
S. pharmamarensis PM267^T	Marine sediment, Mediterranean Sea	Carro et al., 2012
Verrucosispora sedimidis MS426^T	Deep-sea sediment, South China Sea	Dai et al., 2010
*V. maris*AB-18-032^T	Marine sediment from the sea of Japan	Goodfellow et al., 2012

the class *Actinobacteria* including *Micromonosporaceae*, *Nocardiaceae*, *Nocardioidaceae* and *Streptomycetaceae*, spore-forming strains from the *Pseudonocardiaceae* and *Thermomonosporaceae* while the remaining 65 (34.4%) were members of the class *Bacilli* including *Bacillus*, *Pontibacillus*, *Paenibacillus*, and *Laceyella* (Gontang et al., 2007). *Streptomyces*, *Micromonospora*, *Actinocorallia*, *Actinomadura*, *Knoellia*, *Glycomyces*, *Nocardia*, *Nocardiopsis*, *Nonomuraea*, *Pseudonocardia*, *Rhodococcus* and *Streptosporangium* genera were isolated from the shallow water sediments of the Trondheim fjord (Norway) (Bredholdt et al., 2007). *Actinomadura*, *Dietzia*, *Gordonia*, *Micromonospora*, *Nonomuraea*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Salinispora*, *Streptomyces*, "*Solwaraspora*" and *Verrucosispora* were isolated from marine sediments samples collected in the Gulf of California and the Gulf of Mexico (Maldonado et al., 2009). Actinobacteria from marine sediments were isolated from the Valparaíso bay, Chile, containing *Aeromicrobium*, *Agrococcus*, *Arthrobacter*, *Brachybacterium*, *Corynebacterium*, *Dietzia*, *Flaviflexus*, *Gordonia*, *Isoptericola*, *Janibacter*, *Microbacterium*, *Mycobacterium*, *Ornithinimicrobium*, *Pseudonocardia*, *Rhodococcus*, *Streptomyces*, *Tessaracoccus* and one isolate as a novel phylogenetic branch related to the *Nocardiopsaceae* family (Claverias et al., 2015). The distribution of actinobacteria in marine-sediment is summarized in Table 2.

4. SECONDARY METABOLITES AND BIOLOGICAL ACTIVITY OF ACTINOBACTERIA

4.1. Secondary Metabolites and Biological Activity of *Streptomyces*

Marine sediment actinomycete in the genus *Streptomyces* are dominantly found while the rare actinomycetes including genera *Actinoalloteichus*, *Actinomadura*, *Marinactinospora*, *Marinispora*, *Nocardiopsis*, *Saccharomonospora*, *Salinispora* and *Verrucosispora* are isolated. *Streptomyces* strains provided valuable source of new bioactive compounds and biological activity. *S. aureoverticillatus* NPS001583from marine sediment, the strain produced aureoverticillactam, a novel 22-atom macrocyclic lactam, novel anticancer and anti-infective agents (Mitchell et al., 2004). Actinomycete (MAR4) strain CNQ-525 from ocean sediments collected at a depth of 152 m near La Jolla, California produced three new chlorinated dihydroquinones and one previously reported analogue (Soria-Mercado et al., 2005). *S. nodosus* NPS007994 from a marine sediment collected in Scripps Canyon, La Jolla, California, was found to produce lajollamycin a nitro-tetraene spiro-β-lactone-γ -lactam antibiotic and showed antimicrobial activity against both drug-sensitive and -resistant Gram-positive bacteria and inhibited the growth of B16-F10 tumor cells *in vitro* (Manam et al., 2005). *Streptomyces* sp. NPS008187 from a marine sediment collected in Alaska was found to produce three new pyrrolosesquiterpenes, glyciapyrroles A, B, and C, along with the known diketopiperazines cyclo(leucyl-prolyl), cyclo(isoleucyl-prolyl), and cyclo (phenylalanyl-prolyl) (Macherla et al., 2005).

Streptomyces sp. M045 produced two novel antitumor antibiotics, chinikomycins A and B including manumycin A .The compounds exhibited antitumor activity against different human cancer cell lines, but were inactive in antiviral, antimicrobial, and phytotoxicity tests (Li et al., 2005). *Streptomyces* sp. KORDI-3238 from deep-sea sediments produced a new

cytotoxic compound, streptokordin, and four known compounds, nonactic acid, dilactone, trilactone, and nonactin, streptokordin. Streptokordin exhibited significant cytotoxicity against seven human cancer cell lines but showed no growth inhibition against various microorganisms including bacteria and fungi (Jeong et al., 2006). *Streptomyces* sp. QD518 produced a new staurosporinone, *N*-carboxamido-staurosporine, and a new sesquiterpene, (5*S*, 8*S*, 9*R*, 10*S*)-selina-4, 7-diene-8, 9-diol (Wu et al., 2006). A marine-derived actinomyces strain NPS554 from a marine sediment collected from Miyazaki Harbor, Japan, at a depth of 38 m yielded two trialkyl-substituted aromatic acids, lorneic acid A and lorneic acid B. Their structural differences affected inhibition activities against phosphodiesterase 5 (Iwata et al., 2006). *Streptomyces* strain CNQ-085 produced four new cytotoxic compounds, designated as daryamides A, B, and C and (2*E*, 4*E*)-7-methylocta-2,4-dienoic acid amide. The daryamides show weak to moderate cytotoxic activity against the human colon carcinoma cell line HCT-116 and very weak antifungal activities against *Candida albicans* (Asolkar et al., 2006).

Streptomyces sp. CNH990 from marine sediment produced two new cytotoxic quinones of the angucycline class, marmycins A and B. Marmycin A displayed significant cytotoxicity against several cancer cell lines, some at nanomolar concentrations; while compound B, a chloro analogue of A, was less potent. For marmycin A, tumor cell cytotoxicity appeared to coincide with induction of modest apoptosis and arrest in the G1 phase of the cell cycle (Martin et al., 2007). *Streptomyces* sp. KORDI-3973 isolated from the deep sea sediment produced streptopyrrolidine, abenzyl pyrrolidine derivative that exhibited significant anti-angiogenesis activity (Shin et al., 2008). *Streptomyces* sp. CNQ-418 furnished the marinopyrroles A and B that possess potent antibiotic activities against methicillin resistant *Staphylococcus aureus* (Hughes et al., 2008). *Streptomyces*. sp. CNQ-617 produced two novel spiroaminals, marineosins A and B, containing two pyrrole functionalities, showed significant inhibition of human colon carcinoma (HCT-116) in an in vitro assay (IC_{50}) 0.5 μM for marineosin A) and selective activities in diverse cancer cell types (Boonlarppradab et al., 2008). *Streptomyces* sp. 17944 produced three new tirandamycins (TAMs), TAM E, F, and G, along with TAM A and B, that 5 selectively inhibits the *Brugia malayi* AsnRS and efficiently kills the adult *B. malayi* parasite, representing a new lead scaffold to discover and develop antifilarial drugs.

Streptomyces sp. 307-9 produced the novel dienoyl tetramic acids tirandamycin C and tirandamycin D with activity against vancomycin-resistant *Enterococcus faecalis* and also the previous compounds tirandamycins A and B (Carlson et al., 2009; Yu et al., 2011). *Streptomyces albidoflavus* NTK 227 from Atlantic Ocean sediment produced albidopyrone, a new α-pyrone-containing secondary metabolite and found to have a moderate inhibitory activity against protein-tyrosin phosphatase B (Hohmann et al., 2009a). *Streptomyces* sp. NTK 937 produced caboxamycin, a new benzoxazole antibiotic which inhibited against Gram-positive bacteria, selected human tumor cell lines and the enzyme phosphodiesterase (Hohmann et al., 2009b). *Streptomyces* sp. Sp080513GE-23 isolated from a sponge-derived actinomycete, produced tetrapeptides possessing a unique skeleton, JBIR-34 and JBIR-35 (Motohashi, et al., 2010). Actinomycete strain CNQ-509 produced five new farnesyl-*R*-nitropyrroles, nitropyrrolins A – E, several of the nitropyrrolins, nitropyrrolin D in particular, are cytotoxic toward HCT-116 human colon carcinoma cells, but show weak to little antibacterial activity against methicillin-resistant *Staphylococcus aureusus* (MRSA) (Kwon et al., 2010). *Streptomyces tumescens* YM23-260 produced two peptides, tumescenamides A

and B. Tumescenamide A induced reporter gene expression under the control of the insulin-degrading enzyme promoter (Motohashi et al., 2010).

Streptomyces sp. CNQ-027 produced three highly modified peptides, actinoramides A – C (Nam et al., 2011). *Streptomyces antibioticus* H74-18 produced antimycins A_{19} and A_{20}, two new antimycins. All the antimycins showed potential antifungal activities against *Candida albicans* with MIC of about 5 – 10 μg/ml (Xu et al., 2011). *Streptomyces* sp. CNS-575 produced fijimycins A – C, three antibacterial etamycin-class depsipeptides. Fijimycins A – C, and etamycin A, were shown to possess significant in vitro antibacterial activity against three methicillin-resistant *Staphylococcus aureus* (MRSA) strains with MIC 100 values between 4 and 16 μg/ml) Sun et al., 2011). *Streptomyces* sp. SCSIO 01127, isolated from the South China Sea sediment, produced two new spirotetronate antibiotics lobophorins E and F, along with two known analogs lobophorins A and B. The new compound lobophorin F showed antibacterial activities against *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 with MIC values of 8 μg/ml for both the strains, better than that of lobophorin B. Lobophorin Falso displayed better cytotoxic activities than lobophorin B, with IC_{50} of 6.82, 2.93 and 3.16 μM against SF-268, MCF-7 and NCI-H460, respectively (Niu et al., 2011). *Streptomyces* sp. NPS853 from marine sediments, it produced new anthramycin-type analogues, designated usabamycin A – C. The usabamycins show weak inhibition of HeLa cell growth and selective inhibition of serotonin (5-hydroxytrypamine) 5-HT2B uptake (Sato et al., 2011).

Streptomyces spinoverrucosus SNB-032producedfour new anthraquinone analogues including galvaquinones A – C and an isolation artifact, 5,8-dihydroxy-2,2,4-trimethyl-6-(3-methylbutyl- anthra[9,1-de][1,3]oxazin-7(2*H*)-one. Galvaquinone B was found to show epigenetic modulatory activity at 1.0 μM and exhibited moderate cytotoxicity against non-small-cell lung cancer (NSCLC) cell lines Calu-3 and H2887 (Hu et al., 2012). *Streptomyces lusitanus* SCSIO LR32, an actinomycete of deep sea origin produced five new *C*-glycoside angucyclines, named grincamycins B − F, and a known angucycline antibiotic, grincamycin, grincamycin (Huang et al., 2012). A deep-sea-derived *Streptomyces* sp. SCSIO 03032 produced a new bisindole alkaloids spiroindimicins A − D. Spiroindimicins B – D with a [5, 5] spiro-ring exhibited moderate cytotoxicities against several cancer cell lines (Zhang et al., 2012). *Streptomyces* sp. WBF-16 produced two new anthraquinone glycosides strepnoneside A and strepnoneside B, together with chromomycin A3. Chromomycin A3 exhibited cytotoxic activities against HCT 116 cell lines (IC_{50} =300 ± 11 *p*M) (Lu et al., 2012). *Streptomyces* sp. FMA produced streptocarbazoles A and B, two novel indolocarbazoles. Streptocarbazoles A was cytotoxic on HL-60 and A-549 cell lines and could arrest the cell cycle of Hela cells at the G2/M phase (Fu et al., 2012).

Streptomyces sp. CNQ-027 isolated from marine sediment sample collected at a depth of 50 m of the coast of Diego, CA produced a new meroterpenoid, actinoranone that exhibited significantly cytotoxic to HCT-116 human colon cancer cells with an LD_{50}2.0 μg/ml, anti-cancer and tumor cell lines (Nam et al., 2013). *Streptomyces* sp. TP-A0867 from marine sediment sample collected at a depth 38 m near Miyazaki Harbor, Japan, produced akaeolide, a novel polycyclic polyketide, anti-bacteria and cytotoxicity (Igarashi et al., 2013). *Streptomyces* sp. CNH-287 produced chlorizidine, a cytotoxic 5*H*-pyrrolo [2,1-*a*] isoindol-5-one containing alkaloid, cytotoxicity against HCT-116 human colon cancer cells (Alvarez-Mico et al., 2013). *Streptomyces* sp. 7-145, had the potential to produce glycosidic antibiotic,

an elaiophylin derivatives showed good antibacterial activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci pathogens (Wu et al., 2013). *Streptomyces* sp. CNT-372 from Fiji produced farnesides A and B, sesquiterpenoid nucleoside ethers. Farneside A exhibited modest antimalarial activity against the parasite *Plasmodium falciparum* (Iland et al., 2013). *Streptomyces niveus* SCSIO 3406 from a South China Sea sediment sample obtained from a depth of 3536 m produced four new sesquiterpenoid naphthoquinones, marfuraquinocins A − D, and two new geranylated phenazines, phenaziterpenes A and B. Marfuraquinocins exhibited antibacterial activities against *Staphylococcus aureus* ATCC 29213 (Song, et al., 2013). *Streptomyces* sp. RJA2928 obtained from a tropical marine sediment, produced nahuoic acid A, a selective SAM-competitive inhibitor of the histone methyltransferase SETD8 (Williams et al., 2013). In addition, nahuoic acids B – E were produced by the marine *Streptomyces* sp. SCSGAA 0027 (Nong et al., 2016), and were found in the strain RJA2928 (Williams et. al., 2016). Nahuoic acid B – E showed weak antibiofilm activity against Shewanella onedensis MR-1 biofilm. *Streptomyces* sp. SNJ013 isolated from deep-sea sediment collected off Jeju Island, Korea, produced sungsanpin, a new 15-amino-acid peptide. Sungsanpin displayed inhibitory activity in a cell invasion assay with the human lung cancer cell line A549 (Um et al., 2013). *Streptomyces* sp. JAMM992 produced surugamides A − E, cyclic octapeptides with four *D*-amino acid residues (Takada et al., 2013). The metabolites and biological activity of *Streptomycetes* from marine sediments that published on 2010-2016, are summarized in Table 3.

4.2. Secondary Metabolites and Biological Activity of Rare Actinomycetes

Rare actinomycetes are promising sources in search for new drugs, and their potential for producing biologically active molecules, were studied (Bredholdt et al., 2007). A wide variety of secondary metabolites from marine sediment actinomycetes were used in clinical therapy, and some compounds were originally guided for chemical synthesis such as salinosporamide A, isomeric abyssomicin C, and marinomycin A (Endo and Danishefsky et al., 2005; Nicolaou and Harrison, 2007; Nishimaru et al., 2014). For marine natural products, salinosporamide A is a chlorinated polyketide metabolite that belongs to the class of *β*-lactones. The compound is a potent proteasome inhibitor being used as the anticancer agent .It was produced by obligated marine actinomycete, *Salinispora tropica* which was originally isolated from marine sediments (Feling et al., 2003). This compound is currently being evaluated in phase I clinical trials as monotherapy and in combination with dexamethasome in patients with relapsed/refractory MM. Furthermore, the combination therapy of salinosporamide A studies bearing minimally cytotoxic synergistic effects in MM, leukemia, and lymphoma cell lines, this combination significantly decreased viability in tumor cells from five relapsed MM patients and reduced tumor growth in human MM xenograft mouse model without any noticeable toxicity (Chauhan et al., 2008; Gulder and Moore, 2010).

Table 3. Secondary metabolites and biological activity of streptomycetes

Compound	Structural class	Biological activity	Producer	Source/location	References
Actinoramides A-C	Peptide	Not detected	*Streptomyces* sp. CNQ-027	Marine sediment sample collected at a depth of 50 m off the coast of San Diego, CA	Nam et al., 2011
Actinoranone	Meroterpenoid	Anti-cancer and tumor cell lines	*Streptomyces* sp. CNQ-027	Marine sediment sample collected at a depth of 50 m of the coast of Diego, CA	Nam et al., 2013
Akaeolide	Carbocyclic polyketide	Anti-bacteria and cytotoxicity	*Streptomyces* sp. NPS554	Marine sediment sample collected at a depth 38 m near Miyazaki Harbor, Japan	Igarashi et al., 2013
Antimycins A19-A20	3-formami-dosalicylic acid-amide link dilactone ring	Anti-fungal (*C. albicans*) activity	*Streptomyces antibioticus*H74-18	Marine sediment collected at a mangrove zone in the South China Sea, Guangdong province, China	Xu et al., 2011
Chlorizidine	Chlorinated nitrogen-containing carbon skeleton	Anti-cancer and cytotoxicity	*Streptomyces* sp. CNH-287	Marine sediment sample collected at the intertidal zone near San Clemente, CA	Alvarez-Mico et al., 2013
Elaiophylin derivatives	Lactone macrolide glycoside	Anti-bacteria	*Streptomyces* sp. 7-145	Marine sediment sample collected at a depth of 40 m. from Heishijiao Bay, Dalian, China	Wu et al., 2013
Farnesides	Sesquiterpenoid Nucleoside ether	Anti-malaria, anti - bacteria and anti-influenza type A virus	*Streptomyces* sp. CNT-372	Marine sediment sample at a depth of 5 m, Nacula Island in the Yasawa Island chain, Fiji	Iland et al., 2013
Fijimycins A-C	Depsipeptide	Anti-bacterial (MRSA) activity	*Streptomyces* sp. CNS-575	Marine sediment samples collected at a depth of 0.5 m from the Nasese shoreline, Viti Levu, Fiji	Sun et al., 2011
Galvaquinones A-C	Anthraquinone	Anti-cancer activity	*Streptomyces spinoverrucosus* SNB-032	Marine sediment sample collected from Trinity Bay, Galveston, Texas	Hu et al., 2012
Glucopiericidin C	Piericidin glucoside	Anti-cancer and cytotoxicity	*Streptomyces* sp. stB8112	Marine sediment collected from the Laguna de Terminos in the Gulf of Mexico	Shaaban et al., 2011
Grincamycins B-F	Anthraquinone glycoside	Anti-cancer activity	*Streptomyces lusitanus* SCSIO LR32	Marine sediment sample at a depth of 3370 m. collected in the South China Sea	Huang et al., 2012
JBIR-34 and 35	Tetrapeptide	Anti-oxidant (anti-DPPH redical) and anti-cancer cell lines	*Streptomyces* sp. Sp080513GE-23	Marine sponge, Haliclona sp., collected from Tateyama city, Chiba Prefecture, Japan	Motohashi, et al., 2010
Lobophorins E- F	Spirotetronate glycoside	Anti-bacterial and anti-cancer activities	*Streptomyces* sp. SCSIO 01127	Marine sediment sample at the depth of 1350 m in the South China Sea	Niu et al., 2011
Marfuraquinocins A-D	Sesquiterpenoid naphthoquinone	Anti-bacterial and anti-cancer cell lines	*Streptomyces niveus* SCSIO 3406	Marine sediment sample at a depth of 3536 m. collected in the South China Sea	Song, et al., 2013

Table 3. (Continued)

Compound	Structural class	Biological activity	Producer	Source/location	References
Nahuoic acids	Polyketide	Anti -histone methyltransferase SETD8	*Streptomyces* sp. RJA2928 and SCSGAA 0027	Marine sediment collected near the passage Padana Nahua in Papua New Guinea	Williams et al., 2013; Nong et al., 2016; Williams et al., 2016;
Nitropyrrolins A-E	Farnesyl-α-nitropyrrole	Anti-bacteria and anti-cancer cell lines	*Streptomyces* sp. CNQ-509	Marine sediment collected at a depth of 44 m offshore of La Jolla, CA	Kwon et al., 2010
Spiroindimicins A-D	Bisindole alkaloid	Anti-cancer activity	*Streptomyces* sp. SCSIO 03032	Marine sediment sample of the India Ocean at the depth of 3412 m	Zhang et al., 2012
Strepnonesides	Anthraquinone glycoside	Anti-cancer activity	*Streptomyces* sp. WBF-16	Marine sediments sample collected from Wei-hai Sea, China	Lu et al., 2012
Streptocarbazoles A-B	Indolocarbazole	Anti -Hela cells at the G2/M phase	*Streptomyces* sp. FMA	Marine sediments collected from the protected areas of mangrove at Sanya, China	Fu et al., 2012
Sungsanpin	Peptide	Anti-cancer cell lines	*Streptomyces* sp. SNJ013	Marine sediments collected at depths of 108-138 m. from the seafloor off the coast of Sungsanpo on Jeju Island, Republic of Korea	Um et al., 2013
Surugamides A-E	Cyclic octapeptide	Anti- bovine cathepsin B and anti-cysteine protease	*Streptomyces* sp. JAMM992	Marine sediment collected from Kinko Bay, Japan	Takada et al., 2013
Tirandamycins	Dienoyl tetramic acid	Anti-*Brugia malayi* asparagine tRNA synthetase and anti - vancomycin-resistant *Enterococcus faecalis*	*Streptomyces* sp. 307-9 and 17944	Marine sediments collected at a depth of 15 m during a scuba expedition in Salt Cay, US	Carlson et al., 2009; Yu et al., 2011
Tumescenamides A-B	Peptide	Control of the insulin-degrading enzyme promoter	*Streptomyces tumescens* YM23-260	Marine sediments of Big drop-off of the Republic of Palau	Motohashi et al., 2010
Usabamycins A-C	Pyrrolobenzodiazepine	Anti -f HeLa cell and anti -serotonin (5-hydroxyt–rypamine) 5-HT2B	*Streptomyces* sp. NPS853	Marine sediment sample collected at a depth of 20 m. Usa Bay, Kochi Prefecture, Japan	Sato et al., 2011

Atrop-abyssomicin C is the polycyclic polyketide antibiotic that is atropisomer of abyssomicin C. This abyssomicin isomer is produced by the strain in genus *Verrucosispora* (Bister et al., 2004; Riedlinger et al., 2004; Keller et al., 2007). For the abyssomicin C initially discovered with activity against ρ-aminobenzoate (ρABA) in Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* (Bister et al., 2004; Riedlinger et al., 2004), in addition, it showed *in vivo* activity against *Mycobacterium tuberculosis* (Freundlich et al., 2010). Marinomycin A is potent antitumor agent with substantial activities against selected human tumor cells and drug-resistant pathogenic bacteria .This compound derived from marine actinomycete *Marinispora* that isolated from the marine sediments (Kwon et al., 2006). Marinomycin A has highly enhanced *in vitro* activity against six of eight melanoma cell lines, with SK-MEL-5 showing the highest sensitivity)concentration lethal to 50 %of animals tested that showed the LC_{50} at 5.0 *n*M. Marinomycin A also inhibits the growth of human pathogenic bacteria with a minimum inhibitory concentration (MIC) value of 0.1 μM against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faceium* (VREF). Unfortunately, these polyenes are highly photoreactive and undergo polyene isomerization under room light. This reactivity and general long-term instability detracts from their potential for clinical development. The chemical structure of bioactive substances from marine sediment-derive actinomycetes was shown in Figure 1.

Recently, the diversity of rare actinomycetes from sediments and the secondary metabolites with diverse biological activities have been investigated (Bredholdt et al., 2007).

Marinactinospora thermotolerans SCSIO 00652 from the deep South China Sea was found to produce a new sequential tristhiazole-thiazoline-containing cyclic peptide, marthiapeptide A that exhibited antibacterial activity against a panel of Gram-positive bacteria, with MIC values ranging from 2.0 to 8.0 μg/ml, and displayed strong cytotoxic activity against a panel of human cancer cell lines with IC50 values ranging from 0.38 to 0.52 μM (Zhou et al., 2012). This strain produced four new β-carboline alkaloids, designated marinacarbolines A–D, two new indolactam alkaloids, 13-*N*-demethyl-methylpendolmycin and methylpendolmycin-14-*O*-α-glucoside, and the three known compounds 1-acetyl-β-carboline, methylpendolmycin, and pendolmycin .The new alkaloid compounds were inactive against a panel of eight tumor cell lines (IC_{50}> 50 μM) but exhibited antiplasmodial activities against *Plasmodium falciparum* lines 3D7 and Dd2, with IC_{50} values ranging from 1.92 to 36.03 μM (Huang et al., 2011).

Micromonospora rosaria SCSIO N160 from a South China Sea sediment was found to produce three new fluostatins, I − K together with six known compounds, fluostatins C − F, rabelomycin and phenanthroviridone .Rabelomycin and phenanthroviridone exhibited good antimicrobial activities against *Staphylococcus aureus* ATCC 29213 with MIC values of 1.0 and 0.25 μg/ml, respectively. Phenanthroviridone also exhibited significant in vitro cytotoxic activities toward SF-268)IC_{50} 0.09 μM (and MCF-7)IC_{50} 0.17 μM ((Zhang et al., 2012). The marine *Actinomadura* sp. 007 produced the compound ZHD-0501, a novel naturally occurring staurosporine analog showing anticancer activity *in vitro* (Han et al., 2005).

Actinoalloteichus cyanogriseus WH1-2216-6, a mutant could produce three new acyclic bipyridine glycosides, cyanogrisides E – G, and a known cyanogriside H. Cyanogrisides F and G showed cytotoxicities against HCT116 and HL-60 cells, in addition all cyanogriside derivatives showed cytotoxic on K562 cells (Fu et al., 2011). *Actinoalloteichus* sp .NPS702 from marine sediment collected from Usa Bay, Kochi Prefecture, Japan, the strain produced

nine new 26-membered macrolides of the oligomycin subfamily, neomaclafungins A − I.These compounds exhibited significant antifungal activity *in vitro* against *Trichophyton mentagrophytes* ATCC 9533 with MIC values between 1 and 3 μg/ml (Sato et al., 2012). *Marinispora* sp. NPS12745 from a marine sediment collected off the coast of San Diego, California. Strain NPS12745 produced a series of chlorinated bisindole pyrroles, lynamicins A– E, which showed broad-spectrum biological activity against both Gram-positive and Gram-negative bacteria. Significantly, these compounds were active against drug-resistant pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (McArthur et al., 2008). Marine-derived *Marinispora* sp. NPS008920 from a sediment collected in Cocos Lagoon, Guam, produced a series of novel 2-alkylidene -5-alkyl-4-oxazolidinones, lipoxazolidinone A, B, and C. Lipoxazolidinones A – C showed broad spectrum antimicrobial activity similar to that of the commercial antibiotic linezolid (Zyvox), a 2-oxazolidinone .Hydrolysis of the amide bond of the 4-oxazolidinone ring of A resulted in loss of antibacterial activity. The 2-alkylidene-4-oxazolidinone represents a new antibiotic pharmacophore and is unprecedented in nature (Macherla et al., 2007).

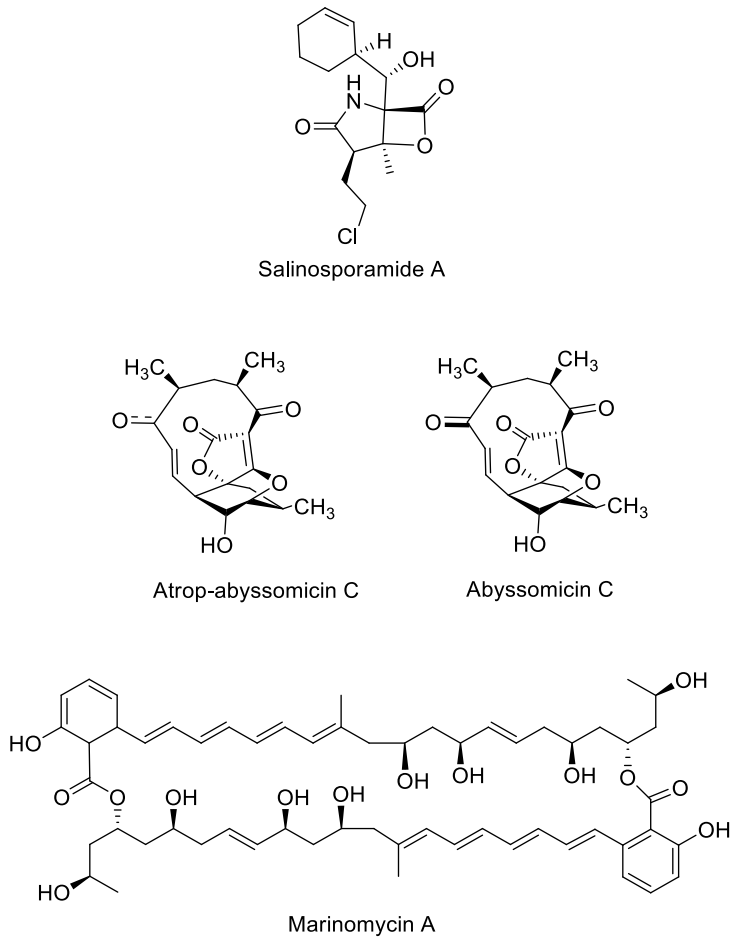

Figure 1. Chemical structure of bioactive metabolites from marine actinomycetes.

Saccharomonospora sp.CNQ490 from marine sediments collected at the mouth of the La Jolla Submarine Canyon, produced the unprecedented alkaloid lodopyridone .This compound is cytotoxic to HCT-116 human colon cancer cells with IC_{50}, 3.6 μM (Maloney et al., 2009). For the obligae marine actinomycete, *Salinispora tropica* CNB-392 produced the potent proteasome inhibitor salinosporamide A and their seven related γ-lactams. The most important of these compounds were a decarboxylated pyrrole analogue, salinosporamide B. The new *structure-activity relationships* (SAR) data for eight compounds, derived from extensive testing against the human colon carcinoma HCT-116 and the 60-cell-line panel at the NCI, indicate that the chloroethyl moiety plays a major role in the enhanced activity of salinosporamide A (Felling et al., 2003; Williams et al., 2005). *Salinispora* sp. CNS103 produced cyanosporasides A and B, chloro -and cyano-cyclopenta[a]indene glycosides (Oh et al., 2006). *Salinispora arenicola* CNR-005 has led to the isolation of two unusual bicyclic polyketides, saliniketals A and B. Saliniketals A and B were found to inhibit ornithine decarboxylase induction, an important target for the chemoprevention of cancer with IC_{50} values of 1.95 ± 0.37 and 7.83 ± 1.2 μg/ml, respectively (Williams et al., 2007). *Salinispora arenicola* strain produced three new macrolide polyketides designated arenicolides A – C (Williams et al., 2007). *Salinispora pacifica* strain led to the discovery of four new polyketides, salinipyrones A – B, and pacificanones A and B (Oh et al., 2008) . *Verrucosispora* sp. MG-37 produced proximicin A, B and C, novel aminofuran antibiotic that showed anticancer compounds. Proximicins showed a weak antibacterial activity but a strong cytostatic effect to various human tumor cell lines (Fiedler et al., 2008). *Verrucosispora* sp. AB-18-032 could be produced abyssomicins G and H and atrop-Abyssomicin C (Keller et al., 2007).

Nocardiopsis dassonvillei HR10-5 produced three new α-pyrones, nocapyrones E − G, and three new diketopiperazine derivatives, nocazines A − C, together with a new oxazoline compound, nocazoline A. Nocapyrones E − G showed modest antimicrobial activity against *Bacillus subtilis* with MIC values of 26, 14, and 12 μM, respectively (Fu et al., 2011) . *Nocardiopsis lucentensis* CNR-712 produced four new 3-methyl-4-ethylideneproline-containing peptides, lucentamycins A – D. Lucentamycins A and B showed significant *in vitro* cytotoxicity against HCT -116 human colon carcinoma (Cho et al., 2007). *Nocardiopsis* strain CNS-653 produced fijiolide A, a potent inhibitor of TNF-R-induced NFκB activation, along with fijiolide B. Fijiolide A is viewed as a promising lead for more advanced anticancer testing (Nam et al., 2010). *Nocardiopsis* sp. CNQ115 from marine sediments collected off the coast of southern California**,** produced two new 4-aminoimidazole alkaloids, nocarimidazoles A and B (Leutou et al., 2015). *Nocardiopsis* sp. CMB-M0232from marine sediment collected off the coast of South Molle Island, Queensland, Australia, yielded two new examples of rare prolinyl-macrolactam polyketides, nocardiopsins C and D, a new highly substituted a-pyrone polyketide, nocardiopyrone A, and the previously reported macrolide polyketides nocardiopsins A and B. PCR amplification of CMB-M0232 genomic DNA revealed the presence of type I and type II polyketide synthase and nonribosomal peptide synthase domains (Raju et al., 2013). The metabolites and biological activity of rare actinomycetes from marine sediments that published on 2010-2016, are summarized in Table 4.

Table 4. Secondary metabolites and biological activity of rare actinomycetes

Compound	Structural class	Biological activity	Producer	Source/location	References
Cyanogrisides A-D	Cyclic bipyridine glycoside	Anti-cancer activity	*Actinoalloteichus cyanogriseus*WH1-2216-6	Marine sediments collected from the seashore of Weihai, China	Fu et al., 2011
Fijiolides	Chloroaromatic glycoside	Induce quinone reductase-1 (QR1) and anti-cancer activity	*Nocardiopsis* sp. CNS-653	Marine sediment sample collected from the Beqa Lagoon, Fiji	Nam et al., 2010
Fluostatins I-K	Polyketide	Anti-bacterial and anti-cancer activities	*Micromonospora rosaria*	Marine sediment sample at a depth of 30 m. collected from a South China Sea	Zhang et al., 2012
Marthiapeptide A	Cyclic peptide	Anti-bacterial and anti-cancer activities	*Marinactinospora thermotolerans* SCSIO 00652	Marine sediment sample collected in the south China Sea	Zhou et al., 2012
Marinacarbolines A-D	*β*-Carboline alkaloid	Anti-malarial and anti-tumor activities	*Marinactinospora thermotolerans* SCSIO 00652	Marine sediment sample collected at a depth of 3865 m. in the northern South China Sea	Huang et al., 2011
Marinactinones A-C	*γ*-Pyrones	Anti-cancer activity	*Marinactinospora thermotolerans* SCSIO 00606	Marine sea sediment sample collected in the northern South China Sea	Wang et al., 2011
Neomaclafungins A-I	Lactonemacrolide	Anti-fungal activity	*Actinoalloteichus* sp.NPS702	Marine sediment collected from Usa Bay, Kochi Prefecture, Japan	Sato et al., 2012
Nocardiamides A-B	Cyclohexapeptide	Anti-acetylcholinesterase (AChE) and anti-bacterial activities	*Nocardiopsis* sp. CNQ115	Marine sediment sample collected at a depth between 18 and 30 m near the La Jolla Canyon, San Diego	Wu et al., 2013
Nocardiopsins C-D	Macrolactam polyketide	Anti-bacterial, anti-fungal and anti-tumor activities	*Nocardiopsis* sp. CMB-M0232	Marine sediment collected off the coast of South Molle Island, Queensland, Australia	Raju et al., 2013
Nocapyrones E-G	*α*-Pyrone	Anti-bacterial activity	*Nocardiopsis dassonvillei* HR10-5	Marine sediments collected from the estuary of Yellow River, Dongying, People's Republic of China	Fu et al., 2011

CONCLUSION

Actinobacteria can be recovered from marine sediments like in the terrestrial soils and play important roles in the decomposition of recalcitrant organic matter in the sea floor. They become recognized as a source of novel antibiotics and anti-cancer agents. A rich biodiversity of culturable actinobacteria recovered from marine sediments belonged to genera *Streptomyces*, *Micromonospora*, *Actinocorallia*, *Actinomadura*, *Knoellia*, *Glycomyces*, *Nocardia*, *Nocardiopsis*, *Nonomuraea*, *Pseudonocardia*, *Rhodococcus*, *Streptosporangium* and *Salinispora.Streptomyces* strains produced diverse core structure of metabolites such as peptides, meroterpenoid, carbocyclic polyketide, chlorinatednitrogen-containing carbon skeleton, lactonemacrolide, sesquiterpenoid nucleoside ether, depsipeptide, piericidin glucoside, anthraquinone, spirotetronate glycoside, sesquiterpenoid-naphthoquinone, farnesyl-α-nitropyrrole, bisindole alkaloid, indolocarbazole, dienoyl tetramic acid, pyrrolobenzodiazepine while the rare actinomycetes, *Actinoalloteichus*, *Marinactinospora*, *Micromonospora*, *Actinoalloteichus*, *Saccharomonospora*, *Salinispora*, and *Nocardiopsis* strains produced cyclic bipyridine glycoside, chloroaromatic glycoside, cyclic peptide, *β*-carboline alkaloid, *α*- and *γ*-pyrones, macrolactone, and lactam polyketide bioactive compounds. To investigate the marine-derived actinomycetes in the world of diverse ecological habitats as the resource for biotechnology, one must understand the extent to which they are capable of growth in the ocean, the degree to which they display specific marine adaptations and the extent to which these adaptations have affected secondary metabolite production.

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Chapter 80

BIBLIOGRAPHY

A biogeographic assessment of seabirds, deep sea corals and ocean habitats of the New York Bight: science to support offshore spatial planning LCCN: 2012452598 Main title: A biogeographic assessment of seabirds, deep sea corals and ocean habitats of the New York Bight: science to support offshore spatial planning/prepared by Center for Coastal Monitoring and Assessment, Biogeography Branch; editors, Charles Menza, Brian P. Kinlan, Dan S. Dorfman, Matthew Poti, Chris Caldow. Published/Created: Silver Spring, Md.: U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Ocean Service, National Centers for Coastal Ocean Science, 2012. Description: ii, 224 p.: col. ill., col. maps; 28 cm. Links: Online version via the NOAA Central Library. http://docs.lib.noaa.gov/noaa_documents/NOS/NCCOS/NY-Bight-report/ http://docs.lib.noaa.gov/noaa_documents/NOS/NCCOS/NY-Bight-report/nycmsp_execsumm.pdf Executive summary in PDF LC classification: QH541.5.C65 B564 2012 Related names: Menza, Charles W. (Charles William), editor. Kinlan, Brian P. (Brian Patrick), editor. Dorfman, Dan S., editor. Poti, Matthew, editor. Caldow, Chris, editor. Center for Coastal Monitoring and Assessment (U.S.). Biogeography Program, creator. National Centers for Coastal Ocean Science (U.S.), sponsor, publisher. Subjects: Sea birds--New York Bight (N.J. and N.Y.) Sea birds--New York Bight (N.J. and N.Y.)--Geographical distribution. Sea birds--New York Bight (N.J. and N.Y.)--Geographical distribution--Maps. Deep sea corals--New York Bight (N.J. and N.Y.) Deep sea corals--Habitat--New York Bight (N.J. and N.Y.) Ocean temperature--New York Bight (N.J. and N.Y.) Marine sediments--New York Bight (N.J. and N.Y.) Notes: "April 2012." Format not distributed to depository libraries. Includes bibliographical references. Additional formats: Online version: biogeographic assessment of seabirds, deep sea corals and ocean habitats of the New York Bight (OCoLC) 844735185 Series: NOAA technical memorandum NOS NCCOS; 141 NOAA technical memorandum NOS NCCOS; 141.

An assessment of chemical contaminants, toxicity and benthic infauna in sediments from the St. Thomas East End Reserves (STEER) LCCN:

2014407153 Personal name: Pait, Anthony S. Main title: An assessment of chemical contaminants, toxicity and benthic infauna in sediments from the St. Thomas East End Reserves (STEER) / Anthony S. Pait, S. Ian Hartwell, Andrew L. Mason, Robert A. Warner, Christopher F.G. Jeffrey, Anne M. Hoffman, Dennis A. Apeti, Francis R. Galdo, Jr., and Simon J. Pittman. Published/Produced: [Silver Spring, Maryland]: NOAA NCCOS Center for Coastal Monitoring and Assessment, [2013] Description: vi, 70 pages; 28 cm. LC classification: QH545.C59 P35 2013 Subjects: Contaminated sediments--United States Virgin Islands--Saint Thomas--Analysis. Marine sediments--Toxicology--United States Virgin Islands--Saint Thomas--Analysis. Benthic animals--Effect of chemicals on--United States Virgin Islands--Saint Thomas. Toxicity testing--United States Virgin Islands--Saint Thomas. Notes: "May 2013." Includes bibliographical references (pages 47-50). Series: NOAA technical memorandum NOS NCCOS; 156

An introduction to hydraulics of fine sediment transport LCCN: 2013000653 Personal name: Mehta, Ashish J. Main title: An introduction to hydraulics of fine sediment transport / Ashish J. Mehta. Published/Produced: New Jersey: World Scientific, [2014] Description: xix, 1039 pages: illustrations; 24 cm. ISBN: 9789814449489 (hbk.: alkaline paper) LC classification: TC175.2 .M44 2014 Contents: Fine Sediment Classification and Characteristic Properties -- Processes of Flocculation, Settling, Deposition, Consolidation, Gelation and Erosion -- Properties and Behavior of Fluid Mud -- Wave-Mud Interaction -- Sedimentation Problems in Coastal and Estuarine Waters as well as Lakes, and Small Tidal Basins. Subjects: Sediment transport. Hydraulic engineering. Marine engineering. Coastal engineering. Coastal sediments. Estuarine sediments. Marine sediments. Notes: Includes bibliographical references (pages 975-1026) and index. Series: Advanced series on ocean engineering; volume 38

Anoxia: evidence for eukaryote survival and paleontological strategies LCCN: 2011935457 Main title: Anoxia: evidence for eukaryote survival and paleontological strategies / edited by Alexander V. Altenbach, Joan M. Bernhard, and Joseph Seckbach. Published/Created: Dordrecht: Springer, c2012. Description: xxxv, 648 p.: ill., ports. (some col.), maps (some col.); 24 cm. Links: Publisher description http://www.loc.gov/catdir/enhancements/fy1316/2011935457-d.html Table of contents only http://www.loc.gov/catdir/enhancements/fy1316/2011935457-t.html ISBN: 9789400718951 (alk. paper) 9400718950 (alk. paper) 9789400718968 (e-ISBN) 9400718969 (e-ISBN) LC classification: QH518.5 .A56 2011 Related names: Altenbach, Alexander V. Bernhard, Joan M. Seckbach, J. (Joseph) Contents: Anaerobic eukaryotes / T. Fenchel -- Biogeochemical reactions in marine sediments underlying anoxic water bodies/ T. Treude -- Diversity of anaerobic prokaryotes and eukaryotes: breaking long-established dogmas / A. Oren -- The biochemical adaptations of mitochondrion-related organelles of parasitic and free-living microbial eukaryotes to low oxygen environments / A.D. Tsaousis et al. --

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Anaerobiosis. Micropaleontology. Anaerobiosis. Eukaryota. Adaptation, Physiological. Biological Evolution. Paleontology. Notes: Includes bibliographical references and indexes. Series: Cellular origin, life in extreme habitats and astrobiology, 1566-0400; v. 21 Cellular origin and life in extreme habitats and astrobiology; v. 21. 1566-0400

Assessment of heavy metal contamination in the marine environment of the Arabian Gulf LCCN: 2012048209 Personal name: Naser, Humood Abdulla. Main title: Assessment of heavy metal contamination in the marine environment of the Arabian Gulf / Humood Abdulla Naser. Published/Produced: Hauppauge, New York: Novinka, [2013] Description: viii, 99 pages: color illustrations, maps; 23 cm. ISBN: 9781624176197 (soft cover) LC classification: GC1451 .N36 2013 Contents: General Introduction -- Valued Ecosystem Components in the Arabian Gulf -- Anthropogenic Sources of Heavy Metals in the Arabian Gulf -- Heavy Metal Levels in Algal Species from the Arabian Gulf -- Heavy Metal Levels in Molluscs from the Arabian Gulf -- Heavy Metal Levels in Fish Species from the Arabian Gulf -- Heavy Metal Concentrations in Seawaters from the Arabian Gulf -- Heavy Metal Concentrations in Sediments from the Arabian Gulf -- Macrobenthic Community Structure as a Bioindicator for Heavy Metal Contamination -- Spatial Distribution of Heavy Metals in Marine Sediments Influenced by Land-based Anthropogenic Sources in Bahrain: A Case Study -- Management of Heavy Metals in the Arabian Gulf -- Conclusions and Recommendations. Subjects: Marine pollution--Persian Gulf. Heavy metals--Environmental aspects--Persian Gulf. Persian Gulf--Environmental conditions. Notes: Includes bibliographical references and index. Series: Environmental Health-Physical, Chemical and Biology Factors

Carbonates: sedimentology, geographical distribution and economic importance LCCN: 2013036362 Main title: Carbonates: sedimentology, geographical distribution and economic importance / Bailey A. Hughes and Thompson C. Wagner, editors. Published/Produced: New York: Novinka, [2013] ©2013 Description: ix, 93 pages: illustrations, map; 23 cm. ISBN: 9781629481784 (soft cover) LC classification: QE471.15.C3 C393 2013 Related names: Hughes, Bailey A., editor of compilation. Wagner, Thompson C., editor of compilation. Subjects: Carbonates. Marine sediments. Notes: Includes bibliographical references and index. Series: Chemical engineering methods and technology Geology and mineralogy research developments

Deep-marine systems: processes, deposits, environments, tectonics and sedimentation LCCN: 2015007967 Personal name: Pickering, K. T. (Kevin T.) Main title: Deep-marine systems: processes, deposits, environments, tectonics and sedimentation / by Kevin T. Pickering & Richard N. Hiscott; with contribution from Thomas Heard. Published/Produced: Chichester, West Sussex; Hoboken, NJ: John Wiley & Sons Inc., [2015] Description: pages cm ISBN: 9781118865491 (cloth) 9781405125789 (pbk.) LC classification: GC380.15 .P54 2015 Related names: Hiscott, Richard N. Subjects: Marine sediments. Plate

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Marine geochemistry LCCN: 2012010712 Personal name: Chester, R. (Roy), 1936- Main title: Marine geochemistry / Roy Chester and Tim Jickells. Edition: 3rd ed. Published/Created: Hoboken, NJ: John Wiley & Sons, c2012. Description: vii, 411 p., [12] p. of plates: ill. (some col.), maps (some col.); 26 cm. ISBN: 9781118349076 (cloth) 9781405187343 (pbk.) LC classification: GC111.2 .C47 2012 Related names: Jickells, T. D. (Tim D.) Subjects: Chemical oceanography. Marine sediments. Geochemistry. Notes: Includes bibliographical references and index.

Marine tephrochronology LCCN: 2014451556 Main title: Marine tephrochronology / edited by W.E.N. Austin, P.M. Abbott, S.M. Davies, N.J.G. Pearce and S. Wastegård. Published/Produced: London: The Geological Society, 2014. Description: 213 pages: illustrations (some color), maps (some color); 26 cm. ISBN: 9781862396418 1862396418 LC classification: QE527.56 .M37 2014 Related names: Austin, W. E. N. (William E. N.), editor. Abbott, P. M. (Peter M.), editor. Davies, S. M. (Siwan M.), editor. Pearce, N. J. G., editor. Wastegard, S., (Stefan), editor. Abstract: This Special Publication includes articles presenting recent advances in marine tephrochronological studies and outlines innovative techniques in geochemical fingerprinting, stratigraphy and the understanding of depositional processes. It represents a significant resource for the palaeoceanographic community at a time when marine tephrochronology is being more widely recognized. It will also serve as a valuable reference to a much wider community of Earth scientists, climate scientists and archaeologists, particularly in highlighting the role of tephra studies in stratigraphy and regional/extra-regional correlations, as well as in tracing the long-term history of regional and global volcanism in the deep-sea archive. Contents: Marine tephrochronology: an introduction to tracing time in the ocean / W.E.N. Austin, P.M. Abbott, S. Davies, N.J.G. Pearce and S. Wastegård -- Marine tephrochronology: a personal perspective / D.J. Lowe -- Preparation of micro- and crypto-tephras for quantitative microbeam analysis / M. Hall and C. Hayward -- Microbeam methods for the analysis of glass in fine-grained tephra deposits: a SMART perspective on current and future trends / N.J.G. Pearce, P.M. Abbott, and C. Martin-Jones -- Physical characteristics of tephra layers in the deep sea realm: the Campanian Ignimbrite eruption / S.L. Engwell, R.S.J. Sparks and S. Carey -- Identification of a MIS6 age (c. 180 ka) Icelandic tephra within NE Atlantic sediments: a new potential chronostratigraphic marker / F.D. Hibbert, S. Wastegård, R/ Gwynn and W.E.N. Austin -- Marine Ash Zone IV: a new MIS 3 ash zone on the Faroe Islands margin / S. Wastegård and T.L. Rasmussen -- North Atlantic marine radiocarbon reservoir ages through Heinrich event H4: a new method for marine age model construction / J. Olsen, T.L. Rasmussen and P.J. Reimer -- Last millennium dispersal of air-fall

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Organic compounds in soils, sediments & sludges: analysis and determination LCCN: 2012036581 Main title: Organic compounds in soils, sediments & sludges: analysis and determination / [editor], T. Roy Crompton, Retired, UK Rivers Authority, UK. Published/Produced: Boca Raton: CRC Press, Taylor & Francis Group, [2013] Description: xii, 263 pages: illustrations; 26 cm ISBN: 9780415644273 (Hbk: alk. paper) LC classification: S592.6.O73 O74 2013 Related names: Crompton, T. R. (Thomas Roy) Subjects: Soils--Organic compound content. Marine sediments. Soil pollution--Measurement. Sewage sludge. Organic water pollutants--Measurement. Notes: "A Balkema Book." Includes bibliographical references and index.

Principles of tidal sedimentology LCCN: 2011939475 Main title: Principles of tidal sedimentology / Richard A. Davis, Jr., Robert W. Dalrymple, editors. Published/Produced: Dordrecht; New York: Springer, [2012] Description: xv, 621 pages: illustrations (some color), maps; 27 cm ISBN: 9789400701229 (hbk) 9400701225 (hbk) 9400701233 (e-book) 9789400701236 (e-book) LC classification: QE471 .P748 2012 Related names: Davis, Richard A., Jr., 1937- Dalrymple, Robert W. (Robert Walker) Subjects: Sedimentology. Marine sediments. Notes: Includes bibliographical references and index.

Reconstructing Earth's climate history: inquiry-based exercises for lab and class LCCN: 2011039577 Main title: Reconstructing Earth's climate history: inquiry-based exercises for lab and class / Kristen St John... [et al.]. Published/Created: Hoboken, N.J.: Wiley, 2012. Description: xlii, 485 p.: ill. (some col.), maps (some col.); 29 cm. Links: Cover image http://catalogimages.wiley.com/images/db/jimages/9780470658055.jpg ISBN: 9780470658055 (hbk.) 9781118232941 (pbk.) LC classification: QC884 .R428 2012 Related names: St. John, Kristen. Summary: "The context for understanding global climate change today lies in the records of Earth's past. This is demonstrated by decades of paleoclimate research by scientists in organizations such as the Integrated Ocean Drilling Program (IODP), the Antarctic Geological Drilling Program (ANDRILL), and many others. The purpose of this book is to put key data and published case studies of past climate change at your fingertips, so that you can experience the nature of paleoclimate reconstruction. Using

foundational geologic concepts you will explore a wide variety of topics in this book, including: marine sediments, age determination, stable isotope paleoclimate proxies, Cenozoic climate change, climate cycles, polar climates, and abrupt warming and cooling events. You will evaluate published scientific data, practice developing and testing hypotheses, and infer the broader implications of scientific results. It is our philosophy that addressing how we know is as important as addressing what we know about past climate change. Making climate change science accessible is the goal of this book. Readership: earth science students at a variety of levels studying paleoclimatology, oceanography, Quaternary science, or earth-system science"-- Provided by publisher. "This project integrates scientific ocean drilling data and research (DSDP-ODP-IODPANDRILL) with education"-- Provided by publisher. Contents: Machine generated contents note: The Authors Acknowledgments Book Introduction for Students and Instructors Geologic Timescale Chapter 1. Introduction to Paleoclimate Records. Part 1.1. Archives and Proxies Part 1.2. Owens Lake - An Introductory Case Study of Paleoclimate Reconstruction Part 1.3. Coring Glacial Ice and Seafloor Sediments Chapter 2. Seafloor Sediments. Part 2.1. Sediment Predictions Part 2.2. Core Observations and Descriptions Part 2.3. Sediment Composition Part 2.4. Geographic Distribution and Interpretation Chapter 3. Microfossils and Biostratigraphy. Part 3.1. What are Microfossils? Why are they Important in Climate Change Science? Part 3.2. Microfossils in Deep-sea Sediments Part 3.3. Application of Microfossil First and Last Occurrences Part 3.4. Using Microfossil Datums to Calculate Rates Part 3.5. How Reliable are Microfossil Datums? Chapter 4. Paleomagnetism and Magnetostratigraphy. Part 4.1. Earth's Magnetic Field Today and the Paleomagnetic Record of Deep-Sea Sediments Part 4.2. Paleomagnetism in Ocean Crust Part 4.3. Using Paleomagnetism to Test the Seafloor Spreading Hypothesis Part 4.4. The Geomagnetic Polarity Time Scale Chapter 5. CO2 as a Climate Regulator during the Phanerozoic and Today. Part 5.1. The Short Term Global Carbon Cycle Part 5.2. CO2 and Temperature Part 5.3. Recent Changes in CO2 Part 5.4. The Long-term Global Carbon Cycle, CO2, and Phanerozoic Climate History Chapter 6. The Benthic Foraminiferal Oxygen Isotope Record of Cenozoic Climate Change. Part 6.1. Introduction Part 6.2. Stable Isotope Geochemistry Part 6.3. A Biogeochemical Proxy Part 6.4. Patterns, Trends and Implications for Cenozoic Climate Chapter 7. Scientific Drilling in the Arctic Ocean: A Lesson on the Nature of Science. Part 7.1. Climate Models and Regional Climate Change Part 7.2. Arctic Drilling Challenges and Solutions Part 7.3. The Need for Scientific Drilling Part 7.4. Results of the Arctic Drilling Expedition Chapter 8. Climate Cycles. Part 8.1. Patterns and Periodicities Part 8.2. Orbital Metronome Part 8.3. A Break in the Pattern Chapter 9. The Paleocene Eocene Thermal Maximum (PETM) Event. Part 9.1. The Cenozoic [delta]13C Record and an Important Discovery Part 9.2. Global Consequences of the PETM Part 9.3. Bad Gas: Is Methane to Blame? Part 9.4. How fast? How long? Part 9.5. Global Warming Today and Lessons

from the PETM Chapter 10. Glaciation of Antarctica: The Oi1 Event. Part 10.1. Initial Evidence Part 10.2. Evidence for Global Change Part 10.3. Mountain Building, Weathering, CO2 and Climate Part 10.4. Legacy of the Oi1 Event: The Development of the Psychrosphere Chapter 11. Antarctica and Neogene Global Climate Change. Part 11.1. What do we Think we Know about the History of Antarctic Climate? Part 11.2. What is Antarctica's Geographic & Geologic Context? Part 11.3. Selecting Drillsites to Best Answer our Questions Chapter 12. Interpreting Antarctic Sediment Cores: A Record of Dynamic Neogene Climate. Part 12.1. What Sediment Facies are Common on the Antarctic Margin? Part 12.2. ANDRILL 1-B The BIG Picture Part 12.3. Pliocene Sedimentary Patterns in the ANDRILL 1-B Core Ch. 13. Pliocene Warmth: Are We Seeing Our Future? Part 13.1. The last 5 million years Part 13.2. Sea Level Past, Present, and Future Chapter 14. Northern Hemisphere Glaciation. Part 14.1. Concepts & Predictions Part 14.2. What is the Evidence? Part 14.3. What Caused It? Index. Subjects: Paleoclimatology. Climatic changes--Observations. Climatic changes--History. SCIENCE / Earth Sciences / Meteorology & Climatology. Notes: Includes bibliographical references and index.

Submarine mass movements and their consequences: 5th international symposium LCCN: 2011939086 Main title: Submarine mass movements and their consequences: 5th international symposium / Yasuhiro Yamada ... [et al.], editors. Published/Created: Dordrecht; New York: Springer, c2012. Description: xxxi, 769 p.: ill. (some col.), maps (some col.); 24 cm. ISBN: 9789400721616 (hbk.: alk. paper) 9400721617 (hbk.: alk. paper) 9789048130702 9048130700 LC classification: QE598 .S83 2012 GC Related names: Yamada, Yasuhiro. Subjects: Mass-wasting--Congresses. Submarine topography--Congresses. Landslide hazard analysis--Congresses. Marine sediments--Congresses. Tsunamis--Congresses. Submarine geology. Conference proceedings. Massenbewegung (Geomorphologie) Meeresboden. Submarine Gleitung. Form/Genre: Austin (Texas, 2009) Kongress-Austin (Texas)-2009. Kongress. Notes: Sixty-five papers from an international symposium held in Kyoto, Japan, in 2011. Includes bibliographical references and indexes. Series: Advances in natural and technological hazards research; v. 31 Advances in natural and technological hazards research; v. 31.

A biogeographic assessment of seabirds, deep sea corals and ocean habitats of the New York Bight: science to support offshore spatial planning LCCN: 2012452598 Main title: A biogeographic assessment of seabirds, deep sea corals and ocean habitats of the New York Bight: science to support offshore spatial planning/repared by Center for Coastal Monitoring and Assessment, Biogeography Branch; editors, Charles Menza, Brian P. Kinlan, Dan S. Dorfman, Matthew Poti, Chris Caldow. Published/Created: Silver Spring, Md.: U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Ocean Service, National Centers for Coastal Ocean Science, 2012. Description: ii, 224 p.: col. ill., col. maps; 28 cm. Links: Online version via the NOAA Central Library.

http://docs.lib.noaa.gov/noaa_documents/NOS/NCCOS/NY-Bight-report/ http://docs.lib.noaa.gov/noaa_documents/NOS/NCCOS/NY-Bight-report/nycmsp_execsumm.pdf Executive summary in PDF LC classification: QH541.5.C65 B564 2012 Related names: Menza, Charles W. (Charles William), editor. Kinlan, Brian P. (Brian Patrick), editor. Dorfman, Dan S., editor. Poti, Matthew, editor. Caldow, Chris, editor. Center for Coastal Monitoring and Assessment (U.S.). Biogeography Program, creator. National Centers for Coastal Ocean Science (U.S.), sponsor, publisher. Subjects: Sea birds--New York Bight (N.J. and N.Y.) Sea birds--New York Bight (N.J. and N.Y.)--Geographical distribution. Sea birds--New York Bight (N.J. and N.Y.)--Geographical distribution--Maps. Deep sea corals--New York Bight (N.J. and N.Y.) Deep sea corals--Habitat--New York Bight (N.J. and N.Y.) Ocean temperature--New York Bight (N.J. and N.Y.) Marine sediments--New York Bight (N.J. and N.Y.) Notes: "April 2012." Format not distributed to depository libraries. Includes bibliographical references. Additional formats: Online version: biogeographic assessment of seabirds, deep sea corals and ocean habitats of the New York Bight (OCoLC)844735185 Series: NOAA technical memorandum NOS NCCOS; 141 NOAA technical memorandum NOS NCCOS; 141.

An assessment of chemical contaminants, toxicity and benthic infauna in sediments from the St. Thomas East End Reserves (STEER) LCCN: 2014407153 Personal name: Pait, Anthony S. Main title: An assessment of chemical contaminants, toxicity and benthic infauna in sediments from the St. Thomas East End Reserves (STEER) / Anthony S. Pait, S. Ian Hartwell, Andrew L. Mason, Robert A. Warner, Christopher F.G. Jeffrey, Anne M. Hoffman, Dennis A. Apeti, Francis R. Galdo, Jr., and Simon J. Pittman. Published/Produced: [Silver Spring, Maryland]: NOAA NCCOS Center for Coastal Monitoring and Assessment, [2013] Description: vi, 70 pages; 28 cm. LC classification: QH545.C59 P35 2013 Subjects: Contaminated sediments--United States Virgin Islands--Saint Thomas--Analysis. Marine sediments--Toxicology--United States Virgin Islands--Saint Thomas--Analysis. Benthic animals--Effect of chemicals on--United States Virgin Islands--Saint Thomas. Toxicity testing--United States Virgin Islands--Saint Thomas. Notes: "May 2013." Includes bibliographical references (pages 47-50). Series: NOAA technical memorandum NOS NCCOS; 156

An introduction to hydraulics of fine sediment transport LCCN: 2013000653 Personal name: Mehta, Ashish J. Main title: An introduction to hydraulics of fine sediment transport / Ashish J. Mehta. Published/Produced: New Jersey: World Scientific, [2014] Description: xix, 1039 pages: illustrations; 24 cm. ISBN: 9789814449489 (hbk.: alkaline paper) LC classification: TC175.2 .M44 2014 Contents: Fine Sediment Classification and Characteristic Properties -- Processes of Flocculation, Settling, Deposition, Consolidation, Gelation and Erosion -- Properties and Behavior of Fluid Mud -- Wave-Mud Interaction -- Sedimentation Problems in Coastal and Estuarine Waters as well as Lakes, and Small Tidal Basins. Subjects: Sediment transport. Hydraulic engineering.

Marine engineering. Coastal engineering. Coastal sediments. Estuarine sediments. Marine sediments. Notes: Includes bibliographical references (pages 975-1026) and index. Series: Advanced series on ocean engineering; volume 38

Anoxia: evidence for eukaryote survival and paleontological strategies LCCN: 2011935457 Main title: Anoxia: evidence for eukaryote survival and paleontological strategies / edited by Alexander V. Altenbach, Joan M. Bernhard, and Joseph Seckbach. Published/Created: Dordrecht: Springer, c2012. Description: xxxv, 648 p.: ill., ports. (some col.), maps (some col.); 24 cm. Links: Publisher description http://www.loc.gov/catdir/enhancements/fy1316/2011935457-d.html Table of contents only http://www.loc.gov/catdir/enhancements/fy1316/2011935457-t.html ISBN: 9789400718951 (alk. paper) 9400718950 (alk. paper) 9789400718968 (e-ISBN) 9400718969 (e-ISBN) LC classification: QH518.5 .A56 2011 Related names: Altenbach, Alexander V. Bernhard, Joan M. Seckbach, J. (Joseph) Contents: Anaerobic eukaryotes / T. Fenchel -- Biogeochemical reactions in marine sediments underlying anoxic water bodies/ T. Treude -- Diversity of anaerobic prokaryotes and eukaryotes: breaking long-established dogmas / A. Oren -- The biochemical adaptations of mitochondrion-related organelles of parasitic and free-living microbial eukaryotes to low oxygen environments / A.D. Tsaousis et al. -- Hydrogenosomes and mitosomes: mitochondrial adaptations to life in anaerobic environments, R.M. De Graaf and J.H.P. Hackstein -- Adapting to hypoxia: lessons from vascular endothelial growth factor / N.S. Levy and A.P. Levy -- Magnetotactic protists at the oxic-anoxic transition zones of coastal aquatic environments / D.A. Bazylinski et al. -- A novel ciliate (Ciliophora: Hypotrichida) isolated from bathyal anoxic sediments / D.J. Baudoin et al. -- The wood-eating termite hindgut: diverse cellular symbioses in a microoxic to anoxic environment / M.F. Dolan -- Ecological and experimental exposure of insects to anoxia reveals surprising tolerance / W.W. Hoback -- The unusual response of encysted embryos of the animal extremophile, Artemia franciscana, to prolonged anoxia / J.S. Clegg -- Survival of tardigrades in extreme environments: a model animal for astrobiology / D.D. Horikawa -- Long-term anoxia tolerance in flowering plants / R.M.M. Crawford -- Benthic Foraminifera: inhabitants of low-oxygen environments / K.A. Koho and E. Piña-Ochoa -- Ecological and biological response of benthic Foraminifera under oxygen-depleted conditions: evidence from laboratory approaches / P. Heinz and E. Geslin -- The response of benthic Foraminifera to low-oxygen conditions of the Peruvian oxygen minimum zone / J. Mallon et al. -- Benthic foraminiferal communities and microhabitat selection on the continental shelf off central Peru / J. Cardich et al. -- Living assemblages from the "dead zone" and naturally occurring hypoxic zones / K.R. Buck et al. -- The return of shallow shelf seas as extreme environments: anoxia and macrofauna reactions in the northern Adriatic Sea / M. Stachowitsch et al. -- Meiobenthos of the oxic/anoxic interface in the south-western region of the Black Sea: abundance and taxonomic composition / N.G. Sergeeva

et al. -- The role of eukaryotes in the anaerobic food web of stratified lakes / A. Saccà -- The anoxic Framvaren Fjord as a model system to study protistan diversity and evolution / T. Stoeck and A. Behnke -- Characterizing an anoxic habitat: sulfur bacteria in a meromictic alpine lake / G.B. Fritz et al. -- Ophel, the newly discovered hypoxic chemolitho-autotrophic groundwater biome: a window to ancient animal life / F.D. Por -- Microbial eukaryotes in the marine subsurface? / V.P. Edgcomb and J.F. Biddle -- On the use of stable nitrogen isotopes in present and past anoxic environments / U. Struck -- Carbon and nitrogen isotopic fractionation in Foraminifera: possible signatures from anoxia / A.V. Altenbach et al. -- The functionality of pores in benthic Foraminifera in view of bottom water oxygenation: a review / N. Glock et al. -- Anoxia-dysoxia at the sediment-water interface of the southern Tethys in the late Cretaceous: Mishash Formation, southern Israel / A. Almogi-Labin et al. -- Styles of agglutination in benthic Foraminifera from modern Santa Barbara Basin sediments and the implications of finding fossil analogs in Devonian and Mississippian black shales / J. Schieber -- Did redox conditions trigger test templates in Proterozoic Foraminifera? / A.V. Altenbach and M. Gaulke -- The relevance of anoxic and agglutinated benthic Foraminifera to the possible Archean evolution of eukaryotes / W. Altermann et al. Subjects: Anoxic zones. Eukaryotic cells--Evolution. Adaptation (Physiology) Extreme environments--Microbiology. Anaerobiosis. Micropaleontology. Anaerobiosis. Eukaryota. Adaptation, Physiological. Biological Evolution. Paleontology. Notes: Includes bibliographical references and indexes. Series: Cellular origin, life in extreme habitats and astrobiology, 1566-0400; v. 21 Cellular origin and life in extreme habitats and astrobiology; v. 21. 1566-0400

Assessment of heavy metal contamination in the marine environment of the Arabian Gulf LCCN: 2012048209 Personal name: Naser, Humood Abdulla. Main title: Assessment of heavy metal contamination in the marine environment of the Arabian Gulf / Humood Abdulla Naser. Published/Produced: Hauppauge, New York: Novinka, [2013] Description: viii, 99 pages: color illustrations, maps; 23 cm. ISBN: 9781624176197 (soft cover) LC classification: GC1451 .N36 2013 Contents: General Introduction -- Valued Ecosystem Components in the Arabian Gulf -- Anthropogenic Sources of Heavy Metals in the Arabian Gulf -- Heavy Metal Levels in Algal Species from the Arabian Gulf -- Heavy Metal Levels in Molluscs from the Arabian Gulf -- Heavy Metal Levels in Fish Species from the Arabian Gulf -- Heavy Metal Concentrations in Seawaters from the Arabian Gulf -- Heavy Metal Concentrations in Sediments from the Arabian Gulf -- Macrobenthic Community Structure as a Bioindicator for Heavy Metal Contamination -- Spatial Distribution of Heavy Metals in Marine Sediments Influenced by Land-based Anthropogenic Sources in Bahrain: A Case Study -- Management of Heavy Metals in the Arabian Gulf -- Conclusions and Recommendations. Subjects: Marine pollution--Persian Gulf. Heavy metals--Environmental aspects--Persian Gulf. Persian Gulf--Environmental conditions. Notes: Includes bibliographical references and

index. Series: Environmental Health-Physical, Chemical and Biology Factors

Carbonates: sedimentology, geographical distribution and economic importance LCCN: 2013036362 Main title: Carbonates: sedimentology, geographical distribution and economic importance / Bailey A. Hughes and Thompson C. Wagner, editors. Published/Produced: New York: Novinka, [2013] ©2013 Description: ix, 93 pages: illustrations, map; 23 cm. ISBN: 9781629481784 (soft cover) LC classification: QE471.15.C3 C393 2013 Related names: Hughes, Bailey A., editor of compilation. Wagner, Thompson C., editor of compilation. Subjects: Carbonates. Marine sediments. Notes: Includes bibliographical references and index. Series: Chemical engineering methods and technology Geology and mineralogy research developments

Deep-marine systems: processes, deposits, environments, tectonics and sedimentation LCCN: 2015007967 Personal name: Pickering, K. T. (Kevin T.) Main title: Deep-marine systems: processes, deposits, environments, tectonics and sedimentation / by Kevin T. Pickering & Richard N. Hiscott; with contribution from Thomas Heard. Published/Produced: Chichester, West Sussex; Hoboken, NJ: John Wiley & Sons Inc., [2015] Description: pages cm ISBN: 9781118865491 (cloth) 9781405125789 (pbk.) LC classification: GC380.15 .P54 2015 Related names: Hiscott, Richard N. Subjects: Marine sediments. Plate tectonics. Notes: Includes bibliographical references and index. Additional formats: Online version: Pickering, K. T. (Kevin T.) Deep-marine systems Chichester, West Sussex; Hoboken, NJ: John Wiley & Sons Inc., [2015] 9781118865422 (DLC) 2015017033

Marine geochemistry LCCN: 2012010712 Personal name: Chester, R. (Roy), 1936- Main title: Marine geochemistry / Roy Chester and Tim Jickells. Edition: 3rd ed. Published/Created: Hoboken, NJ: John Wiley & Sons, c2012. Description: vii, 411 p., [12] p. of plates: ill. (some col.), maps (some col.); 26 cm. ISBN: 9781118349076 (cloth) 9781405187343 (pbk.) LC classification: GC111.2 .C47 2012 Related names: Jickells, T. D. (Tim D.) Subjects: Chemical oceanography. Marine sediments. Geochemistry. Notes: Includes bibliographical references and index.

Marine tephrochronology LCCN: 2014451556 Main title: Marine tephrochronology / edited by W.E.N. Austin, P.M. Abbott, S.M. Davies, N.J.G. Pearce and S. Wastegård. Published/Produced: London: The Geological Society, 2014. Description: 213 pages: illustrations (some color), maps (some color); 26 cm. ISBN: 9781862396418 1862396418 LC classification: QE527.56 .M37 2014 Related names: Austin, W. E. N. (William E. N.), editor. Abbott, P. M. (Peter M.), editor. Davies, S. M. (Siwan M.), editor. Pearce, N. J. G., editor. Wastegard, S., (Stefan), editor. Abstract: This Special Publication includes articles presenting recent advances in marine tephrochronological studies and outlines innovative techniques in geochemical fingerprinting, stratigraphy and the understanding of depositional processes. It represents a significant resource for the palaeoceanographic community at a

time when marine tephrochronology is being more widely recognized. It will also serve as a valuable reference to a much wider community of Earth scientists, climate scientists and archaeologists, particularly in highlighting the role of tephra studies in stratigraphy and regional/extra-regional correlations, as well as in tracing the long-term history of regional and global volcanism in the deep-sea archive. Contents: Marine tephrochronology: an introduction to tracing time in the ocean / W.E.N. Austin, P.M. Abbott, S. Davies, N.J.G. Pearce and S. Wastegård -- Marine tephrochronology: a personal perspective / D.J. Lowe -- Preparation of micro- and crypto-tephras for quantitative microbeam analysis / M. Hall and C. Hayward -- Microbeam methods for the analysis of glass in fine-grained tephra deposits: a SMART perspective on current and future trends / N.J.G. Pearce, P.M. Abbott, and C. Martin-Jones -- Physical characteristics of tephra layers in the deep sea realm: the Campanian Ignimbrite eruption / S.L. Engwell, R.S.J. Sparks and S. Carey -- Identification of a MIS6 age (c. 180 ka) Icelandic tephra within NE Atlantic sediments: a new potential chronostratigraphic marker / F.D. Hibbert, S. Wastegård, R/ Gwynn and W.E.N. Austin -- Marine Ash Zone IV: a new MIS 3 ash zone on the Faroe Islands margin / S. Wastegård and T.L. Rasmussen -- North Atlantic marine radiocarbon reservoir ages through Heinrich event H4: a new method for marine age model construction / J. Olsen, T.L. Rasmussen and P.J. Reimer -- Last millennium dispersal of air-fall tephra and ocean-rafted pumice towards the north Icelandic shelf and the Nordic seas / G. Larsen, J. Eiríksson and E.R. Gudmundsdóttir -- Iceberg-rafted tephra as a potential tool for the reconstruction of ice-sheet processes and ocean surface circulation in the glacial North Atlantic / M. Kuhsk, W.E.N. Austin, P.M. Abbott and D.A. Hodell -- Holocene tephra from Iceland and Alaska in SE Greenleaf shelf sediments / A. Jennings, T. Thordarson, K. Zalzal, J. Stoner, C. Hayward, Á. Geirsdóttir and G. Miller -- Quantifying bioturbation of a simulated ash fall event / J.A. Todd, W.E.N. Austin and P.M. Abbott. Subjects: Tephrochronology. Marine sediments. Submarine geology. Paleoceanography. Volcanic ash, tuff, etc. Notes: Includes bibliographical references and index. Series: Geological Society special publication, 0305-8719; no. 398 Geological Society special publication; no. 398.

Organic compounds in soils, sediments & sludges: analysis and determination LCCN: 2012036581 Main title: Organic compounds in soils, sediments & sludges: analysis and determination / [editor], T. Roy Crompton, Retired, UK Rivers Authority, UK. Published/Produced: Boca Raton: CRC Press, Taylor & Francis Group, [2013] Description: xii, 263 pages: illustrations; 26 cm ISBN: 9780415644273 (Hbk: alk. paper) LC classification: S592.6.O73 O74 2013 Related names: Crompton, T. R. (Thomas Roy) Subjects: Soils--Organic compound content. Marine sediments. Soil pollution--Measurement. Sewage sludge. Organic water pollutants--Measurement. Notes: "A Balkema Book." Includes bibliographical references and index.

Principles of tidal sedimentology LCCN: 2011939475 Main title: Principles of tidal sedimentology / Richard A. Davis,

Jr., Robert W. Dalrymple, editors. Published/Produced: Dordrecht; New York: Springer, [2012] Description: xv, 621 pages: illustrations (some color), maps; 27 cm ISBN: 9789400701229 (hbk) 9400701225 (hbk) 9400701233 (e-book) 9789400701236 (e-book) LC classification: QE471 .P748 2012 Related names: Davis, Richard A., Jr., 1937- Dalrymple, Robert W. (Robert Walker) Subjects: Sedimentology. Marine sediments. Notes: Includes bibliographical references and index.

Reconstructing Earth's climate history: inquiry-based exercises for lab and class LCCN: 2011039577 Main title: Reconstructing Earth's climate history: inquiry-based exercises for lab and class / Kristen St John... [et al.]. Published/Created: Hoboken, N.J.: Wiley, 2012. Description: xlii, 485 p.: ill. (some col.), maps (some col.); 29 cm. Links: Cover image http://catalogimages.wiley.com/images/db/jimages/9780470658055.jpg ISBN: 9780470658055 (hbk.) 9781118232941 (pbk.) LC classification: QC884 .R428 2012 Related names: St. John, Kristen. Summary: "The context for understanding global climate change today lies in the records of Earth's past. This is demonstrated by decades of paleoclimate research by scientists in organizations such as the Integrated Ocean Drilling Program (IODP), the Antarctic Geological Drilling Program (ANDRILL), and many others. The purpose of this book is to put key data and published case studies of past climate change at your fingertips, so that you can experience the nature of paleoclimate reconstruction. Using foundational geologic concepts you will explore a wide variety of topics in this book, including: marine sediments, age determination, stable isotope paleoclimate proxies, Cenozoic climate change, climate cycles, polar climates, and abrupt warming and cooling events. You will evaluate published scientific data, practice developing and testing hypotheses, and infer the broader implications of scientific results. It is our philosophy that addressing how we know is as important as addressing what we know about past climate change. Making climate change science accessible is the goal of this book. Readership: earth science students at a variety of levels studying paleoclimatology, oceanography, Quaternary science, or earth-system science"-- Provided by publisher. "This project integrates scientific ocean drilling data and research (DSDP-ODP-IODPANDRILL) with education"-- Provided by publisher. Contents: Machine generated contents note: The Authors Acknowledgments Book Introduction for Students and Instructors Geologic Timescale Chapter 1. Introduction to Paleoclimate Records. Part 1.1. Archives and Proxies Part 1.2. Owens Lake - An Introductory Case Study of Paleoclimate Reconstruction Part 1.3. Coring Glacial Ice and Seafloor Sediments Chapter 2. Seafloor Sediments. Part 2.1. Sediment Predictions Part 2.2. Core Observations and Descriptions Part 2.3. Sediment Composition Part 2.4. Geographic Distribution and Interpretation Chapter 3. Microfossils and Biostratigraphy. Part 3.1. What are Microfossils? Why are they Important in Climate Change Science? Part 3.2. Microfossils in Deep-sea Sediments Part 3.3. Application of Microfossil First and Last Occurrences Part 3.4. Using Microfossil Datums to Calculate Rates Part 3.5. How Reliable are Microfossil Datums? Chapter 4.

Paleomagnetism and Magnetostratigraphy. Part 4.1. Earth's Magnetic Field Today and the Paleomagnetic Record of Deep-Sea Sediments Part 4.2. Paleomagnetism in Ocean Crust Part 4.3. Using Paleomagnetism to Test the Seafloor Spreading Hypothesis Part 4.4. The Geomagnetic Polarity Time Scale Chapter 5. CO2 as a Climate Regulator during the Phanerozoic and Today. Part 5.1. The Short Term Global Carbon Cycle Part 5.2. CO2 and Temperature Part 5.3. Recent Changes in CO2 Part 5.4. The Long-term Global Carbon Cycle, CO2, and Phanerozoic Climate History Chapter 6. The Benthic Foraminiferal Oxygen Isotope Record of Cenozoic Climate Change. Part 6.1. Introduction Part 6.2. Stable Isotope Geochemistry Part 6.3. A Biogeochemical Proxy Part 6.4. Patterns, Trends and Implications for Cenozoic Climate Chapter 7. Scientific Drilling in the Arctic Ocean: A Lesson on the Nature of Science. Part 7.1. Climate Models and Regional Climate Change Part 7.2. Arctic Drilling Challenges and Solutions Part 7.3. The Need for Scientific Drilling Part 7.4. Results of the Arctic Drilling Expedition Chapter 8. Climate Cycles. Part 8.1. Patterns and Periodicities Part 8.2. Orbital Metronome Part 8.3. A Break in the Pattern Chapter 9. The Paleocene Eocene Thermal Maximum (PETM) Event. Part 9.1. The Cenozoic [delta]13C Record and an Important Discovery Part 9.2. Global Consequences of the PETM Part 9.3. Bad Gas: Is Methane to Blame? Part 9.4. How fast? How long? Part 9.5. Global Warming Today and Lessons from the PETM Chapter 10. Glaciation of Antarctica: The Oi1 Event. Part 10.1. Initial Evidence Part 10.2. Evidence for Global Change Part 10.3. Mountain Building, Weathering, CO2 and Climate Part 10.4. Legacy of the Oi1 Event: The Development of the Psychrosphere Chapter 11. Antarctica and Neogene Global Climate Change. Part 11.1. What do we Think we Know about the History of Antarctic Climate? Part 11.2. What is Antarctica's Geographic & Geologic Context? Part 11.3. Selecting Drillsites to Best Answer our Questions Chapter 12. Interpreting Antarctic Sediment Cores: A Record of Dynamic Neogene Climate. Part 12.1. What Sediment Facies are Common on the Antarctic Margin? Part 12.2. ANDRILL 1-B The BIG Picture Part 12.3. Pliocene Sedimentary Patterns in the ANDRILL 1-B Core Ch. 13. Pliocene Warmth: Are We Seeing Our Future? Part 13.1. The last 5 million years Part 13.2. Sea Level Past, Present, and Future Chapter 14. Northern Hemisphere Glaciation. Part 14.1. Concepts & Predictions Part 14.2. What is the Evidence? Part 14.3. What Caused It? Index. Subjects: Paleoclimatology. Climatic changes--Observations. Climatic changes--History. SCIENCE / Earth Sciences / Meteorology & Climatology. Notes: Includes bibliographical references and index.

Submarine mass movements and their consequences: 5th international symposium LCCN: 2011939086 Main title: Submarine mass movements and their consequences: 5th international symposium / Yasuhiro Yamada ... [et al.], editors. Published/Created: Dordrecht; New York: Springer, c2012. Description: xxxi, 769 p.: ill. (some col.), maps (some col.); 24 cm. ISBN: 9789400721616 (hbk.: alk. paper) 9400721617 (hbk.: alk. paper) 9789048130702 9048130700 LC

classification: QE598 .S83 2012 GC
Related names: Yamada, Yasuhiro.
Subjects: Mass-wasting--Congresses.
Submarine topography--Congresses.
Landslide hazard analysis--Congresses.
Marine sediments--Congresses.
Tsunamis--Congresses. Submarine geology. Conference proceedings.
Massenbewegung (Geomorphologie)
Meeresboden. Submarine Gleitung.
Form/Genre: Austin (Texas, 2009)
Kongress-Austin (Texas)-2009.
Kongress. Notes: Sixty-five papers from an international symposium held in Kyoto, Japan, in 2011. Includes bibliographical references and indexes.
Series: Advances in natural and technological hazards research; v. 31
Advances in natural and technological hazards research; v. 31.

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Chapter 81

OVERVIEW OF SEAWEED BY-PRODUCTS

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ABSTRACT

Seaweed is a feedstocks that is both renewable and available worldwide. It has applications in the food, feed, agricultural, chemical, and pharmaceutical industries. During the industrial processing of seaweed, different waste products are generated and discarded. However, such by-products contain a variety of valuable components capable of independent or joint separation and valorization. This chapter presents an overview of the major marine algal components and applications, as well as examples of valorization of the by-products from different algal classes.

Keywords: seaweed, by-products, wastes, valorization, biorefinery

INTRODUCTION

Seaweed present advantages over terrestrial raw materials, particularly in relation to their rapid growth, yield, assimilation of nutrients and possibility of reducing atmospheric greenhouse carbon and nutrients promoting eutrofication; without competing for land and water (Kraan, 2013; Jaap et al., 2014). Moreover, seaweeds did not require the use of fertilizers or pesticides.

Marine macroalgae are traditionally used for food in Asian countries, and more recently they are being introduced in healthy and novel diets in other parts of the world. They are also commercially exploited for the extraction of hydrocolloids such as agars, carrageenan and

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alginates (Bixler and Porse, 2011). Macroalgae can be also destined for feed or used as fertilizer (Hardouin et al., 2014). Nowadays, they became a valuable feedstock for the development of products with a variety of applications, including nutraceutical, cosmetics, pharmaceutical and energetic uses (Mahadevan, 2015; Tiwari and Troy, 2015; Magnusson et al., 2016). An overview of the potential application sectors is presented in Figure 1.

Figure 1. Sketch of the potential application sectors for the seaweed by-products.

Seaweeds can be collected from both cultivation farms and wildly grown stocks (Barbot et al., 2016). Phycocolloids are one of the main commercial products extracted from seaweed and the wastes coming from their extraction procedure could represent a well characterized, localized and cheaper resource with industrial interest. Also the fractions with lower quality discarded in the food industry could be considered (Quitain et al., 2013).

The valorization of algal wastes can contribute to the optimal valorization of natural resources following a biorefinery approach to achieve an integral utilization of raw materials (Baghel et al., 2015 and 2016; Özçimen et al., 2015; Tiwari and Troy, 2015). In contrast to traditional production processes which are focused on the extraction of a single product and the rest being regarded as a waste, multiproducts processing fits into the biorefinery philosophy for the sustainable utilization of resources (Kumar and Sahoo, 2017; Tedesco and Stokes, 2017; Schiener et al., 2016; Kostas et al., 2017; Baghel et al., 2016; Gajaria et al., 2017). Seaweeds are an ideal feedstock, since they contain a variety of high-value components (Jaap et al., 2014).

SEAWEED COMPOSITION

Macroalgae contain valuable nutritious components such as carbohydrates, proteins, lipids, vitamins and minerals (Holdt and Kraan, 2011). Compared to terrestrial biomass, seaweeds contain higher water contents (90% fresh weight), carbohydrates (25 – 50% dry weight), protein (7 – 15% dry weight) and low lipid (1 – 5% dry weight) contents (Sudhakaer et al., 2018). Table 1 shows the main compositional differences between brown, green and red seaweeds.

The structural polysaccharides (i.e., agar, carrageenan or alginate) in macroalgae can accout for up to 40 - 50% of the dry weight and have gelling properties that makes them important for industrial applications in the food and cosmetic sectors (Sudhakaer et al., 2018).

Table 1. Proximate composition (% dry weight) of seaweeds (Sudhakaer et al., 2018; Rohani-Ghadikolaei et al., 2012)

Components	Average content (%)		
	Brown	Green	Red
Water	80-90		
Carbohydrates	30 - 50	25 - 50	30 - 60
Proteins	3 - 15	9 - 26	10 - 47
Minerals	7 – 38		
Lipids	1.5 - 2.0	2.9 - 3.6	1.8 - 2.8
Ash	28.1 - 29.9	12.4 - 22.4	21.8 - 23.1
Vitamins	A, B, B6, B12, C, D, E		
Chlorophyll-a	12.1 - 13.3	3.5 - 5-6	6.1 - 7-5
Bioactive properties/Activities	Anticoagulant, antithrombotic antiviral, anti-inflammatory, antitumor, antioxidant	Antibacterial, antifungal, anti-microalgae, antioxidant	Antimicrobial, antiviral, antitumor, antihyperlipidemic, immunomodulatory, antioxidant, anticoagulant

Seaweeds also contain storage polysaccharides such as laminarin and fucoidan in brown algae, ulvans in green and porphyrans and floridian starch in red ones (Holdt and Kraan, 2013). Fucoidan has a variety of bioactive properties including anticoagulant, antitrombotic, antioxidant, antiinflamatory, anticancer, cardioprotective, among others (Li and Kim, 2011; Balboa et al., 2013; Mayakrishnan et al., 2013; Kwak, 2014; Flórez-Fernández et al., 2017; Phull and Kim, 2017).

The protein content differs widely amond seaweeds, being found in higher amounts in the red algae, followed by green, whereas brown algae contain a low fraction of their weight (3 - 15%) (Fleurence, 1999). Therefore, red seaweeds can be consider a potential source of proteins (Murata and Nakazoe 2001). In addition, the colored proteins (phycobiliproteins) in red algae, and increasingly demanded as natural colours in food and cosmetics show different bioactive properties (Chandra et al., 2017; Sekar and Chandramohan, 2008). The growing population of the world demands a safe and inexpensive source of proteins, and seaweeds could serve this purpose.

Soluble protein and peptides, produced by hydrolysis of protein by-products, present antioxidant, antimicrobial, antiinflammatory, antihypertensive, anticancer, and hypoglycemic actions (Pangestuti and Kim, 2017).

Low lipids content (up to 4%) is commonly found in seaweeds, but they are particularly rich in polyunsaturated fatty acids (Sudhakaer et al., 2018). These compounds are able to control blood pressure, coagulation as well as to reduce the risk of cardiovascular disease, osteoporosis or diabetes.

Minerals are also a relevant elements group in seaweeds. These compounds have an important role for building human tissues and they are essential in a balanced nutrition. The most common minerals in the algae are Na, K, Ca and Mg, and others trace elements such as Fe, Zn, Mn and Cu (Peng et al., 2015). According to the total content of the minerals, 97% of the dry weight, it can be stated that algae are a good source of these elements.

MAJOR SOURCES OF SEAWEEDS BY-PRODUCTS

Food

Many seaweeds are edible and contain important nutrients such as proteins, amino acids, essential fatty acids like omega-3 fatty acids, vitamins or minerals. They are commercially prepared in dried form, but during processing some low quality speciments can be discarded. Cooking also generates waste streams containing a soluble fraction worth recovering.

Industrial Production of Hydrocolloids

Traditionally the dominant markets for macroalgae has been the food (whole seaweed and phycolloids) and feed industries. However, algal biomass is a renewable source for various products useful for agricultural, bioplastics, dyes, cosmetic, pharmaceutical, energy or bioremediation applications. Emphasis is given to fatty acids, steroids, carotenoids, phycocolloids, mycosporine-like amino acids and halogenated compounds (Cardozo et al., 2007; Holdt and Kraan, 2011).

Different authors have explored the uses of wastes generated after food procesing of seaweeds, including the low quality fractions removed before direct comsumption of the seaweeds and the solid residues discarded after the extraction of phycocolloids.

Production of Fertilizers and Plant Growth Stimulants

Agricultural uses of seaweed are widely known, not only for their high minerals content, but also for the ability of the polysaccharides as soil conditioners to aid in moisture retention. Liquid seaweed extracts are frequently prepared by pressing the fresh seaweed and further clarification and supplementation with preservatives (Mondal et al., 2013; Sing et al., 2016). This liquid phase is usually denoted as sap, and is rich in minerals, amino acids, vitamins and growth-promoting substances (Prasad et al., 2010; Pramanick et al., 2017). It has been

reported its ability to improve the germination, plant height, crop yield and quality (Layek et al., 2016; Raverkar et al., 2016).

The solid after pressing is a residue containing valuable fiber components and therefore is useful for other applications.

Algal Blooms and Beach Wrack

The biomass generated by seaweed washed up on shores either due too storm events or seaweed tides is a cheap biomass source which can be proposed as feed (Holdt and Kraan, 2011), for composting (Negreanu-Pirjol et al., 2011) and to provide nutrients to soil (Possinger and Amador, 2016); Michalak et al., 2017; Tang et al., 2009). However, considerations regarding the seasonal variability on composition have to be considered and also the concentrations of toxic metals should be analyzed before utilization (Villares et al., 2016).

In the next section some examples of valorization of by-products from seaweed processing are summarized. Further detail of selected cases is presented in the next chapters.

VALORIZATION AND UTILIZATION OF SEAWEED BY-PRODUCTS AND WASTES

Separate Utilization of the Constituent Fractions

The separate use of the seaweed consistituent fractions, in terms of alginate, carrageenan, agar or phycolloids, among others, can be found in Tabla 2.

Valorization as a Whole

In addition to the separate valorization of the components, the use of the alga as a whole can be proposed for the production of feed, fertilizer, biofuels or adsorbents.

Biofuel

Seaweeds are regarded as the third generation feedstock for biofuels and seaweed wastes are regarded as the fourth generation biofuels, because their utilization avoids the costs associated to seaweed farming and harvesting (Goh and Lee, 2010; Fernand et al., 2017). These feedstocks that do not compete with food application would be preferable for energetic uses (Mahadevan, 2015; Kositkanawuth et al., 2017).

Different technologies are available for the production of biofuels, including those based on: i) biochemical transformations, such as anaerobic digestion to produce biogas, bioconversion to ethanol, acetone and butanol or the extraction of hydrocarbons to produce biodiesel; and ii) thermochemical transformations by combustion, liquefaction, gasification and pyrolysis (Chen et al., 2015; Sudhakar et al., 2018).

Table 2. Examples of seaweed by-products or underutilized fractions, properties and applications

Commercial product	By-product	Uses	References
Alginate	Residual solid	P	Nguyen et al., 2015
Alginate	Solid wastes to Poly(lactic acid) composites filled with algae	M	Bulota and Budtova, 2016
Alginate	Anaerobic digestion to produce methane	E	Kerner et al., 1991
Carrageenan	Solids to produce ethanol	E	Tan and Lee, 2014
Agar	Leftover pulp to ethanol	E	Kumar et al., 2013
Polysaccharide	Protein and protein hydrolysates	F	Qi et al., 2017
Salts	Protein Energy	F E	Magnusson et al., 2016
Energy	Char (Salts, KCl)	F, A, P	Boake et al., 2018
Ethanol	Ethanol fermentation by-products to biogas	E	Lee and Lee, 2015
Ethanol	Solid to produce biogas	E	Park et al., 2012
Sap	Crude proteins	F	Gajaria et al., 2017
Phycocolloids	Solid wastes to protease hydrolysates enriched in LMW peptides	P	Cian et al., 2012

Sectors: F: food; A: agricultural; P: pharmaceuticals; E: energy; M: materials.

Since seaweed composition differs from that of terrestrial biomass, the required approaches are also different (Michalak, 2018). In comparison to the use of lignocellulosics, the use of seaweed has limitations derived from the high moisture and ash content of seaweeds, aspects limiting the energy-efficient conversion into biofuels (Kraan, 2011; Kumar et al., 2013; Jaap et al., 2014). However, the lack of lignin facilitates some stages, i.e., in the conversion to bioethanol, pretreatment before hydrolysis of polysaccharides to monosaccharides is facilitated in comparison to terrestrial biomass (Goh and Lee, 2010; Schultz-Jensen et al., 2013; Harun et al., 2014; Seghetta et al., 2014; Baghel et al., 2016). However, the carbohydrates are very different among seaweeds. *Saccharomyces cerevisiae*, can ferment glucose, galactose, but also mannitol and laminaran can be utilized (Horn et al., 2000; Lee and Lee, 2012), but the mixture of different seaweeds and adapted microorganisms has also been reported (Sunwoo et al., 2017).

Anaerobic digestion to produce biogas is a highly energy efficient process and seaweeds are suitable for this transformation, offering yields comparable to those attained from land feedstocks (Hughes et al., 2012; Jung et al., 2013). Since the cost of the algae is the highest in this process, the utilization of wastes is recommended for biogas production (Barbot et al., 2016). The combined energetic valorization was also suggested. Park et al. (2012) reported the anaerobic digestibility of the seaweed wastes after saccharification and bioethanol production processes, and found that the energy recovered by anaerobic digestion of the residue was more than twice the value from the ethanol produced in the main process.

The direct combustion of the whole algal biomass is a valid approach, but it is limited by the high ash content (Maceiras et al., 2015), although the high metal content could also act as catalysts (Lee et al., 2014). Pyrolysis was proposed for green tides (Ceylan and Goldfarb, 2015; Roberts and de Nys, 2016) and for waste seaweed fractions (Jung et al., 2016; Poo et al., 2018). Das et al. (2017) valorized the *Kappaphycus alvarezii* solid waste obtained after recovery of sap from fresh alga for energy application using pyrolysis. Jung et al. (2016)

processed *U. pinnatifida* roots, the main waste in farming sites, by pyrolysis to produce an eco-friendly alternative fertilizer. Hydrothermal carbonization, performed under slightly acidic and pressurized conditions, produces a hydrochar with energetic value comparable to that of a low ranking coal (Smith and Ross, 2016).

However, processing of seaweeds for biofuels exclusively is not economical and the joint utilization of the valuable feedstock components is also required to compensate the high production cost of biofuel (Shukla et al., 2016).

Adsorbent

The solid wastes after energetic valorization have been proposed as an adsorbent (Das et al., 2017). The most attractive material for this purpose are invasive seaweeds and blooms, which are low cost adsorbents useful to remove heavy metals and colorants from water, i.e., *Caulerpa racemosa* for methylene blue (Cengiz and Cavas, 2008); *Sargassum* for Cd, Cu and Ni (Padilha et al., 2005; De França et al., 2006; Lodeiro et al., 2006) and for phenols. The stability and adsorption capacity being improved with $CaCl_2$ treatment (Rubín et al, 2006). Combination of *Sargassum sp*. biomass was reported, i.e., coating with hydroxides for removing As from water (Vieira et al., 2017) or combination with clay for hexavalent chromium removal (Aprianti et al., 2017).

When the whole seaweed is used as feedstock, the salt removal is required in order to further utilization for food, feed, fertilizer, feed or fuel applications, since during hydrothermal liquefaction the high mineral content of seaweeds increased mechanical wear on processing equipment and the content of metals in the crude could limit its value and difficult the refining stage; the high salinity also difficults bioconversion to ethanol and to biogas (Zhang et al., 2017; Boakye et al., 2018). The removed salt can be crystalyzed and used for food purposes. Magnusson et al. (2016) and Glasson et al., (2017) reported the water washing of *Ulva sp.* biomass to obtain ulvan and protein fractions and to reduce algal mineral content. Furthermore, the salt of marine algae offers a healthy balance of minerals, K and Mg, and the salts produced after evaporation of the washing water can be used for functional foods. In addition, the remainig biomass composition showed enhanced properties as fuel or feed, since it increased the energy content of the biomass by 20 – 50% up to 18 MJ/kg and protein contents up to 27% (Magnusson et al., 2016).

EXAMPLES OF VALORIZATION OF SEAWEED WASTES

Brown Seaweeds

The extraction of alginate represents an industrial process generating important volumes of brown seaweed wastes. After alginic acid extraction, both the solid residue and a filtrate phase are disgarded as waste. Initially, they were considered as a potential source of iodine (Dave and Sharma, 1974), but other valuable components are focusing interest more recently. Some authors have designed cascade processes for the sequential extraction of fucoidans, alginates and phlorotannins from brown seaweeds (Lorbeer et al., 2017), or fucoidan, alginate, ethanol and antioxidants and antimicrobials from *Laminaria digitata* (Kostas et al., 2017). Likewise, Qi et al. (2017) reported the proteolytic hydrolysis of the by-products

remaining after polysaccharide extraction from *Undaria pinnatifida* to obtain a flavouring product with umami taste.

Brown seaweed by-products have also been proposed for feed purposes, some examples are mentioned here. Choi et al. (2014) supplemented broilers diet with *Undaria pinnatifida* and *Hizikia fusiformis* wastes fermented with *Bacillus subtilis* and *Aspergillus oryzae*, and observed enhanced body weight gain and immune response and lower mortality rate compared to the control group. Hwang et al. (2014) found that supplementation of 2% *Undaria pinnatifida* by-product improved the average daily gain and gain:feed ratio in steers, without affecting composition, quality of meat and carcass yield. Hong et al. (2015) supplemented brown seaweed by-products up to 4% of basal diet in dairy cows without affecting milk yield and composition. Islam et al. (2016) supplemented Hanwoo cows diets at 10% with fermented *Undaria pinnatifida* by-product and found greater weight of their calves. Also, the red seaweed *Gracilaria* wastes supplementation enhanced the carcass characteristics and production efficiency of ducks (Santoso et al., 2016).

Cosmetic applications were tried for the cooking liquid from *Hizikia fusiformis*, a by-product of the food industries, containing large amount of protein, carbohydrates, and phenolic compounds. After irradiation, which can be used to inactivate pathogens, the antiradical and the tyrosinase-inhibiting properties were improved. Since the angiotensin L-converting enzyme inhibitory activity was enhanced, pharmaceutical uses could also be proposed (Kim et al., 2014; Choi et al., 2011). Wijesinghe et al. (2013) confirmed that the phlorotannin-rich extract from the *Ecklonia cava* processing by-product showed anti-inflammatory activity.

The residue after alginate extraction could be valorized as adsorbent (Aderhold et al., 1996; Williams and Edyvean, 1997; Mištová et al., 2010; Costa et al., 2016; Belattmania et al., 2017; Cardoso et al., 2017; Moino et al., 2017).

The polymeric applications were also validated. Poly(lactic acid) composites filled with algae industrial by-product after the industrial scale extraction of alginate were prepared using melt-mixing process (Bulota and Budtova, 2016).

Cherad et al. (2014) proposed the catalytical supercritical water gasification of *L. hyperborea* to produce hydrogen and methane and Lee and Lee (2015) addressed the biogas production from by-products of *Saccharina japonica* after a sludge freezing pretreatment. Also the solid by-product resulting from the energetic valorization were valid adsorbents; *Sargassum* chars produced by pyrolysis and chemically modified were used to capture Hg from flue gas (Yang et al., 2018).

The biorefinery concept is suitable for brown seaweeds. The sequential production of fucoidan, alginate, sugars and biochar was proposed for *Ascophyllum nodosum* (Yuan and Macquarrie, 2015).

Green Seaweeds

After the production of liquid fertilizers, the remaining solids can be an excellent source of food ingredients. Highly digestible crude protein from *Ulva lactuca* was obtained from the sap free biomass with further recovery of minerals, lipids, ulvan and cellulose (Gajaria et al., 2017).

Energy and biofuels can be the final products from green seaweed processing. The whole algae can be used as substrate, as reported by Ben Yahmed et al. (2016). Latter authors proposed an integrated biorefinery for the production of bioethanol and biogas from *Chaetomorpha linum*, including pressurized pretreatment, enzyme production and enzymatic digestion, fermentation to ethanol and valorization of the mycelium from the fungal bioconversion to produce the enzymes the residual solid biomass and all effluents using anaerobic digestion to biogas. The bioconversion was also proposed by Sellami et al. (2013), who explored the lipase production by *Staphylococcus xylosus* and *Rhizopus oryzae* using a culture medium based on a mixture of synthetic medium and supernatants generated from tuna by-products and *Ulva rigida* biomass.

The final use as adsorbent, was reported by Lu et al. (2017), who applied hydrothermal carbonisation and chemical activation of *Ulva prolifera* biochar to produce an efficient adsorbent for the removal of bisphenol A.

The biorefinery of green seaweeds has been proposed (Bikker et al., 2016; Glasson et al., 2017). Later work using *Ulva lactuca* green seaweed was aimed at cascading valorisation of both protein and non-protein seaweed constituents is required to realise an economically feasible value chain.

Red Seaweeds

Red seaweeds have high potential for biorefinery since they contain valuable proteins with nutritious and coloring properties, minerals, lipids, cellulose and agar, which are of considerable commercial value (Kumari et al., 2013; Baghel et al., 2014). Diferent integrated biorefinery approaches for the production of valuable components from red seaweeds have been proposed.

Fresh *K. alvarezii* was used to produce a juice rich in potassium salts and the remaining granular residue is rich in κ-carrageenan. After its extraction can be destined to produce 5-hydroxymethyl furfural, levulinic acid and formic acid and finally be used as a fertilizer (Mondal et al., 2013). Khambhaty et al. (2012) washed this residue with water to remove salt, and proposed and acid hydrolysis and saccharification before fermentation with *S. cerevisiae* for ethanol production and finally, in order to completely utilize the seaweed, biogas production from waste fractions (Ingle et al., 2018).

After the extraction of agar and carrageenan, with application in the food industry as gelling, emulsifying, thickening, and stabilizing agents, 70 – 85% of the seaweed remains as solid or as a waste effluent, susceptible of being valorized (Pereira et al., 2015). These by-products remaining after phycocolloid extraction from red seaweed have been proposed as a source of protein. Cian et al. (2012 and 2013) used the protein from the cold washing stages from the traditional method to obtain phycocolloids and hydrolyzed it with trypsin, alcalase and a combination of both sequentially. The hydrolysates were enriched in peptides with low molecular weight, showing immunosuppressive, antihypertensive and antioxidant properties. Laohakunjit et al. (2014) reported the use of *Gracilaria fisheri* agar extraction by-products as a source of protein and amino acids, after enzyme hydrolysis to obtain a product rich in free amino acids (arginine, lysine, and leucine) and odourant compounds conferring umami taste and seaweed odour, and roasted seafood flavour after thermal processing.

Different processes are also aimed at extracting the valuable proteins from the fresh seaweed. A cascade processing approach of *Gracilaria gracilis* consisted on the extraction of phycobiliproteins and further pyrolysis of the residue to produce bio-oil and biochar (Francavilla et al., 2013 and 2015). Baghel et al. (2015) proposed a scalable process consisting on the aqueous extraction of phycobilins, further purified by ammonium sulphate precipitation. Note here that the supernatant was used as liquid fertilizer. The residues were treated with organic solvent to obtain lipids and the remaining residue was extracted with distilled water to obtain agar and the residual solid remaining after agar extraction were used for hydrolysis and ethanol fermentation. Baghel et al. (2016) developed an aqueous extraction process of *Gracilaria corticata* to obtain R-phycoerythrin, R-phycocyanin, crude lipid, agar, soil conditioner, bioethanol and a mineral rich liquid fertilizer.

Energetic valorization has also been tried. Tan and Lee (2014) reported the enzyme hydrolysis with cellulases and fermentation to ethanol with Saccharomyces cerevisiae either sequential of simultaneous of the seaweed solid wastes remaining after κ-carrageenan extraction from *E. cottonii*. The waste sludges generated after the extraction of alginate from *Laminaria hyperborea* and *Ascophyllum nodosum* were treated by anaerobic digestion to produce methane (Kerner et al., 1991). The pulp after recovering a liquid fertilizer from *Kappaphycus alvarezii* (Khambhaty et al., 2012) and after agar extraction from *Gracilaria verrucosa* (Kumar et al., 2013; Shukla et al., 2016) or from carrageenan extraction (Uju et al., 2015) can be used, after enzymatic hydrolysis, for ethanol production.

The adsorbents prepared from the solid wastes remaining after the ethanol fermentation of some red seaweeds (*Gelidium amansii*, *Gracilaria verrucosa*, *K. alvarezii* and *Eucheuma denticulatum*) was successfully applied to the adsorption of Cd(II), Pb(II) and Cu(II) (Sunwoo et al., 2016). The additional modification of the material was proposed for Pb and Cu sorption (Vilar et al., 2008). Johansson et al. (2016) used slow pyrolysis to obtain a Fe-biochar from Gracilaria waste with high affinity for oxyanions. This product was useful for the adsorptive removal of Se, As and Mo from complex effluents.

Biobased composites prepared with polysaccharides obtained from seaweeds by-products are advantageous regarding renewability and sustainability (Leceta et al., 2014). Madera-Santana et al. (2015) proposed the utilization of the filtration by-product after agar extraction from *Hydropuntia cornea* as a filler for incorporation by melt blending into a polylactic acid matrix. Alamsjah et al. (2017) reused the seaweed waste from *K. alvarezii* and *Gracilaria verrucosa*, to obtain pure fibre, which was mixed with epoxy adhesive and with sawdust to produce medium density fibreboard. Jumaidin et al. (2017a and b) proposed *Eucheuma cottonii* wastes as renewable filler improved the tensile, flexural, and impact properties of the composites as well as the thermal stability and soil biodegradation.

An overview on some industrial applications of by-products above-mentioned from brown, red and green seaweeds with the corresponding sector of use can be found in Table 2.

CONCLUSION

Seaweeds can be used for food and feed or can be industrially processed to obtain high value added products, such as hydrocolloids, food, cosmetics and fertilizers, leaving one or

more fractions as by-products or residues. Since these by-products stil contain compounds susceptible of further valorization, their utilization is promoted for achieving an integral utilization of the natural resources offering a sustainable processing scheme that fits in the concept of biorefinery. However, some challenging aspects have still to be considered, particularly, the variation in the content of bioactives with season, location or processing, both in seaweed by-products and in seaweed biomass from blooms (Adams et al., 2011; Kumar and Sahoo, 2017). This volume presents a survey of the valorization of seaweed by-products into different commercially valuable products.

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Chapter 82

ALGAE AND MICROALGAE BIOREFINERY

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ABSTRACT

In the present work, we present an introduction to biorefinery techniques and their applications in obtaining high, value-added products from algae and microalgae. Extraction and characterization techniques are reviewed, taking into account a Green Chemistry point of view.

Keywords: biorefinery, seaweeds, algae, green, microalgae

INTRODUCTION

In 2015, the European Union published the "action plan for the Circular Economy" (Commission of the European Communities 2015) where the value of products, materials, and resources are maintained in the economy for as long as possible, and the generation of waste minimized. This is to be an essential contribution to the EU's efforts to develop a sustainable, low carbon, resource efficient, and competitive economy.

In this sense, biorefineries play an essential role. Biorefining as defined by the International Energy Agency, is the sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals, materials) and bioenergy (biofuels, power and/or heat). This kind of "refinery" corresponds to classical petroleum refining, where multiple types of fuels and products are obtained from the same raw material. One of the first biorefineries was to produce of bioenergy, biofuels, and biochemicals. Replacing petro-chemical refineries with biorefineries can significantly contribute to mitigating climate change. Estimates from the British government show that the conversion of bio-based wastes into bio-based materials

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alone could reduce emissions by up to 633 million tonnes of CO_2 (Department for Business Enterprise and Regulatory Reform 2009). Biomass energy and material recovery is maximized, if a biorefinery approach is considered, where many technological processes are jointly applied to different kinds of biomass feedstock for producing a wide range of bioproducts. Many biorefinery pathways, from feedstock to products, can then be established, according to the different types of feedstock, conversion technologies, and products (Cherubini 2010).

The ideal Biorefinery should be highly energy efficient and tend to zero-waste production processes, but the "waste" products can be considered as co-products and may be reallocated for added value use or conversion processes. The purpose of the biorefinery is to optimize the use of resources and minimize waste, thus maximizing the benefits and profitability (Ferreira 2017).

Mabee *et al*. emphasized that there is an important choice of biomass and final products in biorefinery design, due to the large-scale production implications. Initial biomass availability, its potential use, and its characteristics need to be considered (Mabee, Gregg and Saddler 2005). Each biomass has its advantages and disadvantages. The biomass from forests, aquaculture, agriculture, and residues from households and industry can be used in the biorefinery, including wood wastes, agricultural wastes, bagasses, waste paper, sawdust, biosolids, grass, and organic residues (e.g., waste from food industries), aquatic plants and algae, animal wastes, among others (Demirbas 2005). Detailed and accurate characterization of biomass feedstocks, intermediates, and products is necessity to understand how the individual biomass components and reaction products interact at each stage in the process. Based on biomass feedstock, the biorefineries can be classified as lignocellulosic or marine (Ferreira 2017).

Another important issue in biorefining is evaluating the economic sustainability, which is extremely important to understand energy, environmental burdens, and costs of any production/conversion system, giving insight into its sustainability. One of the most used methodologies is the life cycle assessment methodology (LCA), which analyzes a product during its lifetime from its production, to its utilization, and end of life, including its recycling process. In this methodology, each step of the processes should be considered, such as biomass feedstock (culture, harvesting, drying, transport, etc.), conversion process (extraction, reactions, fermentation), and use of the product. Moreover, economic viability and evaluation of the costs has to be taken into account, without forgetting socioeconomic costs.

There is a further step in biorefining. The integrated thermo-biorefinery or advanced biorefinery is considered a biorefinery of the future. The concept of advanced biorefineries is similar to a conventional biorefinery, however, in this case, multiple feedstocks, products, and platforms are considered. An integrated biorefinery produces various products, which include electricity produced from thermochemical and bioproducts from the combination of sugar, and other existing conversion technology platforms.

In this chapter, we will focus on marine biorefineries, which are based on marine biomass such as aquatic plants, macroalgae (e.g., seaweed), and microalgae. This type of biomass has some advantages, such as high areal productivities, no need for arable land, and a wide range of biobased products and energy depending on culture conditions. However, industrial processing has a long way to go to be at the level of traditional terrestrial agriculture.

Macroalgae

Macroalgae or seaweed are multicellular organisms from marine sources that are able to carry out photosynthesis, and are characterized by simple reproductive structures. Seaweeds have been extensively utilized as food, animal feed, and fertilizer, and thus farmed commercially in a number of countries (Baghel et al. 2015) (over 30 million tons in 2016) (FAO 2018). Dependingon the seaweed type, whether green, red, or brown, macroalgae and related species can produce different fatty acids, biofuel, proteins, natural pigments, antioxidants, or polysaccharides (Balina, Romagnoli and Blumberga 2017). Furthermore, most macroalgae produce highly valuable metabolites with biological activities due to the adaptation to the extreme environments of light, salinity, and temperature (Ibañez et al. 2012). That is why, the study of a macroalgae biorefinery has attracted lot of attention in the recent years, either from a bioeconomic point of view (Van Hal, Huijgen and López-Contreras 2014; Seghetta et al. 2016), or from the green chemistry approach (Kerton et al. 2013; Herrero and Ibañez 2018).

On one hand, there are biorefinery processes in which more interesting products are obtained through fermentation processes, providing an effective utilization of the remaining by-products. The polysaccharides glucose, rhamnose and xylose from *Ulva lactuca* biomass were enzymatic hydrolyzed and used as substrate for the fermentative production of acetone, butanol, ethanol, and 1,2-propanediol, while a protein-enriched extracted fraction was studied for use in animal feed (Bikker et al. 2016). Another example of this type deals with the enzymatic hydrolysis of *Saccharina latissima* (Marinho, Alvarado-Morales and Angelidaki 2016). In this application, the hydrolysis generates a phenolic fraction, whose fermentation produces succinic acid, a concentrated macro - (Ca, K, Na, Mg, P, N and Fe), and micronutrients solid residue that can be use as fertilizer. On the other hand, downstream processes are found in the literature for the valorization of *Sargassum muticum* (Pérez-López et al. 2014; Balboa, Moure and Domínguez 2015).

In both examples, firstly, there is a supercritical CO_2 ($ScCO_2$) extraction of fucoxanthin, followed by the extraction of polysaccharides, such as alginate or fucoidan, and finally, an antioxidant extract rich in phenols, such as phlorotannins, produced by autohydrolysis.

In the work of Pérez-López *et al*. (Pérez-López et al. 2014), different scenarios were studied from an economic and environmental point of view. It was concluded that the most efficient biorefinery approach is the one that obtains only alginate and antioxidants. Including the fucoxanthin extraction at the beginning of the process implies an electricity consumption that drifts to environmental burdens.

Balboa *et al*. (Balboa, Moure and Domínguez 2015) highlighted that it is possible to carry out a biorefinery approach only using green processes and techniques (Microwave drying, $ScCO_2$ extraction, and membrane microfiltration), in addition to Generally Recognized As Safe (GRAS) solvents (water, ethanol, ethyl acetate and CO_2).

Microalgae

Microalgae are a diverse group of photosynthetic microorganisms, which can be considered as a potential source for the production of several highly valuable and bioactive compounds. They have many advantages since they can rapidly produce biomass using

sunlight and carbon dioxide, and they can also grow in extreme climatological conditions, such as non-arable lands or wastewater (Trivedi et al. 2015).

One of the main applications of microalgae is biodiesel production, because of the high level of triglycerides they contain (Yen et al. 2013). These microorganisms are one of the most promising renewable feedstock within bio-oil production areas, thanks to the inexpensive and effective harvesting of biomass, and the extraction of lipids (Enamala et al. 2018). Other than biofuel, microalgae contain rich compounds such as carbohydrates, polyunsaturated lipids, proteins, and pigments, which are widely used in cosmetic and pharmaceutical industries (Goh et al. 2009; Mimouni et al. 2012; Matos et al. 2017).

Up to now, several researchers have been using microalgae as raw material to obtain these highly valuable compounds using green solvents, not only in single-step processes (Paula R. F. Canela et al. 2002; Soh and Zimmerman 2011; Castro-Puyana et al. 2013), but also in a biorefinery approach. As an example, Gilbert-López *et al*. (2017) developed an efficient method using several green extraction steps to obtain different valuable fractions from *Scenedesmus obliquus*. These steps included supercritical fluid extraction (SFE), gas expanded liquids (GXL), and pressurized liquid extraction (PLE), using $ScCO_2$, a mixture of $ScCO_2$/ethanol, and pure water, respectively. Thus, they could efficiently fractionate triglycerides, and pigments such as lutein and β-carotene (Gilbert-López et al. 2017). A similar study was done where *Isochrysis galbana* was obtained using pressurized green solvents such as $ScCO_2$, mixtures of $ScCO_2$/ethanol, pure ethanol, and water. Furthermore, fucoxanthin, polar lipids, and other bioactive compounds with high antioxidant activity were recovered in different fractions during the whole biorefinery process (Gilbert-López et al. 2015).

It is important to mention that there are around 13,000 species of microalgae, with huge differences in terms of their compositions (Guiry 2012). For example, astaxanthin can be effectively extracted from *Haematococcus pluvialis*, and β-carotene from *Dunaliella salina* (Trivedi et al. 2015). This means that not only is it important to design a suitable integrated green platform to obtain/extract different products from microalgae, but also to design it specifically for each species of microalgae.

CONCLUSION

The biorefinery offers a plethora of opportunities to produce an array of fuels and organic chemicals from biomass. Even though marine biorefineries are still under development, and not currently producing at the industrial scale, there are a wide range of applications, both using micro and macro algae, that are highly promising.

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Chapter 83

POTENTIAL USES OF SEAWEED BY-PRODUCTS IN HIGH-VALUE PRODUCTS AND MATERIALS

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ABSTRACT

Seaweeds are aquatic plants, also known as macroalgae, and they have gained much attention recently, due to them being an alternative renewable feedstock that yield large biomass, as well as due to their fast growth rate, and ability to be produced without the use of any terrestrial land for cultivation. A wide variety of seaweeds, which are also cultivated to extract polysaccharides (i.e., alginate, agar, and carrageenan) and these gelatinous substances (hydrocolloids) are used in the food industry and as biomaterials. Moreover, seaweeds comprise a broad spectrum of functional ingredients (high-valued components) with pharmaceutical and nutraceutical properties. Nevertheless, the by-products from the residual biomass from seaweed processing are recognized to contain bioactive and therapeutic compounds. However, the discovery of new functional components, activities, and applications from seaweed by-products has rarely been discussed in the literature. In this chapter, we will review the application of seaweed by-products with the purpose of obtaining value-added products and materials.

Keywords: seaweeds by-products; biomaterials, bioactive compounds, biofuels, biocomposites

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INTRODUCTION

Algae or seaweed are autotroph unicellular (eukaryotes or prokaryotes) organisms of single structure without complex tissues and low cellular differentiations (tallophytes). They are classified into three groups: Clorophyta (green algae), Phaeophyta (brown algae) and Rhodophyta (red algae) [1, 2]. Furthermore, they can be classified according to their size either as macroalgae, commonly known as "seaweed", and microalgae, microscopic single-cell organisms ranging in size from a few micrometers (μm) to a few hundred micrometers. Seaweeds have been extensively explored since the beginning of civilization and are used in the pharmaceutical, cosmetics, and food industries, and more recently for the production of biofuels. Algae also represent a source of fiber, minerals, pigments, vitamins, steroids, lectins, polysaccharides, proteins, lipids (polyunsaturated fatty acids), and halogenated compounds. Indeed, thus many countries consume algae as a food, and there is interest in the development of new products with high added value [3].

Among the principal advantages of seaweeds is that they do not require agricultural land for their cultivation, and many species can grow in saline water and residual water, and so do not compete for the land and fresh water required for the cultivation of plants [4]. Additionally, their high growth rate produces more biomass yield per unit area than the terrestrial plants [5]; for example, brown seaweeds grown under cultured conditions show yields of ~ 13.1 kg dry weight m-2•yr-1 compared to ~ 10 kg dry weight m-2•yr-1 from sugarcane [6].

Seaweeds are considered among the most potentially significant future sources of sustainable biofuels. Lee et al. (2012) reported high yield of lipids from the microalgae (59 m3 ha-1•yr-1) that can be used to obtain biofuels. Furthermore, seaweeds have been described as potential sunlight-driven cell factories for the conversion of carbon dioxide to biofuels and chemical feedstocks [7, 8]. Nevertheless, despite their obvious potential, there are no economically-viable commercial-scale quantities of fuel from either micro- or macroalgae [9]. This is because it is necessary to adequately control several parameters including the microalgae species, metabolic mechanism, culture and operation conditions, as well as the bioreactor design. The present chapter offers an overview of the products with added value derived from seaweeds and their possible commercial applications.

COMMERCIAL APPLICATIONS OF SEAWEEDS

Food Products

Seaweeds have a wide range of applications in the cosmetics, pharmaceutical, and energy industries, but it is the nutrition sector that seems most promising for algae technology [10]. Seaweeds have been consumed since ancient times. In Japan, seaweed is a traditional food consumed as a seasoning or sea vegetable, and represents 10 - 25% of food intake. Seaweeds, such as *Undaria pinnatifida*, *Monostroma nitidum*, and *Sargassum fusiforme* are consumed in Japan as they contain several nutrients and functional ingredients in their makeup, making them an excellent healthy food source [11]. However, new strategies in food technology open

up the possibility of using sustainable productions with a lower negative impact on the environment. This has increased the focus on seaweeds as a global food source.

Currently, the seaweed market as a global food is very limited and has only two branches: 1) dried microalgae (e.g., *Chorella* and *Spiruline*, rich in vitamin B12 and D2, respectively); and 2) extracted-products from microalgae, such as pigments (astaxanthin), proteins (phycocyanin), antioxidants (β-carotene) and fatty acids (DHA, EPA). Sathasivam and Ki [12] mentioned that pigment extracted from seaweeds (i.e., chlorophyll, phycobiliproteiins, and carotenoids) represent a multi-billion dollar food industry due to the fact that in the USA alone, the food color market is projected to be worth 3.75 billion USD by 2022. Barba et al. [13] reported that seaweeds and microalgae represent an excellent alternative to improve the functionality of food products (e.g., organoleptic, health and technological products), since they can be used to make products with high added value such as emulsifiers, hydrocolloids, proteins, essential amino-acids, polyunsaturated fatty acids, antioxidants and antimicrobials bioactive compounds. Such products not only have nutraceutical importance, but are also important for the pharmaceutical industry. Besides, Agatonovic-Kustrin et al. [14] proposed the seaweeds as a functional food because the diterpenes and fucoxanthin present in *Dictyota dichotoma* they bind with nitric oxide, reducing the threat of cardiovascular diseases (hypertension and heart failures). However, food regulations in the USA and Europe have impeded the launch of new products from microalgae [15].

Agriculture

Since the first green revolution, the use of chemical fertilizers, such as pesticides and herbicides, have been commonly used in modern high-intensity agricultural ecosystems, decreasing soil quality and fertility. In this context, seaweeds are an excellent alternative as a biofertilizer. Bai et al. [16] discussed the beneficial effects of seaweed extracts in agriculture, such as early seed germination and seedling establishment, improved crop yield and quality, increased resistance to environmental stresses, and regulation of soil micro-ecosystems. Wang et al. [17] reported that the use of seaweeds (Sargassum horneri) as fertilizer increased bacterial diversity and shifted the microbial community structure significantly in treated soils. Moreover, the application of seaweed fertilizer can induce soil enzyme activity and microbial activity, thus promoting the improvement of soil quality. Wang et al. [18] investigated the use of seaweeds (*Lessonia nigrescens* and *Lessonia flavicans*) as fertilizer on the growth of apple (*Malus hupehensis* Rehd) and show that it significantly increased plant height and dry weight, promoted the activities of antioxidant enzymes, and inhibited lipid peroxidation in plant cells.

Recently, seaweed extracts have been applied as foliar bio-stimulants in plants, and several reports have indicated beneficial responses, including improved root and shoot growth, higher yields and greater resistance to antibiotic and biotic stress [19]. Other authors such as Stirk et al. [20] and Mosa Salim [21] have reported that hormones, vitamins, amino acids (e.g., cytokinins, auxins, polyamines, abscisic acid, gibberelins and brassinos-teroids) and trace elements (e.g., Fe, Cu, Zn, Co, Mo, Mn and Ni) used together with seaweed extracts for foliar applications, can help to reduce the application use of chemical fertilizers.

Mosa Salim [21] also reported that foliar applications of seaweed extracts improved the growth and yields of wheat plants, due to their ability to enhance cultivation and use sandy soil efficiency by increasing water-holding capacity, ion exchange and nutrient availability.

Moreover, Kasim et al. [22] pointed out that seaweed extract (*Sargassum* or *Ulva*) decreased the damages caused by oxidative stress and provides hormones and micro nutrients essential for wheat growth, especially root depth, shoot height and leaf area. Singh et al. [23] reported the use of *Kappaphycus alvarezii* seaweed extract as a bio-stimulant of sugar cane, enhancing the productivity by 12.5 and 8%, respectively, in plant and root crops. The strategy of increasing sugarcane productivity without any negative implications to the environment is challenging.

Biomaterials

Seaweeds represent an important source of biomaterials due to their structural composition as they contain cellulose, alginates, carrageenans and several sulfated polysaccharides, commonly present in their cell wall. Sulfated polysaccharides (fucans) can be extracted from brown seaweeds while, ulvans can be extracted from green seaweeds. Alginates, carrageenans and agars are commonly obtained from brown and red seaweeds, with a combined market value of 130 million € in Europe alone [24]. Commercial carrageenans have been extracted from *Chondrus*, *Gigartina*, and *Eucheuma sp* and are used as food thickeners in yogurt, ice cream and pudding. Agar is extracted from red seaweeds namely *Gelidium* sp and *Gracilaria sp* and is applied as a hydrocolloid in the food, pharmaceutical, and biological industries [25].

Moreover, Sealy [26] reported obtaining a super-tough biomaterial based on alginate from seaweed that could overcome the shortcomings of conventional polyurethanes used in the repair of damaged or diseased cardiac and vascular tissue. Indeed, due to its biocompatibility, biological, biodegradability [27], one of the main areas of applications of seaweed polysaccharides is in biomedicine, such as carrageenan for drug delivery, tissue engineering and wound healing and dental applications. However, the use of seaweed polysaccharide in biological and biomedical applications is still in its infancy. Moreover, several researchers have performed investigations into the chemical modification of seaweed polysaccharides. These procedures involve oxidation, acetylation, amination, sulfonation, and phosphorylation and provides several advantages in terms of mechanical and biological characteristics that could see them be used as anticancer, antioxidant, antihyperlipidemic, anticoagulation, and anti-inflammation agents [28].

However, one of the current challenges in reducing the costs of biopolymers recovered from seaweed concerns improving extraction methods used. Several novel and interesting techniques to isolate and to characterize seaweed polysaccharides have been developed recently including microwave assisted extraction, enzymatic extraction techniques, conventional methods, ultrasonic assisted methods, and supercritical extraction.

Bioactive Compounds

From seaweeds can be obtained a great variety of bioactive molecules that are not produced by terrestrial plants. The beneficial properties of these bioactive compounds for humans, animals, and plants have been recognized throughout history and are appreciated nowadays in the development of new biotechnological products. Among the most common

are the proteins, carbohydrates, proteins, polyunsaturated fatty acids, omega-3 fatty acids, minerals as well as pigments (phycobilins, chlorophylls, and fucoxanthins), polyphenols, and mycosporine-like amino acids (MAAs). These biomolecules are functional as antimicrobial, antifungal, antioxidant, anti-inflammatory, antivirus, anticoagulant, anticancer, antitumor, anti HIV, antidiabetic, immunomodulatory, even as probiotic and cholesterol-lowering effects [29, 30].

Respect to nutraceutical compounds of seaweeds Wang et al. [31] reported that edible brown (*Undaria pinnatifida*) contain a large number of nutrients such as dietary fiber, proteins, vitamins, minerals, and bioactive molecules; sterols, alkaloids, flavonoids, and fatty acids. Even, mentioned that seaweeds have many advantages due to its non-toxicity compared with chemical synthetic compounds, so it has recently been promoted for use such as a therapeutic agent or as a nutritional supplement. For several years, sulfated polysaccharides with potential pharmacological, nutraceutical, functional food, and cosmeceutical characteristics have been obtained or isolated from brown seaweeds and published in the literature [32]. Sanjeewa et al. [33] mentioned the bioactive potential of sulfated polysaccharides (fucoidans) isolated from seaweed *Sargassum spp*, showing anticancer activity against Lewis lung carcinoma cells and melanoma B16 cancer cells *in vitro* studies. Also, mentioned the antivirus activity and suggested that high molecular weight and the higher levels of sulfation increase the antiviral activity against herpes simplex virus (HSV-1) and HSV-2. The active compounds of seaweeds also have been used in the development of new cosmetic. The phlorotannins isolated from brown seaweeds (e.g., *Ecklonia stolonifera*, *Ecklonia cava*) have an inhibitory effect of the tyrosine, decreasing the melanin production, so showing whitening properties. Besides, they have anti-wrinkling properties, it is due to include a matrix of metalloproteinase (MMP) inhibitory activities and hyaluronidase inhibitory activity [34]. Products with functional properties containing organic compounds derived from natural sources are being increasingly demanded by consumers in contrast to those resulting from heavy organic synthesis [35].

PRODUCTS DERIVED FROM SEAWEED BY-PRODUCTS

Several plant-derived natural products have been recommended as sources of bioactive compounds [36], in spite of the fact that research has already begun on other common natural sources such as macroalgae (or seaweeds) and microalgae. Indeed the marine environment is considered a wealthy, unexploited source of bioactive compounds [37]. Macroalgae and microalgae have attracted the most attention from researcher due to their potential a source of bioactive compounds with useful biochemical properties [38, 39].

Products Obtained by Solvent Extraction

Seaweed extracts can be produced using different strategies yielding interesting new chemical compounds with biological activity that can be used in cosmetics, pharmaceuticals, and food applications. The high abundance of seaweeds in the marine ecosystem means that they have a strong potential to end up as sources of bioactive compounds such as dietary

fiber, omega-3 fatty acids, carotenoids, vitamins, polysaccharides, proteins, and minerals. However, it must be remembered that bioactive compounds are sensitive to extraction strategies based on the use of heat or solvents. These days, researchers in the field of seaweeds have been working to develop novel methods that are more productive in terms of time, yield, and cost, whilst also being environmentally friendly [40]. There are different strategies that can be used to identify these compounds. Firstly, an appropriate extraction procedure should be chosen. For extraction, different mechanical, chemical, and biological procedures can be used. The selection of an appropriate method depends on several variables, including the type of seaweeds involved in the extraction process and the type of bioactive compounds that will be obtained (e.g., polysaccharides, oils, dietary fibers, nutritional elements, etc.). A key issue is the solvent chosen for the extraction. The solvent used will lead to the extraction of different groups of compounds that can have several different end uses. Extraction techniques include the use of single solvents, binary solvent mixtures, and time-based extractions [41, 42]. The main challenge currently is to optimize the extraction conditions to obtain bioactive components and value-added products from seaweeds with high yield and activity.

Table 1. Biocompounds obtained from seaweeds by different extraction methods [45]

Type of solvent or process	Chemical or biological product	Biocompound
Organic	C_3H_6O	Carotenoids
Organic Inorganic	MeOH H_2O	Antibiotics
Organic Inorganic Enzymatic hydrolysis	EtOH, $C_4H_4O_4$, MeOH Na_2CO_3, $CaCl_2$, HCl, Distilled H_2O at pH3, H_2SO_4, HCl, H_3PO_4, H_2SO_4 Dextrozyme, Liquozyme, etc.	Polysaccharides
Organic	$CHCl_3$/MeOH (1/2 or 2/1 v/v) $CHCl_3$/MeOH/ 50 mM phosphate buffer (1/2/0.8 v/v), CH_2Cl_2/MeOH (2/1)	Lipids
Enzymatic hydrolysis Inorganic	Mixtures of cellulose and macerozyme, cellulose, carrageenase, agarose, etc. H_2O	Proteins
Organic Inorganic Enzymatic hydrolysis	MeOH, Hex, $CHCl_3$, EtOH H_2O Proteases, etc.	Phenolics

EtOH: Ethanol; MeOH: Methanol; Hex: Hexane; $C_4H_4O_4$: Acetic acid; C_3H_6O: Acetone; $CHCl_3$: Chloroform.

The different techniques mentioned allow for the extraction of seaweed products for potential application in agriculture. For instance, liquid seaweed fertilizer or biostimulants can be used to treat different crops, replacing commercial chemical fertilizers and reducing production costs. Biostimulants are attractive because they contain large quantities of organic matter, micro and macroelements, vitamins, fatty acids, and growth regulators [43]. The European Biostimulant Industry Council (EBIC) defines an agricultural biostimulant as a substance that acts on the physiology of plants through different pathways to improve crop vigor, yields, quality, and post-harvest shelf life/conservation [44]. The methods employed to

produce seaweed extracts can be divided into three groups: biological methods (e.g., enzymatic degradation method), chemical hydrolysis methods (using organic or inorganic solvents), and physical extraction methods (using high pressure and low temperature, supercritical fluid extraction, etc.). It is important to point out that the method of extraction can have great deal of influence on the active substances and nutrients obtained in the final product. Some bioactive compounds obtained from seaweeds by the different extraction processes using biological and chemical methods are presented in Table 1 [45].

Figure 1 provides a general idea about the treatments that seaweed biomass must be subjected in order to obtain extracts using biological and chemical methods. The commercial seaweed extracts available are solutions (aqueous) and are prepared with different solid content, viscosities, pH (alkaline to acid), odors and colors (from colorless to dark brown or black) [46]. The commercial biostimulant formulated from seaweed extract can contain a dry matter content of 10 – 30% and a pH of 6.4 – 10. Moreover, liquid formulations for foliar applications are produced with a pH of 4 – 5 (acid) using an organic acid and can be fortified with micronutrients with the added advantage of adding the chelating properties developed by the polysaccharides in extracts [47].

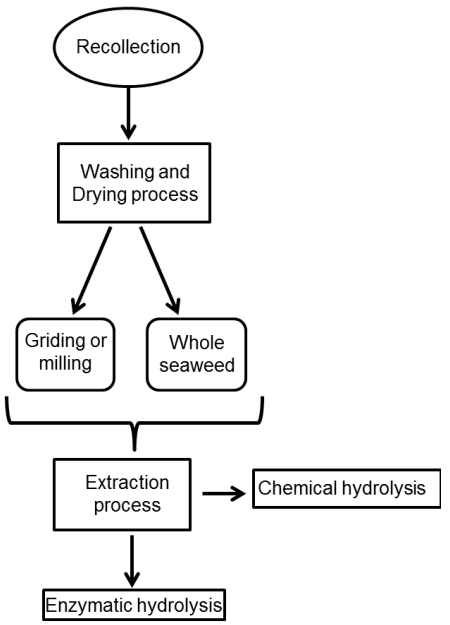

Figure 1. General representation of preparation and extraction process of seaweeds [45].

Products Obtained Using the Enzymatic Extraction Process

The presence of sophisticated cell wall polysaccharides can reduce the efficiency of extraction procedures employed to obtain active compounds from seaweeds. The degradation of cell wall polysaccharide structures could aid the release of such active components. The biological method is based on the principle that several kinds of enzymes are created by microorganisms in the metabolic process that use seaweeds as nutrients. Biopolymers from seaweeds can be degraded into low molecular weight biopolymers and water soluble

biocompounds. According to Lianfeng Bio [48] the conditions that should be used include gentle temperature or pH in order to maximize the retention of the bioactive and alimentary substances in seaweed. The challenge within the extraction of seaweeds is to optimize conditions for extracting bioactive compounds. For instance, the presence of assorted polysaccharides in large quantities within the cell-wall of seaweed is powerfully reduced by the extraction efficiency used in traditional extraction procedures. Therefore, totally different extraction techniques are required. Recently, a novel technique called enzymolysis was developed by Wijesinghe and Jeon [49]. Li et al. [50] have reported that this technique involves chemical process such as catalytic efficiency, high specificity, gentle reaction conditions, and the conservation of the initial efficacy of bioactive compounds.

To perform an enzymatic hydrolysis of biomass, it is important to take into account the suitable hydrolytic enzyme or optimum mixture of enzymes to obtain the expected production and also to select the correct processing conditions to be applied to obtain the maximum retrieval of bioactive compounds. Nevertheless, there are several factors (such as temperature and pH) that could have an influence on the ability of enzymes to degrade cell wall polymers and release bioactive compounds [49]. As reported by Heo et al. [51] enzymatic extraction techniques provide outstanding benefits such as being safe and having excellent water solubility, in addition to being a convenient large-scale method for the production of antioxidants from seaweeds. These authors reported an innovative extraction technique using digestive enzymes in wich they used carbohydrases and proteases to degrade seaweed tissues and to facilitate the release of several bioactive phenolic compounds from different seaweeds (i.e., *Ecklonia cava*, *Ishige okamurae*, *Sargassum fulvellum*, *Sargassum horneri*, *Sargassum coreanum*, *Sargassum thunbergii*, and *Scytosiphon lomentaria*) [51, 52]. The authors report that the enzymes are able to convert water insoluble seaweeds into water-soluble materials. Moreover, Heo et al. [52] reported that enzymatic extracts from seaweeds could be used in applications in the food and pharmaceutical industries, because they exhibited noteworthy inhibitory effects against DNA damage.

In fact, the relatively high antioxidant activities of enzymatic extracts from seaweeds reported by Heo et al. [52] (i.e., *E. cava*, *I. okamurae*, *S. fulvellum*, *S. horneri*, *S. coreanum*, *S. thunbergii*, and *S. lomentaria*) are in contrast to those from commercial antioxidants (e.g., α-tocopherol, BHA -butylated hydroxyanisol-, and BHT -butylated hydroxytoluene). Moreover, the production of alginate from seaweed by the enzymatic method was reported by Choi et al. [53]. As is well-known, alginate is used in different applications (e.g., food, textile, oil, cosmetics, and pharmaceutical applications) and the authors reported the use of liquozyme for liquefaction, while for saccharification they applied dextrozyme, viscozyme, and rapidasee [53]. There are many reports describing the extraction of proteins from several seaweeds such as *Chondrus crispus* by carrageenase and cellulose, *Gracilaria verrucosa* with agarase and cellulose, and *Ulva pertusa* by cellulose and a macerozyme mixture [54, 55].

Biofuels

Seaweeds are a plentiful and varied category of “aquatic plants”, including both uni-cellular and multi-cellular forms, that typically possess chlorophyl, although without true stems and roots. Seaweeds are divided into two groups: macroalgae, commonly known as “seaweed”, and microalgae, referred to as microscopic single cell organisms. The latter

usually have a size of a few micrometers (μm) to a couple of hundred micrometers [56]. In contrast to terrestrial plants cultivated to produce biofuel, seaweeds do not require agricultural land or irrigation for their cultivation. These plants grow in salt water, and therefore do not compete for land and water to grow [57]. Moreover, if we compare the yield of seaweeds per unit area, it is much higher than that of terrestrial plants. Brown seaweeds grown under suitable conditions show yields of ~13.1 kg dry weight m2/yr, compared to ~10 kg dry weight m2/yr from sugarcane [58, 59]. Despite their obvious potential, there are no reports of the production of economically-viable commercial-scale quantities of fuel from either micro- or macroalgae in the literature [60].

It has been reported that microalgae have a low energy return on investment –EROI– compared to crude oil and diesel. Nevertheless, this can be presently addressed by a raft of study initiatives aimed toward considering the whole spectrum of product, from micro to macroalgae to produce biofuel in biorefineries [61]. There has been a lack of studies directed towards producing fuels or developing feedstocks for fuels from macroalgae [62]. However, the use of macroalgal cultivations of non-fuel is currently a hundred times larger in wet tonnage terms in comparison with microalgae. Today, some cities of the world consumes biofuels obtained from macroalgae and it could be a multi-billion dollar business, with Asia being the most important market [63]. Seaweeds are currently used in human foods, fertilizers, cosmetics, pharmaceutical, and phycocolloids. The most important phycocolloids are agars, alginates, and carrageenans, which are widely used by the food industry with a global production is around 86,000 tonnes. About 221 species of seaweeds are known, 66% of which are used as food [64, 65]. Seaweed biomass is derived from a small number of species from five genera, namely *Laminaria* (or *Saccharina*), *Undaria*, *Porphyra*, *Euchema*, and *Gracilaria*, representing 76% of the total tonnage of cultivated macroalgae [66].

Biocomposites and Composite Materials

The substitution of petroleum-derived plastics with novel bio-based materials from inexpensive and renewable natural resources will greatly impact the plastics industry (i.e., coatings, composites, etc.) where biodegradable matrices and seaweed by-products are used as fillers [67]. Bio-based items from renewable resources can display comparable and sometimes superior properties than commercial petroleum-based items. Moreover, they (biocomposites) may have progressed reusing and improve its biodegradability capabilities [68]. PLA is a biodegradable polymer matrix (bioplastic), synthesized by the condensation polymerization of lactic acid, which shows promise as a biodegradable aliphatic polyester. PLA is refered to as a “green” polymer since it is available from renewable agricultural resources; thus, its production requires less fossil fuels in comparison to petroleum-based plastics. Nevertheless, this bioplastic has a few disadvantages such as relatively high resin cost, low impact strength and distortion temperature, and brittleness. To overcome the existing limitations and improve the mechanical performance of PLA, some authors have examined the impact of using renewable and biodegradable fillers (e.g., starch, sisal, hemp, and wood fibers) in the PLA matrix [69, 70]. Beyond conventional fillers, seaweed wastes or by-products have recently been generated through the utilization of novel fillers derived via the phycocolloid industry.

Polymer-matrix composites (PMC) can be classified according to whether the matrix is a thermoset or a thermoplastic polymer. Thermoset matrix composites have traditionally received far more attention, though thermoplastic-matrix composites are currently undergoing rapid development. One advantage of thermoplastic-matrix composites compared over thermoset-matrix composites is the lower manufacturing costs involved (i.e., no curing, unlimited shelf-life, reprocessing, low moisture content, weldability, etc.). Disadvantages of thermoplastic PMCs include high viscosities, high processing temperatures, and the need to perform fiber or particle surface treatments.

During the industrial production of agar, huge quantities of seaweed wastes are produced from the filtration step, in which a "filter cake" is produced and eliminated. According to the literature, the filter cake is around 70% of the raw material used. This filter cake consists mainly of residual seaweeds enriched with cellulose, hemicellulose, agar residues, and small amounts of floridean starch. The filter cake also contains diatomite earth, used as a filtration aid in the filtration step. The seaweed by-product is a material enriched with different polysaccharides and can be used as an inexpensive hybrid filler in biocomposites.

Raw seaweed biomass is used as a reinforcement in biocomposites with PBS, PP, high density PE, and PLA have been reported by several authors [36, 71-73]. The filler used in these biocomposites was formulated using seaweeds (i.e., green, brown, and red algae) in a polymeric matrix. The results reported by the authors indicate that seaweeds could be used to produce composite materials using polymeric matrices. These composite materials have potential applications in the automotive, construction, and packaging industries.

CHALLENGES

Seaweed by-products represent a promising renewable feedstock for applications in the food, energy, pharmaceutical, materials, and environmental industries. It is well known that the industrial production of biopolymers (phycocolloids) from seaweeds produces large amounts of residues, referred to as seaweed wastes (SWWs), which can be used as fillers to produce composite materials or biocomposites. The research in this field is under development and is having a high impact in the environmental and economic fields.

It is important to point out that three aspects that determine the economic viability and environmental sustainability of seaweed production have been highlighted in the literature, namely carbon and energy balance, environmental impact, and cost of production. The production of systems of microalgae under controlled conditions provide good results and the scale-up process is being developed by different research groups and industry. Moreover, during the chemical analysis of the components of seaweed by-products, it is very important to use biomass or its components in different applications such as food, animal feed, agriculture, biodiesel, and cosmetics. The chemical compounds in seaweed by-products include saccharides, proteins, lipids, micronutrients, vitamins, and many other molecules that are yet to be indentified. Some of the algae metabolites, namely lipids, carotenoids, sterols, are compounds that can be extracted by emergent technologies, such as the supercritical carbon dioxide extraction technique. The metabolites can be extracted using co-solvents or different organic solvents, which is sometimes accepted by some regulator agencies when they are used within a limited range. The development of new products for the

pharmaceutical and food industry is representing an opportunity to take advantage of the seaweed by-products of high added value.

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Chapter 84

COLOR STABILITY AND PIGMENT CONTENTS OF POWDERED LAVER (*PYROPIA YEZOENSIS*)

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ABSTRACT

Laver, a type of seaweed, is primarily cultivated in the Far East countries of Japan, Korea, and China. Laver gets its color from several pigments, including chlorophylls, carotenoids, and phycobiliproteins; these pigments strongly affect its grade. Because its color depends on the levels of the pigments, the stability of each pigment during storage is important for maintaining the quality of the laver. In this chapter, the color stability of powdered laver (*Pyropia yezoensis*) and the transient changes in its chlorophyll, carotenoid, and phycobiliprotein levels are examined at different temperatures and relative humidities. The color difference, ΔE, was found to increase with increasing temperature and relative humidity. Additionally, the amount of chlorophyll decreased and that of pheophytin increased. These findings indicate that the magnesium ion in the chlorophyll molecule dissociates during storage. Pheophytin formation resulted in a decrease in the L^* value, which is an index of brightness. The levels of carotenoids, phycoerythrin, and phycocyanin also decreased over time. However, despite the decrease in phycoerythrin levels, the chromatic value, a^*, which indicated red/magenta, increased under certain storage conditions, probably owing to the significant decrease in chlorophyll levels, thereby affecting the green color of the laver.

Keywords: carotenoid, chlorophyll, color stability, phycobiliprotein, laver

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1. INTRODUCTION

Laver is an edible and highly nutritious seaweed; it contains various proteins, minerals, vitamins and functional components, such as polysaccharides and porphyrans [1-3]. It is primarily cultivated in the Far East countries of Japan, Korea, and China, and is examined for utilization as a material for a fermentation culture medium and development of a fermented seaweed sauce [4, 5]. Laver gets its color from several pigments, including chlorophylls (green), carotenoids (yellow), and phycobiliproteins (red and blue), which strongly affect its grade. Chlorophylls are exclusively distributed in green plants as a photosynthetic pigment; chlorophylls a and b are present in higher plants, and the abundance of chlorophyll a in the chloroplast is three times greater than that of chlorophyll b [6]. Chlorophylls have a porphyrin ring with a centrally substituted magnesium ion esterified with a phytol group. Chlorophyll a has a methyl group at the C7 position of the ring, whereas chlorophyll b has an aldehyde group at that same position [7, 8]. Carotenoids are naturally hydrophobic pigments; they are located in intracellular chloroplasts and chromoplasts in colored fruits and green leafy vegetables and belong to the tetraterpene class of hydrocarbons, which contain eight isoprene units [9, 10]. Carotenoids also play important roles in photosynthesis, as do chlorophylls [11]. Phycobiliproteins are hydrophilic pigment-containing proteins and are mainly found in the cytoplasm or stroma of chloroplasts in the Cyanophyceae and Rhodophyceae classes of algae [12]. Phycobiliproteins, which include phycoerythrin (red), phycocyanin (blue), and allophycocyanin (light blue), have different protein structures, phycobilin contents, and chemical properties [13]. Linear tetrapyrrole chromophores, such as phycoerythrobilin and phycocyanobilin, are covalently linked via thioether bonds to a specific cysteine residue in the protein moiety of these molecules [14]. Phycobiliproteins have unique photochemical characteristics [15-18] and various nutraceutical and physiochemical properties, such as antioxidative ability and protective effect on liver injuries and alpha-synuclein toxicity [19-24]. Therefore, many methods for isolation of phycobiliproteins and preparation of phycobilins have been reported [25-34]. Lots of researchers examined various usages of these phycobiliproteins, such as a photoinduced electron transfer device [35], a microcapsule with polysaccharides [36], a rapid assay for virus antibody detection [37] and a biomaterial grafted to N-succinyl chitosan [38]. Because the color of laver depends on the specific levels of these pigments it contains, the stability of each pigment during storage is important for maintaining its quality. In addition, the region wherein the laver is cultivated and its cultivation style may also influence pigment composition and color quality.

In this chapter, the color stability of powdered laver (*Pyropia yezoensis*) and the transient changes in its chlorophyll, carotenoid, and phycobiliprotein contents are examined at different temperatures and relative humidities.

2. MATERIALS AND METHODS

2.1. Materials

Laver (*P. yezoensis*) was cultured in Kawazoe-cho, Saga prefecture, Japan, using a pole-staking method. Acetone, disodium hydrogen phosphate, lithium chloride, methanol,

potassium sulfate, sodium chloride, and sodium dihydrogen phosphate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hexane was purchased from Yoneyama Yakuhin Kogyo Co., Ltd. (Osaka, Japan). All chemicals were of analytical grade.

2.2. Preparation and Storage of Powdered Laver

A specific amount of milled laver powder was placed in flat-bottomed glass cups (1.5-cm internal diameter, 3.0-cm high). Each cup was placed in a plastic container containing a Petri dish filled with saturated lithium chloride, sodium chloride, or potassium sulfate solutions to maintain relative humidities of 12%, 75%, or 96%, respectively. The containers were then stored at 30°C, 45°C, or 60°C in the dark. Each cup was periodically removed from its container, and several measurements of color stability and pigment levels of the powdered samples were carried out.

2.3. Color Analysis of Powdered Laver

For the color analysis of the laver, 0.5 g of powdered sample was placed in glass cups, which were taken from the plastic containers after storage, as described above. Color analysis of the sample was conducted by colorimetry (ZE 2000, Nippon Denshoku Industries Co., Ltd., Tokyo, Japan). The colorimeter was calibrated with a standard white and black reflective plate. The instrumental values L^*, a^*, and b^* were recorded; from these values, the color difference, ΔE ($\sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$), was estimated. All color measurements were carried out in triplicate, and the mean values were calculated.

2.4. Measurement of the Amount of Chlorophyll

First, 0.02 g of the laver sample was placed in glass cups. After storage, each sample was moved into a plastic microfuge tube. One milliliter of methanol was added to the tube, and the tube was fully mixed using a vortex mixer (Vortex Genie 2, Scientific Industries, Inc., NY, US). After resting for 5 min, the mixture was centrifuged at 10,000 rpm for 10 min using a centrifugal separator (MX-100, Tomy Seiko, Tokyo). The absorbance of the supernatant was measured at 650 and 665 nm by spectrophotometry (DR4000, HACH Company, CO, USA), and the amounts of chlorophyll a and b were determined using the following formulas [39]:

$$\text{Chlorophyll a } [\mu g/g] = 16.5 \times A_{665} - 8.3 \times A_{650} \quad (1)$$

$$\text{Chlorophyll b } [\mu g/g] = 33.8 \times A_{650} - 12.5 \times A_{665} \quad (2)$$

All measurements were carried out in triplicate, and the mean values were calculated.

2.5. Measurement of Pheophytin Content

The laver powder (0.05 g) in each glass cup was removed from its respective plastic container after storage and subsequently transferred to a centrifuge tube. After the addition of 10 mL acetone, the tube was incubated in a water bath at 30°C for 30 min with vigorous shaking. The tube was then centrifuged at 3,000 rpm for 5 min, and the absorbance of the supernatant was measured at 534 and 556 nm by spectrophotometry [40]. The amount of pheophytin was estimated from the ratio, *R*, of A_{534} to A_{556}.

2.6. Measurement of Carotenoid Levels

A simple spectrophotometric procedure for estimating b-carotene levels was applied for the measurement of carotenoid levels [41]. After storage, 0.2 g of the laver sample was transferred from the glass cup to a centrifuge tube. Then, 10 mL hexane was added to the tube and mixed using a vortex mixer. After centrifugation at 3,000 rpm for 5 min, the absorbance of the supernatant was measured at 479, 645, and 663 nm by spectrophotometry. The concentration of carotenoid was calculated using the following formula:

$$\text{Carotenoids [mg/L]} = 4.338 \times A_{479} + 1.5835 \times A_{645} + 0.1980 \times A_{663} \quad (3)$$

2.7. Analysis of Phycobiliproteins

The laver sample (0.1 g) in each cup was transferred to a conical beaker after storage and mixed with 100 mL of 0.05 M phosphate buffer (pH 6.5). After centrifugation at 7,000 × *g* for 30 min, the absorbance of the supernatant was measured at 563, 615, and 650 nm by spectrophotometry [42]. The concentrations of allophycocyanin, phycocyanin, and phycoerythrin, which are expressed as C_{AP}, C_{PC}, and C_{PE}, respectively, were calculated using the following formulas:

$$A_{650} = 63.5 \times C_{AP} + 2.5 \times C_{PC} + 0.48 \times C_{PE} \quad (4)$$

$$A_{615} = 45.0 \times C_{AP} + 65.0 \times C_{PC} + 1.45 \times C_{PE} \quad (5)$$

$$A_{563} = 17.2 \times C_{AP} + 30.6 \times C_{PC} + 81.5 \times C_{PE} \quad (6)$$

3. RESULTS AND DISCUSSION

3.1. Effect of Relative Humidity on Color Stability and Pigment Levels

The transient changes in color parameters, L^*, a^*, b^*, and ΔE, for powdered laver at 60°C and three different relative humidities are shown in Figure 1. The psychometric lightness value, L^*, was significantly affected by relative humidity. The L^* value at 12%

relative humidity was nearly constant during storage for 48 h, whereas the *L** value at 96% relative humidity rapidly decreased during the first 5 h. The *a** and *b** values are points in a system of coordinates called color space and represent the positions between red/magenta and green, and between yellow and blue, respectively. A positive *a** value indicates red/magenta, whereas a negative value indicates green. On the other hand, a positive *b** value indicates yellow, whereas a negative value indicates blue. When the relative humidity was high, the *a** value was high but the *b** value was low. The magnitude of the change in both chromatic values during storage were small compared to the *L** value. A transient change in the color difference, ΔE, increased with increasing relative humidity. Low humidity during storage was found to suppress the change in the color of the powdered laver. In addition, a decrease in lightness strongly affected the color difference.

Chlorophyll was found to contribute to the degree of green in the color of the laver. Figure 2 shows the transient changes in chlorophyll a and b levels in powdered laver at 60°C and different relative humidities. The amount of chlorophyll a hardly changed during storage, regardless of the relative humidity level. On the other hand, the amount of chlorophyll b was significantly influenced by the level of humidity, drastically decreasing at 96% relative humidity. These results for chlorophyll b are consistent with the dependence of *a** on relative humidity, as shown in Figure 1b. Chlorophyll b contains an aldehyde group at the C7 position of a porphyrin ring in the molecule, whereas chlorophyll a contains a methyl group at the same position. Therefore, the stability of chlorophyll b is lower than that of chlorophyll a and is thought to be affected by humidity and adsorbed water molecules on the powder due to the polarity of the aldehyde group.

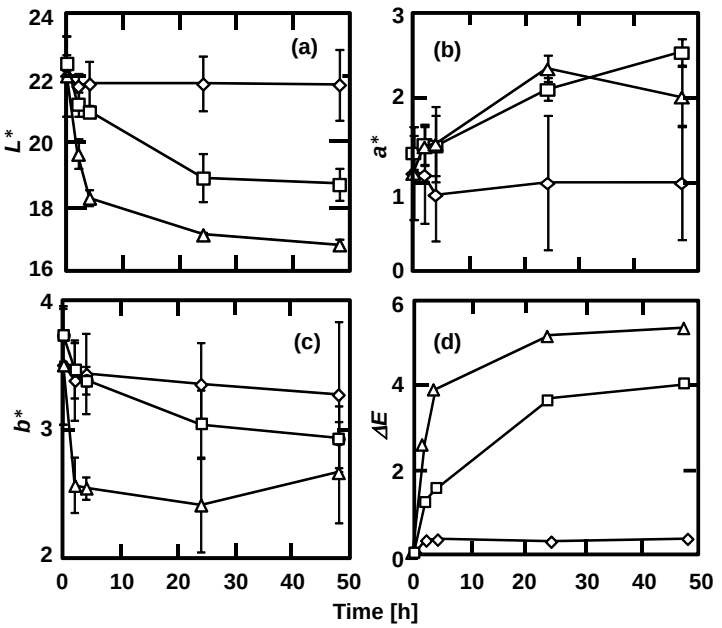

Figure 1. The transient changes in color parameters (a) *L**, (b) *a**, (c) *b**, and (d) ΔE for powdered laver at 60°C and (◇) 12%, (□) 75%, and (△) 96% relative humidities. Statistical significance was determined at $P < 0.05$.

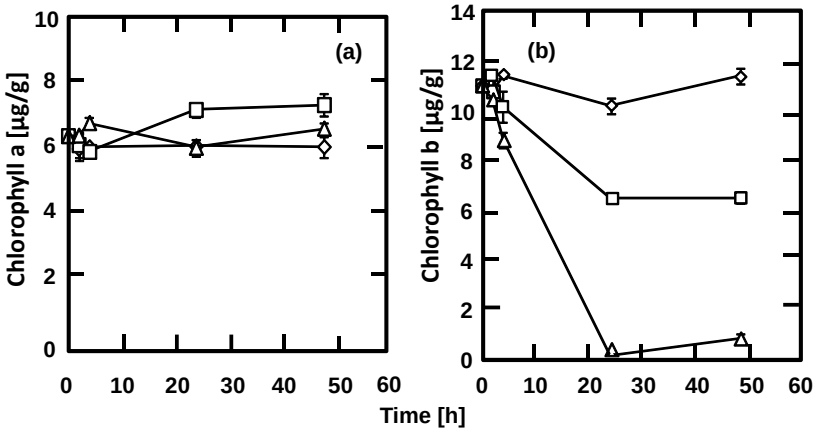

Figure 2. Dependence of (a) chlorophyll a and (b) chlorophyll b in powdered laver on relative humidity at 60°C and (◇) 12%, (□) 75%, and (△) 96% relative humidities. Statistical significance was determined at $P < 0.05$.

Next, the formation of pheophytin in powdered laver was measured under the same conditions used to examine the degradation of chlorophyll in more detail. Figure 3 shows the effect of relative humidity on the formation of pheophytin, which is a chlorophyll molecule lacking a central magnesium ion. The formation of dark green pheophytin resulted in a decrease in the L^* value, as shown in Figure 1a. As pheophytin formation is affected by humidity, formation was greatest at 75% relative humidity. The reason for this finding remains unclear; the magnitude of the contribution of pheophytin formation to the stability of chlorophyll could not be estimated, although the water activity of powdered laver seems to influence pheophytin formation.

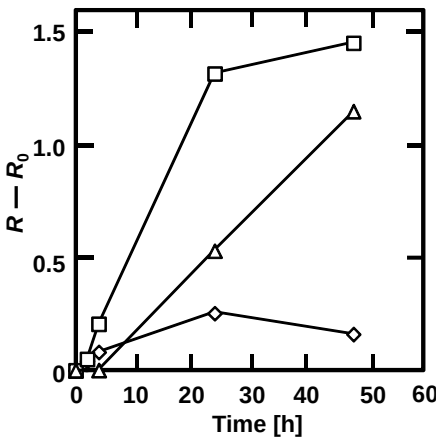

Figure 3. Effects of relative humidity on pheophytin formation in powdered laver at 60°C and (◇) 12%, (□) 75%, and (△) 96% relative humidities. R and R_0 represent the amounts of formed pheophytin and the initial amount of pheophytin, respectively.

Carotenoids are natural yellow pigments that are found in laver as well as in many plants. The stability of carotenoids in powdered laver was measured under the same conditions mentioned above (Figure 4). The carotenoid levels of the laver decreased in a roughly inverse

proportion to the level of relative humidity during storage. The results nearly correspond with those shown in Figure 1c, indicating that carotenoid levels affect the stability of the yellowish color of the laver. Since carotenoids are lipophilic compounds with some conjugated double bonds, their molecular structures and chemical properties are similar to those of polyunsaturated fatty acids. Polyunsaturated fatty acids are quite susceptible to oxidation; their oxidative stability depends on the water activity of the food system in which they are found [43]. Thus, oxidation would be expected to affect the stability of carotenoids in powdered laver. The oxidative stability of the carotenoids was found to be influenced by humidity.

To investigate the effects of phycobiliproteins on color stability, transient changes in the amounts of phycoerythrin and phycocyanin in powdered laver at 60°C and different relative humidities were measured (Figure 5). Both phycobiliproteins were stable during storage; their stabilities were nearly independent of humidity, unlike the other pigments examined. Thus, the high stability of phycobiliprotein in conditions of changing water activity was confirmed. Phycoerythrin and phycocyanin appear red and blue, respectively, but their *a** and *b** values changed over time, despite the consistency of their levels. This finding indicates that the color parameters reflect a relative balance among all the pigments in powdered laver.

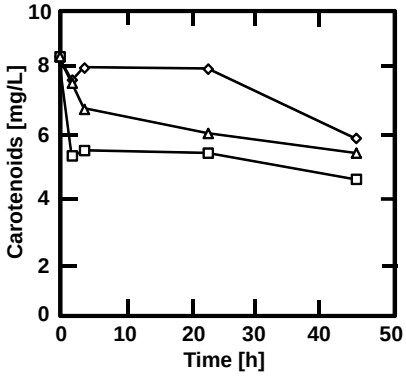

Figure 4. Time course of changes in carotenoid levels in powdered laver at 60°C and (◇) 12%, (□) 75%, and (△) 96% relative humidities.

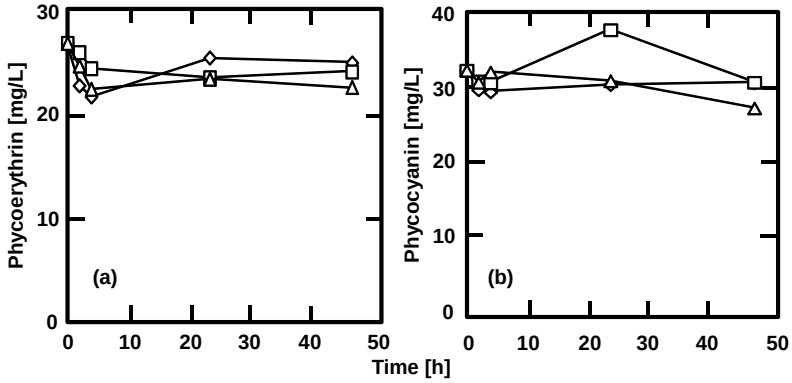

Figure 5. The transient changes in the concentrations of (a) phycoerythrin and (b) phycocyanin in powdered laver at 60°C and (◇) 12%, (□) 75%, and (△) 96% relative humidities.

3.2. Temperature Dependence of Color Stability and Pigment Levels

The changes in the color parameters of powdered laver were measured at different temperatures and 75% relative humidity (Figure 6). Each L^* value promptly decreased over time, plateauing after 24 h. The transient changes in a^* and b^* were found to be temperature dependent, with the a^* value increasing and the b^* value decreasing over time. Larger changes were associated with higher temperatures. The ΔE value, which represents a comprehensive change in color, was also high at high temperatures. The color stability of powdered laver was significantly stable at high temperatures.

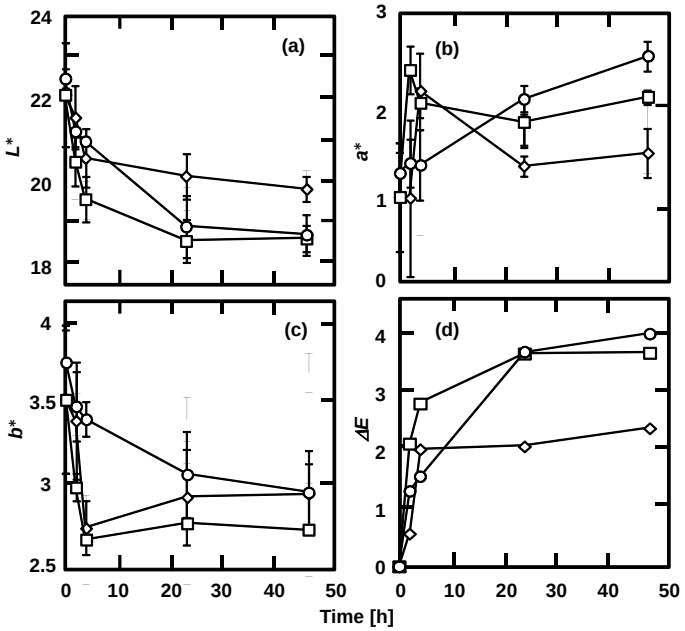

Figure 6. The transient changes in color parameters (a) L^*, (b) a^*, (c) b^*, and (d) ΔE for powdered laver at (◇) 30°C, (□) 45°C, and (○) 60°C and 75% relative humidity. Statistical significance was determined at $P < 0.05$.

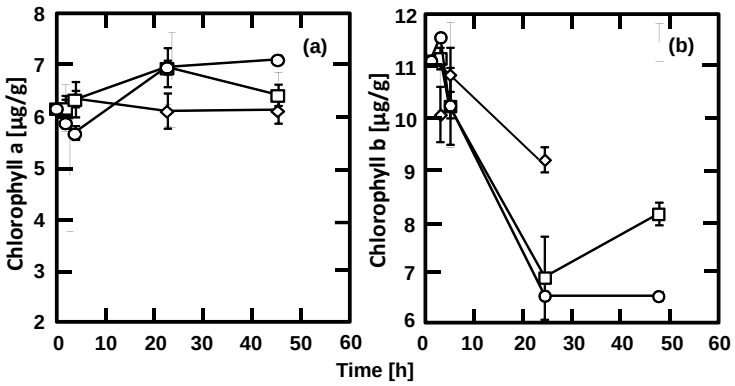

Figure 7. Dependence of (a) chlorophyll a and (b) chlorophyll b in powdered laver on temperature at 75% relative humidity at (◇) 30°C, (□) 45°C, and (○) 60°C. Statistical significance was determined at $P < 0.05$.

The transient changes in chlorophyll a and b levels in powdered laver at different temperatures and 75% relative humidity are shown in Figure 7. Chlorophyll a was stable at all tested temperatures. The decrease in chlorophyll b levels at 30°C was relatively small at 24 h but considerably decreased at 45°C and 60°C. The thermotolerance of chlorophyll b was much lower than that of chlorophyll a.

Figure 8 shows the effects of temperature on pheophytin formation in powdered laver. The formation of pheophytin at 60°C was approximately three-fold greater than at either 30°C or 45°C. Pheophytin was easily formed at high temperatures and did not appear to be associated with the decrease in chlorophyll b levels.

Figure 9 shows the changes in carotenoid levels in powdered laver over time at three different temperatures and 75% relative humidity. Carotenoid levels rapidly decreased at every temperature for first several hours, eventually leveling out. Carotenoid stability depended on storage temperature, decreasing at high temperatures. However, these results were not consistent with those shown in Figure 6c.

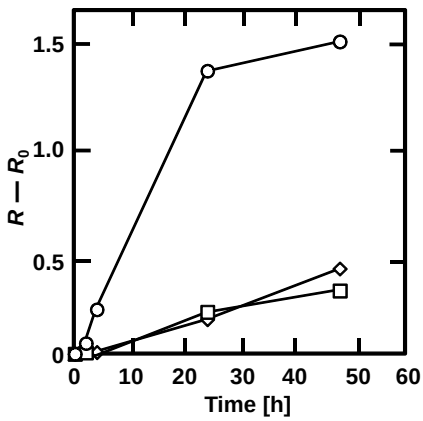

Figure 8. Effects of temperature on the formation of pheophytin in powdered laver at 75% relative humidity at (◇) 30°C, (□) 45°C, and (○) 60°C. R and R_0 represent the amount of formed pheophytin and the initial amount of pheophytin, respectively.

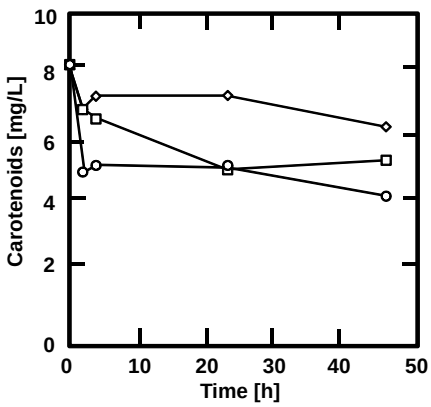

Figure 9. Time course of changes in carotenoid levels in powdered laver at (◇) 30°C, (□) 45°C, and (○) 60°C and 75% relative humidity.

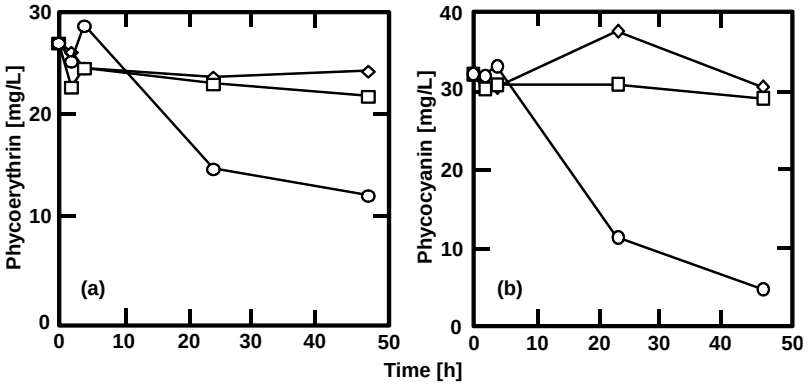

Figure 10. The transient changes in the concentrations of (a) phycoerythrin and (b) phycocyanin in powdered laver at 75% relative humidity at (◇) 30°C, (□) 45°C, and (○) 60°C.

The transient changes in the levels of phycoerythrin and phycocyanin at 75% relative humidity are shown in Figure 10. Both phycobiliproteins were stable at 30°C and 45°C, but their levels significantly decreased at 60°C. These results suggest that the chromophore groups in the phycobiliproteins decompose between 45°C and 60°C and/or the protein moieties denature at these temperatures. Furthermore, the decrease in phycocyanin levels was larger than that of phycoerythrin, indicating the low thermotolerance of phycoerythrin.

CONCLUSION

The effects of humidity and temperature on the color stability of powdered laver and the transient changes in its chlorophyll, carotenoid, and phycobiliprotein levels have been described in this chapter. Both humidity and temperature significantly affect color stability. The color parameters on color space were confirmed to be comprehensive indices for evaluating the color stability of powdered laver, although of all the pigments examined, chlorophyll b levels affected color differences most strongly. Phycobiliproteins were stable in conditions of varying water activity and unstable at high temperatures. A more detailed and quantitative analysis of color parameter values and pigment levels in laver is needed in the future.

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Chapter 85

UNDERSTANDING THE INTERACTIONS OF *SARGASSUM MUTICUM* WITH METALS AS A STARTING POINT FOR THE VALORISATION OF INVASIVE SEAWEED SPECIES

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ABSTRACT

Currently, there is concern about the increasing number of invasive species Worldwide. These species can displace autochthonous flora and fauna leading to significant negative economic and environment impacts. Invasive seaweeds are a good representative example. They proliferate in coastal areas with consequent negative effects on tourism, fisheries and sea-related industries. In order to revalorise invasive seaweed species, it is therefore necessary to find a potential use for them, which would thereby favour their removal from the environment. In this chapter, we review the potential revalorisation of the seaweed *Sargassum muticum*, an invasive species of concern in European waters. This macroalga presents well-known properties as ion exchanger, with an excellent applicability for pollutant removal. There are many biosorption studies investigating interactions between the biomass of *S. muticum* and different ions, such as Ca, Pb, Cd, Cu, Hg or Cr. In addition, its redox properties applied to the synthesis of nanoparticles, and related applications, are also important. A deep understanding of the interactions between metals/protons and *S. muticum* represents the first step for its commercial valorisation either as a biosorbent or a bioreductant material. This review critically analyses what has been done so far regarding crucial factors that must be investigated prior the potential commercial use of *S. muticum*. An overview is provided of its structural and acid base properties, thermodynamic/kinetic aspects of its interactions with metallic and non-metallic compounds, and column or pilot plant applications.

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Keywords: *Sargassum muticum*, biosorbent, bioreductant, acid-base, metals

INTRODUCTION

Marine macroalgae, especially brown seaweed species (Phaeophyta division), contain in their structure pigments such as fucoxanthins and xanthophylls, storage products such as laminaran, and also vitamins and minerals, alongside other bioactive compounds. The presence of these compounds makes the use of brown marine macroalgae very attractive for medical, health and nutritional applications (Liu et al. 2012, Kumar, Sahoo, and Levine 2015). Moreover, brown seaweeds have been tested for many years as biosorbents due to their high retention capacity and selectivity for several different contaminants, such as heavy metals or organic compounds (Mazur et al. 2018, Rubín et al. 2010).

The adsorption properties of macroalgae are usually associated with their rigid skeleton form of cellulose fibres, and especially to the specific chemical compounds present in their cell wall;alginates, and to a lesser extend fucoidans. These polysaccharides are the main chemical groups responsible for the adsorption capacities attributed to brown seaweeds (Davis, Volesky, and Mucci 2003). Alginic acid or its salt, alginate, is formed of 1,4-linked β-d-mannuronic (M) and α-l-guluronic (G) acid residues. The alginate´s structure and its M:G ratio determine its binding capacity (Davis et al. 2003). The main functional groups present in this polysaccharide are carboxylic acids that present a pK of about 3.2 (Rey-Castro, Herrero, and Sastre de Vicente 2004a). Sulfonic acid (pK= 1.5) is the main chemical active group of fucoidans, and the second most abundant acidic functional group present in brown seaweeds, after carboxylic acids (Davis, Volesky, and Mucci 2003). Fucoidans are formed of sulphate esters with l-fucose 4-sulphate units.

The brown macroalga *Sargasum muticum* (Yeldo) Fensholt originated from the northwest Pacific coast of China, Japan and Russia. At present, it is established as an invasive species from Mexico to Canada (East Pacific) and widely distributed around Europe's Atlantic coastal waters, from Portugal to Norway, (Schwartz et al. 2016). *Sargassum muticum* is included in the Global Invasive Species Database, and it is classified as one of the worst invasive alien species on a global scale (Josefsson and Jansson 2011). This seaweed has a high growth and reproduction rate, which favours its competitive behaviour displacing and/or affecting indigenous organisms. Moreover, its uncontrolled proliferation can harm coastal fisheries, aquaculture industries and tourist-based economies (Smetacek and Zingone 2013).

The eradication of *S. muticum* from the coastline by mechanical and manual removal has been unsuccessful. It is important therefore to valorise this seaweed and its by-products in order to make its recovery from the environment a profitable activity. Its by-products, e.g., extracted polysaccharides, polyphenols and fucoxanthins, have been used as antibacterial, antifouling, anticancer, insecticide and cytotoxic compounds, or as sources for the production of active drug substances (Bazes et al. 2009, Vinayaga Moorthi and Balasubramanian 2015, Madhiyazhagan et al. 2015, Casas et al. 2016, Marín et al. 2009). The raw macroalga is also considered an edible substance with nutritional value, but this should be considered cautiously due to its high potentially high content of heavy metals. Moreover, *S. muticum* has been tested as potential biofuel and fertiliser (Balboa, Moure, and Domínguez 2015, Soto et al. 2015, Milledge, Nielsen, and Bailey 2016). Besides these applications, *S. muticum* has

been extensively used in water treatment as an adsorbent for different organic and inorganic species, especially heavy metals (Vieira et al. 2017, López-García et al. 2012, Davis, Volesky, and Vieira 2000, Rubín et al. 2006). Another important application with a broad application range is the use of the aqueous extract from *S. muticum*, which presents high antioxidant potential, for green nanoparticle synthesis (Sanaeimehr, Javadi, and Namvar 2018, Namvar, Rahman, Mohamad, Rasedee, et al. 2015, Madhiyazhagan et al. 2015). Therefore, we review here the potential valorisation of *S. muticum* from two complimentary perspectives: the use of the solid residue of this seaweed as biosorbent, and the use of its liquid extract as reductant agent for nanoparticle synthesis. The economically successful valorisation of seaweed will depend on extensive prior knowledge of its:

1) Structural and acid-base properties
2) Chemical composition and natural environment
3) Binding capacity for metals and other species
4) Physicochemical properties in continuous processes, e.g., column studies at laboratory scale and pilot plants
5) Redox capacity (of the aqueous extract) e.g., for nanoparticle synthesis

The main goal of this work is therefore to review knowledge in each of these fundamental categories numbered above for the specific case of the invasive seaweed *Sargassum muticum*. This will provide a solid background of knowledge regarding the potential revalorisation of this harmful alga species based on its interaction with inorganic compounds (metals and/or metalloids). A schematic view of the topics that will be addressed is shown in Figure 1. This review supplements other works either focused on the analysis and valorisation of liquid extracts obtained from *S. muticum* (Pérez-López et al. 2014, Balboa, Moure, and Domínguez 2015) or addressing general potential uses and applications of this invasive seaweed (Milledge, Nielsen, and Bailey 2016).

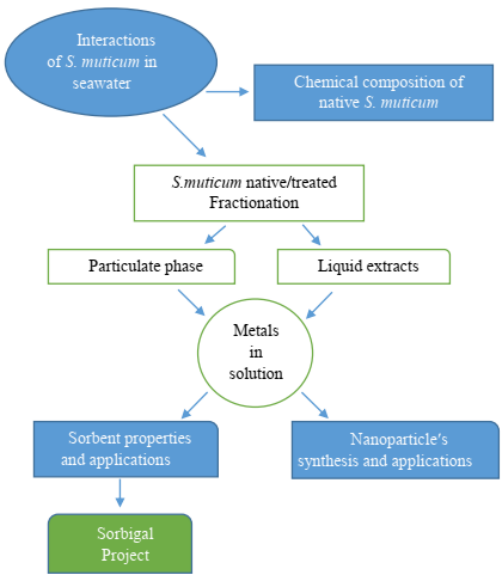

Figure 1. Schematic view of the topics addressed in this review based on interactions of *Sargassum muticum* biomass with metals.

METHODS

The experimental methods normally used on physicochemical studies using *S. muticum* are described below.

Pre-Treatment

After collection from beaches or at sea, *S. muticum* needs to be washed. The superficially bound sea salts, sand and other residuals can be easily removed after extensive washing with tap or deionised water. Afterwards, drying at mild temperature (c.a. 60ºC) should be performed to avoid putrefaction and damage to the algae structure. The next step is to ground and sieve the dried material in order to obtain homogeneous particles (from c.a. 0.10 to 10 mm) with a high surface area, which will have a significant positive effect on their binding properties. The obtained final material is readily to be used. Nevertheless, further improvement to its physical and chemical properties, e.g., better biomass stability and controlled weight losses, can be made by applying additional specific treatments. The native biomass is stabilised with the main cations present in seawater (Na, K, Ca and Mg). These ions can be displaced from the chemical groups where they are bound in order to modify the alga's structure and its chemical properties. The alga *S. muticum* has been modified by protonation, chemical cross-linking with $CaCl_2$, KOH, citric acid and formaldehyde, or esterification of carboxylic groups, by methylene blue, by treatments with acetone, chloroform and methanol to extract the lipid fraction of the algae, and by coating its surface with iron hydroxides (Lodeiro et al. 2004, Carro et al. 2013, Rubín et al. 2010, Vieira et al. 2017). Further details about the experimental design of these treatments can be found in the cited references.

Effect of pH and Ionic Strength

Protons are always present in solution and have a great impact on the electrical charge of the *S. muticum* surface; the study of the effect of pH on the interactions between *S. muticum* and different species should be therefore investigated in detail. Moreover, the presence of light metals such as Na, K, Mg and Ca in solution also influences the availability of chemical groups for binding. The ionic strength (I) is a variable that accounts for the charge and concentration of ions in solution. In addition to the crucial role of pH, the ionic strength has a tuning action on ionic interactions, and should be also considered when investigating potential applications of *S. muticum*. The experimental approach for pH and ionic strength studies is simple. Different experiments at several pHs, ionic strengths and/or electrolyte types should be performed. Moreover, temperature effects should be also taken into account. The pH must be continuously adjusted until a constant value at equilibrium is reached. It is also important to maintain a constant and gentle stirring to ensure the mixing of the solution and avoid damaging the alga structure. Moreover, not only the salts originally present in solution, but also all the acid/base additions must be considered when calculating the ionic strength. A pH range from 1 to 8 has been used to investigate the performance of *S. muticum*

in biosorption studies. This pH range is limited by the algal integrity and the chemical speciation of compounds in solution. For example, basic pH values can result in the precipitation and subsequent unavailability of specific metals in solution, and the swelling and destruction of the algal structure. A few authors have also investigated the effect of ionic strength and electrolyte type on heavy metal removal using *S. muticum*. A range of ionic strength values from 0.001 to 2 M provided by NaCl, KNO_3 and $NaNO_3$ salts were used in these studies (Lodeiro et al. 2004, Carro et al. 2011, Schiewer and Volesky 1997).

Potentiometric Titrations

Potentiometric titration with a glass electrode is a very simple and accurate technique. It measures the electromotive force between a glass and a reference electrode immersed in a solution. Potentiometric titrations have been used to study the proton binding onto *S. muticum*, and to thereby obtain its maximum proton-exchange capacity, and the equilibrium dissociation constants of its chemical groups. The potentiometric titration of *S. muticum* is very similar to acid-base titrations of simple substances. Nevertheless, the experimental set-up requires some additional considerations: for example, a gentle stirring that maintains the alga in suspension but avoids its physical disintegration is required; it is important that the chemical groups of the alga are occupied by protons, and not other cations, since the number of total acid sites can be influenced by the initial protonation state; in addition, the glass electrode readings during a seaweed titration are more unstable than in a common acid-base titration, and as a consequence, the titrations take a long time (c.a. 6-7 hours for protonated *S. muticum*). Titrations should be performed in a close thermostated vessel under inert gas bubbling to remove the effects of CO_2 and O_2. It is also important to use an inert electrolyte in both the suspension containing the alga and the titrant added, in order to minimize the change in proton activity coefficient. The *S. muticum* dose required to obtain quantitative and accurate results is 5 g/L. Please refer to the book chapter Lodeiro et al. (Lodeiro et al. 2018) for further experimental and theoretical details about acid-base properties of biosorbents.

Isotherms and Kinetics

The experimental determination of *S. muticum* adsorption capacity and binding strength is achieved through batch experiments at constant pH, ionic strength and temperature, using increasing concentrations of sorbate. The initial experimental concentration depends on the sorbate type and the applied pre-treatment of the seaweed. For example, for metals the concentration ranges which have investigated are, As: 0.013-0.53 mmol/L (Vieira et al. 2017), Cd: 0.09-3.1 mmol/L (Lodeiro et al. 2005, Freitas et al. 2008), Cr(III): 0.20-9.6 mmol/L (Lodeiro et al. 2008, Vilar et al. 2011), Cu: 0.10-5 mmol/L (Herrero et al. 2011), Hg: 0.25-5 mmol/L (Carro et al. 2009, Carro et al. 2015), Ni: 0.02-2.5 mmol/L (González Bermúdez et al. 2011) and Sb: 0.02-0.41 mmol/L (Ungureanu et al. 2017). In the case of organic compounds the concentration ranges investigated are wider, with 0.042-0.21 and 0.031-31.3 mmol/L for the dyes Basic Violet 10 (Devi, Murugappan, and Rajesh Kannan 2015) and Methylene Blue (Rubin et al. 2005), respectively; and with 0.078-77.8 mmol/L for phenol and 0.11-5.31 mmol/L for chlorophenols (Rubín et al. 2006). Biosorption kinetic

experiments using *S. muticum* are relatively fast. The necessary time to achieve constant adsorption capacities is between 20 and 600 min for metals and organics. The *S. muticum* dosage used on isotherm and kinetic experiments varies between 0.5 and 10 g/L.

Column Studies at Laboratory Scale and Pilot Plants

Continuous column experiments allow a more efficient use of seaweeds on biosorption studies. Moreover, laboratory column design experiments are a required step to extrapolate the obtained results to pilot plants and promote industrial/market applications. A fixed-bed column design has been used with *S. muticum* in laboratory scale experiments. A porous sheet or geotextile material was used to support the biomass, while the top of the bed is usually covered with glass beads to ensure good packing and low material loss. Glass columns of c.a. 25–50 cm length and 1–5 cm of diameter, filled with 5–30 g of *S. muticum* and a flow rate range from 0.5–20 mL/min were tested (Lodeiro, Herrero, and Sastre de Vicente 2006b, a, López-García et al. 2012, Carro et al. 2011, Ungureanu et al. 2017). To the best of our knowledge, the group of M. E. Sastre de Vicente at University of A Coruña is the only one that has used *S. muticum* in a pilot plant (unpublished work). This pilot plant consisted of 4 columns of 2.4 m height each and 5 (2x) – 12 (2x) cm of diameter (Figure 2). A geotextile material was used to support the biomass (up to 15 Kg). The pilot plant was tested with wastewaters from an aluminium factory at flow rates between 0.5 and 5 L/min.

Redox Capacity of the Aqueous Extract: Formation of Nanoparticles

Several authors have studied the synthesis of nanoparticles using the aqueous extract of *S. muticum*. Specifically, this extract was used to obtain ZnO (Azizi et al. 2014, Sanaeimehr, Javadi, and Namvar 2018), Fe_3O_4, (Mahdavi et al. 2013, Namvar et al. 2014), Ag (Azizi et al. 2013, Madhiyazhagan et al. 2015, Moorthi, Balasubramanian, and Mohan 2015) and Au (Namvar, Azizi, et al. 2015) nanoparticles. The aqueous extract was obtained by heating 0.2–10 g of *S. muticum* in 100 mL of deionised water at 60–100ºC for 5–20 minutes. The obtained extract was then filtered and stored frozen until use. Different experimental conditions are required for the preparation of each specific nanoparticle, depending on the nanoparticle type and specific characteristics such as size and shape. Moreover, it is also possible to obtain nanoparticles after direct contact in solution between *S. muticum* and specific metals (Namvar, Rahman, Mohamad, Rasedee, et al. 2015, Lodeiro and Sillanpää 2013b).

STRUCTURAL AND ACID–BASE PROPERTIES

Sargassum muticum is a brown algae that contains anionic polysaccharides, alginates and fucoidans in its cell wall, which is supported by cellulose fibres. The alginate content of *S. muticum* can account for c.a. 15–20% of its dry weight; while in other brown seaweed species (e.g., *Ascophyllum nodosum* or *Laminaria* spp.) the alginate percentages can reach up to 40% (Davis et al. 2003). Despite this relatively low alginate content, *S. muticum* shows other key

characteristics that make it exceptional. For example, the M:G ratio of the Na–alginates extracted from *S. muticum* is c.a. 0.31 (Davis et al. 2003). This low ratio reflects the high content of guluronnic blocks, and represents a unique feature compared to other brown algae. Only the brown seaweed *Laminaria hyperborea* presents a similar ratio; nevertheless, it leaches more polysaccharide matrix at low pH values than *S. muticum*, which makes *L. hyperborea*, and other similar brown seaweeds, less attractive for practical adsorption/desorption applications.

Structural integrity of *S. muticum* is necessary for large scale operations over prolonged time periods. The use of raw alga, after a simple wash, dry, ground and sieve process is therefore not recommended for industrial and practical approaches. Some treatments can improve the raw seaweed mechanical properties and performance through modification of the chemical groups present on the alga surface. Raw *S. muticum* can lose c.a. 30% of its weight and release 122 mg/L of total organic carbon (TOC) in aqueous solution (Table 1). The application of different treatments to *S. muticum* to extract its lipid fraction, cross–link or esterificate its chemical groups has been shown to lead to considerable decreases in the TOC (up to 96%) and mass lost in solution of this invasive seaweed (Table 1) (Lodeiro et al. 2004, Rubín et al. 2010). Despite improving its structural integrity the treatment can however also reduce the alga adsorption capacity. For example, with extraction of Hg or Cr(VI) the dominant removal mechanism is the reduction of the metal ion; treatments that modified or interact with the hydroxyl groups of *S. muticum*, e.g., citric acid, are therefore not recommended. This is because hydroxyl groups, involved in the reduction mechanism, interact with some chemicals used for the treatment. Another example is the use of Ca pre–treated *S. muticum* for Cd removal; the similar ionic ratio of Ca and Cd results in very low selectivity of the alga for the heavy metal, which decreases the adsorption capacity of treated material compared to the raw biomass. The combination of high structural stability and high guluronic acid content is one of the key aspects that makes *S. muticum* a unique material for biosorption processes.

Table 1. Weight loss of native and pre-treated Sargassum muticum after 3-4 h of stirring in water, weight loss due to the treatment, and total organic carbon (TOC) measured in solution after treatment. These values show a considerable improvement of the alga structural integrity. Adapted from Lodeiro et al. (Lodeiro et al. 2004)

Treatment	Weight loss in water (%)	Weight loss due to treatment (%)	TOC (mg/L)
Raw *S. muticum*	30	-	122 ± 1
Formaldehyde	9	39	24.8 ± 0.3
Protonation	12	37	83.9 ± 0.6
Acetone	6	31	5.2 ± 0.2
Methanol	4	30	7.4 ± 0.2
KOH	8	19	13.4 ± 0.2
$Ca(OH)_2$	0.1	6	8.8 ± 0.1
$CaCl_2$	0.2	6	11.1 ± 0.3

Other important structural parameters that can be used for modelling purposes and account for swelling, are the specific surface area and Donnan volume (see acid–base properties below). Experimental determination of these parameters is a challenge and therefore empirical values are usually assumed. The specific surface area of *S. muticum* ($537x10^3$ m^2/Kg) has been experimentally obtained by dye adsorption (Rubin et al. 2005). This value is one order of magnitude lower that the calculated from proton binding (4400–$4800x10^3$ m^2/Kg) by Rey–Castro et al. (Rey-Castro, Herrero, and Sastre de Vicente 2004b). This disparity can be attributed to the different adsorption mechanisms of protons and dyes onto *S. muticum*. Schiewer and Volesky have experimentally established a correlation for the gel volume (total volume) of Sargassum genus algae with pH (Schiewer and Volesky 1997); the values found were between 5 and 10 L/Kg. Rey–Castro et al. obtained similar Donnan volumes for *S. muticum* from fitting proton binding data (5.2–5.7 L/Kg), yet these volumes correspond only to the effective or active volume where the proton binding reaction takes place, and are therefore lower than the ones determined experimentally, which account for the total gel volume (Rey-Castro et al. 2003). Therefore, despite the importance of the Donnan volume and specific surface area for proton binding modelling, these parameters have a limited physical significance.

The research groups of Sastre de Vicente at University of A Coruña (Spain) and Boaventura at University of Porto (Portugal) have investigated the acid–base properties of *S. muticum* using simple potentiometric titrations with glass pH electrodes. The total amount of alginate and other polymers containing active functional groups depends not only on the seaweed species (*S. muticum* in this case), but also on the specific water conditions where the alga was harvested, such as salinity or organic content, and the season, light conditions, etc. The maximum proton binding capacities for *S. muticum* were between 2.40–2.61 mol/Kg of alga (Lodeiro et al. 2004, Vieira et al. 2017). These values were calculated considering only one type of acidic group obtained from the fitting of titration experimental data to a Langmuir–Freundlich (L–F) isotherm or from calculating the inflection point of the titration curves. This approach is valid over a pH range from 2 to 9, which is the pH range of interest in biosorption studies. When two different acidic groups were considered, Lodeiro et al. found 1.78 and 1.33 mol/Kg (Lodeiro et al. 2008), while Ungurenau et al. obtained 1.31 and 1.10 mol/Kg (Ungureanu et al. 2017) of carboxyl and hydroxyl groups, respectively. Values for conditional proton affinity constants (log K or pK) were 3.85, calculated using the Katchalsky model at 0.5 dissociation degree, and 3.80, obtained from least–squares fit of the NICA (L–F) or Langmuir isotherms to the proton binding data. The values of pK obtained using a polyacid expression were 3.41 and 10.2 for carboxylic and hydroxyl groups, respectively, both in 0.05 mol/L $NaNO_3$. Applying a continuous model for the proton binding based on the Langmuir isotherm and Sips distribution Ungurenau et al. (Ungureanu et al. 2017) obtained a pK of 3.38 and 9.8 for carboxylic and hydroxyl groups, respectively, in NaCl 0.1 mol/L. Moreover, the heterogeneity degree parameter was between 0.53 and 0.66, depending on the electrolyte type and expression used to account for the water content of the alga. The ionic strength of the medium does not influence the obtained number of acid sites, but it strongly affects their apparent conditional affinity constants. Moreover, it is worth mentioning that the accuracy of the charge and pH data calculated for the titration curve analysis decreases drastically above pH 10 (Rey-Castro et al. 2003). Therefore, the study of acid groups at high pH values (>9–10) using potentiometric techniques will invariably be affected by large errors.

Rey–Castro et al. proposed the use of the L–F isotherm to account for the specific proton binding, combined with the Donnan model, which describes the electrostatic contribution to the proton binding assuming the interphase to behave as a permeable three–dimensional gel (Rey-Castro et al. 2003). This model, in combination with the master curve approach, accounts for the effect of pH and ionic strength on proton binding to *S. muticum* allowing the estimation of two isotherm parameters describing the intrinsic heterogeneity, and a geometric parameter (Donnan volume) to be calculated independent of the titration conditions. The obtained log K value was 3.18, while the maximum proton binding capacity was 2.61 mol/Kg and the heterogeneity parameter 0.58. In subsequent work, these authors also fitted the proton binding data to a surface charge model that describes the alga as a non–permeable two-dimensional surface with a specific surface area as its main feature (Rey-Castro, Herrero, and Sastre de Vicente 2004b). Both models seemed to describe the titration data equally well, although the Donnan model additionally provides a simple way to account for the effect of activity coefficients and the non–specific binding.

CHEMICAL COMPOSITION AND NATURAL ENVIRONMENT

Different authors have measured the presence of metals, micronutrients and other elements in native *S. muticum* (Tables 2, 3 and 4). The presence of metal and/or metalloids in the algal structure reflects the interactions of *S. muticum* with the natural media where it has grown. The algal elemental composition is in fact also associated with seasonal factors such as temperature, light, salinity and nutrient concentrations. In addition, the chemical composition can also reflect the pollution present in specific sampling sites.

The interactions of metals and algae in seawater are part of a complex system and occur through different mechanisms involving adsorption and redox processes (Morel and Price 2003). Most of the bio–limiting metals (e.g., Fe, Zn, Mn, Ni, Cu, Cd and Co) exist as cations complexed to organic and inorganic ligands that promote the bioavailabity of these essential metals in seawater. Despite their extraordinarily low concentrations (<0.1 μM) in seawater these metals play crucial roles in most cellular processes (Sunda and Huntsman 1998). Algae uptake and store essential metals through membrane transport proteins and specialized enzymes. Nevertheless, these compounds are not entirely specific for essential metals, and toxic metals (e.g., Ag, As and Pb) can be also taken up into cells, rendering proteins and associated metabolic pathways dysfunctional (Lodeiro et al. 2017). In addition, neutral/non–polar compounds such as HgCl or AgCl can directly diffuse across the cellular membranes of seaweeds. The general mechanism underlying metal uptake by seaweeds in a natural environment can be simplistically described in two steps: metal fixation (adsorption) onto the seaweed surface, and consequent metal internalization involving metabolic activity. In some cases the seaweed can reduce metal ions, such as Fe(III) to Fe(II), extracellularly to access otherwise unavailable metals through enzymatic pathways (Morel and Price 2003).

In the absence of metabolic activity (non–living seaweed) the uptake of metal ions process is the so–called biosorption (Volesky 2003). Whereas, if metabolic activity is involved the uptake mechanism is known as bioaccumulation. In natural environments both biosorption and bioaccumulation occur widely and so the chemical elements measured in algae are consequence of both processes (Tables 2, 3 and 4). As mentioned, the chemical

composition of the algae depends on its physiological stage, the season, and physicochemical properties of the environment where it was harvested. This variability, together with the different experimental protocols used for the analysis of elemental composition makes it challenging to quantify, evaluate and compare the chemical composition of *S. muticum*. The inorganic content of *S. muticum* harvested at different locations and seasons has been investigated in a few works (Tables 2 and 3). As expected, the major ions present in seawater, i.e., Na, Mg, Ca and K, are the main elements in the alga structure, with values from c.a. 0.32 to 1.50 mmol/g for Na, 0.43 to 1.09 mmol/g for Mg, 0.23 to 1.74 mmol/g for Ca and 0.35 to 2.1 mmol/g for K (Table 2). Of particular concern is the accumulation level of potentially toxic metals such as Cd, Pb, Ni, Hg or As; for example, *S. muticum* contains As in relatively high concentrations compared to other macroalgae not belonging to the *Sargassum* genus (Whyte and Englar 1983). The concentrations of As, Cd, Hg or Pb have been found in *S. muticum* at levels of 0.099–3.04, 0.0023–0.0133, 0.00011 or 0.0053–0.079 μmol/g, respectively (Table 2). Therefore, potential applications of *S. muticum* as an edible species, fertiliser, animal feed and in other applications closely related to human nutrition, should be considered cautiously due to the risks associated with the presence of toxic heavy metals in its structure and the restrictive legislation regarding these harmful metals (Besada et al. 2009, Rubio et al. 2017). *Sargassum muticum* also exhibits high ash content and this, as stated by Milledge et al. (Milledge, Nielsen, and Bailey 2016), can be a problem for applications in combustion or the gasification of biofuels. Some commercially available seaweeds even present radioactive isotopes in their structure (van Netten et al. 2000), although as far as we are aware no presence of radionuclides has been measured in *S. muticum*. Moreover, macroalgae are considered as a useful biomonitor or bioindicator of environmental contamination due to their high metal uptake capacity (Chakraborty et al. 2014). Essential micronutrients with potential control over *S. muticum* growth were found at relatively high concentrations; for example Fe, Mn and Zn are present at concentrations ranges of 1.22–18.26, 0.15–7.64 and 0.19–4.70 μmol/g, respectively (Table 3).

Light metals, e.g., Na, K, Mg or Ca are electrostatically bound to charged chemical groups in the alga. These hard counterions cannot therefore displace other elements that form covalent bonds such as protons or heavy metals, but can affect their bond by reducing their local concentration. Therefore, the concentration of light metals in *S. muticum* should provide a qualitative indication of its proton binding capacity. For example, the total amount of Na, Mg, K and Ca found in the alga *S. muticum* used in different works of Lodeiro et al. is 2.94 mEq/g (Table 4). This value is c.a. 13% higher than the total proton binding obtained from potentiometric titrations when considering one type of acid group (2.61 mmol/g), and only c.a. 5% lower when considering two main acid groups in the alga (3.11 mmol/g). This small discrepancy can be ascribed to the inaccessibility of some light metals, which are not displaced by protons and/or some multidentism in the proton bound.

Table 2. Chemical composition of *Sargassum muticum* harvested at different locations and seasons for major cations in seawater, toxic heavy metals and other relevant elements

Al	I	Mo	P	S	Se	Ca	Mg	Sr	Ba	Na	K	As	Cd	Hg	Pb	References / notes
												20.8/ 34.0				(Whyte and Englar 1983) Dry weight (µg/g)
						277-953	540-1090			540-661	1770-2160					(Gorham and Lewey 1984) Dry weight (mEq/g*) Different seasons
						27.5	10.4			26.3	82.5					(Csikkel-Szolnoki, Báthori, and Blunden 2000) (g/Kg)
44-550													0.26-1.5		1.1-16.4	(Favero and Frigo 2002) Dry weight (µg/g) Different locations and seasons
	72	0.55			2.15							97.3	0.49	0.022	1.85	(Rey Crespo 2014) Wet weight (mg/Kg)
	0.02		0.23	1.28		1.24	1.88			3.60	7.91					(Sharma et al. 2012) (%)
746-777		0.32-0.72	0.31-0.42a			2.44-5.83a	1.54-2.65a			2.95-4.54a	8.07-10.6a			<0.03	1.4-2.7	(Soto et al. 2015) (mg/Kg) (%TS)a Different seasons
~0.10			2.28			9.18	15.04			34.53	57.56					(Rodrigues et al. 2015) Dry weight (mg/g)
0.23-1.04						16.6-22.1	11.9-13.8	1.12-1.34	0.039-0.052			0.148-0.228	0.55-0.73·10-3		3.4-7.2	(Marzocchi et al. 2016) (mg/g) Different locations
	0.008					24.7-69.6	15.1			7.4-20.1	13.7-38.8	0.007-0.036				(Balboa et al. 2016) (g/Kg) Different seasons

* We suspect that there is a misprint in the original article. The units should be µEq/g and not mEq/g.

Table 3. Chemical composition of *Sargassum muticum* harvested at different locations and seasons for essential micronutrients that could limit the algae growth. Percentages of ash content are also shown for some works

Co	Cr	Cu	Fe	Mn	Ni	Zn	% ash	References / notes
0.22-1.18	0.08-1.31	1.27-44	68-447	8-188	0.339-2.28	23-311		(Favero and Frigo 2002) Dry weight (µg/g) Different locations and seasons
1.21	10.9	5.83	289	61	8.9	140		(Rey Crespo 2014) Wet weight (mg/Kg)
							13.80 ± 0.12	(Zhang et al. 2010)
		0.08	0.29				34.03-41.49	(Sharma et al. 2012) (%)
0.93-8.23	0.75-1.54	1.74-2.5	459-724	28.1-96.9	1.97-8.23	14.3-85.5		(Soto et al. 2015) (mg/Kg) Different seasons
		0.005	0.19	0.011		0.025	22.94 ± 0.06	(Rodrigues et al. 2015) Dry weight (mg/g)
0.58-3.9	0.73-1.9	6.4-57	300-1020	22-420	1.5-42	78-144		(Marzocchi et al. 2016) (µg/g) Different locations
		0.008-0.023	0.2-1.6	0.0121-0.0479		0.0127-0.0408	13.2-30.5	(Balboa et al. 2016) (g/Kg) Different seasons

Table 4. Chemical composition of *Sargassum muticum* harvested at A Coruña bay (Galicia NW Spain) on July 2002. Unpublished data

	Na (mmol/g)	Mg (mmol/g)	K (mmol/g)	Ca (mmol/g)	Cu (mmol/g)	Cd (mmol/g)
Raw *S. muticum*	0.26 ± 0.01	0.444 ± 0.008	1.18 ± 0.05	0.31 ± 0.02	<1.5x10-5	<4.4 x 10-6
Protonated *S. muticum*	<0.002	0.005 ± 0.0002	0.002	0.006 ± 0.0002	<1.5x10-5	<4.4 x 10-6

BINDING CAPACITY FOR METALS AND OTHER SPECIES

The alga *S. muticum* has been used as biosorbent for many different metals and organic compounds (Table 5). The ion–exchange properties of this invasive seaweed make it potentially useful for decontamination or preconcentration processes in aqueous solutions. The binding capacity of *S. muticum* depends on environmental factors such as pH, ionic strength, temperature, the presence of competitive agents and adsorbate´s speciation; moreover, other factors such as the time required to achieve adsorption equilibrium, the alga dose and its specific chemical composition will also affect the binding. Several authors investigated all these factors in order to determine optimal operational conditions (Carro et al. 2015, Freitas et al. 2008, González Bermúdez et al. 2011, Herrero et al. 2011, Lodeiro et al. 2004, Ungureanu et al. 2015, Vieira et al. 2017, Vilar et al. 2011).

To date *S. muticum* has been used to decontaminate solutions containing As, Cd, Cu, Cr, Hg, Ni, Pb, Sb or Zn (Table 5). The alga efficiency, under optimal operational conditions, present a broad range of adsorption values depending on the metal type; for metals that are positively charged in solution over the pH range of study, e.g., Cu^{+2}, Cd^{+2}, Ni^{+2} and $Cr^{+3}/Cr(OH)^{+2}$, the maximum adsorption capacities are 1.12 (Herrero et al. 2011), 1.20 (Lodeiro et al. 2005), 1.29 (González Bermúdez et al. 2011) and 1.42 (Lodeiro et al. 2008) mmol/g, respectively. This series is in agreement with the divalent metal–alginate selectivity (Ni > Cd > Cu), evidencing the role of alginates in the binding. Moreover, these metals present their maximum adsorption capacities at pH values near the dissociation constant of carboxylic acids observed for *S. muticum*. This also demonstrates the key role of these chemical groups that form part of the alginates. The adsorption capacity of alginates for divalent cations depends on the ratio of mannuronic and guluronic acids (M:G) that forms their structure, and their macromolecular conformation. Divalent metals present a higher affinity for guluronic acids due to the formation of the so–called egg–box structure, and therefore the adsorption capacity of *S. muticum* for toxic divalent metals will be increased with its content of guluronic acids.

Other metals such as As and Sb present a neutral or negatively charged form in solution, and their adsorption (0.045–0.093 mmol/g) is therefore not favoured (Vieira et al. 2017, Ungureanu et al. 2017). Nevertheless, if the adsorption mechanism involves not only ionic exchange or ionic/covalent binding but also a redox process, the removal of negatively charged or neutral metal species can be considerably improved. This is the case of Cr(VI), which is present in solution as $HCrO_4^-$, or Hg, which occurs as two neutral forms ($HgCl_2$ and HgClOH) at pH values between 1 and 6. The presence and abundance of chemical groups in *S. muticum* with reduction capacity is critical when a redox process is involved in the removal mechanism (López-García et al. 2012, Carro et al. 2013). This seaweed presents hydroxyl, phenol and amino groups that can act as electron donors. These groups form part of the polysaccharide structure, and the internal cell wall composed of cellulose. Moreover, it should be also considered that the *S. muticum* reduction potential is favoured at acidic pH values, which implies that most chemical groups are protonated and can attract negatively charged compounds.

Table 5. *Sargassum muticum* adsorption values for different metal and organic compounds at optimal pH values. The table also shows valuable experimental information, such as type of experiment, alga treatment and the isotherm and kinetic models for each reference

Adsorbate	Experimental conditions and techniques	Type	Treatment	Isotherm model	Kinetic model	Qm (mg/g) Langmuir	KL (L/mg) Langmuir	Reference
As	pH effect, FTIR, mass titration	Batch	Iron coated	Langmuir, Freundlich	Pseudo– first and second order	4 ± 1 As(III) 7 ± 3 As(V) pH = 7	0.07 ± 0.04 0.08 ± 0.03 pH = 7	(Vieira et al. 2017)
Cd	pH and ionic strength effects, sorbent dosage, titration	Batch	Protonated, several chemical treatments	Langmuir Freundlich L–F	Pseudo–second order	57 – 99 pH = 4.5	0.06 – 0.4 pH = 4.5	(Lodeiro et al. 2004)
Cd	pH effect, titration	Batch	Protonated	Langmuir and NICCA model	Pseudo–second order	95 ± 2 (water) 135 ± 11 0.05 M NaNO3 pH = 4.5	0.388 ± 0.01 0.027 ± 0.01 pH = 4.5 NICCA logK = 3.1 ± 0.1 n = 1.8 ± 0.2	(Lodeiro et al. 2005)
Cd	pH~ 4.5 bed high and flow rate effects	column	Protonated	No	Several column models	No	No	(Lodeiro, Herrero, and Sastre de Vicente 2006b)
Cd	pH ~ 4.5 9 desorbents	Batch and column	Cd–Laden protonated	No	28 days of adsorption/ desorption cycles	No	No	(Lodeiro, Herrero, and Sastre de Vicente 2006a)
Cd	Desorption, pH effect, titrations	Batch	Protonated	Langmuir	No	76.4 pH = 4.5	0.14 pH = 4.5	(Davis, Volesky, and Vieira 2000)
Cd, Zn, Pb	pH effect, FTIR, Porosity	Batch	No	No	Pseudo– first and second order	No	No	(Freitas et al. 2008)
Cd, Pb, Cu, Hg	Natural pH, SEM, EDS, ion exchange	Batch	Ca–pretreated	L–F, ion exchange	Diffusion	No	No	(Carro et al. 2015)
Cu	pH effect, competitive adsorption, acidic desorption	Batch	Protonated	Langmuir, L–F, NICA model	Intraparticle diffusion, LDF model	71 ± 1 pH = 4	0.39 ± 0.03 pH= 4	(Herrero et al. 2011)
Cr(III)	pH effect, SEM, titration, FTIR, TOC, desorption	Batch	Protonated	Langmuir, Freundlich Langmuir competitive	No	74 ± 3 pH = 4.2 Lang.comp. h= 200 ± 6	0.047 ± 0.008 pH = 4.2 Lang.comp. logK = 3.1	(Lodeiro et al. 2008)

Adsorbate	Experimental conditions and techniques	Type	Treatment	Isotherm model	Kinetic model	Qm (mg/g) Langmuir	KL (L/mg) Langmuir	Reference
Cr(III)	pH effect	Batch	No	Langmuir, L–F	Pseudo– first and second order, mass transfer	56 ± 3, 33 ± 1, 19 ± 1 pH= 5, 4, 3	6 ± 1, 12 ± 1, 9 ± 1 pH= 5, 4, 3	(Vilar et al. 2011)
Cr(VI)	pH effect, SEM, EDS, FTIR, titration, extract	Batch	Protonated	Langmuir, Freundlich	Pseudo– first and second order	34.8 pH= 2	0.01 pH = 2	(Belattmania et al. 2017)
Cr(VI)	pH effect, sorbent dosage, temperature, factorial design, SEM, FTIR	Batch	No	Langmuir, Freundlich, Temkin	Pseudo– first and second order, Elovich	196.1, 20 ºC 185.2, 50 ºC pH= 2	0.02, 20ºC 0.03, 50ºC pH = 2	(González Bermúdez et al. 2012)
Cr(VI)	Synthetic, real wastewaters pH effect, FTIR	Batch and column	Protonated	No	Pseudo– first order	No	No	(López-García et al. 2012)
Hg	Desorption, FTIR, SEM, EDS	Batch	No	Langmuir	No	179 ± 10, 15ºC 206 ± 10, 25ºC 226 ± 10, 35ºC 268 ± 12, 45ºC pH= 5	0.0583 0.0603 0.0668 0.0703 pH = 5	(Carro et al. 2009).
Hg + methylene blue (MB) +Acid Blue 25	Competitive adsorption, SEM, EDS	Batch and column	Several chemical treatments	Langmuir L–F	No	174 – 194 pH = 5	0.086 – 0.154 pH = 5	(Carro et al. 2013)
Hg + Cd, Pb, Cu, Ca, MB, AB25, phenols	Competitive adsorption, pH effect, SEM, EDS	Batch and column	Protonated	L–F	Weber–Morris model	No	No	(Carro et al. 2011)
Ni	Titration	Batch	No	Langmuir, Freundlich Temkin,L-F	Diffusion and pseudo–second order	75.6 pH= 3	0.0547 pH= 3	(González Bermúdez et al. 2011)
H	Acid-base modelling titrations	Batch	No	No	No	No	No	(Rey-Castro, Herrero, and Sastre de Vicente 2004)
H	Acid-base modelling titrations	Batch	No	No	No	No	No	(Rey-Castro et al. 2003)
Sb	pH effect, FTIR	Batch	Protonated	Langmuir, Freundlich	Pseudo– first and second order	5.5 ± 0.8 pH = 5	0.7 ± 0.3 pH = 5	(Ungureanu et al. 2015)

Table 5. (Continued)

Adsorbate	Experimental conditions and techniques	Type	Treatment	Isotherm model	Kinetic model	Qm (mg/g) Langmuir	KL (L/mg) Langmuir	Reference
Basic Violet 10	pH effect, temperature, sorbent dosage, FTIR, SEM	Batch	No	Langmuir, Freundlich,Dubinin-Raduskevih, Temkin	Pseudo–second order, Intraparticule diffusion, Elovich	76.92 pH = 3	0.087 pH = 3	(Devi, Murugappan, and Rajesh Kannan 2015)
MB	pH effect, specific surface area	Batch	CaCl2 Protonated Chemical crosslinking	Langmuir, Freundlich	First order Lagergren equation	237 ± 12 CaCl2 279 ± 4, H+ pH = 5.5	0.06 ± 0.01 0.025 ± 0.001 pH = 5.5	(Rubin et al. 2005)
MB	pH effect, specific surface area	Batch	Several Chemical treatments	Langmuir	Pseudo– first second order Intraparticle diffusion	279 – 860 pH = 5.5	0.025 – 0.13 pH = 5.5	(Rubín et al. 2010)
Phenol, Cl-phenol	pH effect, FTIR, BET, titration	Batch	CaCl2	Langmuir, Freundlich	Lagergren equation	4.6 – 251 pH = 1	0.0007–0.006 pH = 1	(Rubín et al. 2006)

Table 6. Synthesis of nanoparticles from Sargassum muticum aqueous extracts. The table also shows the experimental conditions, characterization and biological tests performed in the works cited

Nanoparticle	Extraction conditions	Reaction conditions	Biological tests	Characterization	Other assays	Shape/size	Reference
ZnO	2 g/100 ml water 100ºC	Zn(CH3COO)2·2H2O 70 to 450ºC	Toxicity, apoptotic properties	SEM, optical analysis Antioxidant activity	No	Hexagonal wurtzite 30-57 nm	(Sanaeimehr, Javadi, and Namvar 2018)
Hyaluronan/ ZnO	1 g/100 ml water 100ºC	Zn(CH3COO)2·2H2O and hyaluronan 70ºC	Anticancer activity	XRD, FTIR, TEM, FESEM, UV-Vis, DLS	No	Polygonal shapes 10.2 ± 1.5 nm (mean size)	(Namvar et al. 2016)
ZnO	2 g/100 ml water 100ºC	Zn(CH3COO)2·2H2O 70ºC	Anticancer activity	FTIR, XRD, TEM	No	Hexagonal wurtzite 10-15 nm	(Namvar, Rahman, Mohamad, Azizi, et al. 2015)
ZnO	2 g/100 ml water 100ºC	Zn(CH3COO)2·2H2O 70 to 450ºC	No	XRD, FTIR, FESEM, UV-Vis	No	Hexagonal wurtzite 30-57 nm	(Azizi et al. 2014)
Au	1 g/100 ml water 100ºC	HAuCl4 45ºC	Anticancer activity	UV-Vis, TEM, Zeta potential	No	Spheres <10 nm	(Namvar, Rahman, Mohamad, Rasedee, et al. 2015)
Au	2 g/100 ml water 100ºC	HAuCl4 45ºC	No	XRD, TEM, UV-Vis, Zeta potential	No	Spheres 10.2 ± 1.5 nm (mean size)	(Namvar, Rahman, Mohamad, Azizi, et al. 2015)

Nanoparticle	Extraction conditions	Reaction conditions	Biological tests	Characterization	Other assays	Shape/size	Reference
Au	Variable dosage, water	HAuCl4	No	FTIR, SEM	Kinetic, pH and column studies	Triangles, spheres, hexagons, wires >40 nm	(Lodeiro and Sillanpää 2013b)
Au	Variable dosage, artificial seawater	HAuCl4	No	FTIR, SEM Potentiometric titrations	Kinetic, pH and column studies	Spherical or amorphous 100-500 nm Nanowires 50-250 nm	(Lodeiro and Sillanpää 2013a)
Fe3O4	1 g/100 ml water 100ºC	FeCl3	Anticancer activity	FTIR, XRD, UV-Vis, FESEM, EDXRF, TEM, VSM	No	Cubes 18 ± 4 nm	(Namvar et al. 2014)
Fe3O4	1 g/100 ml water 100ºC	FeCl3	No	FTIR, XRD, UV-Vis, FESEM, EDXRF, TEM, VSM	No	Cubes 18 ± 4 nm	(Mahdavi et al. 2013)
Ag	0.2 g/100 ml water 60ºC	AgNO3 90ºC	Insecticidal activity	UV-Vis, SEM, EDAX, XRD	No	42.3-98.6 nm	(Moorthi, Balasubramanian, and Mohan 2015)
Ag	10 g/100 ml water 100ºC	AgNO3	Toxicity	SEM, FTIR, XRD	No	Cubes 43-79 nm	(Madhiyazhagan et al. 2015)
Ag	1 g/100 ml water 100ºC	AgNO3	No	UV-Vis, TEM, XRD, FTIR	No	Spheres 5-15 nm	(Azizi et al. 2013)

The removal of organic compounds present variable results depending on the *S. muticum* pre–treatment. For example, raw and acetone treated *S. muticum* present a maximum uptake capacity of cationic dyes between 0.16 and 2.69 mmol/g, for Basic Violet 10 (Devi, Murugappan, and Rajesh Kannan 2015) and Methylene Blue (MB) (Rubín et al. 2010), respectively (Table 5). The adsorption of these cationic dyes is favoured at low pH values, for which the seaweed is protonated, and therefore not only pure electrostatic but also hydrophobic interactions account for the adsorption. Moreover, treatments that extract the lipid content (e.g., acetone treatment) of the alga clearly improve the MB adsorption. On the other hand, *S. muticum* adsorption capacity for chlorophenols (1.95 mmol/g) is much higher than for phenol (0.049 mmol/g) (Table 5). In the case of phenolic adsorption hydrophobic and donor acceptor interactions have been suggested to play a key role, and their adsorption is reported to agree with the corresponding octanol–water partition coefficients (Rubín et al. 2006). The donor–acceptor interactions occur between the aromatic ring activated by the -OH and -Cl substituents and the surface groups of *S. muticum*; in addition the interactions of phenols with the algae can also occur due to complex formation or the distribution of these neutral phenols between the aqueous solution and the wall cell structure of the alga. As a result, the more hydrophobic organic compounds are the better will be adsorbed.

COLUMN STUDIES AT LABORATORY SCALE AND PILOT PLANTS

Continuous flow studies constitute an essential step towards large scale/commercial applications of *S. muticum* as a biosorbent. Fixed–bed columns packed with this seaweed have been used in continuous flow experiments. Useful information, e.g., the service and exhaustion times, can be obtained from the characteristic breakthrough curves. For example, breakthrough curve areas were used to calculate sorption and reduction percentages for mercury elimination using *S. muticum* (Carro et al. 2013). Best operational conditions can be found by modelling the dynamic behaviour of the fixed–bed columns, which is also useful to scale–up the system (Mazur et al. 2018). The effect of flow rate and bed depth on the biosorption of metals was investigated in order to obtain the shortest column mass transfer zones (Lodeiro, Herrero, and Sastre de Vicente 2006b, Ungureanu et al. 2017).

Sargassum muticum biomass was used in fixed–bed column experiments to decontaminate solutions containing Cr (López-García et al. 2012), Hg (Carro et al. 2011, 2013), Cd (Lodeiro, Herrero, and Sastre de Vicente 2006b, a), Sb (Ungureanu et al. 2017) and MB (Carro et al. 2013). An improvement on the mechanical, physical and/or chemical properties is recommended for continuous flow operational modes; thus a treatment of the raw seaweed is usually performed. Raw, protonated and ethanol–treated *S. muticum* were used in these continuous flow experiments. For species that show a sorption–reduction removal mechanism, protonated *S. muticum* cleaned up to 3.6 L of a solution containing 0.5 mmol/L of Hg (Carro et al. 2011), or 46.2 L of a 50 mg/L Cr(VI) solution (López-García et al. 2012). Using the raw seaweed the treated volumes for the same Hg solution increased up to 13.5 or 24 L at similar residence times, while using an ethanol–treated biomass 16.7 L were decontaminated (Carro et al. 2011, 2013). Cadmium is positively charged in solution and no–redox reactions are involved in its removal; in this case, a maximum concentration of Cd in the effluent of 0.05 mg/L was obtained after treating 6.2 L of 50 mg/L initial

concentration solution using protonated *S. muticum* (Lodeiro, Herrero, and Sastre de Vicente 2006b). For antimony oxyanions only 2.6 L of a solution containing 1 mg/L Sb were decontaminated obtaining a maximum effluent concentration of 0.1 mg/L using the native seaweed (Ungureanu et al. 2017).

The elution and regeneration of the columns is also essential for continuous operational modes. To the best of our knowledge there is only one paper that shows uninterrupted sorption–desorption cycles in a fixed-bed column using *S. muticum* (Lodeiro, Herrero, and Sastre de Vicente 2006a). In this work, a column was operated for the removal of 50 mg/L of Cd from solution during 605 h for sorption, and 66 h for desorption using HNO_3 0.1 mol/L. This is equivalent to continuous use over 28 days, with no apparent loss of sorption performance, despite the 27% weight lost found at the end of the process.

Briefly, we would also like to highlight the first test of *S. muticum* in a pilot plant carried out by the group of M. E. Sastre de Vicente at The University of A Coruña. Here experiments were performed to investigate the potential use of pre–treated *S. muticum* to decontaminate wastewaters from an aluminium factory. Very promising results (unpublished) were obtained that encourage us to persevere with the idea of revalorising the invasive seaweed *Sargassum muticum* as adsorbent/ion-exchanger material.

REDOX CAPACITY OF THE AQUEOUS EXTRACT: SYNTHESIS OF NANOPARTICLES

The interactions of S. *muticum* biomass with some metals in solution may lead, depending on the physicochemical environment and nature of the system, to the formation of metal nanoparticles (Figure 1). Metal nanoparticles have important applications in many technological areas of interest, i.e., from medicine or pharmacy to electronics. There is therefore a sustained interest in the development of synthetic routes to obtain metal nanoparticles. Currently, there is an unequivocal trend to adopt greener synthesis processes when possible. Thus, researchers are starting to use so–called biological synthesis, biosynthesis or biogenic synthesis methods to manufacture nanoparticles. These eco–friendly syntheses are cleaner, safer and cheaper than the commonly used chemical or physical methods. The main aim is to eliminate or reduce toxic solvents and energy input requirements. Therefore, water is the first choice of solvent and the experiments are performed at ambient pressure and temperature. Moreover, the biological synthesis use renewable resources from inexpensive biomass as feedstock, with adaptation to a wide range of systems and to large–scale possible applications (Asmathunisha and Kathiresan 2013, Vijayan et al. 2016, Dahoumane et al. 2017). Marine biomass meets these criteria and is becoming a promising feedstock material in the area of nanobiotechnology. Algae are rich in bioactive compounds such as polyphenols, polysaccharides, carotenoids and proteins. These chemical species have a high reducing capacity. They are able not only to generate metallic nanoparticles from aqueous soluble salts of metals, but simultaneously supply, non–toxic-stabilizing agents for the nanoparticles formed. The process is commonly performed starting from aqueous extracts of algae biomass.

Nanoparticles with different sizes and shapes were obtained by green synthesis using the invasive seaweed *S. muticum* (Table 6). Moreover, some of these works also included

preliminary experiments on the biological activity (e.g., anticancer, cytotoxic effect or insecticidal activity) of the manufactured nanoparticles (Sanaeimehr, Javadi, and Namvar 2018, Namvar, Rahman, Mohamad, Rasedee, et al. 2015, Namvar et al. 2014, Moorthi, Balasubramanian, and Mohan 2015, Madhiyazhagan et al. 2015). The size and shape of nanoparticles have a crucial role on their properties. Thus, future research should be focused on obtaining reproducible results for homogeneous and monodisperse nanoparticles. Therefore, it is important to investigate different experimental parameters such as temperature, ionic strength, pH, extract concentration, and reaction time that yield high–quality nanoparticles to use for industrial or medical applications.

Finally, it is worth mentioning that there is an urgent need for understanding the mechanism of the biosynthesis of nanoparticles, and to identify and quantify the chemical components involved in the process (Adil et al. 2015). Despite this lack of knowledge, there is already an ongoing effort to extract and quantify natural antioxidant products, e.g., phenolic compounds and polysaccharides, from *S. muticum* (Anaëlle et al. 2013, Casas et al. 2016, Flórez-Fernández et al. 2017, Rodrigues et al. 2017, Mazumder et al. 2016, Balboa et al. 2016).

THE SORBIGAL PROJECT

The SORBIGAL project was the first attempt of large–scale industrial valorisation of the invasive alga in Europe *Sargassum muticum*. The spin–off SORBIGAL project was funded and promoted by M. E. Sastre de Vicente at The University of A Coruña.

The idea for the valorisation of the seaweed *S. muticum* was based on its great properties as an adsorbent/ion exchanger, and the need for an alternative commercial use to subsidise its extremely difficult eradication. Essentially, we wanted to obtain a biosorbent (low cost adsorbent/ion exchanger of biological origin) by harvesting and processing *S. muticum*. A previous solid knowledge on the physicochemical properties of *S. muticum* was crucial for the SORBIGAL project. The group of Sastre de Vicente at The University of A Coruña acquired this expertise after investigating the interactions of this seaweed with metallic and non–metallic compounds in solution from laboratory basic research to pilot plant scale. A brief summary of the itinerary followed is schematically reflected in Figure 2.

The SORBIGAL idea (M. E. Sastre de Vicente, private files of SORBIGAL project; unpublished personal communication) was awarded in 2009 by The University of Santiago de Compostela. As recently pointed out by Milledge (Milledge and Harvey 2016), we thought in that moment that to valorise *S. muticum* as biosorbent was a "golden" opportunity. The marketisation process of using *S. muticum* as a biosorbent however presented many challenges and was finally cancelled after some years of effort. We think that the main possible causes for the lack of success were:

1) The idea of *S. muticum* valorisation as adsorbent/ion exchanger was not good enough for the market.
2) The financial crisis in Spain, which activated a considerable cut in the budget of research and development (R&D) activities. In addition, the absence of private investors and/or seed money.

3) Bureaucratic limitations due to necessary administration permissions for harvesting invasive biomass that is considered an exploitation of resources.
4) The lack of experienced people in management and market related activities.
5) The absence of cooperation with other research groups and industries/companies.
6) The focus in only one specific valorisation of *Sargassum muticum* as biosorbent, and not considering other alternative uses of this invasive seaweed as a whole.

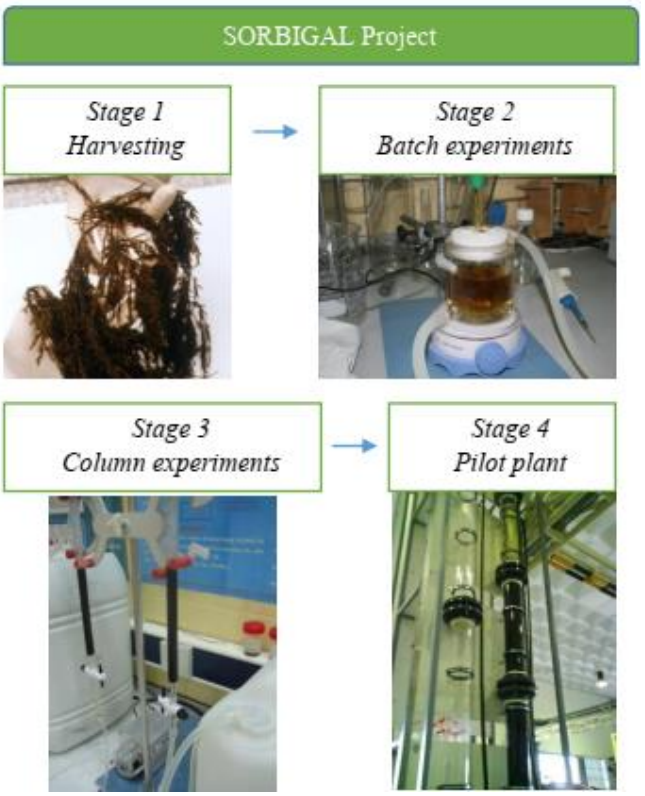

Figure 2. Schematic view of the main experimental stages carried out to revalorise *S. muticum* as adsorbent/ion exchanger material. The SORBIGAL project stopped at the stage 4, before starting the potential commercialization of the pre–treated seaweed.

Looking back, we currently think that the best way to valorise *S. muticum* would be the cooperation of a consortium involving research–companies and universities with the participation of market experts. In addition, the valorisation should be oriented to obtaining multiproducts from *S. muticum* biomass. Is this task easy? Is the effort worth it? We hope to answer these and other related questions in the near future.

CONCLUSION

Currently, there is a considerable amount of information regarding the interactions of *Sargassum muticum* with metals. This is due to several years of investigations by different

research groups worldwide. Most of this work can be easily related with the potential use of *S. muticum* as biosorbent.

The behaviour of *S. muticum* in aqueous solutions has been studied at different levels. For example, initially investigating the fundamental acid–base properties of this biomaterial, and finally running pilot plant tests with apparently successful results.

The biogenic synthesis of nanoparticles from *S. muticum* extracts also presents interesting perspectives. This area has been less developed than the biosorption topic, and thus requires more research. For example, the identification and quantification of the chemical species involved in the formation and stabilization of nanoparticles should be further investigated.

In addition to the valorisation routes described in this article, we think that to success place *S. muticum* on the market the participation of different stakeholders such as administrations, research groups and companies is required. The valorisation should considered *S. muticum* as a whole, exploiting also its properties in medicine, cosmetics and/or its potential use as a fuel.

More than forty years after the entry of *Sargassum muticum* in Europe as an invasive alga there are still many open paths for continuing with new revalorisation research ideas.

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Chapter 86

NEW INSIGHTS INTO SEAWEEDS ON NUTRITION AND FUNCTIONALITY

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ABSTRACT

Epidemiological studies have provided evidence that plant-based foods play a crucial role in the prevention of chronic diseases. The association between dietary vegetable intake and chronic diseases is mainly attributed to vegetables providing dietary fiber, as well as a wide range of secondary compounds called phytochemicals. Seaweed research is an emerging trend in functional foods for the prevention of degenerative diseases. Seaweeds are rich sources of vitamins, minerals and dietary fiber required by the human body for optimal health. In addition, seaweeds are diverse in promising bioactive compounds such as soluble dietary fibers, peptides, phlorotannins, carotenoids, and fucosterol which are amongst the most promising of macroalgae's compounds in terms of functional foods/nutraceuticals. Polyphenols from seaweeds are gaining dietary importance due to their influence over diabetes mellitus and the role as a vital source of high-value nutraceutical. The objective of this chapter is to highlight recent advances made in the search of seaweeds as sources of new functional ingredients, highlighting the new challenges and opportunities regarding healthy effects of the seaweeds.

Keywords: alginate, biomass extraction, fucosterol, fucoxanthine, functional, nutrition, phlorotannins, prebiotics

INTRODUCTION

Seaweeds or macroalgae are classified into three higher taxa: brown (*Class Phaeophyceae*), red (*Phylum Rhodophyta*) and green (*Phylum Chlorophyta*) (Guiry 2013). Seaweeds are harvested and used globally for many different applications, such as ingredients

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in food and cosmetic formulations, and have considerable economic importance (Tierney 2010). At present, around 31 million tons (wet weight basis) of seaweeds and other aquatic plants are produced/captured annually (FAO 2016). Recently, the production, marketing and consumption of seaweeds in food products have increased significantly, not only in countries where there is a traditional use such as Japan and China, but also in many Western countries (Tierney 2010). However, currently seaweed species are still under-exploited bio-resources (Khanw 2009).

Marine macroalgae are gaining attention among the scientific community as a significant source of functional food ingredients. Due to the harsh environments in which macroalgae survive, they produce unique bioactive compounds that are not found in terrestrial plants (Murray 2018). The most promising sources of new bioactive molecules, seaweeds represent valuable, but still underexploited, biofactories for drug discovery and product development (Lopes 2017).

Seaweeds are presently pointed out as the plant-based food of the future, since besides not competing with food crops for the use of arable land and fresh water resources, they are a good supply of key nutrients including carbohydrates, protein and minerals (Lordan 2011). Moreover, they are a rich source of health-promoting compounds capable of acting on a wide spectrum of disorders and/or diseases. This latter fact is becoming particularly evident as macroalgae are presently under the spotlight of many investigations. All these facts are pushing the Western culture to increase the interest in the manufacturing and consumption of high-value products derived from macroalgae, with the main aim of taking advantage of their potential health effects (Cardoso 2015).

Besides, it is worth to mention that seaweeds have been considered over the past few decades as promising organisms for providing both, novel biologically active substances and essential compounds for human nutrition, with high potentially economic impact in food and pharmaceutical industry and public health (Cardozo 2007). In addition, it is stated that much research, such as their role in nutrition and disease prevention, remains to be done before science-based dietary recommendations can be given for edible seaweeds (Smit 2004).

SEAWEEDS AS FUNCTIONAL FOOD SOURCES

Global demand for macroalgal and microalgal foodstuff is growing, and seaweed are increasingly being consumed for functional benefits beyond the traditional considerations of nutrition and health. There is substantial evidence for the health benefits of algal-derived food products, but there are considerable challenges in quantifying these benefits, as well as possible adverse effects. The limits to our understanding fall broadly into three areas. First is the variation of nutritional and functional composition of algae across species, seasons, and different coastal environments. It should be also considered the effect of harvesting, storage, and food processing techniques, which can increase or decrease the nutritional quality. The second, it is quantifying which fractions of algal foods are bioavailable to humans or fraction of nutritional or functional components that actually have effect in relation to their residence time in the digestive system. Another issue is quantifying which fractions of algal foods are bioavailable to humans, and which factors influence how food constituents are released, ranging from food preparation through genetic differentiation in the gut microbiome. There is

an increasing literature on digestive reactor analytical methods. Bioaccessibility will be a complex function of the chemistry of the substance, the processing methods used to prepare the alga as food, the specific algal matrix containing it, the consortium of bacteria and their enzymatic competency, and the presence of other foods that may interfere or enhance uptake. The third limitation lies in understanding how algal nutritional, and particularly functional constituents interact in human metabolism and intermediary metabolic processes. It is needed to highlight the rapidly advancing area of algal science with a particular focus on the key research required to assess better the health benefits of an alga or algal product. There are rich opportunities in this emerging field, requiring exciting new experimental and collaborative approaches, to develop a rich and rewarding collaboration among psychological, nutritional, medical, analytical, and industrial groups investigating algae such as nutritional and functional foods (Wells 2017).

SEAWEEDS AS EMERGENT SOURCES OF PREBIOTICS

Dietary fiber (DF) -polysaccharides known to be resistant to enzymatic hydrolysis by digestive enzymes- in brown seaweeds is essentially composed of four families of polysaccharides, laminaran, alginate, fucan, and cellulose. Laminaran, the reserve polysaccharide in brown seaweeds (Jiménez-Escrig 2000), is a small alfa-glucan of 5kDa, composed of D-glucose with beta-(1,3) linkages and beta-(1,6) interchange branching (Rioux 2010). The major structural component in brown seaweeds is a gelling polyuronide, alginate, outlined as alternating sequences of (1,4)-linked beta-D-mannuronic acid and its C5-epimer alfa-L-guluronic acid residues. The structural polysaccharide fucan, is primarily composed of fucoidan -(1,2)-linked alfa-L-fucose-4-sulphate- linked to D-xylose, D-galactose, D-mannose, and uronic acids (Jiménez-Escrig 2000, Rioux 2010). Among structural polysaccharides, fucans are the most interesting for their potential biological activities, whereas alginate is mostly used as a food ingredient (Rioux 2010).

Algal oligo- and polysaccharides (PSs) show effects on health similar to and sometimes more effective than other oligosaccharides from different sources. Their chemical structures include some of these oligosaccharides and some of the PSs produced by seaweeds that are not degraded by enzymes in the upper part of the GI tract. Therefore, algal PSs present a great potential for emergent prebiotics to be used as dried biomass or nutraceuticals, after extraction from the biomass or from the culture medium. They may be included in food and/or feed, or administered as pills. The development of new enzyme technologies together with new enzymes from marine bacteria and mollusks will enable us to tune these PSs and produce novel prebiotics. (Jesus-Raposo 2016).

Prebiotics enhance immune response through the modulation of intestinal microbial activities, production of short chain fatty acids (SCFA), direct interaction with toll-like receptors and mucin production. These non-digestible food components are utilized as carbon source for the growth of beneficial bacteria population through the process of fermentation. Brown seaweed polysaccharides have been described as emerging prebiotics due to their potential to stimulate gut microbiota activities at *in vitro* and *in vivo* stages (Okolie 2017).

Based on their composition red seaweeds, are good potential functional foods for gut health. Intestinal mucosal barrier function refers to the capacity of the intestine to provide

adequate containment of luminal microorganisms and molecules. The components of red seaweeds play a role on the regulation of mucosal barrier function. Special attention has been paid to unique components of red seaweeds such as proteins and derived peptides (e.g., phycobiliproteins, glycoproteins that contain cellulose binding domains, phycolectins and the related mycosporine-like amino acids) together with polysaccharides (e.g., floridean starch and sulphated galactans, such as carrageenans, agarans and dl-hybrid) and minerals. These compounds have been shown to exert prebiotic effects, to regulate intestinal epithelial cell, macrophage and lymphocyte proliferation and differentiation and to modulate the immune response. Molecular mechanisms of action of peptides and polysaccharides are starting to be elucidated, and evidence indicating the involvement of epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), toll-like receptors (TLR) and signal transduction pathways mediated by protein kinase B (PKB or AKT), nuclear factor-B (NF-B) and mitogen activated protein kinases (MAPK). The need for further research is clear, but *in vivo* experiments point to an overall antiinflammatory effect of these seaweeds, indicating that they can reinforce membrane barrier function (Cian 2015).

FUCOIDAN AS ANTICANCER AGENT IN PRECLINICAL DEVELOPMENT

Fucoidan is a fucose-containing sulphated polysaccharide derived from brown seaweeds, crude extracts of which are commercially available as nutritional supplements. Recent studies have demonstrated antiproliferative, antiangiogenic, and anticancer properties of fucoidan *in vitro*. Accordingly, the anticancer effects of fucoidan have been shown to vary depending on its structure, while it can target multiple receptors or signalling molecules in various cell types, including tumour cells and immune cells. Low toxicity and the *in vitro* effects of fucoidan mentioned above make it a suitable agent for cancer prevention or treatment. However, preclinical development of natural marine products requires *in vivo* examination of purified compounds in animal tumour models. Recent *in vivo* results on the effects on animal models of systemic and local administration of fucoidan on tumour growth, angiogenesis, and immune reaction are likely applicable to the development of fucoidan as a marine anticancer drug.

In addition, diverse potential of fucoidan has created intense interest to review the existing scientific literature, specifically focusing on prevention, associated molecular mechanisms in inflammation related ailments. Fucoidan inhibited inflammatory responses, both *in vitro* and *in vivo* experimental models. Inflammation is a complex response of living systems and involved in pathogenesis of different diseases, including arthritis, cancer and allergies. Sulphated glycans play a crucial role in inflammation related events such as they partake in the regulation of chemokines, rolling of leukocytes along the endothelium at inflammatory sites, and provide the structural assembly of subendothelial basement membrane. These functions make fucoidan as suitable exogenous sulhated polysaccharide to ameliorate the inflammatory events like regulating pro inflammatory targets. Recent studies have been performed to provide the anti-inflammatory properties and underlying molecular mechanisms of this bio-functional molecule (Kwak 2014).

PREVENTION OF SYNDROME METABOLIC BY FUCOXANTHINE

Obesity, which results from an imbalance between energy intake and energy expenditure, has become a major health risk factor worldwide, causing numerous diseases such as diabetes, hypertension, and cardiovascular diseases. Fucoxanthin, a specific carotenoid in brown algae, has garnered much attention for its anti-obesity and anti-diabetic effects attributable to a unique mechanism. Fucoxanthin induces uncoupling protein 1 (UCP1) expression in white adipose tissue (WAT). That inner membrane mitochondrial protein, UCP1, can dissipate energy through oxidation of fatty acids and heat production. Furthermore, fucoxanthin improves insulin resistance and ameliorates blood glucose levels through down-regulation of adipocytokines related to insulin resistance in WAT and up-regulation of glucose transporter 4 (GLUT4) in skeletal muscle. Algae fucoxanthin is a novel beneficial compound for the prevention of the metabolic syndrome (Maeda 2015).

PREVENTION OF OBESITY THROUGH ALGINATE MEDIATED BY ENZYME DIGESTION INHIBITION

Regulation of food intake through modulation of gastrointestinal responses to ingested foodstuff is an ever-growing component of the therapeutic approaches targeting the obesity epidemic. Alginates, viscous and gel-forming soluble fibers isolated from the cell wall of brown seaweeds and some bacteria, are recently receiving considerable attention because of their potential role in satiation, satiety, and food intake regulation in the short term. Enhancement of gastric distension, delay of gastric emptying, and attenuation of postprandial glucose responses can constitute the basis of their physiological benefits. Offering physical, chemical, sensorial, and physiological advantages over other viscous and gel-forming fibers, alginates constitute promising functional food ingredients for the food industry. Therefore, alginates are claimed to exert positive effect in glycemic regulation (El Khoury 2015).

PHLOROTANNINS AS PROMISING FUNCTIONAL INGREDIENT

Fucaceae is the most dominant algae family along the intertidal areas of the Northern Hemisphere shorelines, being part of human customs for centuries with applications as a food source either for humans or animals in agriculture and as remedies in folk medicine. These macroalgae are endowed with several phytochemicals of great industrial interest from which phlorotannins have gathered much attention during the last few years due to their numerous possible therapeutic properties. Phlorotannins are a class of polyphenols that are unique to marine sources. Several biological activities have been attributed to phlorotannins over the years. The range of activity of phlorotannins is remarkable against including antioxidant effects through scavenging of reactive oxygen species (ROS) or enhancement of intracellular antioxidant defenses. Also, including antidiabetic properties through their acarbose-like activity, stimulation of adipocytes glucose uptake and protection of -pancreatic cells against high-glucose oxidative stress; anti-inflammatory effects through inhibition of several pro-inflammatory mediators; antitumor properties by activation of apoptosis on cancerous cells

and metastasis inhibition, among others. Diabetes mellitus is a group of metabolic disorders characterized by hyperglycaemia, and predicted by the World Health Organization as the expected 7th leading cause of death in 2030. Diabetes mellitus type 2 (DMT2) comprises the majority of diabetic individuals around the world (90%-95%). Pathophysiologically, this disorder results from a deregulation of glucose homeostasis, worsened by overweight and by a sedentary lifestyle, culminating in life-threatening cardiovascular events. The currently available anti-diabetic drugs are not devoid of undesirable side effects, sometimes responsible for poor therapeutic compliance. This represents a challenge for contemporary medicine, and stimulates research focused on the development of safer and more efficient anti-diabetic therapies. Phlorotannins are not only promising to avoid hyperglycaemia, through their extraordinary capacity to inhibit glucose absorption, but also they are capable of treating diabetes-associated disorders through their capacity of protecting -cells from glucose-induced toxicity, in diagnosed DMT2 individuals (Lopes 2017). These multiple health properties render phlorotannins great potential for application in numerous therapeutically approaches. Over the past two decades, 20 different bioactive polyphenols/phlorotannins have been isolated and studied from 10 different brown algae. Their assorted beneficial effects on human health include competitive inhibition of digestive enzymes, varying the activity of hepatic glucose-metabolizing enzymes, lowering the plasma glucose levels, and lipid peroxidation, delaying the aging process. Despite the recent advancements in isolating bioactive compounds from seaweeds with potential health benefit or pharmaceutical behaviour, studies on the polyphenol effectiveness on glucose homeostasis in human beings are very few. Further research in this area is required to confirm the close connection of polyphenol rich seaweed-based diet consumption with glucose homeostasis and the exciting possibility of prescribing polyphenols to treat the diabetes pandemic (Chao 2018, Murray 2018).

FUCOSTEROL STILL NEEDS TO REINVENT ITSELF

Recent studies have been focused on the biological and pharmacological activities of seaweeds and their highly bioactive secondary metabolites because of their potential in the development of new pharmaceutical agents. Although several varieties of bioactive novel compounds such as phlorotannins, diterpenes and polysaccharides from seaweeds have already been scrutinized, fucosterol as a phytosterol still needs to reinvent itself. Fucosterol (24-ethylidene cholesterol) is a sterol that can be isolated from seaweeds and diatoms. Fucosterol exhibits various biological therapeutics, including anticancer, antidiabetic, antioxidant, hepatoprotective, antihyperlipidemic, antifungal, antihistaminic, anticholinergic, antiadipogenic, antiphotodamaging, anti-osteoporotic, blood cholesterol reducing, blood vessel thrombosis preventive and butyrylcholinesterase inhibitory activities (Abdul 2016).

SULPHATED POLYSACCHARIDES AS INHIBITORS OF CALCIUM OXALATE RENAL STONE FORMATION

Sulphated polysaccharides (SPSs) from various seaweeds are known to play a significant inhibitory role in calcium oxalate (CaOx) kidney stones. CaOx stone formation is a multistep

process, which includes crystal nucleation, growth, aggregation, and crystal retention. Renal tubular cell injury is one of the determining factors leading to crystal retention and formation of stone in the nidus. The therapeutic mechanisms of action of seaweed SPSs on CaOx renal stone formation are known through the inhibition of crystal nucleation, growth, and aggregation. SPSs prevent renal tubular cell damage because of its antioxidant properties, thereby preventing crystal adherence and internalization. Therefore, seaweed SPSs could be considered promising molecules because of their role in renal stone prevention (Bhadja 2016).

Innovative technology that uses extracts obtained by supercritical CO2 extraction, as a method of isolation of biologically active compounds from algal biomass, is presented (Michalak 2017).

ALGAE INGREDIENTS AS ANTIMICROBIALS

The increasing efforts to use ingredients that are as natural as possible in the formulation of innovative products has given rise to-the introduction of macro and microalgae in food industry. Up to date, scarce information has been published about algae ingredients as antimicrobials in food. The antimicrobial potential of algae is highly dependent on: (i) type, brown algae being the most effective against foodborne bacteria; (ii) the solvent used in the extraction of bioactive compounds, ethanolic and methanolic extracts being highly effective against Gram-positive and Gram-negative bacteria; and (iii) the concentration of the extract. The validation of the algae antimicrobial potential in real food matrices is still a research niche, being meat and bakery products the most studied substrates (Pina-Pérez 2017).

INNOVATIVE EXTRACTION TECHNOLOGY OF ALGAL BIOMASS

Algal extracts are gaining increasing interest due to their unique composition and possibilities in industrial applications. Various extraction techniques are used for conversion of algal biomass into extracts. Recently, scientist attention has been paid to innovative technology, such as enzyme-assisted extraction, microwave-assisted extraction, pressurized liquid extraction, supercritical fluid extraction, and ultrasound-assisted extraction, as novel methods of isolation of biologically active compounds from algal biomass, without their degradation (Michalak 2017).

Polysaccharides obtained from macroalgae have promising prospects and could contribute greatly to the future of a marine based bio-economy. Specifically, laminarin and fucoidan from brown macroalgae have a wide variety of potential industrial applications including functional foods and nutraceuticals, due to their broad range of biological activities. These beneficial biological activities are related to the chemical composition and structure of the macroalgal polysaccharides. The molecular weight, monosaccharide composition and sulphate content of these polysaccharides could be influenced by both macroalgal biology (i.e., variations in polysaccharide composition due to macroalgae species and their biological cycle) and different extraction/purification techniques employed to obtain polysaccharide enriched products (i.e., de-sulphation or fragmentation of sulphated polysaccharides). The

application of innovative extraction technologies (such as ultrasound, microwave and enzyme-assisted extractions), as well as new purification techniques (i.e., membrane separation) are promising technologies for the challenges concerning molecule structure-function relationship and macroalgal variability (Garcia-Vaquero 2017).

The complex structure and distinctive components of seaweed cell walls, which differ significantly from terrestrial plants, presents a major challenge for the effective extraction of bioactive compounds from inside the cells. Enzyme technologies have been used to improve the extraction, hydrolysis and structure modification efficiently with a high degree of environmental sustainability. Future directions in applying enzyme technologies to improve the extraction and processing of bioactive compounds from seaweeds and their potential applications in functional foods and nutraceuticals have been evidenced to assist the extraction by breaking down the seaweed cell walls, and degrade or hydrolyse macromolecules including polysaccharides and proteins. These enzymatic processes can improve the yield and recovery of bioactive compounds and enhance their biological properties with regard to prebiotic, antioxidant, ACE inhibitory, anti-inflammatory, and antiviral effects. Seaweed-derived bioactive compounds from these processes present significant new opportunities in developing novel food applications (Suvimol 2017).

CONCLUSION

It should be highlighted recent advances on the significant health benefits of seaweeds in the nutraceutical field. Seaweed components offer opportunities and a multitude promising health benefits for the construction of novel and enhanced functional food. Future research should focus on new experimental and collaborative approaches, to develop integration among physiological, nutritional, medical, analytical, and industrial groups investigating seaweed as functional foods.

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Chapter 87

MICROALGAE BIOMASS AS A FOOD INGREDIENT TO DESIGN ADDED VALUE PRODUCTS

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ABSTRACT

The use of microalgae as a food ingredient has recently attracted considerable interest worldwide, due to their potential to design value-added products, with remarkable functional activity and health impact. Microalgae are photosynthetic organisms that grow in a wide range of different environmental conditions, from aquatic habitats, including lakes, pounds, or oceans, in wastewater or even on non-arable land. The microalgae culture can be considered as a renewable and sustainable new crop, which can tolerate variable conditions of temperature, salinity, pH or light intensities. Modelling the growth conditions leads to biomasses with different compositions in nutritional terms and bioactive compounds. This crop can be an alternative source of good proteins, polyunsaturated fatty acids and carbohydrates, which may be needed in the near future. Today, microalgae can be considered an underexplored natural source of bioactive ingredients. They are attracting our attention, as they have promising applications in the development of new foods, with improved nutritional profiles and a strong impact on health.

Microalgae are an interesting natural source of bioactive compounds: pigments, enzymes, sugars, lipids with valued fatty acids, sterols, and vitamins, with a recognised positive impact on health. These compounds can be extracted from the microalgae biomass and marketed as nutraceuticals, high added-value products, but the use of the whole microalgae cell, can also be considered, as a functional ingredient. A strong argument to support this approach is that bioactive compounds are naturally encapsulated and protected within the microalgae cell. Thus, it is important to consider the possibility to perform a biomass pre-treatment in order to promote a controlled release of the active compounds trough a partial cell wall disruption. This step can be considered as a biomass

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preparation step. The downstream operations (freeze-drying or spray-drying) can impart different levels of cell wall disruption. In addition, a controlled cell disruption can also be induced by specific methods, such as high-pressure homogenization or microwaves.

Another important issue to address, when the use of microalgae as a food ingredient is considered, is the level of technological processing applied (e.g., temperature, pressure, pH). Examples of food preparations, with different thermal severity, will be discussed in terms of the impact on the bioactivity of the natural compounds. The effect of baking on antioxidant activity of cookies enriched with different types of microalgae is a relevant example. Maintenance of the fatty acid profile, including PUFA's, before and after cooking of pasta enriched with *Isochrysis galbana* and *Diacronema vlkianum* biomass are other examples to be pointed out.

The use of *Arthrospira*, *Chlorella vulgaris*, *Haematococcus pluvialis*, *Diacronema vlkianum*, *Isochrysis galbana* and *Tetraselmis chuii* in different food matrixes – mayonnaise and salad dressings, puddings, pasta and biscuits have been extensively investigated and will be revised. The bioaccessibility of the bioactive compounds *in vivo* and *in vitro* will also be discussed.

Keywords: microalgae, food-rheology, bioactivity, cell wall disruption

1. INTRODUCTION

1.1. What Are Microalgae?

When we take a low tide walk by the sea side, there is a green band where the water stands still, the green is intense and dark, these are microalgae. They have been in the planet from 450 million years ago, at least, and primordial microalgae were very similar to the present ones. Although primordial habitat was totally different, these cells have been able to survive practically unchanged until now, and considering their massive distribution, they are still dominating the planet, being the main part of the planet biomass (Nicoletti, 2016).

Microalgae are amazing beings that survive harsh conditions and can grow almost everywhere, as long as there is light, water (either sweet or salty), enough CO_2, and a few nutrients. In fact, they produce about half of the atmospheric oxygen and they are also great CO_2 capturers, playing a fundamental role in terms of environment balance (Richmond, 2004).

Although they are autotrophic, i.e., they synthetize their own nutrients from inorganic compounds through photosynthesis, they are extremely well equipped and can synthetize the most complex substances. Microalgae cover almost 75% of algae species and despite the apparent simplicity of their cells they spread over at least 40,000 species in phytoplankton (Singh and Dolly, 2011), as they are able to metabolize a myriad of complex substances, this is the key for their success for food supplementation.

The detailed composition of *Chlorella*, a food grade green microalgae, can be seen in Table 1 (gently shared by A4F, Portugal), where all kinds of complex molecules can be found, powerful nutrients from amino acids and vitamins to hormone like and poly-unsaturated fatty acids (PUFA), and this is the reason why *Chlorella* is considered as a superfood.

Nevertheless, the consumption of microalgae in food is still very limited, considered as a nutraceutical, mostly sold in capsules and pills. Though there are some dry powdered *Chlorella* and *Spirulina* commercially available, the price is still very high to be easily generalized as a food ingredient. It is a high potential business that soon will be a huge opportunity.

Table 1. Composition of Chlorella, a food grade green microalgae

NUTRITIONAL COMPOSITION Parameters	Units	Chlorella sp.	NUTRITIONAL COMPOSITION Parameters	Units	Chlorella sp.	NUTRITIONAL COMPOSITION Parameters	Units
Moisture	%	6.00%	Omega 6 (ω6) fatty acids	g/100g	0.83	AABA (alpha-Aminobutyric Acid)	mg/100g
CARBOHYDRATES	g/100g	20.60	Omega 9 (ω9) fatty acids	g/100g	0.69	Superoxide scavenging activity	g/100g
PROTEINS	g/100g	53,4 - 54,5	Other fatty acids			Ratio of Pepsin digestible (crude) protein	
Essential amino acids			Saturated fatty acids	g/100g	2.15	**LIPIDS**	g/100g
Histidine	g/100g	0.97	Unsaturated fatty acids	g/100g	7.75	*Essential fatty acids*	
Threonine	g/100g	2.43	Monounsaturated fatty acids	g/100g	0.91	14:0	g/100g
Arginine	g/100g	3.30	Polyunsaturated fatty acids	g/100g	6.84	palmitic acid 16:0	g/100g
Tyrosine	g/100g	1.81	**VITAMINS & TRACE ELEMENTS**			palmitoleic acid 16:1	g/100g
Valine	g/100g	2.97	Vitamin A (retinol equivalents)	µg/100g	802.00	16:3ω3	g/100g
Methionine	g/100g	0.01	α-Carotene	µg/100g	25,300.00	Estearic acid 18:0	g/100g
Phenylalanine	g/100g	2.43	β-Carotene	µg/100g	3,510.00	Oleic acid 18:1	g/100g
Isoleucine	g/100g	1.78	Vitamin B1 I Thiamine	mg/100g	0.04	linoleic acid (LA) - 18:2ω6	g/100g
Leucine	g/100g	4.34	Viatmin B3 I Niacin	mg/100g	21.00	α-linolenic acid (ALA) - 18:3ω3	g/100g
Lysine	g/100g	3.29	Vitamin B2 I Riboflavin	mg/100g	0.06	γ-linolenic acid (GLA) - 18:3ω6	g/100g
Other amino acids			Vitamin B6 I Pyridoxal phosphate (active form)	mg/100g	0.40	Stearidonic acid (SDA) - 18:4ω3	g/100g
Alanine	g/100g	4.83	Vitamin B12 I Cobalamin	µg/100g	12.00	Gadoleic acid 20:1	g/100g
Glycine	g/100g	3.07	Vitamin C I L-ascorbic acid	mg/100g	92.40	Araquidonic 20:4	g/100g
Proline	g/100g	3.00	Vitamin D	µg/100g	n.d.	Eicosapentaenoic acid (EPA) - 20:5ω3	g/100g
Glutamic Acid	g/100g	5.80	Vitamin D (International Unit)	IU / 100g	n.d.	Behenic acid 22:0	g/100g
Serine	g/100g	2.36	Vitamin E	mg/100g	20.60	Docosadienoic acid - 22:2ω6	g/100g
Aspartic Acid	g/100g	4.59	Vitamin K	µg/100g	n.d.	22:5	g/100g
Tryptophan	mg/100g	21.75	Tryptophan	µg/100g	21,746.00	Docosahexaenoic acid (DHA) - 22:6ω3	g/100g
Cysteine	g/100g	< LoQ	Vitamin B7 I Biotin	µg/100g	28.00	24:0	g/100g
Ornithine	g/100g	0.01	Vitamin B9 I Folic acid	µg/100g	180.00	Omega 3 (ω3) fatty acids	g/100g
Asparagine	g/100g		Vitamin B5 I Panthotenic acid	mg/100g	1.17		
GABA (gamma-Aminobutyric Acid)	mg/100g	402.30	Astaxanthin	mg / 100 g	n.d.	n.d. (not detected)	n.q. (not quantified)

Source: A4F, Portugal.

The biodiversity in microalgae is very large - it is estimated that there are from 200,000 to 800,000 species, of which only about 50,000 species are described, therefore this is a resource almost unexplored. More than 15,000 new compounds have been chemically

identified from microalgae biomass (Cardoso et al., 2018), of which carotenoids, proteins, peptides, fatty acids, polysaccharides, enzymes and sterols are noteworthy. These compounds have specific functional requirements and play important roles in terms of the cellular life, they can be simply natural dyes and/or exhibiting high biological activity and since they are chemically very active, their extraction poses a few problems related to stability against oxidation and other deterior phenomena. Therefore, conveyed by staple foods they can strongly impact health, with major benefits when consumed regularly. For this reason, it is important to address this subject from the point of view of the use of the whole microalgae cell, as a functional ingredient, in common or in staple foods. A strong argument to support this approach is that bioactive compounds are naturally encapsulated and protected within the microalgae cell. Other relevant issues are how these bioactives are going to be released from the cells and affected during food processing, and obviously, what is the final bioavailability and bioactivity of these powerful health improver substances.

With the expectation of a demographic burst from currently seven billion to nine billion people in less than three decades, the production of food, especially proteins, must be increased by about 70% to answer the population nutrition needs. The production of microalgae can be an interesting, complementary food production system, alternative to conventional agriculture, in the context of climate change, and the scarcity of water and farming land, with greater photosynthetic efficiency and low energy consumption. In addition, microalgae can achieve higher biomass productivity, faster growth rate, high rates of CO_2 fixation and O_2 production compared to conventional plants. These microscopic unicellular organisms can be cultivated under strictly controlled environmental conditions (e.g., pH, temperature, salt, light, nutrients), which may stimulate or inhibit the biosynthesis and accumulation of bioactive compounds (e.g., pigments) in amounts greater than those occurring in spontaneous growth situations (Gouveia et al., 2008a).

The commercial production of microalgae is limited to a reduced number of species (Tredici, 2004), and the most popular for food are *Chlorella vulgaris* and *Spirulina platensis*. The production of microalgae in lagoons and other open systems, despite having many advantages in terms of resource utilization, present some strong limitations in terms of safety and quality control of the final product, as they can be easily contaminated even with species that produce toxins. These limitations are especially important for food or pharmaceutical purposes and closed systems – photo-bioreactors (tubular: cylinders and sleeves with transparent walls of glass or plastic), that can be easily controlled, are taking the lead. These systems have much lower risks of contamination and accommodate the development of a specific microalgae, with a tight control of the culture medium, to meet the market specifications.

Microalgae have been used by indigenous populations for centuries (Jensen et al., 2001). However, the cultivation of microalgae has only existed for a few decades and among the 30,000 species that are surveyed, only a few thousand strains are kept in collections, a few hundred are investigated for chemical content, and just a handful are cultivated in industrial quantities (Olaizola, 2003).

Some of the most biotechnologically relevant microalgae are the green algae (*Chlorophycea*) *Chlorella vulgaris*, *Haematococcus pluvialis*, *Dunaliella salina*, *Tetraselmis chuii* and the blue cyanobacteria *Spirulina platensis* which are already widely commercialized and used, mainly as nutritional supplements for humans and as animal feed additives. Many studies are focused on the biological activities of natural products from microalgae (e.g.,

Plaza et al., 2009; Larsen et al., 2011; Freitas et al., 2012; Nicoletti, 2016; López et al., 2017; Sathasivam et al., 2017; Khan et al., 2018). They span from antioxidant to anti-angiogenic, cytotoxic and anticancer activities; reduction of cholesterolemia and diabetes; antimicrobial and anti-obesity activities as well as some toxic metabolites, with strong impacts including death, generally known as cyanotoxins including neuro, hepato and dermotoxins. The food grade microalgae are completely free of toxic effects, the problem can only arise from contaminations with other cyanobacteria and for this reason the use of closed systems for production, and an accurate quality control implemented, are necessary. Nevertheless, some of these toxins are being studied for antitumoral activity.

This section is focused on *Chlorella* and *Spirulina*, the most popular food grade microalgae:

- *Chlorella vulgaris* has been used as an alternative medicine in Asia since ancient times, where it is also known as a traditional food. It is widely produced and marketed as a food supplement in many countries, including China, Japan, Europe, Canada and the US. *Chlorella* is being considered as a potential source of a wide spectrum of nutrients (e.g., carotenoids, vitamins, minerals – see Table 1), being widely used in the healthy food market as well as for animal feed and aquaculture. *Chlorella* is long known as a health promoting factor on many kinds of disorders such as gastric ulcers, wounds, constipation, anemia, hypertension, diabetes, infant malnutrition and neurosis, with a preventive action against atherosclerosis and hypercholesterolemia by glycolipids and phospholipids, and antitumor actions by glycoproteins, peptides and nucleotides (Yamaguchi, 1997 and Plaza et al., 2009). However, the most important substance in *Chlorella* seems to be a beta-1,3-glucan, which is an active immune-stimulator, a free-radical scavenger and a reducer of blood lipids (Spolaore et al., 2006).
- *Spirulina platensis* (Arthrosphira) is a cyanobacteria that grows profusely in certain alkaline lakes in Mexico, Africa and India and has been used as food by local populations since ancient times (Yamaguchi, 1997). It is extensively produced around the world (5,000 tons/year) and broadly used in food and feed supplements, due to its high protein content (up to 70%) and its excellent nutritive value, such as high γ-linolenic acid level. In addition, this microalgae has various possible health promoting effects: the alleviation of hyperlipidemia, suppression of hypertension, protection against renal failure, growth promotion of intestinal *Lactobacillu*s, suppression of elevated serum glucose level (Spolaore et al., 2006, Plaza et al., 2009 and Sathasivam et al., 2017), anticarcinogenic effect and have hypocholesterolemic properties (Reinehr and Costa, 2006). Spirulina is also the main source of natural phycocyanin (blue colour extract), used as a natural food and cosmetic coloring, among other.

Phycocyanin is currently used in Japan and China as a natural coloring, in food products like chewing gums, candies, dairy products, jellies, ice creams, soft drinks (e.g., Pepsi® blue) and also in cosmetics such as lipsticks, eyeliners and eye shadows (Sekar and Chandramohan, 2007). In a previous study, phycocyanin was considered a more versatile blue colorant than gardenia and indigo, providing a bright blue color in jelly gum and coated soft candy, despite

its lower stability towards heat and light (Jespersen et al., 2005). A considerable number of investigations revealed several pharmacological properties attributed to phycocyanin including, antioxidant, anti-inflammatory, neuroprotective and hepatoprotective effects as well as antimicrobial potential. Plaza et al., (2009), Sathasivam et al., (2017) and Raja et al., (2008) demonstrated that this blue pigment stimulates hematopoiesis, emulates the effect of hormone erythropoietin and regulates the production of white blood cells.

1.2. New Trends in Microalgae Food Applications

The commercial production of microalgae for human nutrition is already a reality. In Portugal there are two main companies, one located in mainland, at Pataias, Leiria and the other in the Porto Santo Island, both producing food grade microalgae. The gastronomy people, scientists and chefs, are dedicating their creative knowledge developing attractive dishes with high amounts of microalgae inside. *Chlorella* and *Spirulina* in the dried powder form is readily available on every supermarket in Europe as well. In some countries (Germany, France, Japan, USA, China, Thailand), food production and distribution companies have already started serious activities to market functional foods with microalgae and cyanobacteria (Pulz and Gross, 2004). Food safety regulations for human consumption are the main constraint for the biotechnological exploitation of microalgae resources, but successful cases such as the approval (9 December, 2002) of the marine diatom *Odontella* aurita by Innovalg (France) as a novel food, following EC Regulation 258/97, are encouraging.

Over the last decade, our research group in Portugal has been extensively working to develop a range of novel attractive healthy foods, prepared from microalgae biomass, rich in carotenoids and polyunsaturated fatty acids with antioxidant effect and other beneficial properties (Gouveia et al., 2006; Gouveia et al., 2008a; Gouveia et al., 2008b; Batista et al., 2006; Batista et al., 2012; Batista et al., 2013; Batista et al., 2017; Raymundo et al., 2005). At the same time toxicological studies (Gouveia et al., 2013) involving the microalgae used were also conducted. A strategy to avoid the hassled of changing food habits was used adding the microalgae to staple and traditional foods, like mayonnaises, gelled desserts, biscuits, bread, pasta and breakfast cereals, largely consumed on daily basis on different European diets. The impact of natural substances introduced in the diet via "usual" foods is proved to be efficient at long term and do not present the drawbacks of traditional therapeutic actions based on medicines of short term impact.

The viability of incorporating microalgal biomass in food systems is conditioned by the applied processing type and intensity (e.g., thermal, mechanical), by the nature of the food matrix (e.g., emulsion, gel, aerated dough systems) and to the interactions with other food components (e.g., proteins, polysaccharides, lipids, sugars, salts). Besides colouring and nutritional purposes, introducing microalgae ingredients in food systems, can also impart significant changes in its microstructure and rheological properties (Batista et al., 2012). In addition, the microalgae biologically active compounds, such as the brain active PUFA: EPA and DHA, antioxidant colorants, etc. are naturally very sensitive to degradation when exposed. The option to work with the microalgae biomass and not with the bio-extracts, has two strong reasons. The bioactives are naturally encapsulated within the cell structure, which

contributes decisively to their preservation during food processing and, of course, it is very expensive to extract and preserve these bioactives. However, the question of how effectively/efficiently these bioactives are absorbed and metabolized comes at front. These aspects are particularly focused in our research.

In fact, it is important to highlight microalgae as an excellent source of natural bioactives including colorants, that present a high potential for their culinary application, at home or at industrial level, as a natural food dye. They present a wide diversity of colors and shades, which enhance their use in different types of food and gives an extra appealing strength.

2. MICROALGAE PRE-TREATMENT

The suitability of microalgae for use in various food and feed applications must be based on functionality parameters such as palatability, odour, nutritional profile, digestibility and bioavailability. However, cell wall integrity may significantly limit nutrient availability, since structurally the microalgae are covered with multiple layers of resistant thick cellular walls that hinder the liberation of cell constituents.

The controlled disruption of this cell wall (=cell disruption), resulting from the downstream process of biomass production, or induced by additional microalgae pre-treatment, has an important impact on the bioavailability of microalgae contents, although disruption of microalgae cells may also promote oxidation. Cell rupture have been described by several authors as a spectrum, ranging from minor damage of the cell wall with release of internal components to complete cell fragmentation.

A variety of cell disruption methods is currently available, but depending on the cell wall structure, the size, and the shape of algae, cell disruption can be challenging. Applicability on large industrial scale required cell disruption technologies to be low cost and energy efficient and result in high product quality. Employing an appropriate cell disruption technique, prior to extraction, is undoubtedly one of the most crucial preliminary steps in downstream processing for the maximum recovery of cell contents from microalgae (D´Hondt et al., 2017, Khan et al., 2018, Phong et al., 2018).

Researchers are focusing their interest in algae and algal products that may be used in beverages and functional foods (Khan et al., 2018) and nutraceutical and pharmaceutical products (Phong et al., 2018). To facilitate the release of the cell contents, the complex cell wall structure must be disrupted to allow the liberation of the internal components into the liquid medium. In a recent work, concerning rheological properties of microalgal suspensions as affected by mechanical and thermal processing (Bernaerts et al., 2017), the authors concluded that whereas some microalgae species hardly affect the structural properties of the food product, other microalgae species show large potential for use as a structuring agent in food applications, as it had been previously reported by our research team in different food systems, e.g., emulsions (Batista et al., 2006) and gels (Gouveia et al., 2008a; Batista et al., 2011). Differences in cell rupture were found between the investigated microalgae species, which can be related to size and rigidity of the cells. The effect of subsequent thermal processing on the microstructure and rheological properties is affected by the previous pretreatment, as thermal processing was shown to enhance interactions between the released cell material (Bernaerts et al., 2017).

Besides the structural morphology of each microalgal cell wall, the selection of the cell disruption method depends upon the nature of the desired product or final application. Exposure of the microalgae cells to harsh conditions, such as high temperature, high shear or high pressure, might change the structure of some fragile intracellular compounds, with subsequent impact in their functionality or activity. For example, gentle breakage is necessary to promote the release of bio-functional proteins and peptides from microalgae, while preserving the functionality and biological activity of the molecules. Thus, the current research attention toward microalgae should focus on mild disruption methods, to release various types of intracellular components without degradation (Phong et al., 2018).

2.1. Cell Disruption Methods

Depending on the purpose of the pretreatment chemical, biological, thermochemical, or thermophysical methods are used, sometimes in combination, but there is still no optimal, highly productive method, even for biofuels production (Khan et al., 2018). Recently, the most used and promised methods have been reviewed, for instance, by Günerken et al., (2015), Kim et al., (2016), D´Hondt et al., (2017), Lee et al., (2017), Khan et al., (2018) and Phong et al., (2018). To select the most appropriate method, factors such as yield, cost, quality and bioactivity should be considered, but also sustainability, environmental pollution and residues.

Pretreatment of algae biomass involves the degradation or disruption of biomass (algae wall lysis) to convert, accumulate, and process the constituents it contains. Among those constituents, large-scale utilization of lipids is the most desirable alternative to plant-based lipid sources, although biodiesel production is not yet economically viable (Lee et al., 2017). Fermentation of the algal sugars are used to generate bioethanol. Pretreatment of the algal biomass for fermentation involves the release of the carbohydrates from inside the cells and saccharification of the accumulated sugars to monomeric sugars to be used by fermenting microorganisms. Thus, mechanical methods of pretreatment yield biodiesel, while enzymatic and chemical methods are used in bioethanol production, because fermentative bioethanol production requires degradation of cellulose, hemicellulose and starch (Khan et al., 2018).

Chemicals may influence the quality of the products due to the formation of by-products and, additionally, a pretreatment applied to release one fraction can solubilize and degradate other biomass components. Enzymes achieve high product quality but still remain expensive and oxidizing agents could promote oxidation of sensitive compounds (D´Hondt et al., 2017).

Physical methods for cell wall disruption are preferred, in most cases, as this avoids chemical contamination of the bioproducts and preserves most of the functionality of intracellular material (Khan et al., 2018). Physical pretreatment could be subdivided into thermal: freeze-fracture, freeze-drying and autoclaving (D´Hondt et al., 2017) and mechanical methods.

The most traditional physical pretreatments (Figure 1) include bead milling, high-pressure homogenization, microwave irradiation and ultrasonication. Bead milling and high-pressure homogenization are industrially well known for the extraction of algal products, but are less favourable as mild processes. Microwave and ultrasound greatly reduce working times, increase yields and often the quality of the extract (D´Hondt et al., 2017).

Bead milling causes direct mechanical damages to cells, with high disruption efficiency, high biomass loading, commercial available equipment and easy scale-up operations (Günerken et al., 2015). It is composed of a rotating agitator, in a fixed vessel, filled with beads. The grinding effect is achieved through the physical collision of the solid beads against the cells, through compressive and shear stresses. The disruption efficiency depends on the size and composition of the beads, the speed and design of the agitator, residence time and characteristics of the suspension such as viscosity and concentration (Lee et al., 2012). The energy transfer from the rotating shaft to the beads and the energy conversion into heat due to friction would lead to a rise in temperature. However, with recent design improvements it is possible to control the temperature as the milling chamber is equipped with cooling jackets (Lee et al., 2017, Phong et al., 2018). Thus, bead milling is an interesting cell disruption method with high potential for industrial use. Bead mills have been successfully applied for the disintegration of microalgae for the release of intracellular products under low energy inputs and mild conditions (Postma et al., 2015, 2017).

High-pressure homogenization or French press is one of the earliest techniques developed to disrupt algal cells. Microalgal suspension is pumped through a narrow orifice (80-200 µm) in a valve under high pressure (138-400 MPa), and the suspension is then released into a low-pressure chamber (D´Hondt et al., 2017). The cell suspension flows radially across the valve, strikes an impact ring, exits the valve, and flows to a discharge hole (Günerken et al., 2015). Mechanical effects, including turbulence, shear stress and cavitation promote cell lysis. The disruption efficiency is a function of the pressure at the valve and the cell-suspension properties, such as viscosity, cell size, cell concentration (Lee et al., 2017). Other operating parameters, which affect the disruption efficiency, are the temperature, number of passes, the valve and orifice design and the medium flow rate. In addition, for heat sensitive products, cooling is essential (Lee et al., 2012).

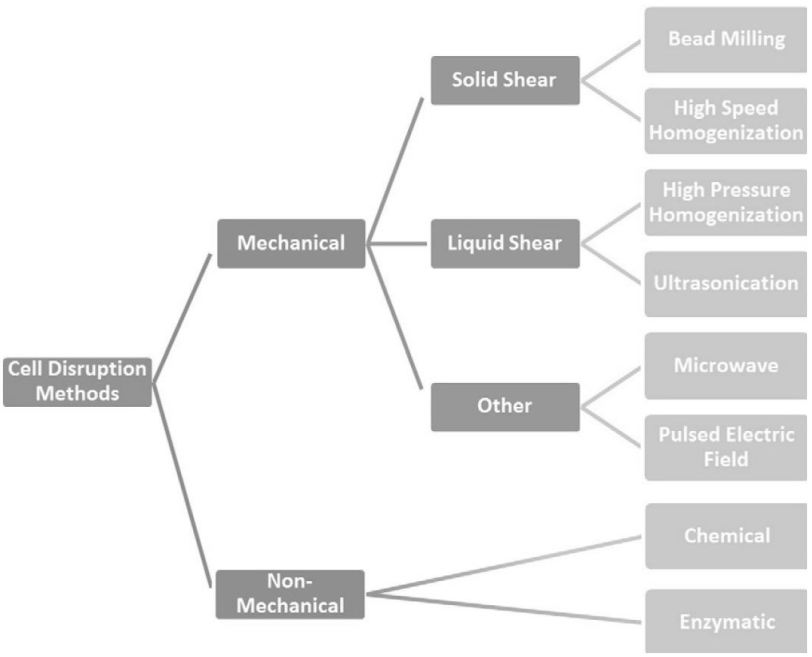


Figure 1. Classification of the cell disruption methods (Günerken et al., 2015).

Microwave treatment is a no-contact method that transfer radiation to the biological material. The mechanism is based on the interaction of electromagnetic waves with dielectric and polar molecules provoking local heating because of frictional forces from inter and intramolecular movements. Biomass concentration, treatment time and power of microwaves are the main operating parameters (Günerken et al., 2015, D´Hondt et al., 2017). The intracellular heating causes the water vapour to disrupt the cells from within - electroporation effect (Lee et al., 2012). Several studies indicate that microwave irradiation is highly effective as a microalgal extraction method.

The severity of thermal treatment induced by some methods depends on several factors, such as particle size, temperature, pressure and time. Severe conditions result in higher digestibility but also in higher bioproduct degradation. Ultrasound pretreatment induces degradation of microalgal compounds through cavitation, although temperature control could reduce this degradation, but also the process effectiveness. Microwave irradiation provides high temperature control but has high operational costs, however, studies for scale-up would be necessary (D´Hondt et al., 2017).

The emerging technologies for cell disruption are continuous explosive decompression, where carbon oxide or nitrogen is introduced in a pressure vessel under high pressure, microfluidization, which functions as a high-pressure homogenizer, and pulsed arc technology, such as pulsed electric fields and high voltage electrical discharges. Extraction and immobilized (bio)chemical agents are also important since they operate at low temperature and avoid solvents (D´Hondt et al., 2017, Phong et al., 2018). From a practical point of view, it is imperative to optimize the balance between product yield, processing cost and energy consumption (Phong et al., 2018).

2.2. Impact of Cell Disruption on Rheological Properties of Wheat Bread with *Chlorella vulgaris* Incorporation

The cell wall of *Chlorella* varies among species and strains and depends on growth conditions. Generally, the inner cell wall has high cellulose content and the outer cell wall may include algaenan which is a highly resistant aliphatic polymer (D´Hondt et al., 2017).

The use of microalgae as a functional ingredient in food has been limited to a narrow niche market and the research on food products with microalgae is still considered as scarce. This work is part of a project that intends to explore the microalgae potential to be a low-carbon-footprint healthy food ingredient for future foods. One of the objectives is to explore the potential to increase nutritional quality of bread by addition of microalgae biomass, while maintaining its high sensory quality.

Bernaerts et al., (2017) suggested that *Chlorella vulgaris* (Cv) find applications as a multifunctional ingredient in food products which are processed mechanically and/or thermally. In our working group, the use of Cv as a food ingredient has been demonstrated to be a promising way to enrich a staple food like bread in bioactive compounds, but a technological limitation for biomass incorporation higher than 3.0 g Cv/100 g of wheat flour was noticed (Graça et al., 2018).

In the present study, Cv biomass (approved as food grade by European Food Safety Authority, with 60.7 Protein, 13.8 Carbohydrates and 2.3g Fat / 100g) was pre-processed

using microwave irradiation as cell disruption method. The morphological changes on the cells were observed by optical and scanning electron microscope (SEM). Since cell disruption promotes the release of intracellular products it can impart structure modifications on bread, resulting from affecting the rheology properties of wheat doughs, therefore breads prepared with addition of microalgae (3% w/w) were investigated. The impact of disruption on the bread antioxidant capacity was also studied.

2.2.1. Morphological Changes on the Cells

Microalgal suspensions can be described as complex systems consisting of algal cells and cell debris that are dispersed in a continuous phase containing water, dissolved salts and polymeric compounds. Gerken et al., (2013) reported for *Chlorella vulgaris* that rigid wall based on cellulose are embedded within a more plastic layer composed of uronic acids, rhamnose, arabinose, fucose, xylose, mannose, galactose, glucose and pectin. The most common skeletal polysaccharide is cellulose, but during maturation the cell gradually increases in thickness due to a chitosan-like layer (Safi et al., 2015). Cv appears as spherical cells of various sizes, ranging from 1 to 3 µm (Lee et al., 2017). When Cv is grown under favourable conditions, it is capable of accumulating 1-2% chlorophyll of its dry weight. This pigment gives it the dense green colour and is in the chloroplast (Safi et al., 2015).

Some other authors studied the impact of microwave in microalgae cells disruption. Heo et al., (2017) investigated the possibility of the utilization of both lipids and glucose from Cv, and microwave irradiation appeared to be a promising method, damaging the cells severely. In other study (Silva et al., 2014), microwaving achieved the highest lipid extraction efficiencies for mixed cultures of microalgae. Cheng et al., (2013), found that pectin and cellulose structures are severely damage. The disruption level of microalgal cell walls was enhanced when temperature increased. The spherical structures of microalgal cells subjected to microwave treatment at 100ºC for 5 min were completely destroyed. The cells size showed negligible changes with treatment time, but the cell wall thickness and pore diameters in cell walls increased, the outer pectin layers of cell walls gradually detached, and the porosity of inner cellulose layers increased with microwave treatment time. After 30 min, the microalgal cell walls were completely disrupted and all the microalgal cell contents flowed out.

From our microscopic images (Figure 2), some damage on the microalgae cell walls is observed in commercial Cv since it was dehydrated via spray-drying during the downstream process. In light microscope images, aggregation of these commercial Cv cells can be observed. After microwave treatment, there was a reduction on the number of cells and disintegration of the aggregates, although small size colonies are still present. Different sizes of cells should be related with a size distribution observed within Cv at various stages of the reproductive cycle. Comparing SEM images with observations of *Chlorella* sp. obtained by Heo et al., (2017), for several cell disruption methods including microwave, and Spiden et al., (2013), after treatment with high-pressure homogenization, our SEM images suggest that the cell walls have not suffered great extent of damage after microwave treatment at 540W-5min and confirm the disintegration of the microalgae agglomerations. However, a small hole in the cell wall is sufficient to extract content from the cells (Heo et al., 2017), but with the magnification used in SEM observations, we were not able to conclude about that.

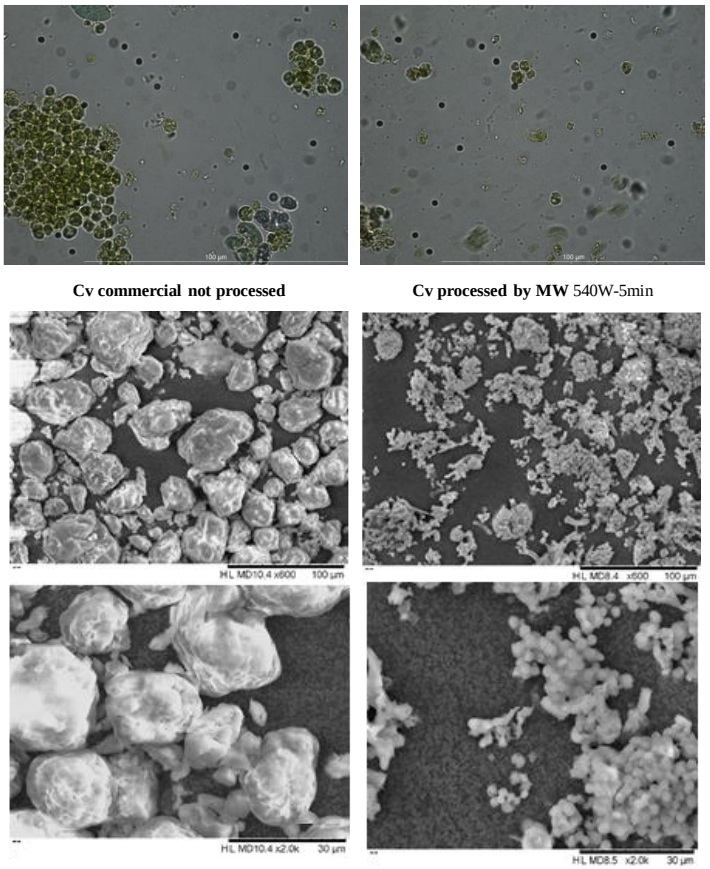


Figure 2. Light microscope (Leica DMR) images (above) and SEM (Hitachi TM 3030 Plus, EDX – Mix mode, after freeze-drying of the suspensions) images (below) of *Chlorella vulgaris* cells suspensions (1g/100mL) processed (images on the right) or not (images on the left) by microwave irradiation.

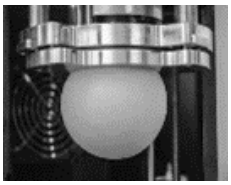
2.2.2. Impact on Rheological and Colour Properties of Wheat Doughs and Breads

Doughs were characterized by empirical rheology methods (alveograph and texturometer – penetration and extensibility tests) and by fundamental small amplitude oscillatory stress (SAOS) rheology through frequency sweeps after fermentation. In the loaves, colour, appearance and puncture tests during the aging process were used to investigate the impact of cell disruption method on bread production.

In the Alveograph test (AACC 54-30.02) the water level added to the flour is fixed (50% hydration), and after mixing, the dough is extruded and shaped. After a resting period, a biaxial extension is applied during dough bubble inflation. The height of the peak relates to the resistance of the dough to deformation (maximum overpressure P), while the length of the curve relates to its extensibility (L). The curve configuration ratio (P/L) expresses the equilibrium between the tenacity and the extensibility. A P/L ratio of about 1.0 is preferred for the baking of raised breads, P/L ratios of about 0.2 correspond to weak and extensible doughs, suitable for cake and cookie production, while P/L ratios of 2 or higher indicate that the dough lacks the desired degree of extensibility (Preston and Williams, 2003). W is the dough baking strength and this area is generally much larger for hard wheat flours than for soft wheat flours. P and W values of wheat dough had a significant decrease ($p < 0.05$) with

the microalgae addition (Table 2), attributed to the reduction of gluten strength (Graça et al., 2018). However, when microalgae cells are disrupted by microwave (Cv-MW) an increase in P and P/L values was observed.

Table 2. Alveograph parameters of wheat doughs with Chlorella vulgaris incorporation, according to AACC 54-30.02 method. Different letters in the same column correspond to significant differences ($p < 0.05$)



	P (mm H_2O)	L (mm)	P/L	W (x 10^{-4} J)
Control	85 a	59 a	1.46 a,b	182 a
Cv	61 b	51 a,b	1.22 a	100 b
Cv-MW	75 c	43 b	1.79 b	109 b

Control wheat flour and Cv doughs were also prepared using the water absorption values from the farinographic analyses (AACC 54-21.02). Water absorption increased from 61.8% for wheat flour to 64.3% with 3% Cv addition. This may be related with the extra protein content from the microalgae, since high water absorbing capacity of microalgae and other protein sources has also been attributed to their ability to compete for water with other constituents in the dough systems (Dervas et al., 1999, Doxastakis et al., 2002, Graça et al., 2018). All the formulations include 1.7% salt, 0.5% SSL (E481), 4.0% dehydrated yeast and 1.0% sugar (in relation to flour + Cv content).

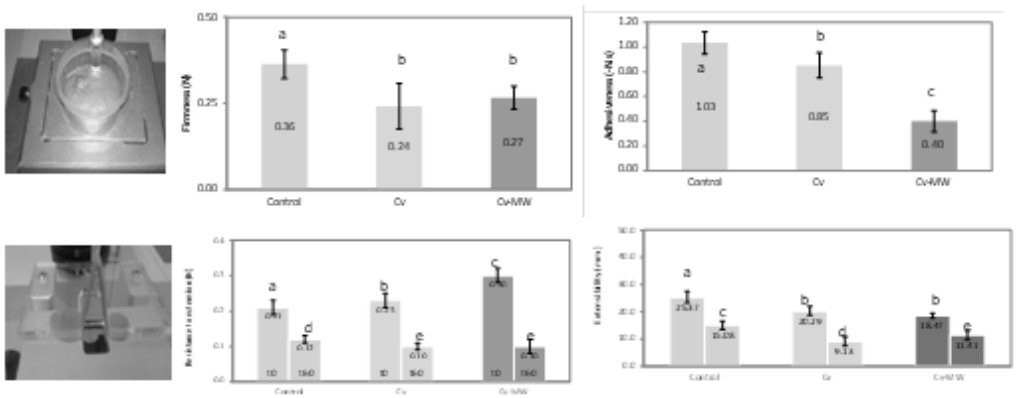


Figure 3. Firmness and adhesiveness values obtained from texture profile analysis (TA.XTplus, 10 mm cylindric probe) after dough fermentation. Resistance to extension and extensibility values obtained from extensibility tests (Kieffer dough rig). Different letters in the same graph correspond to significant differences ($p<0.05$).

After fermentation at 37ºC for 60 min, doughs were characterized by penetration, in a puncture test, and extensibility tests (Kieffer Dough rig) in a texturometer TA. XTplus. Firmness values of control dough are higher than Cv and no significant differences ($p < 0.05$) are noticeable with microwave microalgae cells treatment. However, there was a decrease in adhesiveness values for Cv-MW (Figure 3). Cv-MW dough showed a stronger resistance to extension and similar extensibility at t0 (without fermentation), in accordance to the alveographic results (P and L parameters). Fermented dough (t60) Cv and Cv-MW showed

similar resistance to extension, lower than Control, but Cv-MW presented greater extensibility than Cv, i.e., better bread-making quality. Baking performance may be measured by means of dough resistance and stretching ability ratio – R/E. Fermented loaves Cv-MW and control showed no significant differences ($p < 0.05$) R/E numbers (8.6 and 8.2 × 10^{-3} N/mm, respectively), slightly lower than for the Cv dough (10.8 × 10^{-3} N/mm).

Frequency sweeps were performed to evaluate the impact of Cv microalgae addition on dough structure after fermentation (Figure 4). Doughs have a viscoelastic behaviour with G´>G´´ and a destructuring effect with microalgae addition (3% Cv) was probably due to the disruption of the gluten matrix, as previously suggested by Graça et al., (2018). The frequency dependence of G´ (elastic modulus) and G´´ (viscous modulus) could be described by the power law equation:

$$G' = \alpha' f^{b'} \quad \text{(Eq. 1)}$$

$$G'' = \alpha'' f^{b''} \quad \text{(Eq. 2)}$$

Values of α and b are determined by performing a linear regression on log G' and G´´ versus log frequency, where α´ and α´´ are the y-intercepts and b´ and b´´ are the slopes of the resulting line (Bernaerts et al., 2017). According to b´ and b´´ values all the materials have a similar level of structure, but the magnitudes of α´ and α´´ decreased with Cv incorporation (Table 3), reflecting that the gluten network got weaker, in agreement with firmness and resistance to extension (t60) texture parameters.

In respect to cell disruption, Bernaerts et al., (2017) observed for Cv suspensions no reliable value of G´ upon High-pressure homogenization (HPH) at pH 6, concluding that rheology of the control suspensions was pH-dependent. The pH of our doughs varied between 5.2 and 5.4 and the results are in line with that study since microwave cell disruption had no significant impact ($p < 0.05$) on the dough´s viscoelastic behaviour.

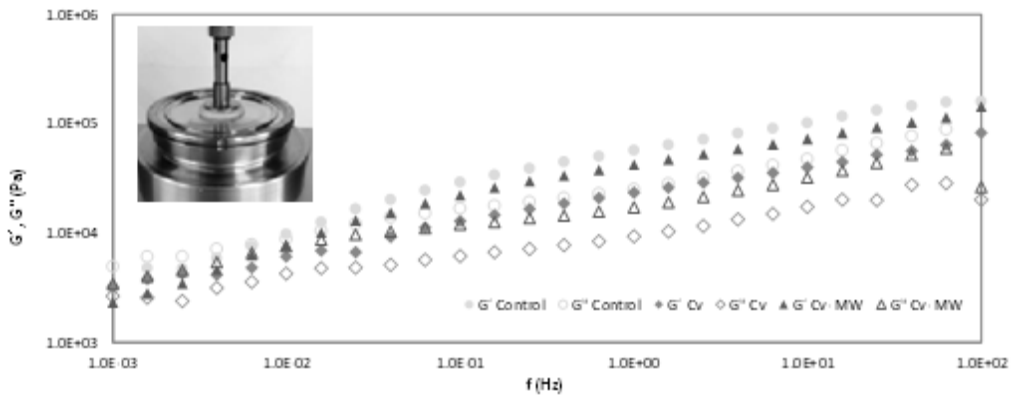


Figure 4. SAOS rheology (Haake Mars III) - Mechanical spectra at 5ºC of fermented dough.

Dough and bread colour was measured with a colorimeter and results are expressed in the CIELAB system - L* (lightness), a* (greenness/redness) and b* (blueness/yellowness) (Table 4). Cell disruption had no significant impact ($p < 0.05$) on bread colour, while Cv dough

presented higher negative a* (green hue) than Cv-MW. The total colour difference ΔE* between Cv and Cv-MW is equal to 3.7 in the dough and 1.2 in the bread. Some authors consider that total colour differences bellow 1 are not obvious for the human eye, for 1<ΔE*<3 values colour differences are not appreciative, being ΔE*>3 the threshold value for obvious colour differences (Francis and Clydesdale, 1975), while others consider than the human eye is only able to differentiate colours for ΔE*>5 (Castellar et al., 2006).

Table 3. Frequency dependence of G´ and G´´ described by the power-law parameters. Different letters in the same column correspond to significant differences ($p < 0.05$) and $R^2 > 0.95$

	a´	b´	a´´	b´´
Control	49256 a	0.318 a	26617 a	0.270 a
Cv	25721 b	0.290 a	12497 b	0.219 a
Cv-MW	30709 b	0.301 a	15206 b	0.235 a

Table 4. CIELAB colour parameters of dough and bread. Different letters in the same column correspond to significant differences ($p < 0.05$)

	Dough colour			Bread colour - Crumb		
	L* (%)	a*	b*	L* (%)	a*	b*
Control	79.68 ± 0.35 a	0.82 ± 0.09 a	20.16 ± 0.63 a	67.48 ± 1.83 a	(-0.80) ± 0.13 a	15.72 ± 1.16 a
Cv	45.57 ± 1.93 b	(-13.04) ± 0.37 b	21.00 ± 0.92 a	40.79 ± 0.56 b	(-3.74) ± 0.13 b	16.16 ± 0.34 a
Cv-MW	44.44 ± 1.29 b	(-11.44) ± 0.55 c	24.18 ± 1.30 b	39.66 ± 0.55 b	(-3.54) ± 0.19 b	16.35 ± 0.39 a

Texture characterisation of breads was performed using a puncture test in penetration mode (Bourne, 2002). The evaluation of crumb firmness during the storage time at room temperature aimed to observe the effect of microalgae biomass on the kinetics of bread aging.

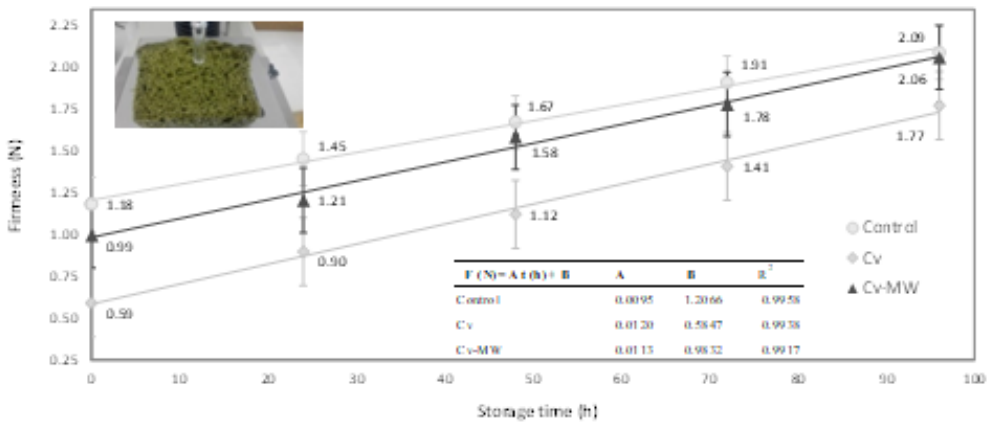


Figure 5. Firmness values obtained from texture profile analysis (TA.XT plus, 10 mm cylindric probe) during bread aging (0, 24, 48, 72 and 96 h).

From Figure 5 a positive linear relation between bread firmness and storage time can be observed, according to a linear equation:

$$\text{Firmness} = A \times \text{time} + B \quad \text{(Eq. 3)}$$

where, A is the aging velocity and B the initial firmness. A similar aging velocity (A) was obtained for all breads, but crumb firmness is significantly ($p<0.05$) affected by microalgae concentration with Control (1.18N) > Cv-MW (0.99N) > Cv (0.59N) at t0.

After 96 h of storage, firmness of bread with microwaved Cv cells is closer to the control and both higher than Cv, demonstrating the positive impact of the microalgae cell disruption in the bread texture. In fact, Bernaerts et al., (2017) observed no reliable value of G´ for Cv suspensions upon HPH at pH 6, but subsequent sterilization of the suspension resulted in a large increase of G´, implying that a stiff network structure is created by an intense thermal treatment. Indeed, thermal processing might damage cell walls and result in solubilization of components and agglomeration/aggregation of cell debris. As Cv biomass contains high protein content, this result was ascribed to aggregation of the denatured proteins.

2.2.3. Impact on the Bread Antioxidant Capacity

Antioxidants are very important substances used by the human body to protect itself from the hazardous effects of free radicals, preventing many severe diseases. Microalgae are one of the richest and most economical sources of natural compounds with a strong antioxidant effect. Green microalgae *Chlorella* has an antioxidant activity thanks to the high content of chlorophylls (a and b) and vitamin E. As reported by Lanfer-Marquez et al., (2005), chlorophylls can inhibit the DPPH radical.

To evaluate the antioxidant compounds from breads, bread samples have been air-dried at room temperature, crumbled fined into homogeneous powder using an electric blender, and shacked during 24 h in 80% methanol at the room temperature. After the filtration, the solvent was evaporated *in vacuo*. Dried extracts were dissolved in DMSO to obtain 100 mg/mL (stock solution) and stored at -4°C until the experiments. Bread extracts were tested for the scavenging effect on the DPPH radical (Sánchez-Moreno et al., 1998). Extract solutions, in 10 μL volume each, were added to 100 μL (90 μmol/L) DPPH solution in methanol and the mixture was diluted with 190 μL of methanol. In control, the exact amount of the extract was substituted with solvent, and in a blank probe, only methanol (290 μL) and extract (10 μL) were mixed. After 1 h absorbance was measured at 515 nm. The percentage of *Radical Scavenging Capacity (RSC)* was calculated using the following equation*:*

$$\text{RSC}\ (\%) = 100 \times (A_{blank} - A_{sample}) / A_{blank} \quad \text{(Eq. 4)}$$

where, A_{blank} was the absorbance of the control reaction and A_{sample} was the absorbance of the examined samples, corrected for the value of the blank probe. Synthetic antioxidants, ascorbic acid and Trolox were used as a positive control.

Compared to the control bread, the incorporation of microalgae led to an increase in the antioxidant capacity of the microalgae-based breads. As expected, bread with *Chlorella* cells pretreated with microwave irradiation showed a higher antioxidant capacity compared with bread with commercial Cv (Figure 6). Even upon baking, the RSC values are interesting, especially when microalgae cells were disrupted by microwave (46.77% compared with

54.27% and 67.27% of Trolox and C-vitamin, respectively). Batista et al., (2017) have studied the incorporation of several microalgae in cookies, concluding that all microalgae-based cookies showed higher total phenolic content, and in vitro antioxidant capacity, compared to the control.

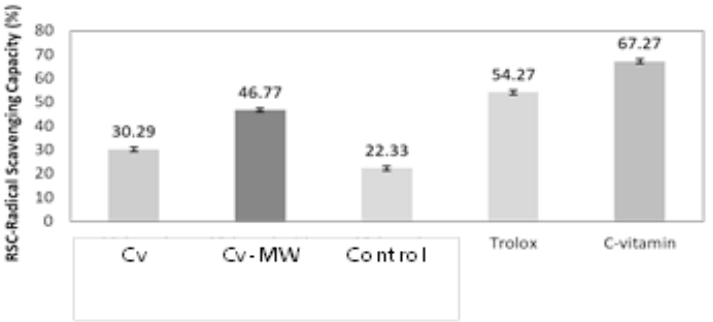


Figure 6. Scavenging effect on the DPPH radical of Cv, Cv-MW and control breads.
Synthetic antioxidants, trolox and ascorbic acid, were used as a positive control. Absorbance was measured at 515 nm. RSC (%) = 100 × ($A_{blank} - A_{sample}$) / A_{blank}.

A recent study of Shen et al., (2018) provides useful information towards understanding and manipulating Maillard Reaction products formation to maximize antioxidant potential in white bread products, which may explain the RSC value obtained for the control bread (22.33%). These authors reported that breads with sucrose or fructose had a good balance of antioxidant capacities.

Concluding this section, it is important to note that a significant improvement in the nutritional composition of the wheat bread with *Chlorella* substitution was observed. This microalgae can be used as one innovative ingredient to enhance functional and technological properties of breads. Differences between bread produced with ruptured cells and bread with microalgae cells, without pretreatment, were found. Thermal processing of breads, after pretreatment of microalgae suspensions using microwave irradiation, should enhance interactions within released cell material.

Microalgae pretreatment contributes to a better baking performance of breads fortified with Cv biomass. An increase in P and P/L alveographic values was observed. Fermented loaves Cv-MW and control sample showed not significantly different ($p < 0.05$) R/E numbers, slightly lower than for the Cv bread. Bread with disrupted Cv cells showed higher firmness than bread with Cv, closer to the control. However, cell disruption by microwave had reduced impact on dough texture, slightly lower adhesiveness and similar firmness, and similar viscoelastic behaviour was found for Cv and Cv-MW doughs.

3. Impact of Processing on the Microalgae Performance as a Food Ingredient

Taking into account the diversity and complexity of bioactive compounds present in microalgae and consequent ability to color food, their incorporation into food matrices determine the final characteristics of the products, such as:

- Color stability, whether in terms of culinary or in terms of industrial processing, it is essential to ensure that the color achieved through the incorporation of a certain microalgae remains stable throughout the shelflife period of the product. This aspect is of great relevance either in industrial products or in the catering industry, since it can be assumed as an important acceptance/rejection factor.
- Considering the high concentrations of macromolecules present in microalgae, namely proteins and polysaccharides, which in some species, such as *Spirulina*, can sum up to 60% of the total weight of the microalgae (Batista et al., 2013), the incorporation of microalgae biomass in food matrices necessarily induces structural changes. Thus, it is fundamental to adjust the recipes or formulations that include microalgae, in order to account for the contribution of these macromolecules to the final structure of the product.
- According to the type of processing involved in the preparation of the food product (time and temperature combinations, mixing processes - mechanical energy, shear and high pressure processing, etc.), the degradation of some of the microalgae bioactive compounds can occur. Therefore, it is fundamental to optimize the culinary or processing technologies to preserving the maximum activity of the bioactive compounds. In this chapter, this issue will be highlighted in foods with very different processing techniques: emulsions (mayonnaises), gels (puddings), biscuits, pasta and bread.

3.1. Antioxidant Activity Microalgae Biomass on Food Emulsions

Food emulsions are thermodynamically unstable systems formed by two immiscible liquids and an interface. Generally, two types of single emulsions can be considered: oil in water (o/w) emulsions, where the oil droplets are dispersed in the aqueous phase and water in oil (w/o) emulsions for which water droplets are dispersed in the oil phase. Combinations of these pairs are possible into multiple emulsions, where emulsions are dispersed concomitantly. Emulsion stabilization is achieved by the addition of emulsifying agents, with an important role in reducing the surface tension of the interface, often including the addition of texture modifiers, with a contribution to the viscosity increase of the continuous phase.

Emulsifiers are surface active molecules with amphiphilic properties - they have both hydrophilic and hydrophobic residues and position themselves in the oil/water interface, therefore reducing the surface tension. In food emulsions, either macromolecules, such as proteins, or smaller surfactant molecules, can be used. When adsorbed at the interface, proteins will form a strong viscoelastic film around the droplet and they will adopt a conformation, which allows the hydrophobic domain to interact with the oil phase and the hydrophilic domain to be inside the aqueous phase, preventing the coalescence of the droplets, contributing to the emulsion stabilization. Traditionally, egg yolk is used as the emulsifier to produce mayonnaise and salad dressings and other hydrocolloids, such as guar gum and xanthan gum, can be used to stabilize the emulsion and to reduce the oil content in low-fat emulsions. However, alternative proteins have been studied as emulsifiers, with advantages in terms of sustainability and nutritional aspects, like pea or lupine proteins (Raymundo et al., 1998). The addition of xanthan gum to reduce the oil content was also

investigated, with a positive impact on the nutritional profile of these emulsions (Raymundo et al., 2002).

The improvement of the nutritional profile of the mayonnaise-like emulsions can be achieved by the addition of several ingredients that contribute to the increase of the bioactive molecules and simultaneously do not disturbed the emulsion stability. Several condiments such as aromatic herbs, oils or seeds can be considered for this purpose. The addition of microalgae as a color ingredient for food emulsions will also contribute to the improvement of their functional behavior. Raymundo et al., (2005) verified that the incorporation of microalgae biomass into food emulsions may not destabilize its structural properties and, depending on the nature of the microalgae, could even present a synergistic effect, increasing emulsions firmness and reducing the oil content of the emulsion, acting as a fat-mimetic agent. Gouveia et al., (2006) prepared pea protein emulsions with incorporation of *Chlorella vulgaris* (green and orange, after carotenogenesis) and *Haematococcus pluvialis* (red, carotenogenic) biomass and evaluated their antioxidant activity. The primary and secondary oxidation products of the emulsions were evaluated, and an enhanced resistance to oxidation was evidenced by emulsions containing microalgae.

It is important to refer that the lipid oxidation is a major cause for quality loss in food emulsions (Mc Clements and Decker, 2000) due to the formation of undesirable volatile compounds, off-flavors (rancidity) and potentially toxic reaction products (Halliwell et al., 1995) which contribute to consumer's rejection.

Microalgae biomass are composed by multi-component antioxidant systems, which are generally more effective due to synergistic or additive interactions between the different antioxidant components (Decker, 1998; Kiokias and Gordon, 2003). Carotenoid pigments, which are the main antioxidant components, under certain conditions, can retard lipid oxidation in a lipidic food matrix, such as a mayonnaise, acting as antioxidants (Bast et al., 1998; Nenadis et al., 2003). The mechanisms associated to the antioxidant performance, in multiphase systems depend on the impact on the interfacial properties and the inhibition of lipid oxidation in bulk (Mc Clements and Decker, 2000).

Considering the results obtained by Gouveia et al., (2006), the impact of microalgae biomass on the formation of primary oxidation compounds (peroxide value) in o/w pea protein emulsions can be illustrated on Figure 7 The addition of *Chlorella vulgaris* (Cv) and *Haematococcus pluvialis* (Hp) to the emulsions tested in strong oxidative conditions (rancimat equipment), yielded smaller values of primary oxidation products in high oxidative environment. However, a considerable increase of oxidation products in the 3rd week for all the emulsions, namely for the control (without microalgae biomass addition) was verified. In the 6th week, primary oxidation compounds decreased, probably due to the conversion into secondary oxidation products. This was confirmed by the results from Table 5, that reveal an increase of secondary oxidation compounds from the 1st to the 6th week, assessed by the p-anisidine value.

From the results of Table 5 it is also possible to observe that the incorporation of Hp and Cv green microalgae biomass provided a higher oxidation stability over time, in comparison with Cv orange. Nevertheless, Cv orange revealed lower peroxide value, suggesting a rapid transformation of the primary oxidation compounds into the subsequent secondary ones.

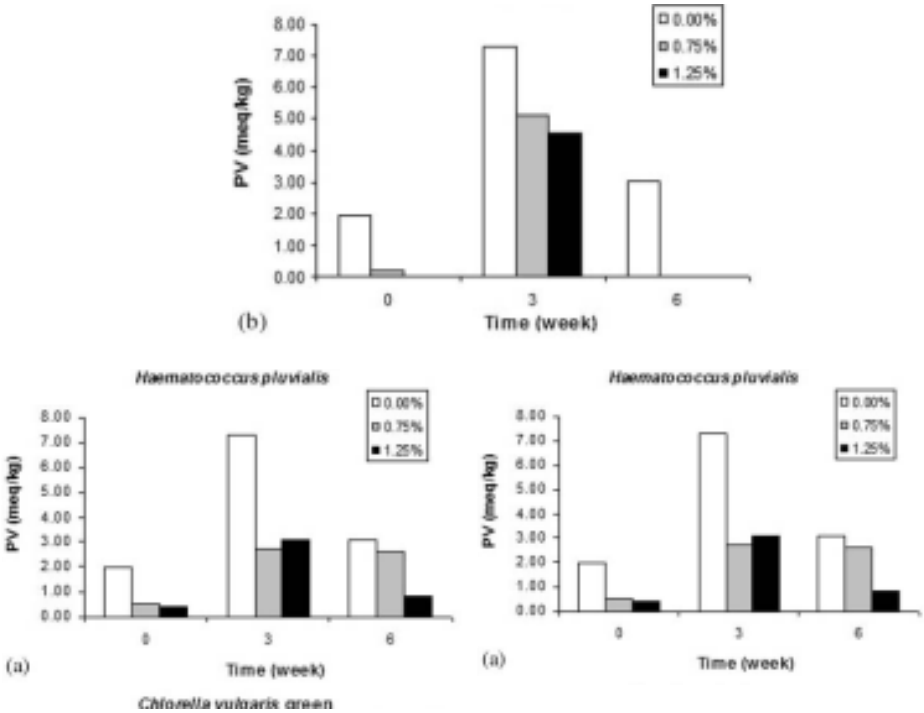


Figure 7. Peroxide value (PV), from oil-in-water emulsions with: (a) *Haematococcus pluvialis,* (b) *Chlorella vulgaris* green and (c) *Chlorella vulgaris* orange biomass (Gouveia et al., 2006 - adapted).

The studied microalgae have a predominance of different pigments, which could justify their different behaviour as an antioxidant. Astaxanthin is the predominant pigment in Hp (Gouveia and Empis, 2003) and is recognized as one of the most effective antioxidant compounds (Yen and Chen, 1995), canthaxanthin is the dominant pigment of Cv (orange), and lutein the main of Cv (green) (Gouveia et al., 2006). Hp showed a better performance as an antioxidant, resulted from the production of lower primary and secondary oxidation compounds, which should be related to the higher pigment concentration and to the effectiveness of astaxanthin as antioxidant agent.

The use of microalgae biomass as an ingredient to produce food emulsions can be an interesting way to prevent the oxidation process associated to this type of products. In addition, these emulsions presented creative colours and a potential positive impact on health, resulted from the presence of the antioxidant compounds.

Table 5. Secondary oxidation products concentration: p-anisidin value, from oil-in-water emulsions, incorporated with Haematococcus pluvialis, Chlorella vulgaris green and Chlorella vulgaris orange biomass (Gouveia et al., 2006 - adapted)

Biomass concentration (% w/w)	1st week		6th week	
	0.75	1.25	0.75	1.25
Haematococcus pluvialis	1.04	0.32	4.05	4.84
Chlorella vulgaris green	0.97	0.95	4.59	2.41
Chlorella vulgaris orange	1.18	1.18	5.73	6.23

The emulsification process occurred at room temperature, as the result of speed and time. For similar emulsifying conditions, the increase of temperature was monitored and 55ºC was the maximum value achieved (Franco et al., 1998). This temperature should have a small impact on antioxidant stability. In addition, the mechanical energy should impart a certain degree of cell wall disruption, which could be associated with a controlled release and availability of the antioxidant compounds.

3.2. Incorporation of Microalgae as a Source of Omega-3 Polyunsaturated Fatty Acids in Pasta Products

Pasta is an Italian word to describe an extruded and dried hard wheat product. After cooking it is a universally enjoyed food, easy to store and manufacture: it is easy to cook, and is rich in proteins and complex carbohydrates. Traditionally, pasta is obtained from a mixture of water and semolina wheat flour, in a specific proportion. Semolina has a lower starch content and a higher protein content than flour, and after cooking is easily digested. Several ingredients can be added to pasta dough to enhance the nutritional profile and to provide functional activity. Eggs are commonly added to improve color and to increase protein content. Vegetables such as spinach, beetroot, tomato, and carrot, can also be added for color and taste. In recent years, the addition of herbs and spices such as garlic, basil, and thyme has become popular. The addition of different species of microalgae biomass has also been investigated, in order to promote pasta bioactivity.

Zouari et al., (2011) evaluated the effects of semolina enrichment with blue-green algae (*Spirulina platensis*) free radical scavenging activity and observed that microalgae addition resulted in higher swelling index and lower cooking loss than the control sample, revealing the structural reinforcement resulted from the biomass incorporation. Similar results were obtained by Özyurt et al., (2015) for the same algae. In addition, an increase in the free radical scavenging activity was observed, comparing to the control (with no microalgae incorporation). This behavior results from the *S. platensis* free radical scavenging activity, due to its higher content on vitamin E, carotenoids, chlorophyll and phycobiliproteins, which are able to decrease DPPH radicals by their hydrogen-donating ability (Gad et al., 2011). Even considering the processing operations for the pasta production and its cooking, it was verified that *spirulina* enriched pasta enhanced the nutraceutical property by increasing the antioxidant activity.

Kadam and Prabhasankar (2010) revised the impact of the incorporation of different marine as functional ingredients in bakery and pasta products and observed that these products have shown considerable improvement in EPA and DHA contents, leading to a reduction of cardiovascular diseases which is a major health concern of the 21st century.

El-Baz et al., (2017) studied the impact of *Dunaliella salina* addition on the nutritional quality of pasta and a reinforcement of the pasta structure, followed by a significant increase of the fatty acids on the final product, was observed. In a previous work (Fradique et al., 2013), the impact of *Isochrysis galbana* (Ig) and *Diacronema vlkianum* (Dv) biomass incorporation in pasta products as PUFA's source was already studied. As an example, on Table 6. the fatty acids profile of raw (R) and cooked (C) pasta prepared with different contents of Ig are presented and similar results were obtained with Dv microalgae.

Table 6. Main saturated, monounsaturated and polyunsaturated fatty acids in raw (R) and cooked (C) control and enriched pasta with 0.5, 1.0 and 2.0 g/100 g DW of Isochrysis galbana biomass (Ig). Expressed as average values (three replicates) of the percent of total fatty acids (Fradique et al., 2013 – adapted)

Fatty acids (%)	Control		Ig (g/100 g)					
	(R)	(C)	0.5 (R)	1.0 (R)	2.0 (R)	0.5 (C)	1.0 (C)	2.0 (C)
14:0	0.14^{a}	0.14^{a}	0.79^{b}	1.35^{c}	2.14^{d}	0.69^{b}	1.22^{c}	2.10^{d}
16:0	21.75^{a}	20.46^{a}	23.11^{a}	23.67^{a}	20.58^{a}	22.98^{a}	17.95^{a}	18.55^{a}
18:0	1.49^{b}	1.26^{ab}	1.56^{b}	1.59^{b}	1.25^{ab}	1.51^{b}	0.97^{a}	1.02^{a}
Other SFA	0.53	0.37	0.57	0.70	0.74	0.75	1.08	0.65
∑SFA	23.96	22.28	26.03	27.31	24.71	25.93	21.22	22.32
16:1	0.13^{a}	0.14^{a}	0.11^{b}	0.11^{a}	0.09^{ab}	0.14^{ab}	0.12^{ab}	0.11^{ab}
18:1	17.65^{d}	16.63^{cd}	15.15^{bc}	13.97^{ab}	13.63^{ab}	14.32^{ab}	13.23^{ab}	12.29^{a}
Other MUFA	2.27	1.82	1.56	0.99	1.11	0.86	0.76	0.80
∑MUFA	21.22	19.64	19.09	18.27	19.46	17.38	17.01	17.65
18:2ω6 – LA	50.11^{ab}	53.01^{b}	48.51^{ab}	46.82^{a}	45.49^{a}	50.68^{ab}	52.86^{b}	46.57^{a}
18:3ω3 – ALA	2.63^{a}	2.99^{a}	2.56^{a}	2.55^{a}	2.53^{a}	2.78^{a}	3.12^{a}	2.83^{a}
20:5ω3 – EPA	–	–	1.19^{b}	2.11^{c}	3.29^{d}	1.12^{b}	2.44^{c}	4.11^{e}
22:6ω3 – DHA	–	–	0.27^{b}	0.48^{c}	0.91^{e}	0.33^{b}	0.63^{d}	1.13^{f}
Other PUFA	0.87	0.72	0.94	0.87	1.57	0.72	1.03	1.77
∑PUFA	53.61	56.72	53.47	52.83	53.79	55.63	60.08	56.41
ω3	3.51^{a}	3.66^{ab}	4.50^{abc}	5.65^{c}	7.65^{d}	4.65^{bc}	7.05^{d}	9.20^{e}
ω6	54.22^{a}	56.49^{a}	48.70^{a}	47.05^{a}	45.80^{a}	50.85^{a}	53.15^{a}	47.00^{a}
ω3/ω6	1:15	1:15	1:11	1:8	1:6	1:11	1:8	1:5

Different letters in the same row correspond to significant differences ($p < 0.05$).

It is possible to observe that palmitic (16:0), oleic (18:1ω9) and linoleic (18:2ω6) acids were the main saturated, mono and polyunsaturated fatty acids found, respectively, in both pastas prepared with microalgae biomass addition. A significant increase of eicosapentaenoic acid (20:5ω3, EPA) and docosahexaenoic acid (22:6ω3, DHA), that were absent in raw control pastas was also found. In addition, it was observed that the cooking process, in boiled water, does not imply a reduction of the omega-3 fatty acids levels, i.e., leaching of the microalgae does not occur, which was retained in the pasta structure. This fact is reinforced by the observation that the incorporation of microalgae implies a reduction of the loss of soluble solids as a consequence of cooking, comparing to the control.

3.3. Gelled Desserts Enriched with Microalgae

The gelled desserts are traditionally obtained from milk, starch and egg yolk, presenting a yellow coloration. The creation of attractive color desserts can be achieved through the incorporation of microalgae, which simultaneously improves the nutritional performance of the products and presents health benefits.

However, it is important to consider that the addition of microalgae biomass can promote modifications on the gel structure, resulting from their high protein content (Batista et al., 2012). Although, it was also verified that the gelling mechanism is ruled by the biopolymers, while microalgae seem to be embedded in the gel network acting as active particle fillers (Batista et al., 2011).

The impact of *Spirulina maxima* and *Diacronema vlkianum* biomass addition to vegetable-based gelled desserts (similar to ‘‘dairy desserts’’) prepared with pea protein isolate, rich in essential fatty acids: omega-3 polyunsaturated fatty acids (PUFA), was investigated by Gouveia et al., (2008a). It was stated that the addition of these microalgae

biomass to vegetable gelled deserts, resulted in a novel alternative food product, with PUFA's (e.g., EPA, DHA and GLA), with favourable texture characteristics, particularly for *Diacronema*, due to the gel structural reinforcement observed, representing a new food market opportunity.

Table 7. Fatty acid composition (percentage of total fatty acids) of the microalgae Spirulina (Sp) and Diacronema (Di), gels prepared with these algae (at 75 and 90 C) and control gel (without alga addition). (Gouveia et al., 2008 – adapted)

	Sp	*Di*	Control gel	*Sp* gel 75 °C	*Sp* gel 90 °C	*Di* gel 75 °C	*Di* gel 90 °C
SFA							
14:0 – miristic	0.2	13.0	0.2	–	0.2	2.7	3.2
16:0 – palmitic	39.7	8.8	14.3	15.8	15.8	13.3	12.2
18:0 – stearic	1.2	–	3.1	3.5	3.5	2.8	2.3
∑ SFA	42.7	23.8	17.7	19.7	19.2	19.4	18.2
MUFA							
16:1ω7 – palmitoleic	7.0	16.1	–	0.5	0.5	3.2	3.8
18:1ω9 – oleic	4.3	–	18.7	19.7	19.2	16.7	15.6
∑MUFA	15.0	24.0	19.1	20.8	20.6	20.7	20.3
PUFA-ω3							
18:3ω3 – α-linolenic	1.5	–	5.8	6.9	6.7	5.8	6.2
18:4ω3	–	7.4	–	–	–	1.2	1.5
20:5ω3 – EPA	–	21.3	–	–	–	3.1	4.1
22:6ω3 – DHA	–	5.5	–	–	–	0.7	0.9
EPA+DHA	–	26.8	–	–	–	3.9	5.0
∑ PUFA-ω3	1.5	35.9	6.6	6.9	6.7	11.1	12.6
PUFA-ω6							
18:2ω6 – linoleic	17.9	0.3	47.1	50.4	49.1	41.9	43.0
18:3ω6 – γ-linolenic	16.8	0.7	–	1.3	1.3	0.2	0.2
22:5ω6	–	6.5	–	–	–	0.9	1.1
∑ PUFA-ω6	35.2	8.9	47.1	51.8	50.4	42.1	43.3
∑ PUFA total	36.7	44.8	53.7	58.7	57.1	53.2	55.9

Notes: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

To prepare protein gels a thermal treatment, previously optimised by Nunes et al., (2006a, b), with some severity, needs to be applied: temperatures ranging from 75ºC to 90ºC, during at least 5 min. This thermal conditions, that are usually used to prepare food gels, had a slight impact on the PUFA's presented in the gel formulations. On Table 7 the impact of thermal treatment on the fatty acids profile is summarised. It can be observed that both microalgae are a rich source of carotenoid omega-3 fatty acids: EPA 20:5ω3 and GLA 18:3ω3, respectively.

The range of temperatures used in the preparation of the gels seems to not affect GLA, EPA and DHA percentage, suggesting that these microalgae cells could resist heavy thermal processing. This resistance of the microalgae bioactive molecules to the wet heat transfer processes evidences the potential of microalgae as food ingredients and/or nutraceutical delivery systems.

3.4. Bioactive Cookies

Cookies are a generalized and convenient food that can be considered as a nutritious dense snack, which can be produced from a wide variety of raw materials. In this baking product, gluten formation takes on less prominence than in bread, so the incorporation of bioactive ingredients (e.g., proteins, fibers, antioxidants) is more widespread.

Regardless of the type of cookies to be prepared, the production process always involves a high temperature heat treatment, for a given time, so that the final product can acquire the typical sensory characteristics, namely the crunchiness. In this case a dry heat transfer mechanism occurs, which will have different implications than the previously analyzed wet process, involved in the gels formation. For cookies, more severe conditions of heat transfer are expected (about 180ºC, 30 min), which may have a more pronounced impact on the bioactive compounds that are incorporated through microalgae biomass.

Functional biscuits enriched with 1 and 3% of *Isochrysis galbana* (Ig), as a source of PUFA-ω3, were developed by Gouveia et al., (2008b). On these biscuits the ω3-fatty acids (DHA, EPA and DPA), coming from the microalgae, remained present after the thermal processing of baking. This can be observed in Figure 8. whereas all the other fatty acids, mainly provided by butter, showed large variations. The authors suggested that the microalgae cells could resist thermal treatment, encapsulating the fatty acid molecules, thus protecting them from oxidation.

Ig biscuits presented PUFA-ω3 levels (EPA+DPA+DHA) of 100mg/100g and 320mg/100 g biscuit, for 1% and 3% microalgae biomass incorporation, respectively. Considering these values, the importance of Ig as a source of PUFA-ω3 should be highlighted, although Ig has not received yet clearance, to be used as food, from EFSA.

In addition to the PUFA's resistance to the heat treatment involved in the biscuits production, it is important to ascertain the stability of other bioactive compounds which are also naturally encapsulated in the microalgae cells. Batista et al., (2017) studied the incorporation of microalgae (*Arthrospira platensis* - Ap, *Chlorella vulgaris* - Cv, *Tetraselmis suecica* – Ts and *Phaeodactylum tricornutum* - Pt) in cookies, as innovative ingredients to enhance its functional properties.

Cookies prepared with *A. platensis* and *C. vulgaris* presented significant ($p < 0.05$) higher protein content compared to the control, which is an important issue in terms of nutritional improvement.

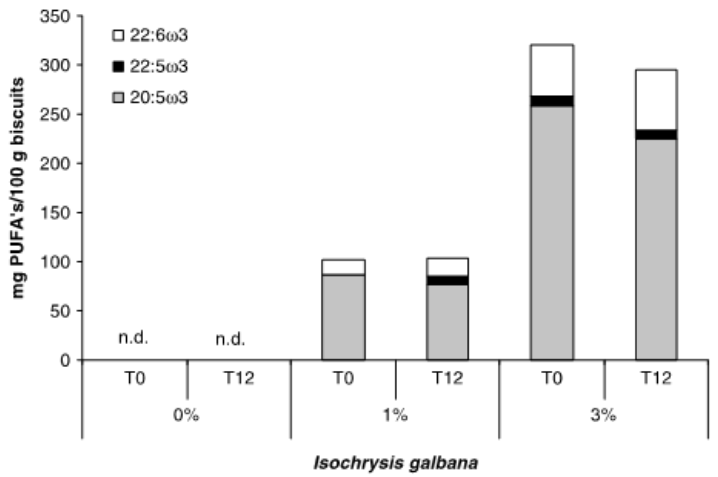
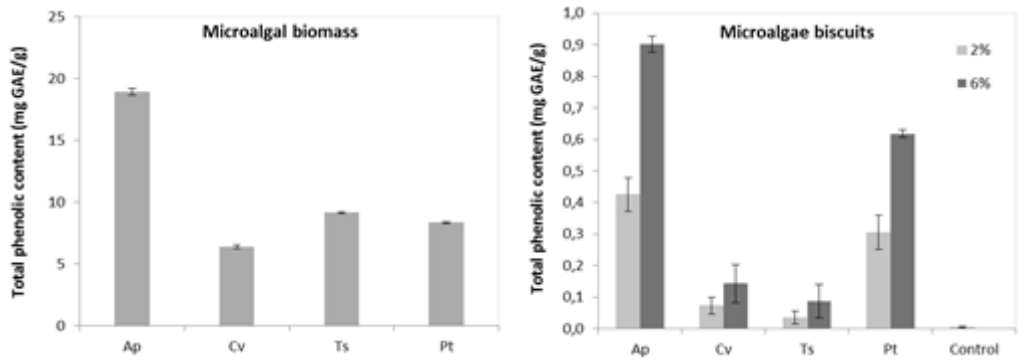


Figure 8. Evolution of ω3-polyunsaturated fatty acids, of biscuits with 0%, 1% and 3% *Isochrysis galbana* biomass incorporation, at 0 and 12 weeks. EPA, eicosapentaenoic acid (20:5ω3); DPA, docosapentaenoic acid (22:5ω3); and DHA, docosahexaenoic acid (22:6ω3). Gouveia et al., (2008b) – adapted.

Phenolic compounds - simple phenols, flavonoids, phenyl-propanoids, tannins, lignins, phenolic acids, and their derivatives, synthesized as secondary metabolites are natural antioxidants and have been extensively incorporated into foods, considering their recognized health benefits (El-Baki et al., 2009; Machu et al., 2015). Total phenolic content (expressed as gallic acid equivalents mg/g dry weight) of four microalgae strains studied and in cookies enriched with different levels was evaluated and can be compared in Figure 9, as well as the respective *in vitro* antioxidant capacity, accessed by the FRAP Method (Ferric Reducing Antioxidant Power) - Figure 10.



(a) (b)

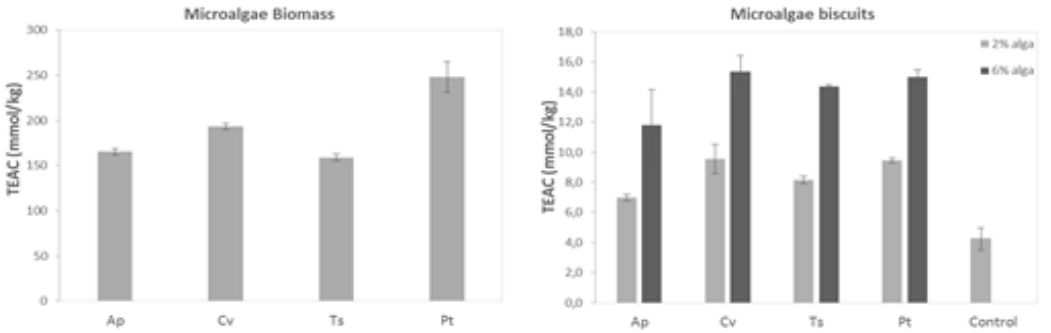
Figure 9. Total phenolic content (expressed as gallic acid equivalents mg/g dry weight) of four microalgae strains (a) and in cookies enriched with different levels of microalgae (b) (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*). Results are expressed as average ± standard deviation (n = 3) Batista et al., (2017) – adapted.

The addition of microalgae resulted in an effective supplementation of phenolic compounds of the biscuits, which are practically absent in the control biscuit. A. *platensis* with 6% (w/w) biscuit presented the highest phenolic content (0.90 mg GAE/g), followed by *P. tricornutum* 6% cookie (0.62 mg GAE/g). Both *A. platensis* and *P. tricornutum* 2% cookies also showed much higher phenolic content than the chlorophyte algae at the highest concentration (*C. vulgaris* and *T. suecica*).

When submitted to baking, at 110°C, during 40 min, *Chlorophyceae* algae presented high phenolic losses: about 50% for *C. vulgaris* and 80% for *T. suecica*. However, *P. tricornutum* cookies showed high final phenolic content and no considerable losses, compared to the initial microalgae biomass, after baking. The authors explained this different behavior based on the differences of cell wall of *P. tricornutum*. This species presents silica bands or an amorphous silica matrix according to the morphotype (Tesson et al., 2009), when compared to the other microalgae. In this case, an additional protection of the phenolics from thermal degradation should be considered.

Comparing the antioxidant capacity of microalgae-enriched cookies and respective biomass (Figure 10), *P. tricornutum* presented the highest ($p < 0.05$) anti-oxidant capacity (248 mmol TEAC/kg), followed by *C. vulgaris* (193mmol TEAC/kg), and by *A. platensis* and *T. suecica* (about 160 mmol TEAC/kg). In addition to phenolic content, *P. tricornutum* has a high content of the carotenoid fucoxanthin with a noticeable antioxidant activity (Gilbert-López et al., 2017; Mikami, and Hosokawa, 2013). The antioxidant activity of green

microalgae - *Chlorella* and *Tetraselmis* results from the high content of chlorophylls (Wang and Wink, 2016) and vitamin E (Zittelli et al., 2006).



(a) (b)

Figure 10. Antioxidant capacity (expressed as mmol of Trolox Equivalent Antioxidant Capacity, TEAC, per kg) of four microalgae strains (a) and in cookies enriched with different levels of microalgae (b) (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*). Results are expressed as average ± standard deviation (n = 3). Batista et al., (2017) – adapted.

All the microalgae-based cookies, even at low levels of incorporation (2% w/w), promoted a significant ($p < 0.05$) increase in the antioxidant capacity, comparing to the control cookie (without microalgae). Generally, cookies with 2% microalgae showed values from 7.0 to 9.5 mmol TEAC/kg1 (+ 65% and + 125% compared to the control cookie, respectively) while 6% cookies showed values from 11.8 to 15.4 mmol TEAC/ kg1 (+ 178% and + 262% compared to the control cookie, respectively). The reported results are in agreement with the studies performed by El Baky et al., (2015) and Singh et al., (2015), which verified that after baking, *S. platensis* cookies showed a high content of phycocyanin, supposedly responsible for the observed anti-oxidant activity.

Batista et al., (2017) also studied the *in vitro* digestibility (IVD) analysis, reproducing the chemical-enzymatic catalysis that occurs in the proximal tract of the monogastric digestive system, according to the procedures described by Boisen and Fernández (1997). The results revealed that *T. suecica* and *P. tricornutum* microalgae biomass presented the lowest IVD (around 50%). The differences among the microalgae tested could be related to their different cell wall structure (e.g., Andersen, 2013). However, no significant difference ($p < 0.05$) in IVD (87–95%) between microalgae cookies and the control was found.

3.5. Bioaccessibility of Microalgae Biomass

The incorporation of microalgae in food products, with different levels of processing, adds bioactive compounds, namely fatty acids and antioxidants, to the final products. The resistance of the bioactivity to different levels of food processing could result from the fact that the bioactive molecules are naturally encapsulated within the microalgae, which gives them a certain degree of protection, makes microalgae a promising functional ingredient.

However, in addition to the activity of the bioactive ingredients in a specific food matrix, it is important to evaluate their bioavailability, i.e., to what extent do conditions exist for the

human body to assimilate the active ingredient. This is the only way to effectively guarantee the alleged health benefits. To perform this type of evaluation, different strategies could be applied. Clinical trials in humans are the most direct method to quantify the real benefit of such ingredient on health, resulting from its regular consumption. However, this type of approach, is expensive and involves ethical issues difficult to overcome. The performance of animal tests leads to the same arguments. As an alternative, the *in vitro* studies can give a realistic prediction of the bioavailability of the functional ingredients.

In the latter described works, the resistance of *Diacronema vlkianum* to the processes associated with the production and cooking of pasta and the preparation of gel desserts was verified. In these two types of products, it was found that, despite the severe heat treatment conditions associated with production, the fatty acids present in the microalgae remain at high levels in the final products, i.e., the process losses are minimal. Mello-Sampayo et al., (2017) evaluated *in vivo* the assimilation of fatty acids present in this microalgae. A single-dose (CD1-mice) studies were followed by 66-days repeated-dose study in Wistar rats with the highest tested single-dose of microalgae equivalent to 101 mg/kg eicosapentaenoic acid + docosahexaenoic acid (EPA+DHA). Microalgae-supplementation modulated EPA and docosapentaenoic acid enrichment at arachidonic acid content expenditure in erythrocytes and liver, while increasing EPA content of heart and adipose tissues of rats. Those fatty acid (FA) changes confirmed the *D. vlkianum*-biomass FA assimilation. Brain was discriminated from other tissues, using a principal component statistical analysis, forming two other groups (erythrocytes, liver, and heart separated from kidney and adipose tissues), pointing to a distinct signature of PUFA deposition for the brain and for the other organs. The improved serum lipid profile, omega-3 index and erythrocyte plasticity support the cardiovascular benefits of *D. vlkianum*. The results obtained in this work support the potential of *D. vlkianum*-biomass to be used as a functional food ingredient, providing a safe source of bioavailable omega-3 PUFA.

Cilla et al., (2018) revised the main issues related to the *in vitro* evaluation of bioaccessibility of bioactive compounds (carotenoids, minerals, ascorbic acid, tocopherols and polyphenols). The authors pointed out some concepts that should be considered. The definition for bioavailability (BAv) is not universally accepted. From a nutritional point of view, it is defined as the fraction of ingested component available for utilization in normal physiological functions. However, two additional terms - bioaccessibility (BAcs) and bioactivity (BAct) are associated with BAv (Fernández-García et al., 2009). BAcs has received two alternative definitions: i) it is considered as the fraction of a compound that is released from its food matrix in the gastrointestinal tract and thus becomes available for intestinal absorption; or ii) it is the fraction of a compound that is released from its food matrix in the gastrointestinal tract and thus becoming available for intestinal absorption including absorption/assimilation into the cells of the intestinal epithelium and, lastly, pre-systemic intestinal and hepatic metabolism (Cardoso et al., 2015). BAct is related to how the bioactive compound has reached systemic circulation and it is transported and reaches the target tissue, interaction with biomolecules metabolism in these tissues, and all the cascade of physiological effects it generates. *In vitro* methods can evaluate BAcs and/or BAct (Fernández-García et al., 2009; Cardoso et al., 2015). Bioaccessibility of elements in green seaweeds from pond aquaculture were recently studied by Afonso et al., (2018), using an innovative *in vitro* digestive model of the human gastrointestinal tract. The study highlighted the importance to take into account bioaccessibility results to estimate the dietary intakes.

Similar work is underway with the same research group to determine the bioavailability of various bioactive compounds of *Chlorella vulgaris* and *Tetraselmis chui* in bakery products.

CONCLUSION

Microalgae are already recognized as an important source of bioactive compounds or phytochemicals, that may benefit health beyond the role of basic nutrition. Nevertheless, the consumption of microalgae in food is still very limited and only a small number of species are allowed for human consumption. The use of whole microalgae as a food ingredient has a high exploitation potential for the production of value-added functional foods.

The bioactive ingredients are naturally encapsulated within the microalgae, which garanties a considerable degree of protection during processing. However, a limited disruption of the cell wall may have advantages in terms of release of bioactive compounds, in particular antioxidants.

The mechanical and thermal food processing conditions may imply a certain degradation of the bioactive compounds. Nevertheless, it was found that due to the natural protection of the cell wall, even for more severe processes, the degradation of fatty acids and antioxidants is limited, in various food matrices - e.g., emulsions, puddings (gels), cookies.

In addition to the processing level and cell disruption associated issues, the bioaccessibility of the different nutrients should also be taken into account for the establishment of recommended dietary intakes when a real impact on health, from the microalgae consumption, is foreseen.

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Chapter 88

SEAWEED BY-PRODUCTS FOR FOOD AND BIOREMEDIATION

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ABSTRACT

Seaweed has received remarkable interest for its potential in assessing the environment, as well as in biomonitoring aquatic pollution of organic and inorganic pollutants. The macroalgae by-products are also used for biological treatment of wastewaters, due to their fast growth, high biomass production and their ability to accumulate and degrade different pollutants. In addition to the environmental applications, the algae produce high added-value compounds that could be used in pharmaceutical industry and for the production of renewable energy. Seaweed biomass is also considered as a potential source of biomass for the aquaculture production sector. In this sense, a considerable attention has been paid, in the last years, to the use of macroalgae as a potential and renewable ingredient for aquafeeds; as a consequence of the rising costs of the two most important ingredients used traditionally in diets for aquatic organisms (fishmeal and fish oil). This chapter aims at discussing the use of seaweed by-products for both heavy metal accumulation and food production for aquatic organisms.

Keywords: seaweed, biomass, bioremediation, aquaculture, fishmeal, fish oil

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1. INTRODUCTION

Seaweeds have attracted considerable attention as potential agents of bioremediation for their capacity to eliminate heavy metals and organic pollutants and for their role in biomonitoring (Ben Chekroun and Baghour, 2013; Ben Chekroun et al. 2013; Ben Chekroun et al., 2014). Similarly, the seaweeds are potential sources of high biotechnological interest due to their production of a great diversity of compounds with a broad spectrum of biological activities (Pereira and Costa-Lotufo, 2012).

Biomonitoring offers an appealing tool for the assessment of metal pollution in aquatic ecosystem (Zhou et al., 2008). Biomonitors can be used to establish geographical and/or temporal variations in the bioavailabilities of heavy metals in the marine environment. The use of a suite of biomonitors allows recognition of the presence of relative magnitude of different metal sources (Rainbow, 1995). With the help of bioindicator organisms, biomonitoring can be regarded as a sensitive tool for the evaluation of the biological and ecological significance of aquatic pollution (Prabhakaran et al., 2017).

Moreover, the phytoremediation is an effective technique that uses plants, fungi or algae to degrade or to remove pollutants from soil, atmosphere or aquatic systems (Baghour et al., 2002; Ben chekroun et al., 2013). The phycoremediation is a cost-effective, eco-friendly, and comparatively safe process. It can effectively reduce the nutrient load of wastewater thereby reducing total dissolved solids (Sriram and Seenivasan, 2012).

The seaweeds can eliminate different pollutants either by biodegradation or by bioaccumulation. The latter occurs when an organism absorbs a toxic substance at a higher rate. Bioaccumulation is a process that allows for binding toxic metals or organic substances inside a cell structure (Zabochnicka-Świątek and Krzywonos, 2014). Several process including uptake, storage and elimination are involved during bioaccumulation (Zhou et al., 2008). Seaweeds are able to accumulate trace metals, reaching concentration values that are thousands of times higher than the corresponding concentrations in seawater (Bryan and Langston, 1992; Föster, 1976; Rai et al., 1981). Seaweeds have been commonly employed to detect the biologically available fraction of trace metals in marine environments (Malea and Haritonidis, 2000; Søndergaard et al., 2014; Bonanno and Bonaca, 2018). The by-products of marine phaeophyceae are even more cost-effective heavy metal biosorbers (Sandau et al., 1996).

On the other hand, seaweed is an abundant biomass with high photosynthetic efficiency, high reproduction rate and strong adaptation ability (Wang et al., 2017), which is an advantage of using seaweeds for accumulation of organic and inorganic pollutants as well as subsequent application of this by-product for the production of renewable energy. Seaweeds are capable of producing lipids and hydrocarbons quickly, and their photosynthetic abilities make them a promising candidate for an alternative energy source (Sivakumar et al., 2012). Studies have demonstrated that seaweed and its spent biomass obtained after pigment and phycocolloid extraction found to be a novel and renewable substrate for bioethanol production (Sudhakar et al., 2017).

An alternative source of biomass is from the aquaculture of seaweed which has seen annual average increases in biomass production of 7.5 % since 2000, with production exceeding 15 million in 2010 (FAO, 2012). Furthermore, global seaweed aquaculture

production occupies approximately 20% of the total world marine aquaculture production by weight, with an annual value of US $6.7 billion in 2013 (Kim et al. 2017; FAO 2017).

Finally, it is important to note that seaweed aquaculture provides ecosystem services, which improve conditions of the coastal waters for the benefit of other living organisms and the environment. Seaweed by-products can be economically valorized in the form of functional ingredients for finfish aquaculture (Wan et al., 2018).

The seaweed biomass offers a novel and added-value dietary ingredient in formulated diets for fish. The seaweed by-products contain a number of pigments and defensive compounds, and secondary metabolites that could have beneficial effects on farmed fish (Wan et al. 2018).

This chapter highlights the significant progress that is achieved in valorization of seaweeds by-products for bioremediation and fish feeds (aquafeeds).

2. SEAWEED BIOMASS AND BY-PRODUCTS FOR BIOMONITORING AND BIOREMEDIATION

Recently, there has been growing interest in the use of seaweed biomass for bioremediation and as bioindicators for environmental assessment. Biological indicators or bioindicators are living organisms such as plants, planktons, animals, and microbes, which are utilized to screen the health of the natural ecosystem in the environment (Baghour et al., 2002; Parmar et al., 2016). Biological indicators can provide information on the long-term effects of metal contamination as well as an indication of the potential for impacts at higher levels as a result of trophic interactions (Farias et al., 2018). Due to their fast growth and high biomass production, s*eaweeds are also considered as* promising candidates for bioremediation of aquatic systems contaminated with organic or inorganic pollutants. The seaweeds are used as an alternative to the direct determination of heavy metals in seawater because of their distribution, size, longevity, presence at pollution sites, ability to accumulate metals to a satisfactory degree and ease of identification (Whitton, 1984; Conti and Cocchetti, 2003; Stengel et al., 2004). Wan Maznah (2010) suggests that algae are ideally suited for water quality assessment because they have rapid reproduction rates and very short life cycles, which make them valuable indicators of short-term impacts.

2.1. Seaweeds as Bioindicators

Marine organisms (e.g., macroalgae, fish, crustaceans and mussels) have been used as bioindicators in order to provide information about concentrations of pollutants in the surrounding environment and to evaluate the status of chemical contamination of aquatic ecosystems and as an alternative to the analysis of water or sediment (Phillips, 1990; Zhou et al., 2008; Azizi et al., 2018). Seaweeds are recognized as accurate bioindicators, as biochemical and physiological changes allied to the response intensities within a population, community, or ecosystem will vary with time and the nature of the disturbance, serving as signals of environmental degradation (Ortega, 2000; Orfanidis et al., 2011, 2014).

Studying the capacity of Mediterranean macroalgae to accumulate and tolerate macro- and micro-elements present in seawater and sediments, Bonanno and Orlando (2018) reveal that seaweeds can accumulate and tolerate high concentrations of chemical elements such as Pb, Cd, Zn and Cu, which allow them to be exploited as bioindicators for biomonitoring of aquatic pollution by heavy metals and organic pollutants. Several species of the green algae *Enteromorpha* and *Cladophora* (Figure 1), occurring in fresh water, brackish and/or marine conditions, have been utilized to measure heavy metals in many parts of the world (Al-Homaidan et al., 2011).

In other study, Akcali and Kucuksezgin (2011) reported that the brown algae *Cystoseira* sp., the green algae *Ulva* sp., and *Enteromorpha* sp. possess high potential as cosmopolitan biomonitors for trace metals in the Aegean Sea (Figure 1).

Figure 1. (a) *Cladophora glomerata* (Safari et al., 2016), (b) *Enteromorpha intestinalis* (Ibrahim et al., 2014) and (c) *Ulva* sp. (Baghour, 2017).

More recently, Squadrone et al. (2018) show that Al, Be, Pb and Zn levels in seaweeds from Giglio Island were found to be significantly higher than in macroalgae from other islands of the Tuscany archipelago. These authors reported that the removal operation realized by these seaweeds confirmed the suitability of these marine organisms in signaling an environmental perturbation due to anthropogenic activities. Seaweeds by-products are considered bioindicators of water pollution due to their ability to accumulate metals at several orders of magnitude higher than in the environment in which they live.

2.2. Seaweeds By-Products for Bioremediation

Bioremediation is the use of living organisms such as bacteria, algae and other organism to remove or degrade pollutants and toxins from soil and aquatic ecosystems. Biomass by-products demonstrated specific binding abilities, allowing them to be potential adsorbents for the treatment of aquatic ecosystems contaminated with heavy metals (Schneegurt et al., 2001).

Seaweeds and other benthic organisms such as sponges and gastropods were shown to accumulate high level of heavy metals from seawater and, can therefore, serve as biological materials for decontaminating polluted sites (Ben Chekroun and Baghour, 2013; Azizi et al., 2018; Ferrante el al., 2018).

Recently, Fazal et al., (2018) suggests that algae can be effectively employed to bioremediate environmental pollutants and to produce biodiesel. This process of integration (bioremediation-Energy production) can potentially improve biodiesel production and wastewater treatment.

The metal ion uptake by living and dead cells can consist of two different modes. The mechanisms of uptake by living materials (bioaccumulation) and removal by dead ones (biosorption) are entirely different. Biosorption is a physiochemical process that occurs naturally in certain biomass, which allows it to passively concentrate and bind contaminants onto its cellular structure (Volesky, 1990). Biosorption that use living organisms have long been reported as an alternative technology for combating the water pollution caused by heavy metals.

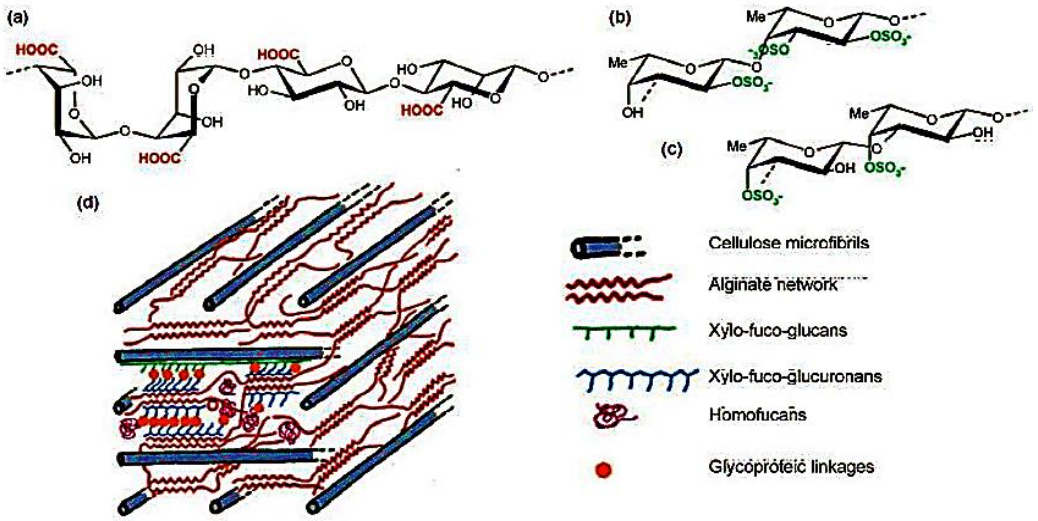
Recent biosorption experiments have focused attention on waste materials, which are by-products or the waste materials from large-scale industrial operations (Abbas et al., 2014). However, this biosorption could be affected by many parameters such as cell size and morphology, pH, ions concentrations in the external media and temperature (Wase and Forster, 1997). Mohan et al. (2007) showed that by-product chars from bio-oil production can be explored as cost effective adsorbents for arsenic removal from contaminated water system.

The by-products of marine seaweeds are even more cost-effective heavy metal biosorbers. To optimize the heavy metal biosorption by seaweed by-products, Sandau et al. (1996) found that under optimum conditions seaweed by-products, which is cheap and easily available, show high sorption capacities and efficiencies of heavy metal cations, as well as favorable sorption kinetics (Table 1). Sandau et al. (1995) reported that the algal by-products, obtained for example in the preparation of valuable biomaterials: which are practically free of cost, have a biosorption capacity, which is only negligibly lower than the capacity of the original biomasses. Since viability is not necessary for biosorption, dried algae or even algal extraction residues (by-products from the high-pressure extraction of valuable algal components) can be used as biosorptive material (Sandau et al., 1996; Soltmann et al., 2010).

Table 1. Seaweed species used for their by-products in biosorption of heavy metals

Species	Group	References
Codium sp	Chlorophyceae	Abbas et al. (2014)
Enteromorpha sp.	Chlorophyceae	Abbas et al. (2014)
Halimeda opuntia	Chlorophyceae	Kuyucak & Volesky (1989)
Ulva lactuca	Chlorophyceae	Zeroual et al. (2003)
Ulva rigida	Chlorophyceae	Sandau et al. (1996)
Ceramium rubrum	Rhodophyceae	Sandau et al. (1996)
Gigartina acicularis	Rhodophyceae	Sandau et al. (1996)
Gracilaria spp.	Rhodophyceae	Roberts et al. (2015)
Palmaria palmata	Rhodophyceae	Kuyucak & Volesky (1989)
Porphyridium cruentum	Rhodophyceae	Sandau et al. (1996)
Ascophyllum nodosum	Phaeophyceae	Sandau et al. (1996)
Ascophyllum nodosum	Phaeophyceae	Kuyucak & Volesky (1989)
Bifurcaria bifurcata	Phaeophyceae	Abbas et al. (2014)
Ecklonia maxima	Phaeophyceae	Nigro et al. (2002)
Fucus vesiculosus	Phaeophyceae	Sandau et al. (1996)
Laminaria digitata	Phaeophyceae	Sandau et al. (1996)
Laminaria saccharina	Phaeophyceae	Sandau et al. (1996)
Laminaria pallida	Phaeophyceae	Nigro et al. (2002)
Lessonia nigrescens	Phaeophyceae	Cid et al. 2018
Sargassum filipendula	Phaeophyceae	Nishikawa et al. (2018)
Sargassum filipendula	Phaeophyceae	Abbas et al. (2014),

Figure 2. *Gracilaria* sp. (Haryatfrehni et al., 2015).



Source: Michel et al., 2010.

Figure 3. Structures of the main polysaccharides typical of brown algae: (a) alginate; (b) sulphated fucan from Fucales; (c) sulphated fucan from Ectocarpales. (d) Hypothetical model of the biochemical organization of cell walls of brown algae.

Roberts et al. (2015) reported that the potential use of *Gracilaria* waste is as a biosorbent for the removal of metalloids waste effluents (Table 1). *Gracilaria* spp. (Figure 2) by-product is a sustainable substrate for iron-based sorbents (IBS) production and can be used to treat a costly waste problem. The IBS can be produced from waste biomass that remains after the commercial extraction of agar from farmed seaweed.

The cell wall of red macroalgae species contains high concentrations of sulfated polysaccharides such as agar, which are characterized by negatively charged functional groups such as carboxyl. This gives red macroalgae a high affinity for dissolved metals (Davis et al. 2003). The brown seaweeds contained the greatest number of acidic functionalities on the seaweed surface (Figure 3). These carboxylic groups are thought to be responsible for metal absorption (Murphy et al., 2007).

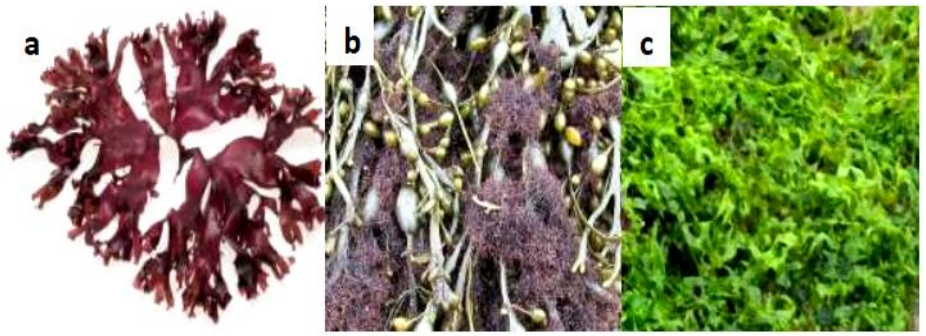


Figure 4. (a) *Palmaria palmata* (Mouritsen et al., 2012), (b) *Polysiphonia lanosa and* (c) *Ulva reticulate* (Kumar et al., 2018).

Murphy et al. (2007) studied the biosorption performance of Cu (II) by the dried biomass of the two red seaweeds *Palmaria palmata* and *Polysiphonia lanosa*, and they found that carboxyl and sulphonate functionalities involved in binding Cu(II) in both species (Figure 4). However, the amino and hydroxyl groups took part in Cu(II) binding in *P. lanosa*.

Vijayaraghavan et al. (2005) showed that the marine green alga *Ulva reticulate* was found to be an effective biosorbent for the removal of copper (Figure 4), cobalt and nickel from aqueous solutions, because the cell wall composition of green algae provides binding sites such as carboxyl hydroxyl amino and sulphate for metal ions (Deng et al., 2007; He and Chen, 2014; Neori et al., 2003).

3. SEAWEED BIOMASS FOR FEEDING FISH AND BIOREMEDIATION IN INTEGRATED AQUA CULTURE SYSTEMS

Recently, there has been a growing interest in the use of seaweed biomass in formulated diets for fish, in addition to their use for the direct determination of heavy metals in seawater (among other applications). Protein and lipid derived from fishmeal and fish oil respectively are the most expensive ingredients in fish feeds (aquafeeds). Any reduction and replacement of these ingredients by a less expensive protein and lipid sources which will produce the same growth performances will contribute significantly to the reduction of fish production cost.

One of these ingredients is seaweed by-products that contain high valuable protein, lipid and carbohydrate contents. However, like terrestrial plants, nutritional content in macroalgae can vary greatly amongst species. Seasonality and geographic locality can also play an important role in influencing the nutritional composition found in the algal species. In addition to their basic nutritional value (protein and lipid of high quality), seaweeds contain a number of pigments and defensive compounds, and secondary metabolites that could have beneficial effects on farmed fish (Wan et al. 2018). With such promising attributes, seaweeds may fill not only a nutritional role in aquafeeds, but also they may promote fish health and fish welfare. Shpigel et al. (2017) suggested that replacing 100% of the fishmeal by poultry meal and algae (*Ulva lactuca*), while keeping the level of all other ingredients constant, *Sparus aurata* performances were similar to those of fish fed the control feed containing fishmeal.

An Integrated Multi-Trophic Aquaculture (IMTA) system is a practice in which excretions of one or more organisms are utilized by other cultured organisms from different trophic levels. Seaweeds are frequently used in IMTA systems as biofilters of fishpond effluents (Shpigel and Neori 1996; Neori et al. 2004), saving the cost of water treatment while being at the same time a reliable feed source throughout the year. Shpigel et al. (2017) suggested that protein-rich *Ulva lactuca* used as biofilter in an IMTA system was evaluated as a dietary ingredient for gilthead seabream (*Sparus aurata*), at up to 30% replacement ratio of fishmeal enhancing growth performance of fish and reducing nitrogen loads from the effluents by Ulva biofilter, saving on water treatment costs, an additional advantage of integrated multi-trophic aquaculture (IMTA) system. Several studies have reported the beneficial qualities of these macroalgae compounds and their potential for exploitation in commercial finfish feeds.

3.1. Seaweeds as a Sustainable and Functional Aquafeed Ingredient

Commercially formulated diets represent more than 60% of the production costs in finfish aquaculture (Naylor et al. 2009; Shpigel et al. 2017). A continuous stagnation in the production of fish meal (FM) and fish oil (FO) from fisheries and a rising demand for finfish diets has led to an overall increase in feed costs (Shepherd and Jackson 2013). As result, both feed manufacturers and research institutions are seeking novel economically and environmentally sustainable sources of feed ingredients as replacements for FM and FO. In this context, restrictions on diets based on farmed animal tissue, issues of source availability and low-cost production have seen plants as the principal alternative to FM and FO use in aquafeeds (Gatlin et al. 2007). More recently, processing plant based meals, such as soybean and lupin, through applying exogenous enzymes, chemicals and physical treatments, have allowed manufacturers to overcome the effects of antinutritional factors (ANF) and digestibility issues, which are commonly present in plant-derived ingredients. Unfortunately, an increasing global demand for food (FAO 2009) together with of the use of plants to produce biofuel (Rathmann et al. 2010; Harvey and Pilgrim 2011) will probably increase the price of plant meals and oils in the future, making it less attractive as an alternative to FM and FO into aquafeeds.

Seaweeds by-products offer a novel and added-value dietary ingredient in formulated diets for fish and shellfish aquaculture. Production of biomass can be achieved without dependence on expensive arable land, since seaweed may be collected from coastal regions (in the intertidal, subtidal coastal zones and estuarine habitats) or farmed (Macroalgae aquaculture). The beneficial qualities of the macroalgae compounds and their potential for exploitation in commercial finfish feeds were reported in various studies, with enhancing trends in fish growth, physiology, stress resistance, immune system and fillet muscle quality (Wan et al. 2018).

In an early study, Nakagawa et al. (1984) showed that the inclusion of 10% of *Ulva pertusa* into formulated diet for black sea bream *(Acanthopagrus schlegeli)* produced elevated protein efficiency; while other measured growth performance remained in variable. After that, a numerous studies have been performed testing a wide range of fish and seaweeds species. Many of these studies have focused on fish species of high commercial interest, such as rainbow trout, salmon, seabream and sea bass; and centered on several genera of macroalgae,

such as *Ulva*, *Gracilaria* and *Porphyra* species. This was the case of Wassef et al. (2013) who suggested that the inclusion of 5% of *Pterocladia capillacea* in the diet for European seabass (*Dicentrarchus labrax*) resulted in an increase in body weight and weight gain. In the same way, 5% *Pyropiayezoensis* inclusion in diet for red seabream (*Pagrus major)* resulted in increase in fish final weight, weight gain, specific growth rate (SGR) and protein efficiency ratio (PER) Kalla et al. (2008).

Valente et al. (2006) recommended that macroalgae such as *Gracilaria* and *Ulva* can be incorporated up to 10 % in European sea bass feeds without affecting the performance of fish.

In addition, *Gracilaria cornea* and *Gracilaria bursa-pastoris* (Valente et al. 2006), *Porphyra* (Soler-Vila et al. 2009), and *Ulva lactuca* (Wassef et al. 2001) were added to the feed as partial substitutes for dietary fishmeal and increased the fish growth rate and improved protein assimilation.

Other studies using seaweed have suggested that kelp meal works as an excellent additive (attractant, agglutinant and binder) in pelleted feeds for penaeid shrimps and thus improved feed utilization efficiency in this slow feeding species (Cruz-Suarez et al. 2009; Silva-Neto et al. 2012).

3.2. Seaweeds Biomass for Bioremediation in Integrated Aquaculture Systems

In the last years, intensive fed aquaculture (e.g., finfish and shrimp) throughout the world has developed rapidly; making aquaculture the fastest growing global food production sector (Figure 5). Nevertheless, this increase in the aquaculture activities is associated with concerns about the environmental impacts of such often mono-specific practices (Chithambaran, 2016). One of the main environmental impacts is the direct discharge of significant nutrient loads into coastal waters from open-water systems and with the effluents from land-based systems. For this, the aquaculture industry should develop adequate and responsible practices that ensure the remediation of the consequences of its activities in order to maintain the health of coastal waters.

The integrated aquaculture is proved to be potentially more sustainable than monoculture practices, because of the reutilization of waste products of one species by another (Neori et al., 2004). Canada has been a leader in developing seaweed culture techniques as a part of integrated multi-trophic aquaculture (IMTA), where aquatic organisms (fish and shellfish), and seaweed are all grown together in an ecologically-based aquaculture farm design.

The IMTA-produced *U. lactuca* represents multiple advantages for the aquaculture sector by considerably reducing nitrogen loads in the effluents (a significant source of environmental pollution), saving water treatment costs, and turning into an additional valuable crop (Bunting and Shpigel 2009; Holdt and Edwards, 2014).

Various strategies for integrating seaweed into fish culture have been proposed. Buschmann et al. (1994) find that the effluents from intensive tank culture of salmon in Chile favor the production of *Genypterus chilensis* in tank culture. Similarly, Haglund and Pedersen (1993) found that *Gracilaria tenuistipitata* shows a better growth performance in co-cultivation with rainbow trout, especially in warm months of the year.

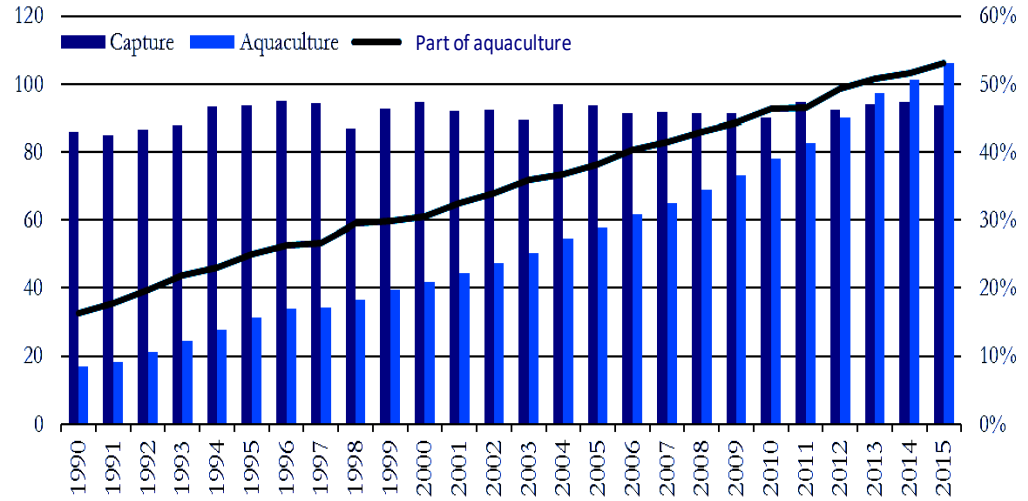


Figure 5. Evolution of the world production resulting from the aquaculture activity (FAO, 2016).

Results indicated that the seaweed is suitable as a good candidate for seaweed/fish integrated mariculture for bioremediation and economic diversification. The integration can benefit economy and environment in a sustainable manner in warm seasons in coastal waters of north China.

From the above-mentioned studies, it seems that the seaweed compounds might show the beneficial qualities, being potential for exploitation in commercial finfish feeds and act as biofilter. As such, these functional compounds could present an attractive incentive to feed manufacturers and fish farmers.

CONCLUSION

Seaweeds play a significant role in marine ecosystems. They are an important renewable source in marine environments and have received great attention for their high bioremediation capabilities. By-products from seaweeds are currently being used as an alternative adsorbent in removal of heavy metal ions from polluted aquatic ecosystems. Seaweed contains various biologically active components, after the extraction of these molecules; the by-product can be used in formulated diets for fish.

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Chapter 89

ETHANOL AND MACRO ALGAE: WHERE TO NEXT?

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ABSTRACT

An overview on the ethanol production using macro algae is presented including future developments needed to develop the Industry. The potential of algae blooms, species and production, cultivation systems and bottlenecks, as well as processing and fermetation of macroalgae biomass are dealt with. Some representative examples for the biotheanol production are evaluated.

Keywords: biorefinery, bioethanol, macro algae

INTRODUCTION

Seaweeds or macroalgae and ethanol production have somewhat followed a similar pathway as microalgae and biodiesel. Over the last 10 years a lot of effort, funding (EU projects such as Macrocascade, Biomara and Macrofuels), subsidies and investment have gone into this area of research. The current bottleneck for seaweed for biofuel production is adequate upscaling methodologies of seaweed production and mechanisation of seeding, harvesting and processing as at the current level seaweed cultivation for bioethanol is not economically feasible yet (Soleymani and Rosentrater, 2017). These cultivation upscaling technologies are needed in order to establish a seaweed aquaculture industry in Europe. In Western Europe this hasn't happened yet in respect of seaweeds for biofuel production although has happened and is starting to happen at a much smaller scale for food and value added products. Nevertheless in the Western world even for food production there is still a bottleneck which is caused not only by a lack of upscaling cultivation techniques, but also

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Marine licensing issues, multi-user bay management issues, environmental issues and processing issues (van Oirschot et al., 2017; Hasselström et al., 2018).

Bioethanol has attracted attention for many years as a possible alternative to hydrocarbon fuels. Seaweeds have been often proposed as some of the most promising raw materials for bioethanol production because they do not contain lignocellulose. Seaweeds are a feedstock which is abundant and carbohydrate-rich. It is a crop that can be grown sustainable, uses no agricultural inputs (pesticides, fertilizer, land, water), and is in general not part of the human or animal food chain (Kraan, 2011; 2012; 2013; Obata et al., 2016). One of the most critical aspects of biofuel sustainability from land plants such as corn stand in the way in which biofuel's raw materials are produced, especially with respect to their CO_2 balance and to the competition with food agriculture areas that they create (Kraan, 2011; Obata et al., 2016). Macroalgae and aquatic fresh water biomass represent major progress in obtaining a feedstock that has high carbohydrate levels and biomass yields. These resources are widespread and available, do not compete with food or agricultural surfaces, produce high quality by-products, can be used as a means to capture CO_2 and are suitable for integrating in wastewater treatments to reduce pollution. This makes marine aquatic resources one of the most attractive renewable sources for a sustainable energy production (Wargacki et al., 2012).

Rapid growth rates also mean seaweed absorb significant amounts of CO_2. One hectare of seaweed can fix about 66t of CO_2 from the atmosphere. Depending on the species, it will take between 10-100 years before the CO_2 contained in a combusted tree is recaptured in regrowth. For seaweed, taking about six months to mature, it absorbs CO_2 at a much quicker rate and the carbon released from its combustion is thereby sooner reabsorbed (Obata et al., 2016; Andersen 2017).

Several species of the brown and red algae are used for industrial applications, such as extraction of alginate, and agar or for food e.g., Kombu, Wakame and Nori. Global industrial production of seaweeds is about 25 million tonnes wet weight representing a value of 6 billion dollars (FAO, 2018).

Bioethanol could be produced from seaweeds that are not used for these applications or from the waste generated by the food Industries such as the holdfasts from Wakame processing which are the main waste in farming sites and account for 40-60% of annual production (Jung et al., 2016). Cultivated seaweeds and seaweeds that are not utilized in the marine ecosystem would be suitable for use as raw materials for bioethanol production. Large scale bioethanol production needs highly productive seaweed biomass that either grows fast or can form blooms, Macrocystis pyrifera (Linnaeus) C. Agardh (Rassweiler et al., 2018) and Ulva Linnaeus (Yanagisawa et al., 2013) respectively. Experiments on small-scale cultivation of green seaweeds such as Ulva, Chaetomorpha Kützing and Cladophora Kützing have suggested around 50 tons volatile solids ha/y (Hansson 1983). Habig et al., (1984) have determined that the volatile-solid contents of Ulva spp. are approximately 70% on a dry-weight basis indicating about 7 kg dryweight/m2/y. According to Gao and McKinley (1994) the productivity of brown seaweeds such as kelp (Macrocystis C. Agardh, Laminaria J. V. Lamouroux, Ecklonia Hornemann) or Sargassum C. Agardh ranges from 3,3-11,3 dry $kg/m^2/y$ with red seaweeds exhibiting a similar range.

ALGAE BLOOMS

Productivity is generally a factor 2-3 higher in Aquatic biomass compared to land biomass. Besides seaweed waste from the processing industries, other sources like Ulva from green tides seems to be a good target for bioethanol production (Yanagisawa et al., 2013). Green tides pose a nuisance for tourism, causes anoxic zones and produces sulphur emissions when washed up and rotting (Shimada et al., 2003; Charlier et al., 2007). It has been estimated that in China, around 2 million wet tonnes of green Ulva seaweeds were produced as a result of a green tide along the shores of Qingdao in 2008 and 2015, and every year with a minimum of 90,000 tonnes in 2012 (Hu et al., 2017). The introduced species Gracilaria salicornia (C. Agardh) E. Y. Dawson in Hawaii in the 1970s to research its potential for an agar industry has now become invasive (Smith et al., 2004). Large decomposing piles of this seaweed have drawn complaints from residents and has driven tourists away (Wang et al., 2011). The adequate disposal of these seaweed wastes, which are regarded as a nuisance, most probably involves high energy input and costs. The production of bioethanol from these seaweed wastes could lead to their effective disposal in addition to their economically advantageous use. Moreover, large Sargassum blooms in the Atlantic Caribbean have become a huge problem (Louime et al., 2017). The genus Sargassum includes many species that are often attached with a holdfast. Those common in the Gulf and western Atlantic are *Sargassum natans* (Linnaeus) Gaillon and *Sargassum fluitans* (Børgesen) Børgesen which are free floating. Huge Sargassum blooms occurred in 2011, 2014, 2015 and as recently as 2018 (Laoime et al., 2017; Putman et al., 2018). When waves of Sargassum washed up on Eastern Caribbean shores seven years ago, people hoped it was a one-off as piles swamped coastlines from Tobago to Anguilla. Nevertheless remote sensing in the summer of 2018 detected more than 323km^2 of Sargassum in the Caribbean basin, which is about three times larger than the previous record bloom in 2015 (Putman et al., 2018).

The reason for this alga to bloom remains unclear but possible causes include ocean pollution and the effects of climate change (Wang et al., 2018). As the alga reproduce asexually by fragmentation; when wind, waves, animals or boats break up the floating mats, small pieces grow into larger ones. Normally floating Sargassum form mats that provide food, refuge, breeding grounds and nursery habitat for an array of marine life from fish to sea turtles and birds. Its role as a nursery for commercially important fish such as mahi-mahi and amberjacks earned Sargassum designation as essential fish habitat. After a few years, mats lose their buoyancy and sink to the bottom of the ocean, becoming food for creatures in the deep Ocean. Washed ashore by wind and currents, the algae add nutrients to the beach ecosystem and help build and strengthen dunes (Putman et al., 2018). Blooms, however, can trap sea turtle hatchlings and thicker-than-normal rafts of it on the water can block needed sunlight for coral reefs. Caribbean fishermen report local flying fish disappearing from affected areas. Thick accumulations can entangle fishing gear and block water intake on fishing boat motors (Louime et al., 2017).

Monthly mean integrated Sargassum biomass in the Caribbean Sea and Central West Atlantic reached at least 4.4 million tons in July 2015. The average % C, % N, and % P per dry-weight are 27.16, 1.06, and 0.10, respectively (Wang et al., 2018).

The month of October 2018 showed a total Sargassum coverage of 323 km2 as compared with a historical mean of 103 km2 between 2011 and 2017, for the area bounded by 8 - 23°N

and 89 - 58°W. This indicates that there was approx. 12 million tonnes of free floating Sargassum available. The bloom extent in 2018 is still the highest during 2011-2018 for the Caribbean and the central West Atlantic (Wang et al., 2018).

Using algae blooms and waste material from the seaweed industry would make the food versus fuel debate redundant and macroalgae defacto provide the third generation biofuels (Graham-Rowe, 2011; Anderson, 2017). Moreover seaweeds (including Sargassum sp.) can ecologically play an important role as a biosequester that utilizes the atmospheric carbon dioxide for the assimilation as effectively as ligno-cellulosic biomass (Widyaningrum et al., 2016). Perez et al., (2018), demonstrated that with the aid of bacterial laminarinase ethanol can be produced from Sargassum using halotolerant yeast (without pre-treatment).

SPECIES AND PRODUCTION

Several species of macroalgae accumulate high levels of carbohydrates (Holdt and Kraan, 2011), which are all suitable as substrate for direct conversion into ethanol (Li et al., 2014; Ji et al., 2016; El-Sayed et al., 2016). In Western Europe kelp is the species of choice although the fast growing brown alga *Saccharina latissima* (Linnaeus) C. E. Lane, C. Mayes, L. Druehl & G. W. Saunders, has become the species of choice (Forbord et al., 2012). The ability to release spores by controlling light and temperature in tandem with a well-established protocol for gametophyte cultivation has made continuous cultivation a possibility (Forbord et al., 2012). Biomass productivity converted to carbon ranges from 1 to 3.4 kg carbon m-2 year-1 (Gao & McKinley, 1994; Mann, 1982; Mohammed & Fredriksen, 2004) and is in line with annual productivity of approximately 2 kg C per m2 for seaweed communities of the European coasts (Löning & Pang, 2003; Mohammed & Fredrikson, 2004), and 2.8 times higher than for example sugar cane (Gao & McKinley, 1994). There are many different numbers on seaweed production, but worldwide commercial production today is widely recognised to be 12-14Mt dry matter per hectare per year (Chen et al., 2015). For the North Atlantic, cultivation experiments with sugar kelp predict production potentials in the range 170-340t wet weight per hectare (Skjermo et al., 2014). The completed BioMara research programme estimated a seaweed bioethanol production potential of 18927.5 litres per hectare per year (Biomara, 2018).

Sugar kelp (*S. latissima*) is the fastest-growing macroalgal species of the kelp species in the European Atlantic Ocean. This species is similar to S. japonica (Areschoug) C. E. Lane, C. Mayes, L. Druehl & G. W. Saunders of which 3 million ton fresh weight is harvested annually from aquaculture in northern China, and 1 million ton fresh weight additionally in Japan (Wu & Pang, 2006; Ohno & Largo, 2006). Saccharina Stackhouse has as a rule approximately 40-50% carbohydrates of the dry weight (Adams, 2009). Laminaran (a glucose polymer) and mannitol are energy storage compounds, resembling starch in land plants, while alginates are structural compounds and correspond to cellulose and lignin in land plants. The seasonal variations of the carbohydrate composition are considerable (Schiener et al., 2015). The content of storage carbohydrates has a maximum in autumn. During the winter season, with nutrients in excess, the stored carbohydrates are utilized as energy source for protein synthesis reproduction and growth. Growth conditions and harvesting time may therefore strongly affect the quality of the seaweed biomass as substrate for microbial production

processes. As a general rule the highest yields of laminarin and mannitol coincided with the lowest yields in ash, protein, moisture and polyphenols (Schiener et al., 2015).

CULTIVATION SYSTEM BOTTLENECK

Over the last couple of decades different cultivation systems for seaweeds have been developed and improved ranging from intertidal fixed and floating bottom farms for Eucheuma J. Ahardh, Kappaphycus Doty and Gracilaria Greville (e.g., Philippines, Vietnam and Thailand) to elaborate floating net structures for Porphyra C. Agardh and long-line systems for kelp in China, Korea and Japan (Critchly et al., 2006). Modifications of long-line systems have been tested at small scale in Europe (Buck & Buchholz, 2004). These cultivation systems show that there is potential to develop large-scale ocean cultivation of seaweeds. However, existing cultivation and harvesting technology is labour intensive and need to be optimised to reduce costs and energy demand (Troell et al., 2009). Several EU funded initiatives have researched production and new structures e.g., the "Atsea project" (AtSea, 2015) has demonstrated a complete novel approach by using textiles in various forms and shapes creating a secondary seafloor. Using this approach seeding and harvesting could be mechanised and production increased up to 15 kg wet weight per m2. Nevertheless, seaweed cultivation has not become a mainstream activity yet due to cost and lack of mechanisation even with the knowledge to manipulate conditions to produce year-round sorus induction in *S. latissima* by applying short-day treatment of adult sporophytes throughout the year, and by the removal of the basal blade meristem (Forbord et al., 2012). The artificially induced and released zoospores formed viable sporelings at all seasons. This is of huge importance in order to develop a commercial mass scale cultivation and industrial scale-up and continuous production of kelp biomass (Forbord et al., 2012). This indicates that funding and EU initiatives should be directed at up scaling technologies.

PROCESSING AND FERMENTATION OF MACROALGAE BIOMASS

The water content of macroalgae is higher than for terrestrial biomass (80-85%; Holdt and Kraan, 2011), making seaweeds more suited for microbial conversion than for direct combustion or thermo-chemical conversion processes, which is an alternative for land-based biomass (Horn et al., 2000; Ross et al, 2007). The carbohydrates from seaweeds can be used for microbial conversion into a wide range of fuels and chemicals although ethanol seems to be a very straightforward approach (Kraan, 2013).

Ethanol production from hexose sugars, derived from e.g., corn stover or sugar cane, is a well-known process (USDA, 2006). However, hexose-based polysaccharides constitute only about 30-40% of the carbohydrates in kelp. The remaining fraction is composed of C-5 sugars that until now have not been applied as substrates for microbial production processes. However, recent breakthroughs have been made in C5 sugar fermentation technology allowing up to over 80% of the available carbohydrates to be fermented (Takeda et al., 2011; Wargacki et al., 2012; Ji et al., 2016). The potential ethanol production from seaweeds can be calculated and is based on the following assumptions: A carbohydrate content of 50% of the

dry weight and an 80% conversion ratio to ethanol. Through fermentation one gram of sugar can yield 0.4 g ethanol. This will yield 0.2 kg or 0.24 l ethanol from 1 kg dry weight seaweed biomass, corresponding to approximately 0.05 l ethanol per kg wet weight. Nevertheless if we take complex seaweed polysaccharides, levels of 25-40 gram ethanol per litre are achievable using bioengineered microorganisms (Kawai and Murata, 2016). Nevertheless Ji et al., 2016 managed to obtain 0.25 kg ethanol per kg of none pre-treated kelp biomass using a thermophilic bacteria. Moreover, high ethanol yields of 0.44, 0.47, and 0.3 g/g of sugars were obtained by fermenting glucose, mannitol, and alginate at 60°C, respectively (Ji et al., 2016).

SEAWEED POLYSACCHARIDES FOR BIOETHANOL PRODUCTION

Macroalgae are made up of several polysaccharides such as laminarin, mannitol, cellulose, fucoidan, agar, Carragenan and alginate (Holdt and Kraan, 2011). Except for cellulose and laminarin, all other polysaccharides have been utilized industrially. Mannitol, a sugar alcohol, has wide medical applications such as in osmotherapy and in food products where it is used as a sweetener for individuals suffering from diabetes due to its low glycemic index. Fucoidan, a sulfated polysaccharide, is widely used as dietary supplement. Alginate has the most industrial importance. Its utilization ranges from textile printing to pharmaceutical, medical, and food applications (Kraan, 2012).

ETHANOL PRODUCTION

Macroalgae are good candidates for the conversion of polysaccharides into sugars which can then be converted into ethanol (Goh and Lee, 2010; Table 1). However, it is often difficult for one microorganism to convert all components of brown algae with different oxidoreduction potentials to ethanol (Ji et al., 2016; Jung et al., 2013).

The conversion of biomass to bioethanol usually requires different stages (Harun et al., 2014; Widyaningrum et al., 2016):

- Pretreatment of feedstock to release complex carbohydrates
- Hydrolysis of polysaccharides to monosaccharides
- Fermentation
- Product recovery

Nevertheless, Ji et al., (2016) demonstrated that the thermophilic bacterium *Defluviitalea phaphyphila* sp. nov.is the first characterized thermophilic bacterium capable of direct utilization of brown algae. It can simultaneously utilize mannitol, glucose, and alginate to produce ethanol.

Table 1. Some examples of seaweeds utilized for the production of bioethanol

Seaweed or seaweed waste	Pretreatment/Hydrolysis (conversion, %)	Fermentation (ethanol yield, %)	Reference
Alariacrassifolia	Thermal acid/enzymatic (0.82)	*Saccharomyces cerevisiae* (0.38)	Yanagisawa et al., 2011
Gelidia dura residue after agar extraction	Alkaline/acid treatment (0.85)	*Saccharomyces cerevisiae* (0.46)	Baghel et al., 2015
Gelidiumamansii	Thermal acid/enzymatic (0.65)	*Saccharomyces cerevisiae* (0.49)	Sunwoo et al., 2016
Gelidiumamansii	Thermal/acid (0.72)	*Pichia stipitis* (0.50)	Sukwong et al., 2018
Gellidiumpusillum residue after agar extraction	Enzymatic (0.84)	*Saccharomyces cerevisiae* (0.47)	Baghel et al., 2015
Gracilaria verrucosa	Thermal acid/enzymatic (0.75)	*Saccharomyces cerevisiae* (0.48)	Sunwoo et al., 2016
Gracilaria verrucosa residue after agar extraction	Enzymatic (0.87)	*Saccharomyces cerevisiae* (0.43)	Kumar et al., 2013
Gelidiumelegans	Thermal acid/enzymatic (0.67)	*Saccharomyces cerevisiae* (0.38)	Yanagisawa et al.,2011
Eucheumadenticulatum	Thermal acid/enzymatic (0.53-0.80)	*Saccharomyces cerevisiae* (0.47) *Scheffersomycesstipitis* (0.48)	Sunwoo et al., 2016 Ra et al., 2017
Eucheuma spinosum	Thermal acid/enzymatic (0.49)	*Candida tropicalis* (0.40)	Ra et al., 2015
Kappaphycusalvarezii	Thermal acid/enzymatic (0.53)	*Saccharomyces cerevisiae* (0.47)	Sunwoo et al., 2016
L*aminaria*digitata	Acid (0.28) Acid/enzymatic (0.94)	*Saccharomyces cerevisiae* (0.38) *Saccharomyces cerevisiae* (0.94)	Schiener et al., 2016b Kostas et al., 2017
Laminaria japonica	Acid/enzymatic (0.37)	*Saccharomyces cerevisiae* (0.41)	Kim et al., 2011
Laminariahyperborea	Acid (0.35)	*Saccharomyces cerevisiae* (0.29)	Schiener et al.,2016b
Chaetomorpha linum	Hydrothermal treatment (1.00)[1] Wet oxidation (0.98) Steam explosion (0.92) Plasma assisted treatment (1.00) Ball milling (0.91)	*Saccharomyces cerevisiae* (0.39)[2] *Saccharomyces cerevisiae* (0.44) *Saccharomyces cerevisiae* (0.38) *Saccharomyces cerevisiae* (0.41) *Saccharomyces cerevisiae* (0.44)	Schultz-Jensen et al.,2013
Gelidiellaacerosa residue after agar extraction	Enzymatic (0.83)	*Saccharomyces cerevisiae* (0.47)	Baghel et al., 2015
Kappaphycusalvarezii	Thermal acid (0.31)	*Saccharomyces cerevisiae* (0.40)	Khambhaty et al., 2012
Saccharina japonica	Thermal acid (0.21) Thermal acid/*Bacillus* sp (0.31) Thermal acid/enzymatic (0.69)	SSF – *Bacillus* sp. + *Pichia angophorae* (0.33)	Jang et al., 2012
Saccharina latissima	Acid (0.26)	*Saccharomyces cerevisiae* (0.13)	Schiener et al.,2016b
Ulva fasciata	Thermal + enzyme (0.91)	*Saccharomyces cerevisiae (*0.45)	Trivedi et al., 2013
Ulva pertusa	Thermal acid/enzymatic (0.59)	*Saccharomyces cerevisiae* (0.38)	Yanagisawa et al., 2011
Ulva rigida	Thermal/acid (0.64)	*Pachysolentannophilus* (0.50)	El Harchi et al., 2018

PRETREATMENT

Several pretreatments consist of hydrothermal pretreatment, wet oxidation, steam explosion, plasma-assisted pretreatment and ball milling (Schultz-Jensen et al., 2013; Harun et al., 2014; Table 1). Wet oxidation of *Chaetomorpha linum* (O. F. Müller) Kützing caused more than 50% biomass loss (Schultz-Jensen et al., 2013) which indicate the need for an appropriate pre-treatment. Acidic pretreatment at high temperature is widely used, but requires certain chemicals which are difficult to recover and can generate non-sugar byproducts, with inhibitory potential on further biological conversion. Alternatively, gamma irradiation is an effective method for the depolymerization of complex polysaccharides and structural breakage of the seaweed cell wall (Yoon et al., 2012). Other pretreatments proposed for altering enzymatic digestibility and ethanol potential for the green macroalgae are ethanol organosolvents and liquid hot water, causing more drastic structural changes than pretreatments with alkaline media and ionic liquids (Jmel et al., 2018).

HYDROLYSIS

Dilute-acid hydrolysis is a typical physicochemical method to treat raw macroalgal biomass (Yanagisawa et al., 2011; Meinita et al., 2012a; Lee et al., 2013; El-Sayed et al., 2016; Soliman et al., 2018), and is highly influenced by acid concentration and hydrolysis time which need to be optimized to maximize concentrations of mono-sugars and ethanol (Meinita et al., 2012a; Soliman et al., 2018). Combination with ionic liquid pretreatment increased the enzymatic saccharification of seaweed waste pretreated (Uju et al., 2015). The decomposition of sugars caused by acid treatment at elevated temperatures can lead to the formation of degraded products, susceptible of causing inhibition of fermenting microorganisms or inducing a prolonged lag phase, i.e., furfural, 5-hydroxymethylfurfural and levulinic acid. Other inhibitory substances could be the metals in macroalgae, which show a higher content (1–1.5%wt.) than for terrestrial biomass (0.5–1.1%wt.; Jung et al., 2013; Fakhrudin et al., 2014). Degraded products in pretreated hydrolyzate can be removed prior to fermentation, and activated charcoal and calcium hydroxide have been frequently proposed (Meinita et al., 2012b; Ra et al., 2015 and 2017), as for example is done in the detoxification of hydrolyzates from lignocelullosic biomass (Soto et al., 2011). Less often used is the alkaline treatment, i.e., for galactan extraction (strong alkaline solution, 70–90°C, 5 h), but the major advantages are the low polysaccharide yields and generation of waste streams (Goh and Lee, 2010). The efficient hydrolysis to monosaccharides or polysaccharides without a fermentation inhibitor was reported using an integrated pretreatment with hydroxyl radicals and hot water (Gao et al., 2015). Alternatively, the production of platform-chemicals and sugar production by dilute-acid-catalyzed hydrothermal reaction was proposed. *Kappaphycus alvarezii* was used for the production of glucose, galactose, levulinic acid and 5-hydroxymethylfurfural (Lee et al., 2016a).

ENZYMATIC HYDROLYSIS

This is a milder saccharification tool for both whole seaweeds or for residual fractions, but the lack of specific enzyme activities for seaweed polysaccharides limits this approach and the contribution of molecular bioengineering would be interesting since new microorganisms would be required (Wargacki et al., 2012; Jung et al., 2013; Kim et al., 2015; Shukla et al., 2016; Obata et al., 2016; Kawai and Murata, 2016).

The hydrolytic enzymes used for lignocellulosics (cellulase and cellobiase) and other multienzyme complexes are frequently proposed for seaweeds (Yanagisawa et al., 2011; Trivedi et al., 2013). Other specific enzymes for the degradation of brown algal polysaccharides, are alginate lyases, laminarinases or β-glucanases, isolated from marine microorganisms (Wargacki et al., 2012; Jung et al., 2013; Kim et al., 2013; Ji et al., 2016; Obata et al., 2016), seaweed compost (Tang et al., 2009) or sandbar substratum (Kang and Kim, 2015), and could be suitable to saccharify macroalgae, although they showed low hydrolysis efficiency, requiring additional pretreatment (Schaumann and Weide, 1990; Adams et al., 2011; Jung et al., 2013). The enzymes represent an important cost of the process, therefore reutilization is encouraged (Trivedi et al., 2013). Combinations of treatments can be successful, such as mechano-enzymatic (Amamou et al., 2018) or chemical and enzymatic hydrolytic treatments (Ge et al., 2011; Jung et al., 2013; Ra et al., 2015; Sunwoo et al., 2016). The absence of pretreatment prior to fermentation yielded lower ethanol values, but the direct application reduces the product cost.

FERMENTATION

Fermentation to ethanol is generally performed by Saccharomyces cerevisiae, commonly used for industrial fermentation of glucose, but also can ferment galactose (Goh and Lee, 2010; El-Sayed et al., 2016; Renita and Utharalakshmi, 2017), however, macroalgae contain specific carbohydrates with different degree of utilization, i.e., mannitol and laminaran (Horn et al., 2000; Lee and Lee, 2012), and in some cases, specific adaptation of bacterial strains to these specific sugars and to high salinity was required (Cho et al., 2013; Sunwoo et al., 2016). Furthermore the utilization of seaweed mixtures and different microorganisms has been reported, i.e., a mixture of red, brown, and green seaweed wastes treated with acid, saccharified with enzymes and further fermented with a co-culture of adapted S. cerevisiae and Pichia angophorae (Sunwoo et al., 2017). A novel approach consists of the use of metabolically modified bacteria (Takeda et al., 2011). Conditions for hydrolysis and fermentation in studies published from 2000 till 2015 can be found (Fernand et al., 2017) and are summarized and updated in Table 1. The bioconversion strategies widely used are separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Furthermore, consolidated bioprocessing, consisting of performing enzyme production, hydrolysis, and fermentation in a single unit (Harun et al., 2014; Fernand et al., 2017). Most processes were successfully scaled up (Shukla et al., 2016). After the biomass is converted to ethanol, the solid protein residue could be used as an ingredient for fish feed or other protein applications (Seghetta et al., 2016a).

Defluviitalea phaphyphila sp. Nov. is the first characterized thermophilic bacterium capable of direct utilization of brown algae (Ji et al., 2016) and can simultaneously utilize mannitol, glucose, and alginate to produce ethanol. Ethanol yields of 0.47 g/g-mannitol, 0.44 g/g-glucose, and 0.3 g/g-alginate were obtained. A rational redoxbalance system under obligate anaerobic condition in fermenting brown algae was revealed in *D. phaphyphila* Alga l strain through genome and redox analysis. The excess reducing equivalents produced from mannitol metabolism were equilibrated by oxidizing forces from alginate assimilation. Furthermore, *D. phaphyphila* Alg1 strain can directly utilize untreated kelp powder, and 10 g/L of ethanol was accumulated within 72 h with an ethanol yield of 0.25 g/g-kelp. Microscopic observation further demonstrated the deconstruction process of brown algae cell by *D. phaphyphila* Alg1 strain (Ji et al., 2016).

CONCLUSION

The growing importance of seaweeds for the production of alcohol as fossil fuel replacement to abate global issues in respect of global warming, CO_2 reduction and climate change are obvious. The integrated biomass deconstruction system of *D. phaphyphila* Alg 1 strain, as well as its high ethanol yield, provides an excellent alternative for brown algae bioconversion at elevated temperature (Ji et al., 2016). Currently seaweed production from longline systems is not economically feasible. The lowest production level of dry seaweed to meet 0.93($/L) for ethanol fuel and 0.07$/kW-h for electricity was found to be 0.68 and 3.7 million tons (drybasis), respectively (Soleymani and Rosentrater, 2017). However looking at the volume of algae blooms with 2 million tonnes of Ulva and 4.4 million tons of *Sargassum* (Hu et al., 2017; Wang et al., 2018) ethanol production or electricity generation is a reality. Achieving economic cultivation may be possible by lowering production costs and increasing the area under cultivation. Logically society should tap into the availability of massive algae blooms as a feed stock to make seaweed bio-refineries a reality. Seaweed bio-refineries should be supplemented with cultivated seaweeds and it is recommended that time and effort are focused on R&D of seaweed farming in Europe in parallel with exploiting algae blooms. Solving specific bottlenecks in upscaling and mechanical harvesting technologies in order to mechanise large seaweed cultivation at a European level has priority in order to fulfill the promise of macroalgae seaweed bioethanol.

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Chapter 90

THE BROOK LAMPREY (*LAMPETRA PLANERI*) AND UKRAINIAN LAMPREY (*EUDONTOMYZON MARIAE*) IN THE CZECH REPUBLIC: GENERAL BIOLOGY, ECOLOGY, DISTRIBUTION AND STATUS WITH RECOMMENDATION FOR CONSERVATION

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ABSTRACT

At present two native lamprey species occur in the Czech Republic, namely the brook lamprey (*Lampetra planeri*) and the Ukrainian lamprey (*Eudontomyzon mariae*). Both species are listed under Annex II to the EU Habitats Directive (Directive 92/43/EEC), thereby requiring Member States to designate Special Areas of Conservation (SACs) for their conservation. The paper summarizes knowledge about biology, ecology, distribution, monitoring of occurrence and action plans/recovery programmes for the mentioned species.

1. INTRODUCTION

The Czech Republic is situated in the very heart of Europe, and it is sometimes called the „roof of Europe“, as this region is wholly dependent upon precipitation coming in the form of rainfall or snowfall. The Czech Republic´s territory was historically inhabited by four

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lamprey species belonging to the family Petromyzontidae: the sea lamprey (*Petromyzon marinus*), the river lamprey (*Lampetra fluviatilis*), the brook lamprey (*Lampetra planeri*) and the Ukrainian lamprey (*Eudontomyzon mariae*). The occurrence of the first two mentioned species has been historical. The last sea lamprey specimen was caught in Bohemia in 1902 and the river lamprey was reported there for the last time in 1897 (Hanel and Andreska 2016). Nevertheless, both of the lamprey species could appear again in the Czech Republic, as there have been recent reports of their findings in the German reaches of the Labe/Elbe River, not far from the Czech border. Systematic monitoring of the brook lamprey and Ukrainian lamprey in the Czech Republic has been carried out within the Lampetra project implemented by many mappers since the 1990s (Hanel 1994a). Therefore, current knowledge of the topic is presented in this chapter. The taxonomic position of the Ukrainian lamprey in the Czech Republic has been unclear (some authors synonymized it with *Eudontomyzon vladykovi*, others believe that both the species are valid taxons and *Eudontomyzon mariae* does not inhabit the Danube basin, see Hanel et al. (2015), Levin et al. (2016)). For the purpose of this chapter, the lamprey population in the Račí potok/Crayfish Brook (the Danube River basin) in the Czech Republic is classified within the species *Eudontomyzon mariae* (Hanel and Lusk 2005, 2006, Hanel and Andreska 2016).

2. RESULTS

2.1. Lamprey Population Monitoring

Distribution data on both of the lamprey species (i.e., *Lampetra planeri, Eudontomyzon mariae*) are principally based on various sources from the past with some new additions. The former data was gathered from literature (scientific/technical articles, regional contributions, local chronicles), data based on materials kept at museums and universities were also used for the study (Hanel 1994a, c, 1996). The following methods were applied in gathering the new data:

- living adult specimens seen with a naked eye during the spawning period (e.g., Hanel et al. 2015);
- digging the larvae out of the suitable sediments using a shovel (e.g., Hanel 1994a);
- electrofishing (e.g., Hanel et al. 2015);
- searching for dead adults after spawning (e.g., Hanel and Jankovský 1996);
- searching for the remnants of lampreys´ bodies in the digestive tract of piscivorous vertebrates (e.g., the black stork - Pojer and Hanel 1996, european eel - Bílý 2000, common kingfisher - Čech 2017);
- detection of dead ammocoetes (when the stream bed dries out, e.g. Hanel 2000);

Electrical fishing is the preferred technique used for juvenile lampreys (also called ammocoetes) sampling in waters less than 1 m in depth (APEM 2004, Harwey and Cowx 2003, Dunhama et al. 2013, Hanel et al. 2015). The dual wave operation “on-off” sequence is used to irritate ammocoetes out of the substrate. While the gear is operated, the anode is slowly pulled backwards in the water to cause lampreys to emerge from burrows as a result of

electro-taxis. This procedure is repeated throughout each operation. The time sequence for "on" and "off" states during electrofishing can be variable under different natural conditions (water temperature, conductivity, sediment depth). For quantitative surveys, some authors use a delimiting framework (equivalent to a quadrat base area 1m^2), see e.g. Harvey and Cowx (2003), Persat and Copp (1990). Two basic methods for quantitative sampling are used in the Czech Republic: Point Abundance Sampling or continuous electrofishing in the selected brook/river sections. Matěnová (2003) and Matěnová and Matěna (2003) compared both mentioned methods in the Malše River (Southern Bohemia). Using continual electrofishing the reached estimate was 2.5-17 ammocoetes of the brook lamprey per 100 m^2, whereas up to 10,000 ammocoetes per 100 m^2 were found using the PAS method. The total ammocoetes estimation in delimited section of the brook/river is variable due to heterogeneous sediments.

Successive removal method in brook lamprey electrofishing is usually used for quantity estimation. The study for accuracy verification of quantitative estimation was carried out on a smaller lowland trout brook in Central Bohemia (the Losinský potok Brook) in 1996 (Hanel and Müller 1997) and 2015. Characteristics of the Losinský potok Brook are as follows: coordinates 49°50′44,15″ N, 15°7′49,7″ E, length 14.3 km, watershed area 40.5 km^2, average discharge at opening 0.23 m^3.s^{-1}, water conductivity approx. 330 μS.cm^{-1}. The selected stretch was 60 m long, its mean width was 4.0 m, the water column depth 30-40 cm. Pools up to 1 m deep were present in the area of study. The brook bed was not artificially modified, ground was sandy to gravely, with some large stones. Both riversides were vegetated with shrubs and trees. A belt of fine sediments, about 1 m wide, was found along riversides, in depths up to 20 cm. The first test (in 1996) was conducted using electric aggregate IG200/2 (made by Hans Grassl, Germany, direct current, 130-250 V, to 2.5 A, to 250 W), the second one in 2015 was carried out using electric aggregate SEN (made by Radomír Bednář, the Czech Republic, direct current, 180–220 V, 1.2–1.7 A, to 95 Hz). It was used as a method of energising the electrode for 3-5 seconds bursts of pulsing direct alternative current. A brief 2-3 second pause was made between the bursts, allowing lampreys to emerge when the power stopped.

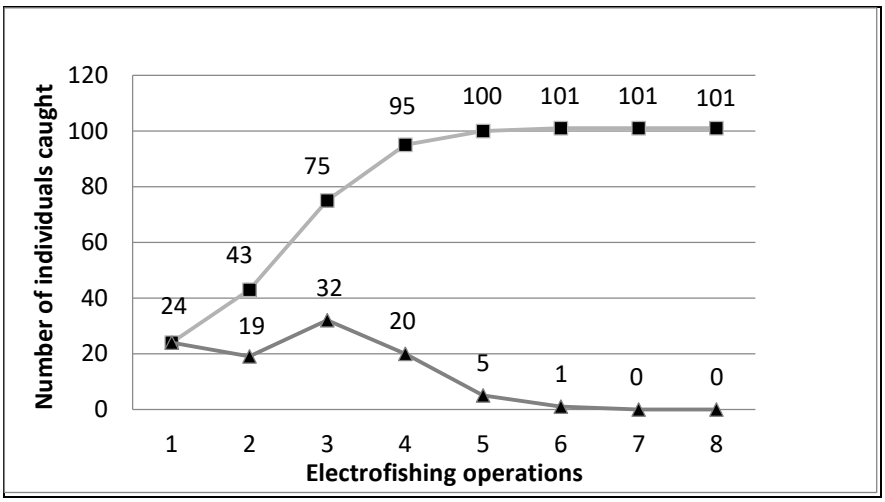

Figure 1. Result of a successive removal electrofishing exercise on lamprey capture in the Losinský potok Brook (the Labe/Elbe River basin, Central Bohemia) in March 1996. Catch at each fishing (below curve), cumulative catch (upper curve), n = 101. The theoretical total number of larvae calculated by linear regression was to be 131 (see Hanel and Müller 1997).

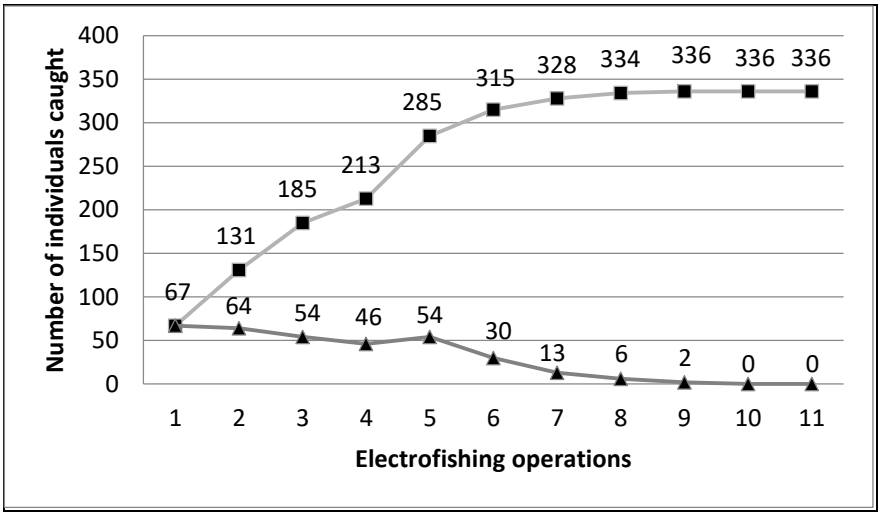

Figure 2. Result of a successive removal electrofishing exercise on lamprey capture in the Losinský potok Brook (the Labe/Elbe River basin, Central Bohemia) in May 2015. Catch at each fishing (below curve), cumulative catch (upper curve), n = 336. The theoretical total number of larvae calculated by linear regression was estimated to be 417.

The goal of the experiment was to estimate how many times hunting by electric aggregate should be repeated before no more larvae are found at the given site. A hunting period lasted 30 minutes, followed by a 15 minute long pause. Hence, the next hunt started 45 minutes after the beginning of the foregoing hunt. Once in the water column, further bursts of electricity immobilised the ammocoetes and allowed them to be netted and removed for subsequent analysis. Immobilized lamprey were captured by a fine-mesh net, and transferred to a water-filled container on the brook bank. Only larvae longer than 50 mm were recorded. The sampling process was repeated several times until there were no more larvae.

The experiment showed that lamprey abundance can be significantly underestimated if catching is not repeated several times at the same site (see Figures 1 and 2). The large number of electrofishing operations required confirms the difficulty of obtaining quantitative data by lamprey larvae electrofishing. Our results correspond with the data cited by Igoe et al. (2004). After using successive removal electrofishing in the Irish Black River (monitored area of the bottom as for 20 x 2 m) there were no brook lamprey larvae caught, except for the seventh catch (but only 8 individuals were caught in total).

To estimate the number of brook lamprey larvae, Visible Implant Elastomer Tags in the river Ostružná (tributary of the Vltava River, the Labe/Elbe River basin) were also tested, see Křížek (2003). Based on the tagged and nontagged larvae ratio the abundance was estimated to range between 5.9 and 6 ind.m^{-2} of the examined sediments.

A laboratory experiment in aquarium with known number of ammocoetes was also carried out. In total 20 larvae (8-12 cm in length) were placed in a glass aquarium 100 x 50 x 50 cm and with a 10 cm depth of fine sediment from the Losinský potok Brook. After the first hunt using electric aggregate (type SEN) 4 larvae appeared on the sediment surface.

Moreover, an estimate of the size of the lamprey population by electrofishing alone is difficult from another point of view. Small juvenile lampreys (smaller than about 5 cm) are only rarely caught during electrofishing (Igoe et al. 2004, Hanel et al. 2015).

Using continual electrofishing, numbers of the brook lamprey usually varied between 1–24 ind.m^{-2} in brooks and river sediments in the Czech Republic. Big floods can cause exceptional conditions for lampreys. After a flood in the Štěpánovský potok Brook (a tributary of the Sázava River, Labe/Elbe River basin) Hanel and Pešout (1989) found a site with 47 larvae per m^2 in the depth of the silt reaching 30 cm (70-120 mm in length). The ammocoete number ranged between 311 and 7,067 individuals per hectar (calculated from the total surfaces of the bottom of the flowing waters which were studied) in the Czech Republic (Hanel and Lusk 2005).

Based on all the data about brook lamprey larvae numbers in the Czech Republic the following categories can be established: above average abundance – more than 5 ind.m^{-2}, average abundance 0.5-5 ind.m^{-2}, below average abundance – less than 0.5 ind.m^{-2} in sediments with optimal structure (Hanel et al. 2015).

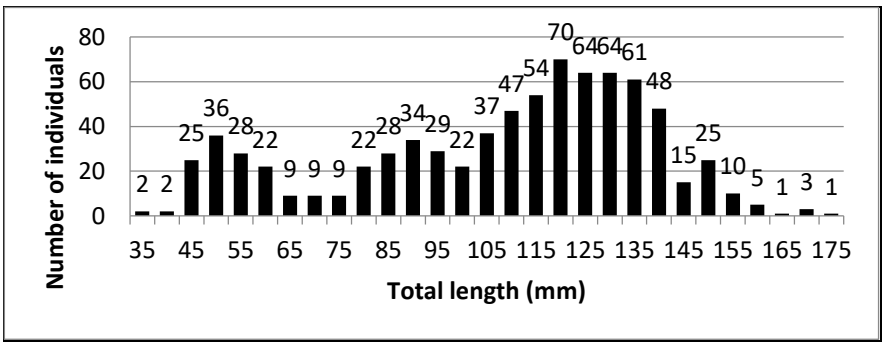

Figure 3. Length composition of brook lamprey dead larvae collected in a mill-race of the Padrťský potok Brook on July 1998 (Labe/Elbe River Basin, Western Bohemia), n = 782, see Hanel (2000).

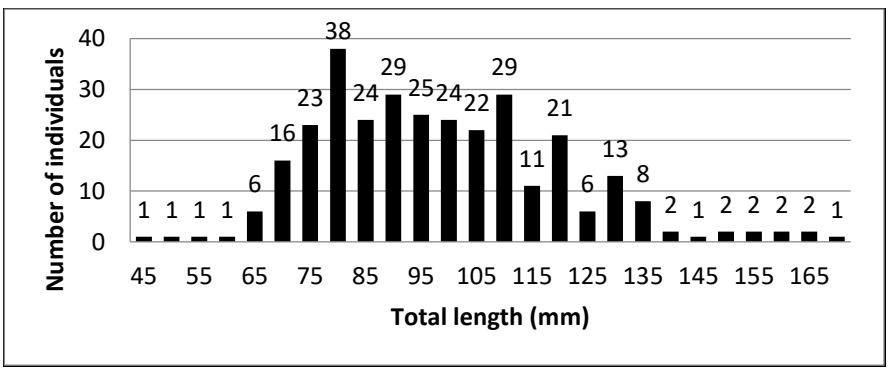

Figure 4. Length composition of electrofished sample of the brook lamprey larvae in the Losinský potok Brook (Labe River basin, Central Bohemia) in May 2015, n = 331.

2.2. Growth of Larvae and Population Structure of the Brook Lamprey Population

Ranges in length/weight growth of brook larvae from various Czech sites were presented by Kux (1985): age 0+ (25–80 mm, 0.03–1.2 g), age 1+ (39–125 mm, 0.25–2.5 g), age 2+

(69–160 mm, 1.0–5.5 g), age 3+ (100–190 mm, 1.5–10.6 g), age 4+ (126–185 mm). In Figure 3 the length structure of the brook lamprey dead larvae collected in the mill-race of the Padrťský potok Brook is depicted. Figure 4 shows the length structure in living ammocoetes of the same species collected in the Losinský potok Brook.

2.3. Habitat Selection by Lampreys and Food of Ammocoetes

Poulíčková (1996, 1998) found over hundred diatoms in total in sediments of 40 running waters with the occurrence of the brook lamprey in the Czech Republic. The following taxa are among the most common species: *Achnanthes lanceolata, A. minutissima, Cymbella silesiana, Fragillaria* sp., *Gomphonema parvulum, Meridion circulare, Navicula avenacea, N. gregaria, N. lanceolata, Nitzschia* sp., *Surirella ovata*. It is interesting that sometimes diatoms can survive passage through lamprey digestive tract. The proportion of surviving diatoms depends on the season (it is higher in winter than in summer) and length of lamprey. The lowest value was found in the genus *Achnanthes* (10-20%), on the contrary the highest surviving share for the genera *Cyclotella* a *Amphora* (65 % and more), see Špačková and Jasenská (1985) and Špačková and Poulíčková-Jasenská (1987). Diatoms as long as 380 μm were observed in the intestinal content, in the genus *Closteria* even 610 μm (with a width of 60 to 70 μm). Fragments of plant vessel bundles reached a length of 350 to 580 μm and width of 40 to 120 μm. The longest organic fragments were 700 to 3960 μm long and 2 to 4 μm wide. The size of sand grains was about 400 μm (Poulíčková 1994).

Poulíčková et al. (2000) studied microphytozoobenthos and macrozoobenthos in 14 brooks with the occurrence of the brook lamprey larvae in the Šumava/Bohemian Forest Mts. region (Southern Bohemia). A total of 69 diatom taxa have been found, the most frequent species being *Achnanthes lanceolata*, *A. minutissima*, *Diatoma mesodon*, *Gomphonema parvulum* and *Meridion circulare*. A total of 31 macrozoobenthos taxa were found, the most frequent species being *Ancylus fluviatlis*, *Drusus* sp., *Limnephilus* sp. and *Rhyacophila* sp. The gut in the larvae of the brook lamprey can also contain small nematodes (Hanel and Pešout, 1989, Poulíčková 1994). Poulíčková and Merta (1998) presented variability of water quality in the whole Račí potok/Crayfish Brook, the only site of occurrence of the Ukrainian lamprey in the Czech Republic (from the spring to the month of September 1995) as follows: water temperature 9.7–12°C, pH 6.97–7.44, dissolved oxygen level 10.2–11.0 $mg.l^{-1}$, conductivity 168–235 $\mu S.cm^{-1}$, total nitrogen content 0.5–1.2 $mg.l^{-1}$.

Merta et al. (2000) analyzed silts in the Račí potok/Crayfish Brook and in the main stream of the Morava/Moravia River (a stretch between the villages of Postřelmov and Bludov), see Table 1. With the habitat requirements of lamprey larvae in mind, a rapid decrease in the oxygen dissolved in the water in various silt depths should be mentioned. Average protein proportion in dry silt substance in the depth 0-20 cm was 13.7 % in the Račí potok/Crayfish Brook and in the Morava/Moravia River only 8.04 %. Average silt particle in the Račí potok/crayfish Brook was 3.049 mm while in the Morava/Moravia River it was 0.438 mm. Brook lamprey larvae were found in the waters of the Czech Republic, mostly in sediments with particle sizes between 0.1–0.7 mm (Merta et al. 2000; Hanel et al. 2015).

Table 1. Physical and chemical characteristics of sediment with the occurrence of the Ukrainian lamprey larvae (Račí potok/Crayfish Brook, November 20, 1998) and the brook lamprey larvae (Morava/Moravia River, June 1, 1999), see Merta et al. (2000)

	Račí potok Brook			Morava River		
Silt depth (cm)	0	10	20	0	10	20
Temperature (ºC)	2.5	2.2	3.2	15.2	16.3	16.6
Dissolved oxygen level ($mg.l^{-1}$)	13.8	5.5	2.4	11.3	4.0	2.6
Dissolved oxygen level (%)	101	42	24	116	41	28
Potential redox (mV)	179	181	99	_	_	_
pH	7.2	6.7	6.47	7.36	6.75	6.83
Conductivity ($\mu S.cm^{-1}$)	183	186	295	212	1031	980

Average values of water quality from three brooks with the Brook lamprey occurrence (Štěpánovský potok Brook, Polánecký potok Brook, Částrovický potok Brook, Central Bohemia, Labe/Elbe River basin) are as follows (the values are in $mg.l^{-1}$ unless stated otherwise): calcium 54.9-63.7, magnesium 16.2-19.5, iron 0.19-0.26, ammonia 0.34-0.68, chloride 39.2-45.4, sulphate 84.9-94.2, nitrite 0.03-0.089, nitrate 33.3-53.28, o-phosphate 0.042-0.12, pH = 7.32-7.50, conductivity 438-507 $\mu S.cm^{-1}$, total hardness 2.02-2.26 $mmol.l^{-1}$, BOD_5 2.67-4.03, total suspended solids 11.7-13, total dissolved solids 307-357.6. The level of heavy metals in sediments (range of average values from the sites with larvae occurrence within the brooks studied) was as follows (in $mg.kg^{-1}$ of dry sediment): Cd 0.09-0.21, Cr 1.4-3.2, Cu 1.9-9.6, Zn 8.9-65.1, Pb 4.5-14.6, Ni 2.5-10.1, Hg 0.005-0.021 (Hanel 1994b).

In a study by Slavík et al. (1996) season preference of microhabitat in brook lamprey ammocoetes in the Vltava River was reported. Using Physical Habitat Simulation they found that the larvae migration to offshore sand zones with slow water flow happens in June.

2.4. Breeding

The brook lamprey typical breeding site is a small stream with water 10-20 cm deep over the nests, the redds were usually just upstream from rapids or riffles, often under bridges but also in sunlit areas (author's data). During mating, the oral disc in males usually attaches to the head or branchial region, and the male´s tail almost immediately coils around the female, bringing the urinogenital papilla toward the cloaca of the female. Moreover, Dyk (1949) described atypical breeding, during which the male was not attached to the female.

Our data shows that the most frequent spawning group consisted of a single female with 2 or 3 males. Lohniský (1975) reported that the spawning act was repeated every ½ to 2 minutes, and for individual females, the spawning cycle lasted for 0.5 to 1 hour. Dyk (1949) and Lohniský (1966) described „aggressive behaviour" in which a male may appear to remove a rival from the nest.

According to Lohniský (1975), the whole spawning period from the beginning of the upstream migration to the spawning grounds to the end of spawning lasts about 23 days at water temperature of 14–15°C, 15 days at 11–17°C, and 8 days at 12.0–19.5°C. The death of spent individuals occurred within 33 days of the completion of spawning. For the record, the first author mentioned observed a solitary female, which spontaneously spawned 438 eggs

using convulsive body movement (water temperature was 8°C) after attaching itself on an aquarium wall. The maximum number of eggs per female was 2,696 in an individual with the length of 158 mm, originating from the Mastník Brook (the tributary of the Slapy water reservoir, Central Bohemia), see Hanel and Pešout 1989.

2.5. Metamorphosis

Metamorphosis begins in native waters during the end of the summer. According to the authors observations, complete transformation in the brook lamprey was confirmed in an individual caught on October 23, 2001 in the Štěpánovský potok Brook (the Labe/Elbe River basin), in two males caught in the Losinský potok Brook on November 11, 2016 and in a male and a female captured on November 30, 2001 in the Strašický potok Brook (the Labe/Elbe River basin).

2.6. Bioindication by Lampreys

The saprobity index of the brook lamprey reaches oligosaprobity (Si = 1.1) at a high significance level (I = 4), Sládeček (1976). Morever, Poulíčková et al. (1998) clearly showed that only half of the sites studied matched the expected level, i.e., oligosaprobity. Other localities were found to be within the range of beta-mesosaprobity. The lamprey larvae seem to be able to tolerate a higher organic pollutant level than expected. The saprobity index based on calibration of the sediment diatoms was in good agreement with the results obtained by zoobenthos analysis. The actualized saprobic index (Si), i.e., the indication of an organic pollution level was calculated using analyses of diatoms and benthos from water streams inhabited by the brook lamprey. The values found were Si = 1.3 (indicator weight = 4, oligosaprobic zone - 7, beta-mezosaprobic zone - 3), for more details see Hanel (1997). The results show that the brook lamprey is not fixed to only oligosaprobic zones but it tolerates the beta-mesosaprobic conditions. They can also tolerate the wide ranges in the trophic state, pH and oxygen content (Poulíčková et al. 1998).

The brook lamprey can be found in running waters from trout to barbel fish zones (mostly in trout and grayling ones). The fish zones correspond with better/worse oligosaprobity and better beta-mesosaprobity (BOD_5 to 4 $mg.l^{-1}$) – the extreme value is related to brook lamprey populations. Brook lamprey are specific bioindicators of water quality and environmental conditions. The well balanced length and age structure of larvae confirm long-term high quality in environmental conditions. The observed abundance of ammocoetes in Czech waters varied between 311-7,067 individuals per hectare. These relatively broad ranges may be influenced by various electrofishing methods, anthropogenic factors or various study sites (optimal or sub-optimal muddy sediment habitats). Low number in ammocoetes or total absence of some age groups indicate influence of some negative factors during the past breeding period. Annual observations of breeding population and comparison of adult specimen numbers between years can be a good and helpful bioindicative parameter. Absence of breeding adult specimens or their remarkable low number indicate the influence of some negative factor in the past. Blind larvae are found on the bottom of muddy sediments and due to their specific life strategy they are not suitable bioindicator of short-time

deterioration of water quality. The life strategy of larvae during contamination of their habitats is limited to the lower parts of the substrate and it is necessary for the larvae to withstand these adverse conditions. It is known that the brook lamprey population in the Polánecký potok Brook (Central Bohemia) survived the pollution of the brook with diesel oil which lasted for several weeks. It turned out that the lamprey larvae can survive even a water pollution accident when other ichtyofauna become extinct. Such an event was studied in the Polánka Brook (Central Bohemia, Sázava/Elbe River basin, 49° 41′ 51″N, 14° 51′ 14″ E) in 1992. At that time the quantity of oil substances in the water reached 0.3 $mg.l^{-1}$ and the level in the upper layers of bottom sediments ranged between 155 to 363 $mg.l^{-1}$ (Hanel 2004). Ammocoetes survived because of their underground way of life, as they are continuously buried in soft sediments in the brook bed during their larval stage. The lamprey has been occurring in the Polánka Brook (ammocoetes were confirmed there in 2014 as well). The strategy of larvae during water pollution is therefore to remain in the substrate as long as possible. Only if toxicants penetrate into the substrate, the larvae leave it and tend to get in more suitable sites (author's observations). Therefore, the brook lamprey seem to be a good bioindicator of long-term good quality of the water environment, but its larvae can sometimes survive short-term pollution.

2.7. Occurrence of Lampreys

In the Czech Republic the mapping method for evidence of wild animal findings is the most used. Grid size is 10 degrees East longitude and 6 degrees North latitude. The grid is coded by four digits, for example 6670, where "66" is the row and "70" is the column of the map (see Figure 5).

2.7.1. Lampetra planeri

Hanel and Lusk (2005) gathered more than 400 brook lamprey samples in the Czech Republic in 1961-2005. Thus, it was confirmed that the species is the most common lamprey there. The species can be found in the Labe/Elbe and Odra rivers basins (98% of the known localities). Only a few isolated populations are found in the Morava/Moravia River basin (the Danube River system), see Merta (2000). All the findings in 1949-2017 are presented in Figure 5, where 301 mapping grids display the occurrence (i.e., 45% from the total number of the mapping grid).

This lamprey inhabits brooks and rivulets in trout and grayling zones at the elevation of 130-900 m above sea level, but most of the findings come from 300-600 m a.s.l. Most findings were made in short streams less than 40 km in length. In total 2/3 of the findings were recorded in natural streams, the rest in streams modified to a various extent. The stream gradient was mostly about 1.5-2 $m.km^{-1}$, the prevalent annual average flow rate in the river mouth of the analysed watercourses was lower than 1 $m^3.s^{-1}$. In the Czech Republic, both sexes participate in the nest building usually in shady stream parts about 1-8 m in width and 0.8 m in depth. The spawning occurs usually in May/June in water temperatures of 9-19.5°C (Hanel 1997, 2004).

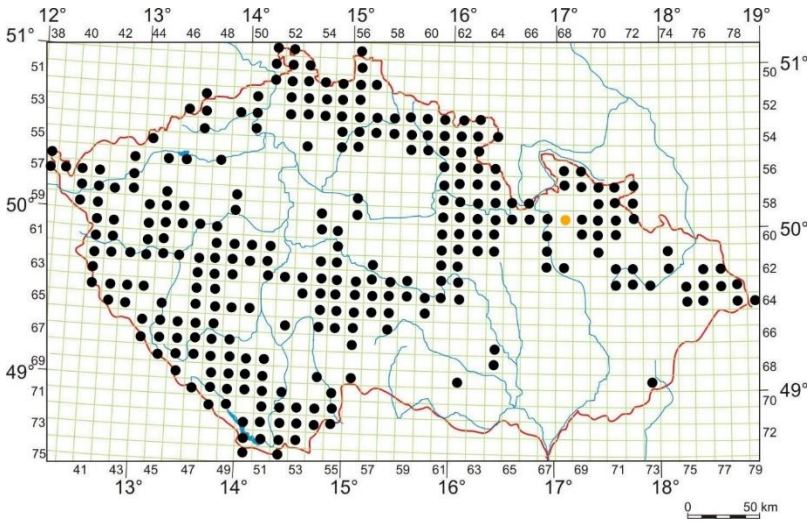

Figure 5. Occurrence of the brook lamprey (*Lampetra planeri*) and the Ukrainian lamprey (*Eudontomyzon mariae*) in the Czech Republic in 1949-2017. Black dots are *Lampetra planeri*, the yellow dot is *Eudontomyzon mariae*.

Based on surveys carried out in 33 brooks, rivulets and rivers in the Czech Republic were found, together with the brook lamprey, the brook trout (*Salmo trutta*) at 83% of the localities studied, bullhed (*Cottus gobio*) at 53%, stone loach (*Barbatula barbatula*) at 37%, gralying (*Thymallus thymallus*) at 24%, minnow (*Phoxinus phoxinus*) at 28%, gudgeon (*Gobio gobio*) at 19%, chub (*Squalius cephalus*) at 17% and dace (*Leuciscus leuciscus*) at 14%. Ammocoetes were found in those brook sections where the fish number (particularly salmonids) on average was 13,084 ind.ha^{-1} and the biomass on average was 234 kg.ha^{-1} (Hanel 1996). Together the Ukrainian lamprey, the brook trout and the bullhead were reported from the Račí potok/Crayfish Brook (Hanel and Lusk 2006).

2.7.2. Eudontomyzon mariae

The occurrence of the Ukrainian lamprey in the Račí potok/Crayfish Brook at Velké Losiny (District of Šumperk, Northern Moravia) has been known for almost half a century. This is the only known population of the species in the Czech Republic found in 1968 (Kux 1969). At that time, it inhabited the brook section of about 1.5-2.0 km: the author found 2.5-3.2 ind.m^{-2} in suitable sediments there. The status of the site and population size was monitored in 1997-2010 (Hanel and Lusk 1998, 2000, 2002, 2004, 2006, Merta and Křesina 2017).

Since its discovery (1968), the section of the brook inhabited by lampreys has gradually been reduced due to a number of negative factors. These include channel modification and fragmentation, water pollution and high predation. In July 1997, extreme floods occurred in streams of the Morava/Moravia River basin, i.e., in the Račí potok/Crayfish Brook. Thus, in a lower part of the reach where the larvae had occurred, sediment deposits were drifted away by extreme high flows. In this way, the reach containing the larvae was shortened by about 250 m (Hanel and Lusk 1998).

The restoration measures taken in 2005-2006 (wooden dams and artifitial damage to banks causing suitable meandering and new deposits of fine detritus) appeared to be

insufficient for the population survival. In order to save the Ukrainian lamprey population, it is necessary to formulate and implement a recovery programme. Purposeful measures must be taken to create, in the adjacent waterlogged meadow, a new meandering brook bed approx. 250 m in length, to provide stabilised drift sedimentation. As a part of monitoring the population, the larval lampreys should be transferred from the lower part into parts harbouring stabilised sediments until the migration barrier is removed. Every year, brown trout should be removed from the brook section in which the larvae occur. Additional modifications in the brook bed should be carried out in order to improve the conditions for the presence of larval lampreys. Perspectively, it is necessary to consider strengthening the Ukrainian lamprey population under study by translocation of larvae gathered from populations living in Slovakia. The donor population should be selected on the basis of assessing the extent of genetic identity with that in the Račí potok/Crayfish Brook population.

Křesina and Hradecký (2009) estimated that about 750 Ukrainian lampreys can live in the restored stretch of the Račí potok/Crayfish Brook (river km 0.62-2.81). The lampreys has become completely extinct from all the historically known sections of the brook by 2013 at the latest. However, in the same year, the presence of ammocoetes was discovered on the previously unexplored lower section of the Račí potok/Crayfish Brook. Between 2014 and 2016, many activities were carried out in order to save the lampreys, particularly re-introduction and recovery transfers of the ammocoetes to the historically inhabited sections in the brook. At present, the population size is estimated at 450–600 individuals of all ages (Merta and Křesina 2017).

2.8. Main Threats to Lampreys and Their Conservation and Management

Lampreys prefer natural and seminatural channels with sites of fine sediments within the suitable qualities (lamprey sites), but also flowing stretches with coarse-grained sand or with gravel (spawning grounds). Lampreys can sometimes survive in the stream even after its modification. The larvae can be threatened by sand exploitation when muddy sediments are taken with the sand. Upstream spawning migration becomes more difficult by various barriers in the channel (e.g. wires, steps etc.) which often are insurpassable for lampreys. Further important factors limiting or preventing the occurrence of the brook lamprey are the following: water pollution (particularly long-term), inappropriate stream modifications, e.g. building of firm sides, removing mud, water loss in parts of the stream due to small hydropower plants, etc., excessive fish stock and, to some extent, predation by some piscivorous birds or mammals (Hanel and Andreska 2006, Hanel at al. 2015). The occurrence of Ukrainian lamprey in the Račí potok/Crayfish Brook is threatened not only by numerous bigger trout (*Salmo trutta*) but by terrestrial ichtyophagous predators occurring there: the black stork (*Ciconia nigra*), the grey heron (*Ardea cinerea*), the Eurasian otter (*Lutra lutra*) and as of recently the American mink (*Mustela vison*). Their imprints and excrements were often found near the fine-grained sediments and potential spawning sites.

The lamprey numbers may also be influenced by extreme water drainage**.** Minimum summer flows, or the incorrect operation of a hydroelectrical power station, can cause the channel to dry which can have lethal effects on larvae because they cannot temporarily survive during a decreased water drainage in bottom depressions, where sufficiently wet mud is maintained. Likewise, extremely increased water flows caused by heavy impact rains

during or after the lampreys´ spawning can damage spawning grounds and drift eggs to unsuitable sites. According to Lusk et al. (1998) significant shifts downstream in the species associated with bottom sediments have been found. These bottom materials are regularly shifted during spates, consequently causing significant shift downstream in the brook lamprey.

The Nature Conservation Agency of the Czech Republic coordinates monitoring habitats and species under the EU Habitats Directive. Both species are listed under Annex II to the EU Habitats Directive (Directive 92/43/EEC), thereby requiring EU Member States to designate Special Areas of Conservation (SACs) for their protection. SACs for the brook lamprey and Ukrainian lamprey were established based on the present knowledge of sites preferred by the viable populations. Altogether 21 sites (sections) of running water–were identified for the brook lamprey (17 of them belonging to the Labe/Elbe River basin, 3 sites to the Odra River basin and one site located in the Morava/Moravia (Danube) River basin. The single locality with the occurrence of the Ukrainian lamprey (the Račí potok/Crayfish Brook) was also included into the SACs.

Fundamental principles to protect, conserve and manage habitats inhabited by the brook lamprey in the Czech Republic are as follows:

- maintaining naturally meandering brooks and rivulets, bank and bottom diversity with alternating gravel-sand bottom (breeding sites) and fine deposits (occurrence of larvae)
- continuous water discharge
- preservation of longitudinal migration permeability in streams for adult specimens (elimination of migrating barriers restraining upstream breeding migrations).
- optimal water quality is distinguished by the oligosaprobic level with the maximum limits typical for better beta-mesosaprobity which corresponds with unpolluted water (surface water that has not been substantially affected by human activities, water quality characteristics do not exceed the standard values in surface waters) or slightly polluted waters (surface water having been affected by human activities, however, water quality has been supporting diverse assemblages/communities in a viable ecosystem)
- admissible values of water quality for long-term viable brook lamprey populations are the following: saprobity of macrozoobenthos lower than 2.2, biological oxygen demand, five days (BOD_5): lower than 4 $mg.l^{-1}$.
- in the Special Areas of Conservation (SAC) proposed for conservation of the brook lamprey and managed for fish it is necessary that the fish stock should be in accordance with natural feeding grounds (elimination of big salmonid or other predatory fishes specimens is recommended to reduce predation on breeding adult brook lamprey specimens).
- rescue transfers of the lampreys with respect to restoration of fishponds, brooks and rivers can be applied (Dušek et al. 2003).

In the most recent edition of the Red List of Threatened Species in the Czech Republic, the Ukrainian lamprey has again been classified as Critically Endangered (CR) while the brook lamprey has been listed as Endangered (Lusk et al. 2017). In accordance with Ministry

of the Environment of the Czech Republic Decree No. 395/1992, as amended later, both the species are classified as critically endangered. Nowadays, the brook lamprey is more widely distributed in the Czech Republic than it has previously been assumed.

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Chapter 91

LAMPREY BLOOD CLOTTING: A 60-YEAR JOURNEY FROM FIELD WORK TO GENOMICS

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ABSTRACT

Mammalian blood clotting is an extremely complex phenomenon involving more than two dozen proteins interacting in a series of limited proteolytic events partitioned into two calcium-dependent pathways, one *intrinsic* to the blood alone and another initiated by a tissue component (*extrinsic pathway*). The two pathways converge and culminate in the thrombin-catalyzed polymerization of a large molecular weight protein called fibrinogen, yielding a space-filling gel ("clot") called fibrin. A long-term study on blood clotting in lampreys has revealed a simpler system in which the extrinsic scheme dominates the process. An evolutionary scenario based on a series of gene duplications accounts for the differences that have arisen since the divergence of cyclostomes from other vertebrates. The earliest of those studies, begun six decades ago, were limited to simple methods dependent on fresh blood plasma, suitable chemical agents to bind calcium to arrest the process, and a stop-watch for measuring clotting times upon the restoration of calcium. Gradually these studies became more sophisticated with the introduction of technological advances like amino acid sequencing, SDS polyacrylamide gels, cloning and DNA sequencing. Even so, some clotting proteins in mammals are present in only trace amounts, and proving their absence or presence in lampreys was not possible until the era of whole genome sequencing. This chapter retraces some of this development from a personal standpoint, beginning with having to capture lampreys by hand and bleeding them at streamside and continuing over the decades to the bioinformatic analysis of complete genomes.

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INTRODUCTION

My interest in the evolution of blood clotting, and the important role lampreys played in unraveling the process, began in the late 1950's. At the time I was a graduate student in a laboratory mainly devoted to the study of blood proteins, including certain aspects of blood clotting in humans. It was an era when not all graduate students were supported by year-round fellowships, and in the summer after my first year of graduate study I found outside employment working for an ophthalmologist conducting a summer project at the Marine Biological Laboratory in Woods Hole, Massachusetts. My job was to measure the chemical composition of eye fluids and blood plasmas from various fish. One perplexing problem I encountered was that in some fish the blood clotted very readily, even before I could get it out of the syringe, whereas in some others, I had no difficulty at all.

I did a lot of reading to find what was known about clotting in fish, and, although there were a few scattered but outdated articles (e.g., Zunz, 1933), in the end there were enough unanswered questions that I decided to center my PhD research on the evolution of clotting (Doolittle, 1961). My plan was to find representative creatures in each of the major vertebrate classes, get blood from them, and see how complex or not the process was compared with humans. In particular, it seemed that no one had ever studied blood clotting in the jawless fish, although I did find one off-hand comment in a report about hagfish hemoglobin that "no anticoagulant was needed since the blood of this animal has little clotting ability" (Manwell, 1958).

So the jawless fish – hagfish and lamprey – were an obvious place to begin except for one problem: obtaining fresh blood from live specimens to work on. Through good fortune I learned that one of my recent instructors, an anatomist named Will Roth, worked on the neuro-anatomy of the lamprey "third eye" and its relationship to the pineal gland. Roth generously offered to give me a lesson in lamprey collection. In New England, it turns out, this is an operation that can only be undertaken for a few months in the Spring. Unfortunately, when I first became aware of this sporadic availability, the season had just passed, and I had to bide my time in the summer of 1959 working with available cartilaginous and bony fish, the eye fluids of both of which I had studied for the ophthalmologist. I froze away blood plasmas so that I'd have something to work on in the fall and winter, periods during which I also read a great deal. These first faltering steps were useful in that I became more adept at handling blood and performing assays, and when the next spring finally came around, I was prepared.

Catching Lampreys

One rainy day late in April, 1960, Will Roth and I drove north from Boston to one of the many small rivers that drain into the Atlantic Ocean near Exeter, New Hampshire. The equipment we had was decidedly "low tech": two pairs of waders to keep us dry, a few old pillowcases, some simple cloth garden gloves, and a plastic barrel in which to store any lampreys we might catch. The secret, Roth confided, was to understand lampreys and their life style.

As is doubtless detailed elsewhere in this book, sea lampreys, *Petromyzon marinus*, live most of their lives in the open ocean, but they are anadromous and return to the fresh water breeding grounds of their birth to spawn. Adults, which are about two feet long, are not great swimmers against the current, and they need to stop and rest a good deal. They do this by fastening their round mouth – the "sucker" – on to a rock or some other firm object in the stream.[1]

A good place for collecting is any small dam across one of these streams where the lampreys tend to get stuck temporarily. Most often there are "fish ladders" around these dams, and the lampreys and other migrating fish eventually find their way into those boxy stairs filled with water that allow them to get by the dam.

Usually, all we had to do was grope our way along the bottom of the dam itself, trying not to get too wet from the water spilling over its top. The cloth gloves were needed because lampreys are slippery (but not nearly as slimy as hagfish!). We would simply grab a lamprey behind the head region and guide it into a pillowcase. When enough of them were in the pillowcase, we would waddle back to shore and transfer the specimens into the barrel.

As an anatomist, Will Roth didn't really have to worry about keeping the creatures alive; he could simply cut off their heads and drop them into a suitable fixative. But in order to study blood clotting I needed to get the blood out of the creature while it was still alive, and that took some learning on my part. In the long run, I became very adept and have bled – without exaggeration – well more than a thousand lampreys since that first trip.

Bleeding Lampreys

There were several little tricks that mattered. First, it was best accomplished as a two-person operation, one to hold the lamprey and one to do the bleeding (Figure 1). This was true even though we would anesthetize the creatures by putting an anesthetic (e.g., tricaine) in the barrel water. The holder would grasp the animal using disposable diapers to get a good grip.

Figure 1. A 1962 photo of the author preparing to collect blood from a sea lamprey.

[1] The word "lamprey" is from the Latin "sucking stone" (LaBonte (1991).

A single-edged razor blade was then used to cut off the tail a few centimeters behind the cloaca, exposing the main caudal vein and artery in cross-section. A large syringe fitted with a big-bore needle with the bevel cut off was pushed gently into the vein. If the bevel were left on, the vein wall would be pulled down on it and close off the system. The syringe had been rinsed well with sodium citrate solution for binding up calcium ions. On my very first attempt, red blood began to fill the syringe as I pulled back slowly on the plunger, and I knew I had a fool-proof method.

The next challenge was to separate the cells from the plasma. Running a centrifuge at streamside is neither convenient nor safe, and whenever possible I'd take the animals back to the lab in Boston and do the bleeding there. The alternative was to do the bleeding in the field, keep the blood cold and well decalcified for the journey back to Boston, and then centrifuge it to separate the plasma from the cells.

BLOOD CLOTTING IN MAMMALS

At this point we need to take a detour from the lamprey experiments to describe, however briefly, what was known at the time about mammalian blood clotting, even then acknowledged to be an exceedingly complex process involving numerous blood plasma proteins, blood platelets, and tissue components. Under ordinary physiological conditions there was an obvious need to strike a balance between keeping the blood fluid in the circulation while at the same time maintaining the potential for rapid gelation in response to injury, the balance being struck by a host of forward and backward compensating reactions. It had long been recognized that a plasma protein called fibrinogen can be converted into a gelatinous material called fibrin by the action of an enzyme called thrombin. Thrombin itself was known to exist in a precursor form called prothrombin, changed into its activated form by two different processes referred to as the *intrinsic* and *extrinsic* systems. The activating complexes – called *thromboplastins* – were calcium-dependent. As such, it was a simple matter to conduct clotting assays in the laboratory if calcium were removed from blood when it was drawn, the simple re-addition to either blood or cell-free plasma leading to the formation of fibrin clots.

The intrinsic scheme involved what were then a set of still unidentified plasma proteins, as well as prothrombin, fibrinogen and calcium. The extrinsic system needed a tissue extract – lung and brain were especially effective – and a somewhat different set of plasma proteins and prothrombin, fibrinogen and calcium.

Extrinsic system:

tissue extract + Ca++ + plasma ------→ tissue thromboplastin

Intrinsic system:

platelets + Ca++ + plasma---------→ plasma thromboplastin

thromboplastin

prothrombin ----------→ thrombin
thrombin
fibrinogen ----------→ fibrin

Most of the evidence for distinguishing the two pathways was based on simple clotting time assays after the restoration of calcium ions to different mixtures of the various components.

Nomenclature of Clotting Factors

By mid-century more than a dozen additional clotting "factors" had been identified in human blood plasma, largely protease precursors, protease substrates and protease inhibitors. Because of the confusion that arose with the casual nomenclature of the day, a commission was charged with assigning each a Roman numeral, as in "factor V" or "factor XIII." The previously known reactants were given numerals I-IV: fibrinogen designated factor I, prothrombin as factor II, tissue factor-thromboplastin as factor III, and calcium as factor IV.

What emerged was that, depending on the nature of an injury, either or both of two parallel series of *limited proteolysis* could be initiated, one for the intrinsic scheme and the other for the extrinsic. As depicted above, the two pathways converge and culminate in the thrombin-catalyzed polymerization of fibrinogen. A third pathway, also involving limited proteolysis, led to the subsequent destruction of fibrin. Notably, thrombin activated several factors besides fibrinogen, and the overall process was further regulated by an antithrombin and some other protease inhibitors.

All this was becoming known during an exciting period in molecular biology when the concept of "one gene-one polypeptide" had just become firmly established, and the question presenting itself was, how could such a complicated process with its numerous forward and backward components ever have evolved?

BACK TO LAMPREYS

My first laboratory experiments on lamprey plasma were performed in early May, 1960. As it happened, simple re-calcification of the decalcified plasma resulted in very long clotting times, of the order of 30-40 minutes instead of the two or three minutes needed for human plasma to clot, suggesting a diminished if not absent intrinsic pathway. The obvious way to speed the system up was to add tissue extract to activate the extrinsic system. The commercial tissue factor ("thromboplastin") used in most clotting labs at the time was prepared from rabbit brains, and because it was already recognized that there is a "species specificity" associated with the reaction (Dorst & Mills, 1923; Mann & Hurn, 1952)), I wasn't awfully surprised when addition of the rabbit brain extract had no significant effect in speeding up lamprey clotting. It was obvious that I needed lamprey tissue extract. Lamprey brains are beebee-sized, however, and it was impractical to even think about using them as a source. Instead, I used a mortar and pestle to grind up a few grams of lamprey skin with some sand and water. I poured off the soluble fraction and mixed a small amount of it with the calcium to be added to the plasma. On my first try the clotting time dropped to 27 seconds, more than

50-fold faster than without the extract. I repeated the observation numerous times and ran all sorts of controls. As I write this I still have that laboratory notebook of 58 years ago beside me, and I can remember the pleasure of the moment.

The first impulse was to think that lampreys didn't have an intrinsic clotting system at all, but I realized that there had to be more to the story. Lampreys, like all infra-mammalian vertebrates, do not have platelets or the platelet precursor called the megakaryocyte. Instead they have a class of nucleated white blood cells called *thrombocytes*. The long clotting times may simply have been because the thrombocytes were removed when the blood was centrifuged to obtain the plasma. In the case of humans and other mammals, platelets are less dense than blood cells and do not sediment readily, usually remaining suspended in the plasma. Accordingly, in an effort to keep the lamprey thrombocytes from sedimenting with the denser red cells, I layered lamprey blood on to various concentrations of sucrose solutions and spun them gently. A concentration of sucrose was readily found with a density between that of lamprey red cells and white cells (which include thrombocytes), allowing the latter to be cleanly separated and easily removed (Doolittle & Surgenor, 1962). When an extract of those white cells was added during the re-calcification of lamprey plasma, the clotting times were almost faster than I could measure: of the order of 10 seconds, even better than skin extract on the basis of estimated protein content. Did this imply there was an intrinsic scheme after all? Or did the thrombocytes contain the equivalent of a tissue factor? The answer remains murky to this day.

Nonetheless, it was certain that jawless fish have clotting systems that are basically similar to those found in other vertebrates. The question remained, did they have all the many components that make up the human clotting apparatus? Certainly they had fibrinogen, because the clot had all the characteristics of fibrin. And they must have a calcium-dependent thrombin generating system because calcium removal completely blocked the system, and certainly they had a tissue factor and/or a thrombocyte component. How was I to find out about all the other factors?

Working backwards, I began by purifying the most abundant protein in the system, fibrinogen, and showing that it had the same physical and chemical properties as mammalian fibrinogen, including its sedimentation and diffusion constants. More important, mammalian thrombins clotted the purified lamprey fibrinogen and removed peptide material (fibrinopeptides) proteolytically, just as was known to occur in mammals (Doolittle, 1965).

The next step was to purify lamprey prothrombin, the second most abundant protein in the system. To that end, I treated lamprey plasma with barium sulfate, an insoluble substance known to adsorb mammalian prothrombins and, as we shall see, other vitamin-K dependent clotting factors. Anything that stuck could be eluted by simply changing the solvent conditions. When the eluted material from lamprey plasma was mixed with lamprey thrombocyte extract, to my delight, a powerful thrombin activity was rapidly generated.

SPECIES SPECIFICITY

At this point I had at my disposal purified lamprey and human fibrinogens and very active lamprey and human thrombin preparations. Knowing that calcium was not required for the thrombin-catalyzed conversion to fibrin, I could simply prepare the two fibrinogens at the

same concentrations and adjust thrombin dilutions to the point where the homo-specific clotting times were the same in both systems:

lamprey thrombin	+	lamprey fibrinogen	15 seconds
human thrombin	+	human fibrinogen	15 seconds

The reciprocal experiments with lamprey thrombin clotting human fibrinogen and human thrombin clotting lamprey fibrinogen gave the following clotting times:

human thrombin	+	lamprey fibrinogen	40 seconds
lamprey thrombin	+	human fibrinogen	450 seconds

Although the lack of exact reciprocity in clotting times was puzzling at first, the general phenomenon of species specificity, whereby thrombin from a given species clotted fibrinogen from the same species faster than it did fibrinogens from another species, was certainly clear.

In 1962, when revelations about mutations and amino acid replacement were on everyone's mind, the following explanation was offered as a model of how species specificity develops in general between any set of interacting proteins:

> "Mutations can result in amino acid substitutions in either of the protein reactants which may be (a) helpful, (b) without effect, (c) slightly harmful, or (d) harmful. All of these judgments are made in terms of the rate of reaction between the two proteins. Substitutions of the last type would not persist, but the fact that slightly harmful point mutations must persist long enough for selection pressures to realize complementary (helpful) mutations in the other protein reactant is the crux of 'species specificity' based on a rate of reaction criteria. Category (b) embraces those mutations which result in amino acid changes which do not distort the steric or electronic conditions involved in the protein-protein interactions under study. Such changes result in what might be called a 'secondary species specificity,' one which might be detected physically or chemically but which does not influence the rate of physiological rate of reaction." (Doolittle et al.., 1962)

It was possible to describe this sequence of events pictorially with a model I called "make a hole and fill it." As shown in Figure 2, a mutation may occur that gave rise to a small "hole" on one side of the interface between the two interacting proteins, the loss of interaction energy being quite small. Subsequently, however, a compensatory mutation could occur that allowed the other protein to "fill the hole," restoring the full binding force. However, if one now tries to interact the two mutant forms (in the test tube), the effectiveness of the interaction will be greatly compromised by the clashing residues.

It was quite gratifying when a few years later population biologists brought forward the notion of "neutral evolution" on the one hand (Kimura, 1968), and the advantage and impact of "slightly deleterious" mutations, on the other (Ohta, 1973), in complete accord with the diagrammatic depiction of the kinds of mutational events that lead to "species specificity" (Figure 2).

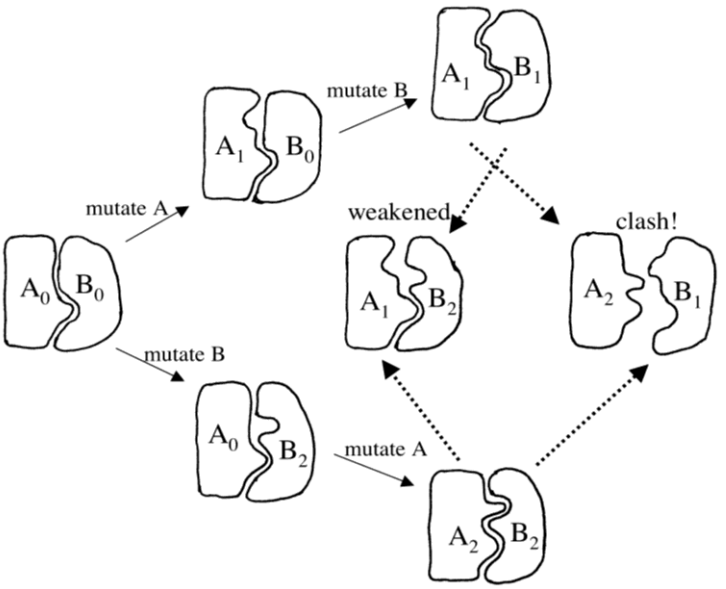

Figure 2. Diagrammatic depiction of how "species specificity" arises for the interaction of a pair of proteins. Initially, the two proteins (A_0 and B_0) share a fully complementary interface. Different mutations along divergent lineages lead to small losses in complementarity, often by replacement of an amino acid by one with a smaller side chain. Subsequent mutations in the gene for the other protein can fill the gap and restore the full interface. As a result of different patterns of mutation along different lineages, later versions of A and B lose their effectiveness of interaction. Note the lack of reciprocity in the simple example offered: A_2 and B_1 are badly compromised but A_1 and B_2 are less so (from Doolittle, 2013).

IDENTIFYING THE LESS ABUNDANT CLOTTING FACTORS

The downside of "species specificity" was that it precluded the use of simple complementation assays with genetically defective human plasmas. For example, the clotting activity of blood from persons with hemophilia can be restored, not only by the addition of small amounts of normal human plasma but also some other mammalian plasmas, thereby showing that the non-human plasma contained the active factor. But the more distant the non-human relationship, the less effective was the correction, to the point where none would occur at all.

Accordingly, even though panels of defective human plasmas were becoming available for virtually all the known human clotting factors, assessing the occurrence of those same factors in a wide range of species by this approach wasn't at all feasible. It was also out of the question to think one could find mutant lampreys with bleeding diseases.

On the other hand, a few factors could be identified in lamprey plasma simply by what they did. As noted above, tissue factor in skin was relatively easy to demonstrate for the extrinsic system, and the presence of insoluble cross-linked fibrin under appropriate conditions indicated that the transglutaminase designated factor XIII was present in lamprey plasma. Beyond that, a simple thrombin generation test showed that an anti-thrombin was also present, although the relative activity was weaker than in the case of humans (Doolittle & Surgenor, 1962). The fact that fibrinolysis occurred when clots were left to stand showed that

the precursor protein plasminogen was being converted to plasmin. But determining the presence or absence of the majority of the more than two dozen clotting factors of interest would have to be postponed for several decades.

During that long interval my lab continued to focus on lamprey fibrinogen, comparing it with those from other vertebrates. In this regard, the introduction of SDS gel electrophoresis (Shapiro et al., 1967) provided an elegantly simple method for determining the subunit structure of human and other mammalian fibrinogens, fibrin and cross-linked fibrin (McKee et al., 1970), and we followed the same course with lamprey fibrinogen (Doolittle & Wooding, 1974). These studies led to some unexpected differences, including one of the subunits having a significantly larger size, for one, and the finding that the fibrinopeptide B was a glycopeptide, for another (Doolittle & Cottrell, 1974). On the whole, however, lamprey fibrinogen had the same unique structure found in other vertebrates, namely, a dimer of three disulfide-linked polypeptide chains that gave rise to an extended structure with terminal globular regions connected by three-stranded coiled coils to a central region.

By this time I had re-located to Southern California where I found lamprey collecting to be not nearly as rewarding as it had been in New England. Accordingly, I began making annual three-day expeditions back to my old collecting places, flying out to Boston on a Friday, renting a car and driving up to the University of New Hampshire (UNH) in Durham where I could stay at their pleasant New England Center, collect specimens on Saturday, use a centrifuge at their Biochemistry Department, freeze the plasma and ship it back to California, before returning on Sunday. By good fortune one of my former graduate students, Andy Laudano, joined the faculty at UNH and not only helped me collect the lampreys, but became so proficient at every stage that I no longer needed to go myself; Andy did it all.

There was a period after cDNA cloning became available when it was only necessary to ship frozen livers from which we could prepare mRNA. During the 1980's we managed the cDNA sequences of the three homologous chains of lamprey fibrinogen (Strong et al., 1985; Bohonus et al., 1986; Wang et al., 1989), as well as a number of other proteins. More than a decade later we were even able to determine the crystal structures of some important fibrinogen and fibrin fragments (Yang et al., 2002a, Yang et al., 2002b).

Although the similarities and differences found were interesting and informative, they didn't really shed much light on the original question: to wit, how did vertebrate blood clotting ever become so complicated? From the start, the most likely course of events depended on a series of gene duplications, similar to what had recently been shown for the α and β chains of human hemoglobin (Ingram, 1961). When a full set of those serine proteases that are clotting factors in humans became available, it was possible to reconstruct the history of duplicative events by the construction of phylogenetic trees (Doolittle & Feng, 1987), but this approach on its own could not provide direct information on *when* the various gene duplications occurred.

DOMAIN SHUFFLING

It became clear during the 1970's that a level of organization exists in proteins beyond the simple concept of primary-secondary-tertiary, sets of consecutive amino acid residues folding into discrete *domains* or folds. Domains are often eponymously named for the protein

in which they were first observed, such as "fibronectin type 1 domain" (Fn1, Fn2 or Fn3)), or "epidermal growth factor domain" (EGF), and so forth. Although some proteins are completely embodied in a single domain, many others are made up of combinations that reflect a heritage of having been shuffled about during evolution. Certainly that is a feature of many of the blood clotting proteins. In this regard, several of the clotting factors are made up of a serine protease domain with various other domains occurring at their amino-terminals, including, for example, EGF or kringle domains. In many cases, these domains serve as good signposts for identifying homologous factors.

Among them is an important domain responsible for the tight calcium-binding and non-biological absorption on to substances like barium sulfate mentioned earlier. The sequence motif characterizing these domains involves a skein of uncommon γ-carboxyglutamic acid residues – often shortened to "GLA" – and the domains dubbed "GLA-domains." The biosynthesis of GLA residues is the result of post-translational modification promoted by vitamin K, and GLA-domain-containing proteins are often referred to as vitamin-K dependent. A phylogenetic tree of an assortment of clotting factor proteases with amino-terminal GLA-domains from a variety of species – including seven from lamprey – is depicted in Figure 3. It is reassuring that the assignments based on domain arrangement are completely borne out, even though only the serine protease sequence data were used to calculate the tree.

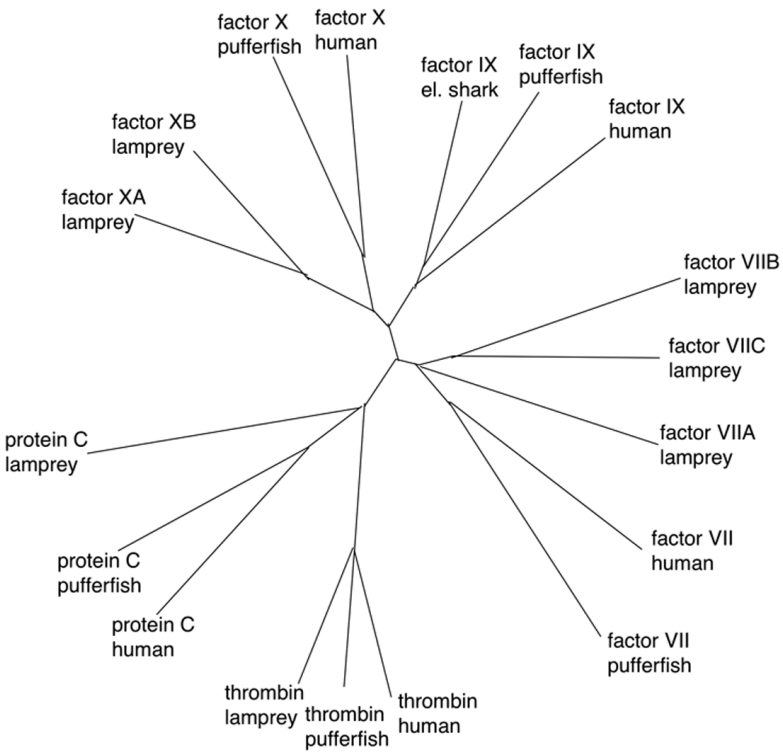

Figure 3. Phylogenetic tree (unrooted) of 18 vitamin K-dependent clotting factor sequences (serine protease domains only) from lamprey (*P. marinus* and/or *L. japonicum*), elephant shark (*Callorrhincus milii*) (only factor IX), pufferfish (*Takifugu rubripes*) and human. The seven lamprey entries include three factors VII, two factors X and one each for thrombin and protein C (from Doolittle, 2015).

Another important domain in some non-protease clotting proteins is the ferroxidase domain, a founding version of which occurs in an intestinal transport protein called hephaestin, as well as a copper-transporting protein found in vertebrate blood plasma called ceruloplasmin (Vulpe et al., 1999). In mammals and other later diverging vertebrates these domains also occur in factors V and VIII. Invariably these domains occur in sets of three, the structures of which form tripods. Hephaestin is readily distinguished from the others because it has a membrane-spanning segment at its carboxy-terminal, whereas factors V and VIII both have a pair of discoidin domains at their carboxy-terminals. Ceruloplasmin has no other domain at its carboxy-terminus and circulates freely in the blood plasma. Also, in factors V and VIII the second and third ferroxidase domains are separated by non-descript segments that are removed by thrombin during an activation process that allows the third leg of the tripod to fall into place.

Ferroxidase domains, like GLA domains, are in keeping with the bulk of the protein remaining on a membrane localized at the site of injury. Similarly, tissue factor, the activation of which ignites the clotting process, is made up of a pair of fibronectin type-3 domains (Fn3) and a membrane-spanning domain. Just as is the case for the serine protease factors, a phylogenetic tree based only on the ferroxidase domain sequences is in perfect accord with the assignment of hephaestins, ceruloplasmins and clotting factors V and VIII (Figure 4).

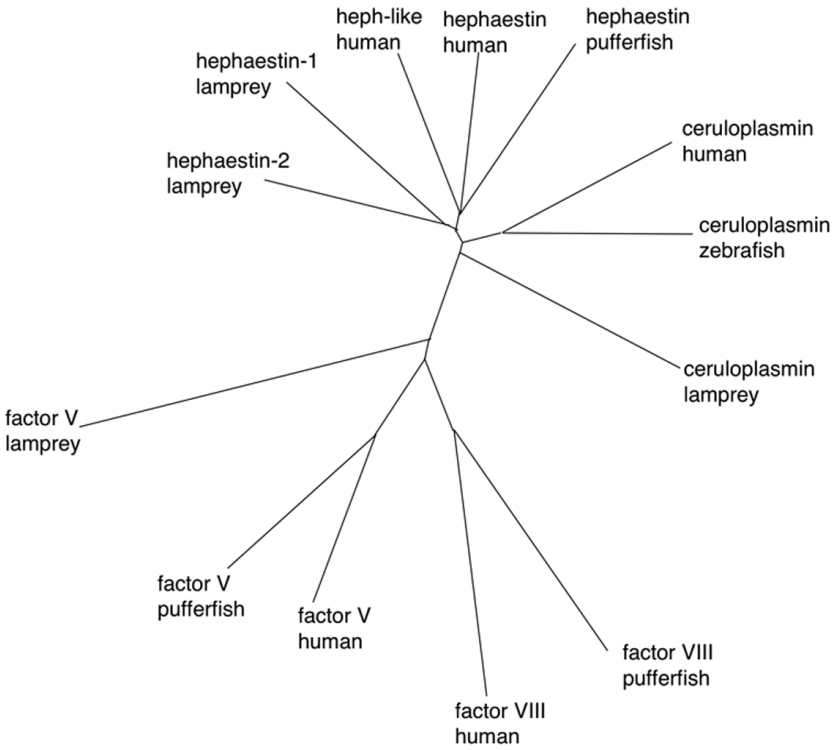

Figure 4. Unrooted phylogenetic tree of 13 ferroxidase family proteins (coagulation factors V and VIII, hephaestins and ceruloplasmins) from human (5 entries), lamprey (4 entries), pufferfish (3 entries), and zebrafish (1 entry). Each entry depicts three consecutive ferroxidase domains. (from Doolittle, 2015).

BIOINFORMATICS

As the decades slipped by, the number of fully characterized clotting factors in lamprey slowly grew (Kimura et al., 2009; Ragg et al., 2009), but not to the extent observed in other vertebrates. They *appeared* to have a simpler clotting system (Doolittle et al., 2008), but how could it be proven beyond a doubt? It is much more difficult to prove the absence of a gene than to document its presence. It was the era of complete genome sequencing ushered in at the millennium that provided the means to that end.

Or did it? Whereas complete genome sequences for a variety of vertebrates were soon available, lamprey and hagfish seemed lost in a backwater. Part of the reason was that much of their genomes are highly repetitive, greatly impeding efforts directed at full genome assembly (Smith et al., 2013). There was an alternative strategy, however; one could use complementary data from partially assembled genomes in combination with the all-inclusive trace sequence archive maintained by the National Center for Biotechnology Information (NCBI), and indeed this approach allowed a complete inventory of the lamprey clotting factors that at the very least confirmed the absence of genes for factors VIII and IX (Doolittle, 2015).

LAMPREY CLOTTING AND THE 2R HYPOTHESIS

The fact that vertebrate genomes were typically much larger than the genomes of protochordates from which they had descended led Susumo Ohno to propose that the enlargement was the result of at least two rounds of whole genome duplication (Ohno, 1970). He based this idea on the fact that polyploidy is common not only in plants but also occurs frequently in fish. This bold proposal became known as the 2R hypothesis. The lamprey data bear on the matter because it was thought likely that one of these duplications occurred before the divergence of cyclostomes and the other after. In passing, it can be noted that it has been suggested – without any consideration of lampreys – that the duplications responsible for clotting factor genes may have been linked to 2R whole genome duplication events (Davidson et al., 2003).

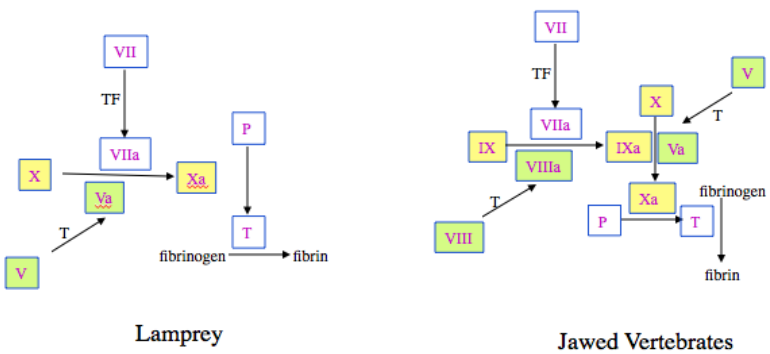

Figure 5. Scheme showing how a putative single block duplication event after the divergence of lampreys from other vertebrates gave rise to a more complex clotting scheme. Colors emphasize sets arising through concerted gene duplication. Note that thrombin (T) interacts with other proteins besides fibrinogen. TF = tissue factor. (adapted from Doolittle et al., 2008).

The 2R hypothesis has been much debated over the years, and two recent lamprey genome assemblies seem to have yielded two different interpretations (Mehta et al., 2013; Smith and Keinath, 2015); the most recent comments on the subject seem to favor replacing the second "R" with several large-scale duplications (Smith et al., 2018).

Even so, the blood clotting data remain in full accord with a second round of whole genome duplication occurring after the divergence of cyclostomes from the main line of vertebrates (Figure 5). The distinction between a full genome duplication and a number of partial duplications is difficult to make, however (Escriva et al., 2002), and the matter is hardly settled. In the wake of that divergence the lineage leading to jawed vertebrates must have experienced a large scale block duplication – even if less than genome wide – that simultaneously gave rise to genes that were destined to encode factors VIII and IX, quite apart from several independent duplications that would give rise to additional genes for factors VII and X on the lineage leading to lampreys.

FIBRIN CLOTS PRE-DATE LAMPREYS

Up to this point I have tried to emphasize the prominent role played by lampreys in our understanding of how vertebrate blood clotting evolved. Its importance stems from its having diverged from the main line leading to other vertebrate groups at an early stage when the blood clotting scheme was simpler. Lampreys lack genes for coagulation factors VIII and IX, both of which are critical for the "intrinsic" clotting system and defects in which are responsible for hemophilia in humans. On the other hand, lampreys have three each of the genes for factors VII and X, participants in the "extrinsic" clotting system.

Even though the clotting system leading to fibrin clots in lampreys--and likely hagfish (Banfield & MacGillvray, 1992)-- is less complex than what exists in other vertebrates, it is hardly simple (Figure 5). It is important to note, also, that fibrin clots have never been observed in any protochordate, although in ascidians circulating white cells are known to clump in response to injury. It is notable that these same creatures have a protein that is remarkably similar to vertebrate fibrinogen but which lacks a thrombin-vulnerable site of the sort that leads to fibrin formation, i.e., the elongated molecules have holes, but there are no knobs to fit in them that would allow polymerization (Doolittle, 2012).

These same protochordates have other proteins that have similar domain arrangements as some key vertebrate clotting factors, including proteases with amino-terminal kringle domains, but none of them has a GLA domain or any arrangement similar to that of prothrombin. Intriguingly, there is also a hephaestin-like protein with a set of three consecutive ferroxidase domains and a pair of discoidin domains at the carboxy-terminus, just like the activated configuration found in vertebrate factors V and VIII (Doolittle, 2015). In any case, it wouldn't have taken much in the way of domain shuffling during the interval between the divergence of protochordates and cyclostomes to give rise to the first thrombin-catalyzed fibrin clots.

AFTERTHOUGHTS

Interest in lamprey blood clotting remains high even today as evidenced by a recent article on how the allosteric activation of factor VII by tissue factor is the same in lampreys as in all other vertebrates (Biller et al., 2018). Doubtless, future studies will explore the detailed mechanism of other parts of the clotting scheme as well.

For my own part, there are a number of experiments I would like to have done myself if time and resources had allowed. For example, I have wondered if the three different factors VII that occur in lampreys interact preferentially with one or the other of the two factors X, as might be expected if the same kinds of change occur that lead to the "species specificity" shown in Figure 2? And, if so, how much "cross-talk" exists among them? It would also be of interest to know whether the different versions of those factors are differentially expressed at different stages of lamprey development; does the larval stage express these factors differently from the mature adults?

There is also the matter of the powerful clotting activity associated with lamprey white blood cells (thrombocytes). Is it only because these cells are rich in tissue factor? Or, is it because factor V is bound to these cells and accelerates the process more than occurs in free solution? There are simple experiments I could have done to find this out, but now that it is too late for me, I'm hoping other clotting enthusiasts will take up the challenge and find themselves some lampreys.

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Chapter 92

MORPHOMETRIC AND MERISTIC VARIABILITY IN LAMPREYS OF THE GENUS *LETHENTERON* (PETROMYZONTIDA: PETROMYZONTIFORMES) IN SAKHALIN ISLAND RIVERS

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ABSTRACT

The occurrence of lampreys of the genus *Lethenteron* (Cephalaspidomorphi, Petromyzontida, Petromyzontiformes, Petromyzontidae) in nineteen rivers and one lake in Sakhalin Island is documented in the chapter. Morphometric parameters in larvae and adult specimens from the selected areas are compared using statistically significant differences. Sex differences in adults in respect to morphometric parameters are also presented.

ABBREVIATIONS

TL	total length
MBD	maximum body depth
HD	head depth
PBL	prebranchial length (the distance from the anteriormost edge of the disc to the anterior edge of the first branchial opening)
SL	snout length (preocular length)
EL	eye length (horizontal diameter of the eye)
POD	postorbital distance, postocular length (the distance from the posteriomost edge of the eye to the anterior edge of the first branchial opening)
DL	disc length (the length of the closed disc measured longitudinally and including the oral fimbriae)
DB	the length from the anteriormost edge of the disc to the posterior edge of the last branchial opening)
PDL	predorsal distance
PD	preanal distance
IDD	interdorsal distance
1DL	first dorsal fin length
1DH	first dorsal fin height
2DL	second dorsal fin length
2DH	second dorsal fin height
LCF	length of the caudal fin (the distance between posteriormost edge of the second dorsal fin and posteriormost tip of the caudal fin)
TAL	tail length (the distance between the posterior edge of the cloacal slit and the posteriormost tip of the caudal fin
ID	internasal distance
BW	maximum body width
LDS	longitudinal diameter of closed mouth slit
HW	head width
UPL	urogenital papilla length
1BOC	the length from the anteriormost edge of the first branchial opening to the end of the caudal fin
7BOC	the length from the posteriormost edge of the last branchial opening to the end of the caudal fin
SN	length from the anteriormost edge of the snout to nostril

PB	maximum perimeter (girth) of the body
17BO	the length from the anteriormost edge of the first branchial opening to the posteriormost edge of the last branchial opening
POL	the length from the anteriormost edge of the snout to the anteriormost edge of the eye, preocular length
S1BO	the length from the anteriormost edge of the snout to the anteriormost edge of the first branchial opening
MD	longitudinal diameter of the mouth disc
S7BO	length from the anteriormost edge of the snout to the posteriormost edge of the branchial opening
WS	width of the skull
2DC	length from the end of the second dorsal fin to the end of the caudal fin
LA	length of anus
PAD	postanal distance, length from the posteriormost edge of the anus to the end of the caudal fin
M	mean
S.E.M	standard error of the mean
+	statistically significant difference
W	weight

1. MATERIAL AND METHODS

Archive specimens of caught lampreys and our unpublished data, as well as data from the Sakhalin State University and Sakhalin Research Institute of Fisheries and Oceanography (Sakhalin, Russia) covering the period of 1998-2005 were used for the study. Material caught by electrofishing was preserved in 70% alcohol and later in 2-3% formalin solution. Methods published by Pravdin (1966), Holčík (1986) and Renaud (2011), were used for morphometrical studies. For the morphometrical analysis common statistical evaluation was used (mean, standard error of the mean /S.E.M./, Student´s test, level of significance $P \geq 0.95$, see Matsueva, 2003).

In total 1228 individulas of lampreys of the genus *Lethenteron* from Sakhalin waters (adults: Nitui River – 245 individuals, Kopilka River – 31, Novikovka River– 33, Pugachovka River – 24, Bol‘shoi Garomai River - 153; larvae: Nabil‘ River - 6 specimens, Yasinge River - 28, Bol‘shaya Aleksandrovka River - 25, Bol’shoi Matchi River - 13, Moguchi River - 85, Malyi Garomai River - 74, Kura River - 49, Ol´chovka River - 10, Taranai River - 113, Krasnodonka River - 16, Fevralevka River - 29, Bol´shaya River - 23, Bol‘shoy Chibisan Lake - 54, Pugachovka River - 29, Nitui River - 89, Ostrovka River - 48, Novikovka River – 51) were studied , see Figure 1.

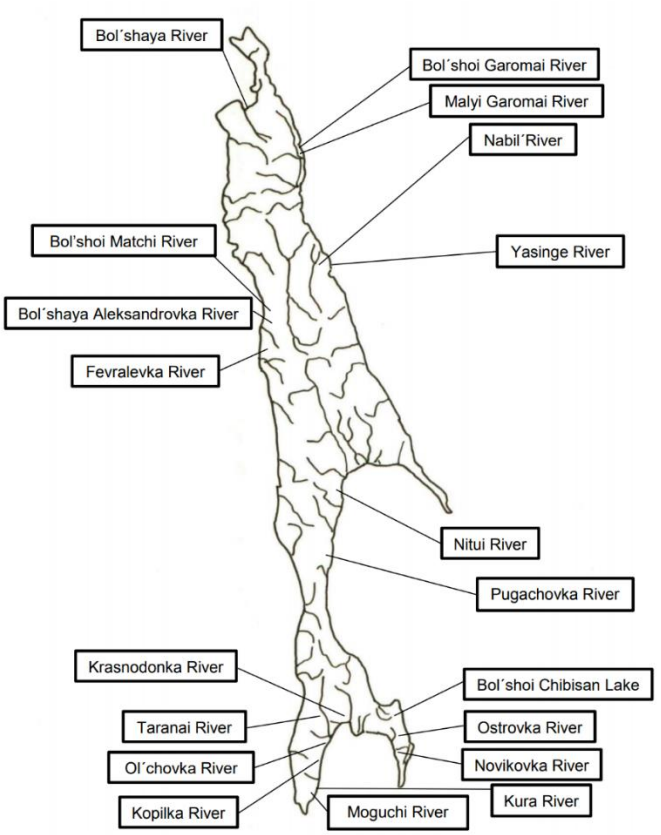

Figure 1. Studied rivers of Sakhalin Island where occurrence of larvae and adult lampreys of the genus *Lethenteron* were confirmed.

2. TAXONOMIC NOTES

As of now the taxonomic list includes seven species in the genus *Lethenteron* (Renaud 2011), but the relationships among them has been controversial. Renaud (2011) recognized one parasitic species, *Lethenteron camtschaticum* (Tilesius, 1811) (i.e., the stem species) and six nonparasitic species, *Lethenteron alaskense* Vladykov & Kott,1978, *L. appendix* (DeKay,1842), *L. kessleri* (Anikin, 1905), *L. ninae* Naseka, Tuniyev & Renaud, 2009, *L. reissneri* (Dybowski, 1869), and *L. zanandreai* (Vladykov, 1955) (i.e., the satellite species) in the genus, along with two additional undescribed nonparasitic species from Japan, and noted that the taxonomic limits of the nonparasitic *Lethenteron reissneri* were unclear. Authors have either treated these satellites of *Lethenteron camtschaticum* as distinct species (e.g., Potter et al. 2015) or as synonyms of the species. For example, Artamonova et al. (2011) and Makhrov et al. (2013) have suggested that *Lethenteron ninae*, *L. reissneri*, and *L. kessleri* are synonyms for *Lethenteron camtschaticum* and that life strategy (i.e., parasitism versus nonparasitism) is not a valid criterion for specific distinctiveness. Li (2014) summarized data on taxonomy of *Lethenteron camtschaticum.*

The original description of the Far Eastern brook lamprey *Petromyzon reissneri* Dybowski,1869 was very short, incomplete and based on material from the upper Amur River basin, Russia (type locality: Onon and Ingoda rivers). In the following years a number of authors conducted morphological studies on what they referred to as the species *Lampetra reissneri* and anadromous *Lethenteron japonica* (= *Lethenteron camtschaticum*) from the lower Amur River basin (Berg 1931, 1948; Abakumov 1960, Hensel 1963). Mina et al. (2006) mentioned that the *Lethenteron japonicum* and *L. camtchaticum* being synonyms is in accordance with the International Code of Zoological Nomenclature (ICZN).

The Siberian brook lamprey *Lethentheron kessleri* was described by Anikin (1905) based on 16 specimens (160-210 mm TL) collected in the vicinity of Tomsk, mostly in the Tom River, including its basin in the Kirgizka River of Western Siberia. According to Holčík (1986) the external morphology, including the dentition of the oral disc, is the same as that of *L. japonicum*. According to the original description of Anikin (1905) the tip of the caudal fin is darkly pigmented, and the top of the second dorsal fin bears a typical dark blotch, a "fine ashy-grey color." The area of distribution according to Reshetnikov et al. (1997) stretches throughout Siberia to the Anadyr and Sakhalin. The species was reassigned to the subspecies category (Berg 1948). Poltorykhina (1979) suggested that the taxon must be classified within the species category (see also Chereschnev 1996; Safronov and Nikiforov 2003). According Yamazaki and Goto (2016) *L. reissneri* and *L. kessleri* were recognized as being conspecific, the name *L. reissneri* taking priority of the ICZN.

The Arctic lamprey *Lethenteron camtschaticum* (Tilesius, 1811) is an anadromous parasitic lamprey species, widely distributed in the Arctic and the North Pacific. Now, its distribution range covers waters of the seas of the Arctic Ocean**.** In the North Pacific, it includes areas from the Bering Strait to the eastern part of the southern Korean Peninsula (the Sea of Japan), as well as those from the central part of Honshu Island (Japan) in the Pacific waters along the Asian coastline to the Kenai Peninsula in the Gulf of Alaska along the United States coastline (Orlov et al. 2014). Several studies focused on the genetic population structure in *L. camtschaticum.* Yamazaki et al. (2011) described establishing a fluvial non-parasitic population of *Lethenteron camtschaticum* as the first step in petromyzontid speciation. Landlocked fluvial non-parasitic populations in the upper region of water reservoirs dams were genetically divergent from anadromous parasitic ones. Yamazaki and Nagai (2013) found discrepancies in allelic frequencies of Lspn013 as being remarkable between anadromous and landlocked-fluvial populations of *L. camtschaticum*, leading to high population genetic divergence between them. Based on a homology search, Lspn013 was estimated to be located near the vasotocin precursor gene, which plays an important role in osmoregulation. Life history differences in lampreys have resulted from habitat difference in the adult phase, which have probably led to recent natural selection on the gene responsible for osmoregulatory functions. Yamazaki et al. (2014) studied migration ecology, genetic population structure and gene flow pattern in anadromous Arctic lamprey (*L. camtschaticum*) by analyzing polymorphic microsatellite loci. Their results suggest that *L. camtschaticum* has considerable ability to migrate long distances in the sea and has lower homing abilities. For resource management of *L. camtschaticum*, unconstrained gene flow across all populations should be maintained.

Currently, there have been conflicting opinions on the validity of the so-called "landlocked" lamprey forms of lampreys. This, according to the opinion of some authors (Bugaev 2007; etc.), is based on their joint spawning in the rivers of Kamchatka. *L. reissneri*

is only a freshwater (landlocked) form of the anadromous species *L. camtschaticum*. At the same time, Gritsenko (1990, 2002) pointed out that the joint spawning of *L. camtschaticum* and *L. reissneri* within the same spawning area does not violate the reproductive isolation of these forms. Other authors, on the basis of genetic similarity of *L. kessleri* and *L. reissneri* according to the Principle of Priority of ICZN, consider it possible to regard *L. kessleri* as a junior synonym of *L. reissneri* (Shed'ko 2001, 2002; Yamazaki et al. 2006; Gritsenko 2002). There are also other points of view. Thus, recent results of genetic studies have shown that freshwater forms of *L. reissneri* from the waters of Kamchatka are identical to *L. camtschaticum* (Artamonova et al. 2011; Nazarov et al. 2011) or that *L. reissneri* is synonymous with *L. camtschaticum* (Artamonova et al. 2015). However, these conclusions have not been accepted by a number of other researchers (Renaud 2011; Gritsenko 2012; Renaud and Naseka 2015). Molecular genetics methods have shown a great similarity between the Far Eastern brook (*Lethenteron reissneri*) and the Siberian lamprey (*L. kessleri*), see Yamazaki et al. (2006), Yamazaki and Goto (2016), but Romanov et al. (2017) accepted *L. camtschaticum* and *L. kessleri* as valid species. Acording to Freyhof and Kottelat (2008), lampreys identified as *L. kessleri* from the Arctic basin are *L. reissneri* and a part of those from Sakhalin, Japan and rivers of the Sea of Japan belong to *L. reissneri* and two unnamed species. Two species (*L. camtschaticum, L. reissneri*) are classified under "Least concern" in the IUCN Red List (Freyhof and Kottelat 2008; NatureServe 2013).

This chapter deals with lampreys of the genus *Lethenteron* inhabiting rivers on the island of Sakhalin. Berg (1948) recognized in Sakhalin three lamprey taxa: *Lethenteron* (*Lampetra*) *japonicum* (Martens, 1868), *Lethenteron* (*Lampetra*) *reissneri* (Dybowski, 1869) and *Lethenteron* (*Lampetra*) *kessleri* (Anikin, 1905). Abakumov (1960) and Poltorykhina (1971, 1974) also recognized here three species, but Voronov (1982) only two species: *Lethenteron japonicum* and *Lethenteron reissneri*. Safronov and Nikiforov (2003), Bogutskaya and Naseka (2004) and authors of the Atlas of Freshwater Fishes of Russia (2003) distinguish three species there, namely *Lethenteron japonicum*, *L. reissneri* and *L. kessleri*. Efremova (2006) distinguished three lampreys species (*L. camtschaticum, L. kessleri, L. reissneri*) in Sakhalin rivers. According to Dyldin and Orlov (2016) the Arctic lamprey (*L. camtschaticum*) can be found along the whole coast of Sakhalin in marine waters (in almost all bays), where it swims in many rivers for spawning, including the largest of them, such as Tym', Poronai, and Lyutoga (see also Berg 1948; Taranetz 1937). The occurrence of the species in Sakhalin was reported by Gritsenko (1990, 2002), Nikiforov (2001); Safronov and Nikiforov (2003), Safronov et al. (2008), Pietsch et al. (2012).

The Far Eastern brook lamprey (*L. reissneri*) can be found mainly in southern and central parts of Sakhalin Island, including the Tym' River basins, the Pil'tun Bay, and the Nevskoe and Tunaicha lakes (Berg 1948; Lindberg and Legeza 1959; Gritsenko 1990, 2002). Some authors report its occurrence, as *Lethenteron kessleri*, e.g., Pietsch et al. (2001), Nikiforov (2001), Safronov and Nikiforov (2003) Safronov et al. 2008). Gritsenko (1990, 2002) suggests that there are three lamprey forms on Sakhalin Island: *Lethenteron camtschaticum, L. reissneri*, and, possibly, a landlocked form of *L. camtschaticum*. Furthermore, it should be noted that, instead of *L. camtschaticum* and *L. reissneri*, he specified *L. japonicum* and *L. kessleri*, which is due to the views of lamprey taxonomy of the time (see, Gritsenko, 2012). Zhivoglyadov (2014) mentioned from Sakhalin only the genus *Lethenteron* without specific determination. Dyldin and Orlov (2016) distinguished two species in the genus *Lethenteron* (*L. camtschaticum* and *L. reissneri*) in Sakhalin rivers. The occurrence of *L. reissneri* is

supposed in Sakhalin rivers of Novikovka, Yasynge, Krasnaya, Pugachovka, Mereya, Taranai, Ostrovka and B. Chibisan Lake, and *L. kessleri* in the Melkaya and Bol'shoi Garomai rivers and *L. camtschaticum* in the Nitui River (Efremova, 2006).

3. RESULTS AND DISCUSSION

From the above mentioned data it is unclear whether the species position within the genus *Lethenteron* in Sakhalin waters is evident. Therefore we present our results based on the samples studied without specific species determination (as *Lethentheron* sp.). In the next text morphometric and meristic parameters in the lamprey samples examined from various areas are analysed. Berg (1948) found in *L. camtschaticum* the following dentition: supraoral lamina – 2 teeth, infraoral lamina - usually 6, sometimes 7, bicuspid lateral teeth, single row of posterial teeth. Safronov (2000) analyzed dentition in 25 individuals (as *L. camtschaticum*) from the Lazovaya River in Sakhalin: supraoral lamina – 2 teeth, infraoral lamina – 6-7, rarely 5 teeth, endoratelal formula – 2-2-2, total number of anterials, 18-22, total number of posterials, 19-23, number of trunk myomeres 70-76. Matsueva (2003) analyzed dentition in 30 specimens (as *L. camtschaticum*, total length 154-183 mm) from the Sakhalin river of Nitui: supraoral lamina – 2 teeth, infraoral lamina – 6, rarely 7 teeth, endolateral formula - 2-2-2, number of trunk myomeres - 68-75. The occurrence of the species was supposed in the Nitui River (see Efremova 2006). Meristic data in lampreys (as the Siberian brook lamprey, *L. kessleri*) based on 140 adults taken mainly from the Ob' River and its tributary, the Irtysh (Anikin 1905, Ioganzen 1935, Poltorykhina (1966, 1971, 1974) are as follows: the supraoral lamina bears two teeth on its edges, the infraoral lamina usually has 6 to 8 teeth, of which the marginals are bicuspid, endolaterals bicuspid, three on each side of the disc, anterial range from 17 to 27, their size decreases towards the disc margin, one row of unicuspid posterials contains 16 to 25 teeth. The dentition of the tongue is known from the original description by Anikin (1905): transerse lingual lamina has one large, sharp tooth flanked by 8 to 9 small cusps, the size of which decreases towards the lamina margin. Longitudinal lingual laminae have minute cusps. The number of trunk myomeres counted by Poltorykhina (1966, 1971, 1974) ranges from 67 to 72. Altogether 121 adults were examined in respect to morphometrical characteristics (Ioganzen 1935, Poltorykhina 1966, 1974). The following are expressed as % of TL: prebranchial length 8.6-14.6, branchial length 8.3-11.1, trunk length 47.4-47.7, tail length 25.5-33.3, disc length 4.4-6.8, diameter of eye 1.5-3.0 (see also Holčík 1986). According to Renaud (2011) ranges of trunk myomeres in the species was 65-73, for resident *L. camtschaticum* form *sensu* Kucheryavyi et al. (2007) as 57-78. Berg (1931, 1948) stated that the key character to distinguish *L. reissneri* and *L. kessleri* is the lack of posterials or their weak development in the former and their presence in the latter. Posterial teeth were not mentioned in the original description, but Berg (1931) reported that they were sometimes absent, which he later (Berg 1948) changed to usually absent, based on material (some of which were re-identified as parasitic *Lethenteron camtschaticum*) from far outside of the type locality. The latter view has been widely accepted by subsequent authors. Unfortunately, the poor condition of the two adult syntypes did not permit verification of the character. However, a row of posterials was clearly visible in six of the seven topotypic metamorphosing ammocoetes and indicates their usual presence in the species (Renaud and

Naseka 2015). In view of the fact that they are not unabiguous morphometric and metric differences among the supposed lamprey species (see Tables) all samples of larvae and adults examined are in this chapter designated as *Lethenteron* sp. The occurrence of the Siberian lamprey is supposed in Sakhalin River Melkaya (Efremova 2006). Dentition and morphometric characters according to A. Safronov were as folllows (9 individuals, 158-220 mm) from the Melkaya River: supraoral lamina – 2 teeth, infraoral lamina – 5-7 teeth, endoratelal formula – 2-2-2, total number of anterials, 14-21, total number of posterials, 15-24, number of trunk myomeres 68-72. Matsueva (2003) studied dentition in 49 individuals from the Bol'shoy Garomai River (120-148 mm): supraoral lamina – 2, infraoral lamina – 6-7, endoratelal formula – 2-2-2, total number of anterials, 12-20, total number of posterials, 10-16. A number of trunk myomeres in adult lampreys from the above river Bol´shoy Garomay was determined as 67-74 (mean 70.6), see Table 1. Morphometric characters in lampreys from the Bol'shoy Garomai River are presented in the Table 2. The occurrence of the Far Eastern brook lamprey, *Lethenteron reissneri,* is supposed in the following Sakhalin Rivers: Bol'shoi Garomai, Yasinge, Krasnaya, Fevralevka, Pugachovka, Taranai, Mereya, Ostrovka, Novikovka and Bol'shoi Chibisan Lake. Safronov (see Matsueva 2003) studied 9 specimens from the Tym' River with the following results: supraoral lamina – 2 teeth, infraoral lamina – 5-7 teeth, endoratelal formula – 2-2-2, total number of anterials 17-20, total number of posterials 19-21, number of trunk myomeres 57-67. Ioganzen (1935) also mentioned occurrence of the species in Sakhalin Island. The lowest average number of myomeres in ammocoetes within the genus *Lethenteron* was counted in the Ol´chovka as 66.5, the highest in Moguchi (as 69.1), Pugachovka (as 69.9) and Novikovka (as 69.4) rivers.

Table 1. Number of myomeres in larvae and adult lampreys of the genus Lethenteron from Sakhalin waters by Matsueva (2003)

River/Lake	Larvae			Adults		
	n	ranges	M ± S.E.M.	n	ranges	M ± S.E.M.
Pugachovka R.	29	68-72	69.9 ± 0.15	24	68-73	70.7 ± 0.23
Novikovka R.	51	64-75	69.4 ± 0.35	33	65-74	69.4 ± 0.39
Moguchi R.	85	67-72	69.1 ± 0.1			
B. Matchi R.	13	66-71	68.9 ± 0.29			
Fevralevka R.	29	66-72	68.8 ± 0.18			
Nitui R.	89	66-72	68.8 ± 0.13	146	66-71	68.8 ± 0.09
Yasynge R.	28	67-71	68.7 ± 0.16			
Nabil´R.	6	67-70	68.6 ± 0.34			
Krasnodonka R.	16	66-71	68.5 ± 0.35			
B. Aleksandrovka R.	25	66-71	68.4 ± 0.32			
Kura R.	49	66-71	68.3 ± 0.23			
Taranai R.	113	66-71	68.2 ± 0.1			
Kopilka R.	31	65-69	67.8 ± 0.14			
Ostrovka R.	48	63-74	67.7 ± 0.32			
M. Garomai R.	74	66-69	67.3 ± 0.08	153	67-74	70.6 ± 0.11
Bol'shaya R.	23	64-69	66.8 ± 0.25			
B. Chibisan Lake	154	63-71	66.6 ± 0.14			
Ol'chovka R.	10	64-69	66.5 ± 0.39			

Table 2. Morphometric parameters found in larvae of the genus Lethenteron from four Sakhalin rivers (Taranai, n = 30, 130-165 mm TL; Fevralevka, n = 25, 133-187 mm TL; Nitui, n = 30, 132-197 mm TL; Pugachovka, n = 29, 96-162 mm TL; Novikovka n = 20, 76-162 mm TL (Efremova, 2006)

River	Taranai	Fevralevka	Nitui	Pugachovka	Novikovka
Parameter	M ± S.E.M.	M ± S.E.M.	M ± S.E.M.	M ± S.E.M.	M ± S.E.M.
TL	148.6 ± 1.76	159.0 ± 2.77	155.4 ± 3.38	127.0 ± 0.32	118.5 ± 0.25
In % of total length					
1BOC	92.5 ± 0.17	92.8 ± 0.22	92.2 ± 0.43	93.4 ± 0.32	91.0 ± 0.18
7BOC	97.3 ± 1.24	81.0 ± 0.20	76.8 ± 0.59	79.8 ± 0.30	77.6 ± 0.16
MBD	5.3 ± 0.09	5.0 ± 0.07	5.1 ± 0.08	5.4 ± 0.08	5.08 ± 0.06
BW	3.9 ± 0.07	3.7 ± 0.07	4.0 ± 0.07	4.2 ± 0.08	4.6 ± 0.06
ID	2.4 ± 0.05	2.4 ± 0.04	2.0 ± 0.04	2.3 ± 0.05	2.5 ± 0.07
PB	15.6 ± 0.23	15.6 ± 0.22	16.0 ± 0.24	16.5 ± 0.15	16.6 ± 0.09
HD	3.8 ± 0.07	3.3 ± 0.07	4.3 ± 0.07	4.3 ± 0.06	5.4 ± 0.05
PBL	7.1 ± 0.10	7.4 ± 0.08	6.9 ± 0.12	7.2 ± 0.07	7.6 ± 0.08
17BO	11.6 ± 0.18	11.9 ± 0.15	11.6 ± 0.14	13.2 ± 0.16	10.5 ± 0.07
DL	2.7 ± 0.04	2.5 ± 0.05	2.6 ± 0.05	2.3 ± 0.06	3.6 ± 0.32
LDS	3.5 ± 0.06	3.4 ± 0.06	3.9 ± 0.06	2.7 ± 0.05	4.0 ± 0.33
DB	18.9 ± 0.18	18.8 ± 0.13	18.5 ± 0.23	18.0 ± 0.14	17.0 ± 0.81
HW	2.0 ± 0.03	1.9 ± 0.03	2.1 ± 0.03	4.3 ± 0.07	4.9 ± 0.43
PDL	47.3 ± 0.33	47.4 ± 0.28	48.0 ± 0.36	49.0 ± 0.21	50.8 ± 1.07
PD	71.1 ± 0.21	70.2 ± 0.22	70.6 ± 0.69	68.1 ± 0.26	71.6 ± 0.16
IDD	2.9 ± 0.05	2.7 ± 0.05	2.0 ± 0.03	2.8 ± 0.09	2.8 ± 0.10
1DL	14.4 ± 0.25	14.0 ± 0.18	14.5 ± 0.26	13.2 ± 0.16	11.9 ± 0.09
1DH	0.7 ± 0.01	0.6 ± 0.01	1.5 ± 0.03	0.7 ± 0.03	0.7 ± 0.07
2DL	23.7 ± 0.40	23.2 ± 0.28	24.7 ± 0.42	23.6 ± 0.18	21.8 ± 0.10
2DH	1.4 ± 0.02	1.6 ± 0.03	1.3 ± 0.02	1.6 ± 0.05	1.6 ± 0.06
LCF	11.4 ± 0.21	12.0 ± 0.22	8.8 ± 0.16	10.5 ± 0.12	10.2 ± 0.08
2DC	34.5 ± 0.28	34.4 ± 0.22	33.6 ± 0.39	32.8 ± 0.19	31.9 ± 0.13
LA	1.4 ± 0.02	1.3 ± 0.02	1.3 ± 0.02	1.4 ± 0.05	1.7 ± 0.06
PAD	27.6 ± 0.19	28.9 ± 0.23	26.5 ± 0.32	25.8 ± 0.17	24.9 ± 0.11
In % of prebranchial length					
ID	32.8 ± 0.55	32.5 ± 0.58	29.5 ± 0.44	32.5 ± 0.77	36.4 ± 0.94
HD	45.3 ± 0.78	45.1 ± 0.88	62.8 ± 1.10	63.8 ± 0.95	40.0 ± 0.67
WS	28.3 ± 0.43	26.2 ± 0.43	29.2 ± 0.42	56.6 ± 1.01	32.3 ± 0.66

3.1. Larvae

Differences in morphometric parameters among larvae samples of the *Lethenteron* lampreys in five Sakhalin rivers are presented in Table 3. The smallest frequency differences among samples (statistically significant difference /SSD/ in ten morphometric parameters) was found between the Taranai and Fevralevka rivers, as well as between the Taranai and Nitui rivers. On the contrary, the biggest frequency difference (SSD in 22 morphometric parameters) was confirmed between the Taranai and Novikovka rivers).

Table 3. Differences in morphometric parameters among larvae samples of the Lethenteron lampreys in Sakhalin waters. Abbreviations: 1 - Taranai, 2 - Fevralevka, 3 - Nitui, 4 - Pugachovka, 5 - Novikovka), + statistically significant difference (Efremova, 2006)

Rivers	1-2	1-3	1-4	1-5	2-3	2-4	2-5	3-4	3-5	4-5
TL	+		+	+		+	+	+	+	+
In % of total length										
1BOC				+			+			+
7BOC	+	+	+	+	+	+	+	+		+
MBD				+		+	+		+	+
BW				+	+	+	+		+	+
ID		+			+			+	+	
PB			+	+		+	+			
HD	+	+	+	+	+	+	+		+	+
PBL				+	+				+	+
17BO				+		+		+	+	+
DL	+		+	+			+	+	+	+
LDS		+	+		+	+		+		+
DB			+			+				
HW			+	+	+	+	+	+	+	
PDL			+	+		+	+			
PD	+		+			+	+	+		+
IDD	+	+			+			+	+	
1DL			+	+		+	+	+	+	+
1DH	+	+			+	+		+	+	
2DL				+	+		+		+	+
2DH	+	+		+	+			+	+	
LCF		+	+	+	+	+	+	+	+	
2DC			+	+		+	+		+	+
LA	+	+		+			+		+	+
PAD	+		+	+	+	+	+		+	+
In % of prebranchial length										
ID		+		+	+		+	+	+	+
HD		+	+	+	+	+	+		+	+
WS	+		+	+	+	+	+	+	+	+

Matsueva (2003) studied total length/body weight (W) relationship in the sample of lampreys caught in the Nitui River during 1999-2002 (larvae: total length 14-21.5 cm, ave. 17.9 ± 0.15, n = 161; adults: total length 12.5-20 cm, ave. 17.1 ± 0.12, n = 245). Formulated equations have the following form:

$W = 0.0031TL^{2.725}$ (larvae)
$W = 0.0026TL^{2.794}$ (adults)

3.2. Adults

Sex differences in adult lampreys from the Bol'shoi Garomai river are presented in Table 4. From the 26 morphometric parameters studied, 18 had statistically significant differences. Females had (in % of the total length) longer maximum body depth, body width, internasal distance, maximum body perimeter, head depth, prebranchial length, snout length, longitudinal diameter of closed mouth slit, prebranchial + branchial length, first dorsal fin length, first dorsal fin height, second dorsal fin height, caudal fin length and total length. At the same time, males had (in % of the total length) longer branchial + postbranchial length, postbranchial length, distance between eye and first gill opening.

Table 4. Sex differences in adult lampreys of the genus Lethenteron from the river Bol'shoi Garomai (females, n = 25, 123-140 mm TL; males, n = 24, 120-148 mm TL), + statistically significant difference (Efremova, 2006)

	Females	Males	
	M ± S.E.M.	M ± S.E.M.	t-test
TL	130.4 ± 0.92	131.9 ± 1.64	
In % of total length			
1BOC	88.1 ± 0.21	87.2 ± 0.19	+
7BOC	78.5 ± 0.21	77.1 ± 0.15	+
MBD	5.2 ± 0.06	5.7 ± 0.10	+
BW	4.0 ± 0.06	4.3 ± 0.06	+
ID	5.8 ± 0.09	6.8 ± 0.10	+
PB	16.4 ± 0.13	17.3 ± 0.20	+
HD	4.7 ± 0.05	5.1 ± 0.09	+
PBL	11.8 ± 0.09	12.7 ± 0.16	+
17BO	10.0 ± 0.13	10.0 ± 0.15	
POL	6.6 ± 0.11	7.6 ± 0.10	+
EL	2.3 ± 0.02	2.3 ± 0.03	
S1BO	3.1 ± 0.02	2.9 ± 0.06	+
DL	3.6 ± 0.07	3.8 ± 0.06	
LDS	5.6 ± 0.06	6.2 ± 0.11	+
DB	21.2 ± 0.19	22.2 ± 0.18	+
HW	3.4 ± 0.06	3.4 ± 0.06	
POL	49.7 ± 0.29	48.5 ±-0.40	
PD	70.8 ± 0.30	68.9 ± 0.34	+
1DL	12.8 ± 0.18	13.9 ± 0.22	+
1 DH	2.3 ± 0.02	3.2 ± 0.06	+
2DL	25.5 ± 0.21	24.9 ± 0.32	
2DH	5.1 ± 0.09	5.8 ± 0.10	+
LCF	10.6 ± 0.17	11.8 ± 0.21	+
2DC	36.2 ± 0.27	36.5 ± 0.32	
PAD	27.8 ± 0.30	29.7 ± 0.33	+

Tables 5 and 6 present the differences between morphometrical parameters in adults from four Sakhalin rivers. There were 25 morphometric parameters (in % of total length) examined in total. When samples were mutually compared, the smallest frequency of differences among the samples (statistically significant difference /SSD/ in 16 morphometric parmeters) was found between the samples from the Pugachovka and Bol'shoi Garomai rivers. On the contrary, the biggest frequency difference (SSD in 24 morphometric parameters) was confirmed between the Novikovka and Bol'shoi Garomai rivers.

Table 5. Morphometric characters in adult lampreys (Lethenteron sp.) from four Sakhalin rivers (Nitui, n = 30, 156-220 mm TL; Pugachovka n = 24, 116-150 mm TL; Novikovka n = 20, 133-179 mm TL; Bol'shoy Garomai n = 49, 120-148 mm TL), Efremova (2006)

River	Nituy	Pugachovka	Novikovka	B. Goromay
Parameter	M ± S.E.M.	M ± S.E.M.	M ± S.E.M.	M ± S.E.M.
TL	186.9 ± 2.96	133.0 ± 0.14	155.9 ± 0.25	131.1 ± 0.93
In % of total length				
1BOC	89.2 ± 0.37	87.1 ± 0.14	93.2 ± 0.18	87.6 ± 0.15
7BOC	80.4 ± 0.34	78.5 ± 0.13	82.3 ± 0.16	77.8 ± 0.17
MBD	5.2 ± 0.09	5.7 ± 0.09	5.5 ± 0.06	5.4 ± 0.06
BW	4.2 ± 0.06	4.1 ± 0.07	5.1 ± 0.06	4.2 ± 0.05
ID	5.2 ± 0.09	6.0 ± 0.07	3.7 ± 0.07	6.2 ± 0.07
PB	15.1 ± 0.22	18.2 ± 0.13	18.2 ± 0.09	16.9 ± 0.13
HD	4.3 ± 0.07	4.6 ± 0.06	4.1 ± 0.05	5.3 ± 0.06
PBL	10.1 ± 0.14	12.1 ± 0.06	8.5 ± 0.08	12.2 ± 0.11
17BO	9.1 ± 0.11	11.1 ± 0.06	10 ± 0.07	10.0 ± 0.10
POL	5.8 ± 0.11	7.3 ± 0.06	4.6 ± 0.07	6.9 ± 0.08
EL	2.0 ± 0.04	1.8 ± 0.03	1.6 ± 0.06	2.3 ± 0.02
S1BO	2.7 ± 0.05	2.9 ± 0.03	2.7 ± 0.04	2.8 ± 0.03
DL	2.9 ± 0.05	4.6 ± 0.06	2.0 ± 0.06	3.7 ± 0.05
LDS	4.0 ± 0.05	5.6 ± 0.06	2.5 ± 0.06	5.9 ± 0.08
DB	18.9 ± 0.16	23 ± 0.08	18.2 ± 0.09	21.7 ± 0.15
HW	3.0 ± 0.05	4.2 ± 0.06	3.3 ± 0.05	3.5 ± 0.05
POL	47.7 ± 0.31	48.8 ± 0.10	47.4 ± 0.11	49.1 ± 0.26
PD	69.7 ± 0.44	68.5 ± 0.14	68.9 ± 0.16	69.9 ± 0.26
1DL	13.7 ± 0.21	14.1 ± 0.07	14.2 ± 0.09	13.3 ± 0.16
1DH	1.7 ± 0.03	2.9 ± 0.08	1.5 ± 0.07	2.4 ± 0.03
2DL	24.0 ± 0.29	24.3 ± 0.10	22.8 ± 0.10	25.2 ± 0.19
2DH	2.8 ± 0.05	5.6 ± 0.06	2.5 ± 0.06	5.4 ± 0.07
LCF	9.7 ± 0.17	10.5 ± 0.08	9.8 ± 0.08	11.2 ± 0.16
2DC	34.4 ± 0.29	36.1 ± 0.12	32.7 ± 0.13	36.3 ± 0.21
PAD	28.6 ± 0.24	28.2 ± 0.12	26.7 ± 0.11	28.7 ± 0.26
POL	57.1 ± 0.92	60.4 ± 0.05	45.6 ± 0.88	57.7 ± 0.63
EL	18.9 ± 0.33	15.4 ± 0.03	14.5 ± 0.77	18.7 ± 0.20
ID	51.6 ± 0.77	49.8 ± 0.04	36.4 ± 0.94	51.5 ± 0.56
HD	42.3 ± 0.78	40.0 ± 0.04	40.0 ± 0.67	39.8 ± 0.48
HW	30.4 ± 0.50	34.7 ± 0.3	32.3 ± 0.66	28.5 ± 0.37

Table 6. Differences in morphometric parameters among adult lamprey (Lethentheron sp.) samples in Sakhalin waters. Abbreviations: 1 – Nitui River, n = 30; 2 - Pugachovka River, = 24; 3 – Novikovka River, n = 20; 4 – Bol'shoi Garomai River, n = 49; + statistically significant difference (after Efremova, 2006)

Rivers	1-2	1-3	1-4	2-3	2-4	3-4
TL	+	+	+	+		+
In % of total length						
1BOC	+	+	+	+		+
7BOC	+	+	+	+	+	+
MBD	+	+			+	
BW		+		+		+
ID	+	+	+	+		+
PB	+	+	+		+	+
HD	+		+	+	+	+
PBL	+	+	+	+		+
17BO	+	+	+		+	+
POL	+	+	+	+	+	+
EL	+	+	+	+	+	+
S1BO	+			+		
DL	+	+	+	+	+	+
LDS	+	+	+	+	+	+
DB	+	+	+	+	+	+
HW	+	+	+	+	+	+
POL	+		+	+		+
PD					+	+
1DL					+	+
1DH	+		+	+	+	+
2DL		+	+	+	+	+
2DH	+	+	+	+		+
LCF	+		+	+	+	+
2DC	+	+	+	+		+
PAD		+		+		+
POL	+	+		+	+	+
EL	+	+			+	+
S1BO			+		+	+
ID		+		+	+	+
HD	+		+			
HW	+		+	+	+	+

Sex differences in adults from the Novikovka and Pugachovka rivers are presented in the Table 7. From the 26 studied morphometric parameters, 9 had statistically significant differences. Females had (in % of the total length) longer maximum body depth, internasal distance, maximum body perimeter, head depth, prebranchial length, prebranchial + branchial length, second dorsal fin height, total length. On the other hand, males had (in % of the total length) longer predorsal length.

Table 7. Differences in morphometric parameters between sex in the lamprey (Lethenteron sp.) samples originating from the Novikovka and Pugachovka rivers (females, n = 19, 123-179 mm TL; males n = 27, 116-162 mm TL). Abbreviation: M – mean, S.E.M – standard error of the mean, + statistically significant difference, Efremova (2006)

Character	Females	Males	t-test
	M ± S.E.M.	M ± S.E.M.	
TL	148.6 ± 3.00	146.1 ± 2.09	
In % of total length			
1BOC	90.4 ± 0.27	89.7 ± 0.22	
7BOC	80.5 ± 0.24	80.1 ± 0.21	
MBD	5.4 ± 0.11	5.9 ± 0.05	+
BW	4.4 ± 0.13	4.5 ± 0.08	
ID	4.4 ± 0.09	4.9 ± 0.11	+
PB	17.0 ± 0.17	17.8 ± 0.09	+
HD	4.2 ± 0.06	4.5 ± 0.05	+
PBL	9.8 ± 0.08	10.2 ± 0.11	+
17BO	10.4 ± 0.07	10.4 ± 0.06	
POL	5.8 ± 0.11	6.0 ± 0.12	
EL	1.7 ± 0.05	1.7 ± 0.05	
S1BO	2.8 ± 0.04	2.8 ± 0.03	
DL	3.0 ± 0.13	3.2 ± 0.15	
LDS	3.8 ± 0.13	3.9 ± 0.17	
DB	19.6 ± 0.08	20.4 ± 0.09	+
HW	3.7 ± 0.05	3.8 ± 0.07	
POL	47.5 ± 0.14	47.0 ± 0.11	+
PD	68.9 ± 0.21	68.6 ± 0.17	
1DL	13.7 ± 0.11	13.6 ± 0.08	
1DH	2.0 ± 0.35	2.0 ± 0.34	
2DL	24.6 ± 0.11	24.9 ± 0.11	
2DH	1.9 ± 0.06	2.4 ± 0.13	+
LCF	3.7 ± 0.13	4.1 ± 0.17	
2DC	10.3 ± 0.09	10.3 ± 0.08	
PAD	34.1 ± 0.14	35.0 ± 0.12	+

Comparison of the anadromous and resident lampreys (*L. camtschaticum*) shows that there are no morphologic differences between these forms (Makhrov et al. 2013). *L. kessleri* is distinguished from *L. reissneri* by the presence of distinct posterial teeth, by the pointed supraoral lamina, by the larger eye diameter, and by the larger number of trunk myomeres (Iwata et al. 1985). *Lethenteron reissneri* differs from *L. kessleri* by reaching a number of 57-65 (ave. 60.4) myomeres versus 65-73 (ave. 69.0), *L. reissneri* and *L. kessleri* showed an overlap in all body measurements. The number of myomeres in *L. camtschaticum* (as *L. japonicum*) ranges from 68-77 (ave.72.1), see Iwata et al. (1985).

Renaud (2011) elaborated on the determination key for all lamprey species, including the genus *Lethenteron*. Gular region unpigmented (this characteristic requires verification in *Lethenteron kessleri* but the region is definitely unpigmented in the other species referred to

below as *L. camtschaticum*, and *L. reissneri*). The second dorsal fin unpigmented; trunk myomeres, 57–65; 38–44 anterials in *Lethenteron reissneri*. The second dorsal fin usually with a dark blotch; trunk myomeres, 65–77; 15–38 anterials are in *L. camtschaticum* (total length, 110–625 mm; parasitic) and *L. kessleri* (total length 112–230 mm; nonparasitic, 15–28 anterials). The key is not suitable to identify the lamprey samples from Sakhalin Island studied in the chapter because of overlapping ranges in the metric parameters.

In the supraoral lamina adults were always found with 2 teeth (the Lazovaya River, 25 species, Safronov 2000; the Nitui River, 30, Matsueva 2003; the Melkaya River, 9, Safronov 2000; the Bol'shoi Garomai River, 49, the Tym´ River, 9, Safronov 2000). Infraoral lamina bears mostly 5-7 teeth (the Lazovaya, Melkaya, Tym´ rivers), and in the Nitui and Bol'shoi Garomai rivers 6-7 teeth. The endorateral row formulae is identical (2-2-2) in the Lazovaya, Nitui, Bol'shoi Garomai and Tym´ rivers. Ranges in the number of anterior teeth (anterials) is overlapping in the Lazovaya (18-22), Melkaya (14-21), Bol'shoi Garomay (12-20) and Tym´ (17-20) rivers. Posterior disc teeth (posterials) range from 19-23 (the Lazovaya River), 15-24 (the Melkaya River), 10-16 (the Bol'shoi Garomai River), 19-21 (the Tym´ River). The number of myomeres varies between 70-76 (the Lazovaya River), 68-75 (the Nitui River), 68-72 (the Melkaya River), 67-72 (the Bol'shoi Garomai River), 55-67 (the Tym´ River).

The dentition in lampreys from the above mentioned Sakhalin rivers is analogous to the data on *L. camtschaticum* (the Eurasian Arctic) published by Makhrov et al. (2013) exept for the Bol'shoi Garomay river, where the posterial number range is evidently lower (10-16/the Bol'shoi Garomai river/compared to 15-24/data by Makhrov et al. 2013/).

CONCLUSION

Samples of larvae and adult lampreys of the genus *Lethenteron* from 19 Sakhalin rivers and one lake were studied in respect to meristic and morhometric parameters and evaluated by the standard statistical methods. We confirm statistically significant differences in many morphometric parameters among the samples studied and between sexes, the ranges of trunk myomeres in all samples varied between 63-75. Based on our own results, it is not possible at present to classify the studied samples to definite species within the genus *Lethenteron* without doubts (see also Khusainova and Karpenko 2017). This question requires further research based on numerous materials from various areas focused on examining clinal variability in meristic and morphometric parameters, ecology and completion of genetic characteristics. Genetic studies are needed to better define the differences among populations inhabiting Sakhalin rivers. Results could also be applied in protecting, conserving and managing the particular species or populations with individual genetic differences originating from various catchment areas in Sakhalin Island (see also Futuyma and Kirkpatrick 2017).

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Chapter 93

BIOLOGY OF COMMON CARP IN NATURAL AND FARMED HABITATS FROM A GLOBAL PERSPECTIVE

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ABSTRACT

Common carp (*Cyprinus carpio* L.) is an important food fish in many Asian and European countries, where it is considered as an important aquaculture species. Common carp account for approximately 4.1% of the total global aquaculture production and 9% of the global freshwater aquaculture production. The bulk of world's common carp production (97%) comes from aquaculture. It is the most cultured fish species in Central and Eastern Europe, with production levels reaching more than 80% of total fish production in some countries. Aquaculture of this species is not observed in North America, New Zealand and Australia, where it is considered as a potential invasive and noxious aquatic species. Common carp belongs to the largest family (Cyprinidae) of freshwater fishes in the world. Their high adaptive capacity in a wide range of conditions and habitats accounts for their widespread distribution in most countries of the world. Presently, two subspecies exist in the world: the Asian (*C. carpio haematopterus*) and the European subspecies (*C. carpio carpio*). Common carp live in lakes, ponds, reservoirs, natural depressions and rivers. They are bottom dwellers, generally preferring to live near the soft vegetated sediments. They prefer benthic organisms particularly chironomids, oligochaetes, gastropods and other larval insects available in and on the sediment of natural habitats. When preferred foods become depleted, they shift their food habits, feeding niche and behavior. The behavior of common carp is consistent with classical optimal foraging theory. They readily adapt when inter- and intra-specific competition are pronounced. Common carp significantly influence the behavior of other species (e.g., *Labeo rorita*) without showing any aggressive interactions. This article reviews the

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origin, habitats, distribution, growth, production, food habit, spawning, maturation, fecundity and behaviour of common carp focusing through a global lens.

Keywords: common carp, origin, distribution, habitat, growth, production, food habit, reproduction, fecundity, behavior, b–value, sexual maturity

INTRODUCTION

Common carp can dominate in any freshwater body due to their fast growth, early maturity, high fecundity and high adaptive capacity. These characteristics have enabled them to persist and proliferate in a wide array of environments, making them one of the major exotic species to have spread throughout the world. In some countries, particularly North America, New Zealand and Australia, common carp is considered as an invasive and destructive aquatic pest due to a combination of their potential to be invasive and their ecological impacts on ecosystems (Koehn 2004; Oyugi et al. 2011). In natural habitats, carp is considered a pest fish because of its tendency to destroy aquatic macrophytes (through uprooting of aquatic macrophytes, direct consumption of tubers and young shoots), increase water turbidity and alter ecosystem function for local species. It is one of eight fish on the IUCN list of the World's worst 100 invaders (Lowe et al. 2000).

Many countries consider the management of common carp as a key target for mitigating detrimental impacts on freshwater ecosystems (Britton et al. 2011a). In natural habitats, particularly in lakes, river and reservoirs in Asia, the population of this species is decreasing rapidly due to overexploitation of water resources, climate change and various anthropogenic activities. In Asia and Europe, common carp is an important food fish and is accepted as a naturalized species that poses little or no threat to the environment (Britton et al. 2011b). Due to the high importance of common carp as food fish, aquaculture of this species is a common practice in many Asian and European countries, where it is an important candidate for continued research.

Considering its local importance, most of the research conducted in Europe and Asia has focused on the development of common carp aquaculture, while most of the research in North America, New Zealand and Australia has focused on management of common carp in natural habitats particularly how to mitigate various detrimental impacts of common carp on local fish species (Koehn 2004). However, there has been a limited focus on the global aspects of common carp literature. Consequently, this chapter reviews the origin, habitats, distribution, growth, production, food habit, spawning, maturation, fecundity and behaviour of common carp with a global focus.

ORIGIN, HABITATS AND DISTRIBUTION

Common carp has a high DNA content and about 52% of its enzymes have shown a pattern consistent with duplication (Ohno et al. 1967). These findings support the hypothesis that common carp is an evolutionary tetraploid species that may have originated through species hybridization. Recent genetic evidence (sequence analyses of duplicate loci) indicates

that the tetraploidization in common carp occurred around 16–19 million years ago (Larhammar and Risinger 1994). David et al. (2003) determined the time of occurrence of this tetraploidization to around 12 million years ago using microsatellites analysis. According to Berg (1964), modern common carp came from a Caspian Sea ancestor and initially spread into the Black and Aral Sea basins. Under favorable conditions of the postglacial thermal increase they dispersed as far as the Danube and the East Asia (Balon 1995). Common carp appeared in the River Danube about 8,000 to 10,000 years ago and, therefore, it is unlikely that the carp occurred naturally in waters of Central and Western Europe outside the River Danube at the beginning of the Christian era (Balon 1995). Wild carp then reached the delta of the Rhine, somewhat later, probably in 12th century.

Assuming the long cultivation history of common carp in Asia, some scientists consider that the ancestor of the European domestic common carp came from Asia during the periods of ancient Greece and Rome (Vooren 1972; Chistiakov and Voronova, 2009). Others scientists believe that a German domestic strain of common carp to be the first improved carp, which appeared after the domestication of a wild common carp collected from the Danube River during the 17th and 18th centuries (Kirpichnikov 1981, Chistiakov and Voronova 2009). However, recent molecular genetics of wild common carp in some Turkish lakes supports the hypothesis of a single origin of present European domesticated and wild carp to have been from a common ancestor of Central Asian carp (Memis and Kohlmann 2006).

According to Kirpitchnikov (1967), there are at least four subspecies of wild common carp in the world: (i) the European and Transcaucasian *Cyprinus carpio carpio,* (ii) the Middle East *C. carpio aralensis,* (iii) east Asian C. *carpio haematopterus* and (iv) the south Chinese and Vietnamese *C. carpio uiridiviolaceus.* However, according to Balon (1995), two subspecies are present worldwide: *C. carpio carpio* and *C. carpio haematopterus. Cyprinus carpio carpio* is known as the European wild common carp, which originated from the western dispersant. *Cyprinus carpio haematopterus* is known as the Asian wild common carp, which originated from the eastern dispersant. In the duration of a long history of domestication, common carp has been subjected to various kinds of adaptation and genetic interventions particularly chromosome manipulations, selective breeding, sex reversal, and transgenesis (Chistiakov and Voronova 2009). Therefore, many common carp researchers believe that presently all common carp populations in the world may be hybrids. The pure breed of the wild common carp is presently faced with extinction resulting from various genetic interventions, serial domestication, and habitat modifications (Chistiakov and Voronova 2009; Oyugi 2012). In many countries, particularly those in Asia, studies on genetic distances among common carp populations are rare. There is a common belief in a number of Asian countries that wild populations are now dominated by hatchery common carp. Presently, introduction of hatchery–reared common carp is a serious threat for the conservation of wild carp, particularly in Asia and Europe, where common carp are domesticated as an important food fish. In several areas of Europe, the wild carp populations particularly the Danubian wild form is rare or may even be extinct (Piria et al. 2016).

Common carp belong to the Cyprinidae (minnow and carp family), which is the largest family of freshwater fishes in the world. The family Cyprinidae has nearly 2.5 thousands species. Common carp can be easily recognized by its small eyes, thick lips with two barbels at each corner of the mouth. The color of common carp is variable based on the environment, geographical distribution and genetics. However, their color is often olive–green to silvery–grey dorsally, fading to silvery yellow on the belly. Based on the arrangement of scales on the

body, common carp has four varieties throughout the world: scaly common carp, mirror carp, linear carp and leather carp. Two genes, S (scaly) and N (nude) present on two chromosomes are responsible for the arrangement of scales on the body. The arrangements of scales are dependent on the character of these genes, whether recessive (S, N) or dominant (s, n). Common carp that are coved with scales are called scaly carp (genes: SSnn, Ssnn). Mirror carp (ssnn) are partially scaled along their sides. Some common carp have no scales and are called leather carp (ssNn). Scales arrangement of linear carp (SSNn, SsNn) is along the lateral line. The genotypes SSNN, SsNN and ssNN do not exist in common carp.

When reared under unfavorable conditions, mirror carp are generally less resistant and achieve lower growth rates compared to scaly carp but both have similar viability (Kirpitchnikov 1999). Leather and linear carp are less viable even under favorable conditions. When common carp become mature, the operculum and the pectoral fin's upper surface of male common carp develop tubercles and turn rough compared to the female common carp. Mature males often release white milt, which oozes out from its genital opening on slight pressure on the abdomen. The belly of mature female are swollen compared to males (Oyugi 2012). Wild common carp has a typical life span of about 17 to 24 years but their lifespan can be up to 47 years in captivity (Froese and Pauly 2002).

The common carp is native to Europe and Asia. The natural distribution of this fish is very wide. Their high adaptive capacity in a wide range of conditions and habitats distribute them almost in all countries in the world. The ecological spectrum of carp is very wide. It normally lives in a temperature range of around 15–32°C, but are able to survive in a wide range of temperatures (2–40°C). They can easily survive winter in an ice–covered lake, reservoir or river if some free water remains below the ice. Therefore, it is considered a eurythermal fish species. Common carp can also live in water with low dissolved oxygen. They may be found in waters with dissolved oxygen concentrations less than 2.0 mg/L and able to survive in water with dissolved oxygen concentrations levels as low as 0.5 mg/L for several days (Panek 1987; Zhou et al. 2000; Butler and Wahl 2010). They take atmospheric oxygen by gulping at the water surface at very low dissolved oxygen concentrations in the water.

Lakes, ponds, natural depression and rivers are natural habitats of common carp (Barus et al. 2001; Rahman 2015a). Common carp are mainly bottom dwellers, generally preferring to live near the soft vegetated sediments. In rivers, they are generally found in standing water or moderately flowing water. According to Butler and Wahl (2010), common carp prefer littoral zones with low current velocity compared to lotic areas with high current velocities in rivers. During the colder months, common carp, particularly adults, stay in the deeper areas of lakes and river channels and during the warmer months they migrate toward shallow, heavily vegetated floodplain areas (Bajer et al. 2011; Taylor et al. 2012). The optimal pH for common carp is 6.5–9.0. Water pH of <5.0 and >10.5 are harmful or lethal to common carp. Common carp can tolerate salinity up to about 5‰. However, sometimes they are found in brackish–water estuaries and bays, where their growth and production are normally very low.

GROWTH AND PRODUCTION

Common carp is one of the most important food fish in many Asian and European countries. In 2015, it contributed over 4.4 million metric tons to fish production worldwide (FAO 2017). The bulk of the world's common carp production (97%) comes from aquaculture (Figure 1). Asia contributes the majority of world's common carp production. In 2015, Asia contributed 92% of the world's aquaculture production and half of the world's capture production (Figure 2). Among all countries, Mexico contributed the highest common carp capture production and China contributed the highest common carp aquaculture production (Table 1). Common carp account for approximately 4.1% of total global aquaculture production and 9% of the global freshwater aquaculture production. The common carp also accounts for the world's third highest farmed fish production, mainly from polyculture. It is the most cultured fish species in Central and Eastern Europe, with production levels reaching more than 80% of total fish production in some countries (Woynarovich et al. 2010; Adamek et al. 2012; FAO 2017).

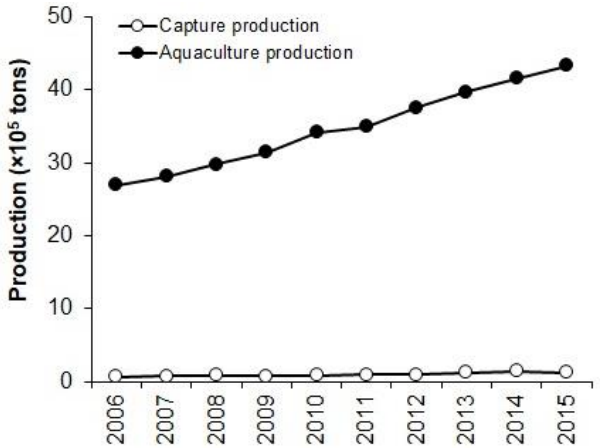

Figure 1. Global capture and aquaculture production of common carp (data source: FAO 2017).

Common carp is well recognized as one of the oldest domesticated fish species. In Asia, domestication of common carp first commenced in China in the 5th century BC, whereas their domestication in Europe started during the period of the Roman Empire (Balon 2006). Common carp is normally cultured in various grow–out systems, which include conventional pond culture, large–scale commercial farms, duck cum fish, cage culture, irrigated area culture and rice field culture. In many Asian countries, common carp is most commonly cultured as a bottom–dwelling fish in polyculture ponds. For example, in south Asian countries, the traditional pond polyculture mostly includes a bottom dwelling fish like common carp with a surface (e.g., catla, *Catla catla*) and a column dwelling fish (e.g., rohu, *Labeo rohita*). As in Asia, common carp is also cultured in earthen ponds in Europe, applying extensive and semi–intensive management regimes, thereby allowing use of natural food resources for growth (Anton–Pardo et al. 2014).

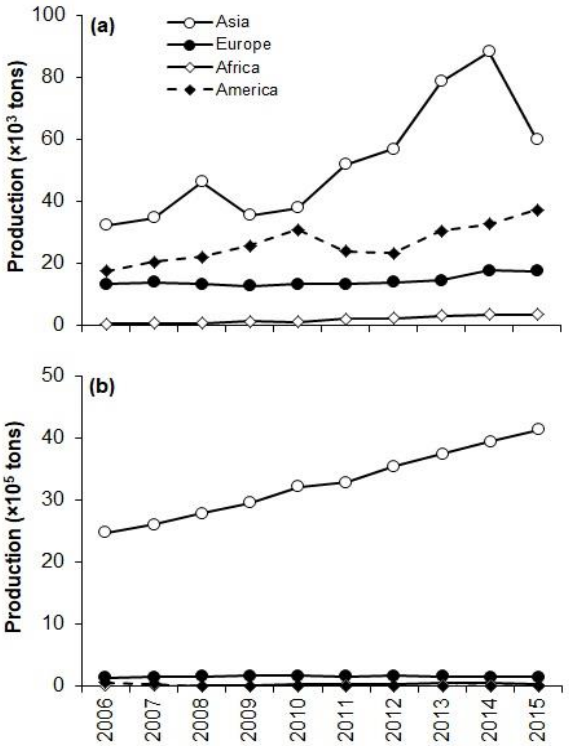

Figure 2. Continent-wise capture (a) and aquaculture (b) production of common carp (data source: FAO 2017). The production of common carp in Australia is very low compared to other continent and therefore, it has been ignored.

Table 1. Top 15 capture and aquaculture producing countries and their production (tons) in 2015

Capture production		Aquaculture production	
Mexico	35,779	China	3,357,962
Iran	15,520	Indonesia	461,107
Iraq	15,397	Viet Nam	94,273
Indonesia	13,480	Bangladesh	76,484
Hungary	7,307	Russia	57,983
Turkey	7,223	Iran	46,016
Thailand	4,900	Egypt	30,000
Russia	3,524	Iraq	22,303
Czechia	3,014	India	18,800
Kenya	2,302	Myanmar	18,441
Uzbekistan	1,680	Czechia	17,860
Slovakia	1,466	Poland	17,749
Ethiopia	1,349	Hungary	10,725
South Korea	1,206	Ukraine	9,640
Ukraine	1,127	Laos	7,950

Source of raw data: FAO (2017).

A typically semi–intensive production system generally utilizes a combination of supplementary feeding and occasional fertilization with animal manure to increase natural food availability, particularly phytoplankton, zooplankton and various aquatic invertebrates.

In Asian semi–intensive polyculture systems, a larger fraction of the natural food available in the pond is used by various species. In some cases, common carp enhance the food available for other species, as a consequence of nutrients re–suspension from their feeding activities, thus increasing further the total fish yield per unit area. The supplementary feed applied in semi–intensive systems usually varies from a high protein containing diet (Rahman et al. 2006) to cheap and locally available raw ingredients such as wheat, triticale or rye (Markovic et al. 2009; Anton–Pardo et al. 2014). The specific growth rate (SGR) of common carp can be 0.3 to 2 percent of body weight per day (Table 2). Carp generally reach 0.5 to 1.0 kg body weight within 6 months in the polyculture fish ponds in tropical and subtropical areas, particularly in Asia. Growth is much slower in ponds in the temperate zone (Europe), where the fish reach the 1 to 2 kg body weight in a three–year cycle. The growth of common carp is greatly dependent on culture location and management strategies particularly density of fish, and the quality and quantity of supplementary feed (Table 2). However, a strong understanding of growth and condition, food habit, stocking density, and environmental effects of common carp is essential for a sustainable and cost–effective semi–intensive aquaculture of this fish.

Table 2. Reported growth of common carp in ponds applying various management strategies

Average SGR*	Culture system	Pond management	Reference
0.37	Monoculture	S: 363 kg ha^{-1} (461–800g); A: No feed; L: Czech Republic	Anton–Pardo et al. (2014)
1.59	Two species polyculture	S: 0.5/m^2 (fingerlings); A: half of the total ponds; L: Bangladesh	Rahman et al. (2006)
1.38	Two species polyculture	S: 1/m^2 (fingerlings); A: half of the total ponds; L: Bangladesh	Rahman et al. (2006)
1.75	Two species polyculture	S: half of the total 0.5/m^2 and other half 1/m^2 (fingerlings); A: all ponds; L: Bangladesh	Rahman et al. (2006)
1.22	Two species polyculture	S: half of the total 0.5/m^2 and other half 1/m^2 (fingerlings); A: No; L: Bangladesh	Rahman et al. (2006)
1.46	Three species polyculture	S: 0.5/m^2 (fingerlings); A: half of the total ponds; L: Bangladesh	Rahman et al. (2008a)
1.47	Two species polyculture	S: 0.5/m^2 (fingerlings); A: half of the total ponds; L: Bangladesh	Rahman et al. (2008a)
1.65	Half of total ponds three species and other half two species	S: 0.5/m^2 (fingerlings); A: all ponds; L: Bangladesh	Rahman et al. (2008a)
1.28	Half of total ponds three species and other half two species	S: 0.5/m^2 (fingerlings); A: No; L: Bangladesh	Rahman et al. (2008a)
1.6	Two species polyculture	S: 1/m^2 (fingerlings); A: all tanks; L: Bangladesh	Rahman et al. (2008b)

*,% body weight day^{-1}; S, stocking density; A, artificial feed; L, location reach the 1 to 2 kg body weight in a three–year cycle. The growth of common carp is greatly dependent on culture location and management strategies particularly density of fish, and the quality and quantity of supplementary feed (Table 2). However, a strong understanding of growth and condition, food habit, stocking density, and environmental effects of common carp is essential for a sustainable and cost–effective semi–intensive aquaculture of common carp.

LENGTH–WEIGHT RELATIONSHIP AND CONDITION FACTORS

Published information on length–weight relationships indicates that common carp have negative allometric growth (b < 3.0) in most natural habitats and aquaculture ponds (Table 3). This indicates that length increment of common carp is higher than weight increment. Common carp do not show any habitat–wise distinct growth coefficient (b value) and condition factor (Table 4).

Table 3. Regression co–efficient (b value) of length–weight relationships of common carp reported from various habitats globally

b–values	Location of study	Reference
3.14	Omerli Dam lake, Turkey	Tarkan et al. (2006)
3.07	Pag island, Croatia	Piria et al. (2016)
3.06 (M), 3.14 (F)	Bhamanghata river, India	Singh et al. (2015)
3.00 (M), 3.00 (F)	Murray river, Australia	Brown et al. (2003)
2.97	Fincha reservoir, Ethiopia	Degefu et al. (2012)
2.95	Karamık lake, Turkey	Balık et al. (2006)
2.92	Amerti reservoir, Ethiopia	Hailu (2013)
2.92	Polyculture pond, Albania	Bllaca and Spaho (2014)
2.90 (M), 2.90 (F)	Irrigation channels, Australia	Brown et al. (2003)
2.90	Vransko lake, Croatia	Treer et al. (2009)
2.88 (M), 2.96 (F)	Ziway lake, Ethiopia	Abera et al. (2015)
2.871	Cayumapu river, Chile	Prochelle and Campos (1985)
2.86	Carl Blackwell lake, USA	Mauck and Summerfelt (1970)
2.83	Lake Iznik, Turkey	Tarkan et al. (2006)
2.80	Marmara Lake, Turkey	Ihan and Sari (2015)
2.79	Lake Sapanca, Turkey	Tarkan et al. (2006)
2.75	Pond, Sri Lanka	Nathanael et al. (1998)
2.71 (M), 2.53 (F)	Apa Dam lake, Turkey	Mert et al. (2008)
2.68	Polyculture pond, Albania	Bllaca and Spaho (2014)
2.50	Victoria reservoir, Sri Lanka	Nathanael et al. (1998)
2.35 (M), 1.95 (F)	Lake Naivasha, Kenya	Aera et al. (2014)

M, male; F, female.

Table 4. Reported condition factor of common carp in various habitats globally

Condition factor	Location of study	Reference
1.40–1.62	Lake Vransko, Croatia	Treer et al. (2009)
F: 0.6–1.3, M: 2.1–2.2	Lake Naivasha, Kenya	Aera et al. (2014)
F: 0.5–1.0, M: 1.7–1.8	Lake Naivasha, Kenya	Aera et al. (2014)
1.10–1.37	Pag Island, Croatia	Piria et al. (2016)
M: 1.20, F: 1.21	Lake Carl Blackwell, USA	Mauck and Summerfelt (1970)
M: 0.9–1.1 , F: 1.0–1.2	Bhamanghata river, India	Singh et al. (2015)
M: 1.3–1.6, F: 1.5–1.8	Lake Ziway, Ethiopia	Abera et al. (2015)
1.42–153	River Cayumapu, Chile	Prochelle and Campos (1985)
1.88–2.15	Apa Dam Lake, Turkey	Mert et al. (2008)
1.50	Polyculture pond	Bllaca and Spaho (2014)
1.52	Polyculture pond, Albania	Bllaca and Spaho (2014)
1.652–2.695	Lake Karamık, Turkey	Balık et al. (2006)
M: 1.22–1.46, F: 1.23–1.77	Amerti Reservoir, Ethiopia	Hailu (2013)
1.78	Pond, Sri Lanka	Nathanael et al. (1998)
1.89	Victoria reservoir, Sri Lanka	Nathanael et al. (1998)

M, male; F, female.

FOOD HABIT

Various research on food habit of common carp (e.g., Khan 2003; Adamek et al. 2003; Rahman et al. 2006; Rahman et al. 2008a; Saikia and Das 2009; Anton–Pardo et al. 2014) indicates that adult common carp prefer benthic organisms particularly chironomids, oligochaetes, gastropods and other larval insects associated with the benthos of natural habitats. In most cases, a variety of foods are identified in their stomachs. Therefore, they are omnivorous, with a tendency towards the consumption of animal foods, such as chironomid larvae, gastropods, oligochaetes, water insects, larvae of insects, worms, mollusks, and zooplankton (Table 5). Additionally, the common carp consume various plant–originated foods such as fresh and decayed seeds, leaves and stems of various aquatic and terrestrial plants. Phytoplankton is also found rarely in the stomachs of common carp.

Among a wide variety of factors that can influence the diet of common carp, the most important factors are age/size, availability of natural food resources, season and food competition (Rahman et al. 2009; Kloskowski 2011). Like many other fishes, common carp food preferences differ across their different age and /or size structures (Table 5). This ontogenetic food habit of common carp is greatly dependent on anatomical structures, behavior, physiological demands, habitat and food supply. Changes in the size of the mouth and oral anatomy may also correspond to ontogenetic dietary shifts (Rahman et al. 2009; Rahman 2015a). Various studies on food habit of common carp indicate that common carp prefer zooplankton when they are small, whereas larger fish prefer benthic macroinvertebrates over zooplankton (Adamek et al. 2003; Rahman et al. 2009). There is also some evidence of zooplankton avoidance by large common carp. For example, Rahman et al. (2009) observed that common carp of up to 15.4 cm total length preferentially selected zooplankton, but those larger than 18.9 cm total length avoided them. Phytoplankton ingestion by common carp is likely accidental given common carp of all sizes tend to avoid its direct consumption (Rahman et al. 2009). According to Al–Lamy and Taher (2016), common carp do feed directly upon phytoplankton in their larval and juvenile stages.

Common carp consume different food organisms in different amounts depending on their availability in the surrounding environment (Rahman et al. 2009). When preferred foods become depleted, common carp shift their food habits. For these fish, both availability of preferred food items and the presence of competitors are equally important. When low abundance of preferred food items (benthic macroinvertebrates) or density of common carp is high, they switch to their next most–preferred food items (zooplankton) (Rahman et al. 2009; Rahman 2015a). Zooplankton can be a very dominant dietary item in the absence of benthic macroinvertebrates. Zooplankton consumption can be particularly dominant in fish ponds where the stocking density of common carp is high. However, in the absence of preferred food, common carp follows the classical optimal foraging theory, whereby they broaden their feeding niche to maximize their food intake (Rahman and Meyer 2009). The results of various research indicates, however, that common carp has excellent adaptive capabilities in the presence of low food availability. For example, at high density, when there are insufficient natural foods, common carp eat the fry of other fish (Weber and Brown 2011; Rahman 2015b). In ponds supplied with artificial feed, common carp shifts its preference from zooplankton and benthic macroinvertebrates to artificial foods (Rahman et al. 2006; Rahman and Meyer 2009; Jurajda et al. 2016). Common carp grow better in ponds supplied

with pelleted artificial feed than with cereals. Pelleted artificial feed acts as a source of nutrients for common carp growth, but it also indirectly maintains ecological stability of the surrounding environment due to less disturbance from carp feeding on natural foods (Rahman 2015b).

Table 5. Dominant food items of common carp at various life–stages, globally

Size (total length, cm)	Food items	Dominant food	Study area
<2	Cladocera, Copepoda	Cladocera, Copepoda	Lake Colac and Lake Modewarre, Australia (Khan, 2003)
>2	Cladocera, Copepoda, Benthic food resources	Cladocera, Copepoda	
(≤15	Cladocera, Copepoda, Benthic food resources	Cladocera, Copepoda	
>15	Cladocera, Copepoda, Gastropoda, Ostracoda, Amphipoda, Detritus	Detritus	
9.5–9.9	Detritus, Benthic macroinvertebrates, Rotifera, Cladocera, Copepoda, Phytoplankton	Detritus, Benthic macroinvertebrates	Polyculture ponds, Bangladesh (Rahman et al. 2009)
14.7–15.4	Detritus, Benthic macroinvertebrates, Rotifera, Cladocera, Copepoda, Phytoplankton	Detritus, Benthic macroinvertebrates	
18.9–19.2	Detritus, Benthic macroinvertebrates, Rotifera, Cladocera, Copepoda, Phytoplankton	Detritus, Benthic macroinvertebrates	
22.3–22.6	Detritus, Benthic macroinvertebrates, Rotifera, Cladocera, Copepoda, Phytoplankton	Detritus, Benthic macroinvertebrates	
24.2–24.4	Detritus, Benthic macroinvertebrates, Rotifera, Cladocera, Copepoda, Phytoplankton	Detritus, Benthic macroinvertebrates	
25.7–25.9	Detritus, Benthic macroinvertebrates, Rotifera, Cladocera, Copepoda, Phytoplankton	Detritus, Benthic macroinvertebrates	
<20	Insects, detritus, Macrophytes, Zooplankton,Ostracods, Phytoplankton	Insects, Detritus	Lake Koka, Ethiopia (Dadebo et al. 2015)
20– 29.9	Insects, detritus, Macrophytes, Zooplankton,Ostracods, Phytoplankton	Insects, Detritus	
30– 39.9	Detritus, Insects, Macrophytes, Zooplankton,Ostracods, Phytoplankton	Detritus, Insects	Lake Koka, Ethiopia (Dadebo et al. 2015)
> 40	Detritus, Insects, Macrophytes, Zooplankton,Ostracods, Phytoplankton	Detritus, Insects	
Small carp	Cladocerans, Ostracods, Chironomids, Detritus, Plant Debris, Diatoms, Seeds, Amphipods, Phantom midge larvae	Cladocerans, Ostracods, Small chironomids	Lake Banyoles, Spain (Garcia–Berthou 2001)
Large carp	Cladocerans, Ostracods, Chironomids, Detritus, Plant Debris, Diatoms, Seeds, Amphipods, Phantom midge larvae	Phantom midge larvae, Large chironomids	
Spring			
2–5	Rotifera, Cladocera, Copepoda, Ostracoda, Trichoptera , Ephemeroptera, Chironomids, Zygoptera, Nematoda, Oligochaeta, Hydracarina	Chironomids, Cladocera	Ponds, Poland (Kloskowski 2011)
12–14	Cladocera, Copepoda, Ostracoda, Trichoptera , Ephemeroptera, Chironomids, Zygoptera, Nematoda, Oligochaeta, Hydracarina	Chironomids, Cladocera	
21–24	Cladocera, Copepoda, Ostracoda, Trichoptera, Ephemeroptera, Chironomids , Zygoptera, Nematoda, Oligochaeta, Hydracarina	Chironomids, Cladocera	

Size (total length, cm)	Food items	Dominant food	Study area
Summer			
6–10	Rotifera, Cladocera, Copepoda, Trichoptera, Ephemeroptera, Chironomids , Zygoptera, Nematoda, Oligochaeta, Hydracarina	Chironomids, Ephemeroptera	Ponds, Poland (Kloskowski 2011)
16–20	Cladocera, Copepoda, Trichoptera, Ephemeroptera, Chironomids, Zygoptera, Nematoda, Oligochaeta, Hydracarina	Chironomids, Trichoptera	Ponds, Poland (Kloskowski 2011)
28–31	Cladocera, Copepoda, Ostracoda, Trichoptera , Ephemeroptera, Chironomids, Zygoptera.	Chironomids, Trichoptera	
1.0–1.9	Algae, Diatom, Copepods	Diatom	Ponds, Iraq (Al–Lamy and Taher 2016)
2.0–2.9	Algae, Diatom, Copepods	Diatom	
3.0–3.9	Algae, Diatom	Algae	
4.0–4.9	Algae, Diatom	Diatom	
6.0–7.0	Algae, Diatom	Diatom	
461–800 g	Cladocerans, Copepods, Rotifers, Insects, Hydracarina, Chironomids, Ostracoda, Oligochaeta, Nematoda, Plant debris, Detritus, Seeds	Detritus, Plant debris, Cladocerans	Ponds, Czech Republic (Anton–Pardo et al. 2014)
0.5–1.0 kg	Detritus, Macrophyte tissue, Crustacea, Insects, Fish, Mollusc, Oligochaetes, Protozoa, Rotifera, Algae	Detritus, Macrophyte tissue, Crustacea	Dal Lake, India (Naik et al. 2015)

SPAWNING, MATURATION AND FECUNDITY

Among a variety of factors that influence the reproductive biology of common carp, strain of fish, water temperature and habitat are the most important. Fish of the Asian strain mature faster than the European strain. In tropical and subtropical environments, this fish is able to mature around 6 months of age, whereas it takes more than 3 years age for common carp to reach maturity in temperate systems, particularly in northern Europe or North America. For example, in pond culture in India, males matured at 6 months of age and females at 8 months, whereas in Canada, in the wild, male common carp matured at age 3–4 years (35.6 cm fork length), and females at age 4–5 (43.2 cm fork length) (Parneswaran et al. 1972; Tempero et al. 2006). The size at first sexual maturity typically is around 30 cm in natural environments but they can be mature as small as 11 cm (Table 6). Water temperature and sexual maturity are highly correlated and geographical location (latitudes) of habitats, therefore, play an important role into at age of maturity of common carp (Fernandez–Delgado 1990; Tempero et al. 2006). Males also generally mature earlier than their female counterparts.

Apart from influencing maturation, temperature also has a large influence on the carp spawning period. The spawning season of Asian common carp populations may start when the water temperature is >15°C, although they generally spawn between 18 and 26°C. The spawning activity of this fish generally decreases above 26ºC and ceases entirely above 28ºC (McCrimmon 1968; Fernandez–Delgado 1990). The European carp start their spawning when water temperatures reach 17–18°C. According to Horvath (1985), under pond conditions the optimum spawning temperature for common carp in Europe is between 18°C and 22°C, although spawning of this fish may take place as low as 14°C. In Canada, carp spawned

between 16.5°C and 28°C, with a peak of spawning activity between 17°C and 23°C (McCrimmon 1968). In USA particularly in Minnesota, common carp spawns when water temperatures exceed 16–20°C (Bajer and Sorensen 2010; Chizinski et al. 2016).

Table 6. Size at first sexual maturity of male (M) and female (F) common carp in various habitats, globally

Size at first maturity (cm)	Habitat	Reference
M: 32.2, F: 19.8	Lake Naivasha, Kenya	Aera et al. (2014)
M: 27.2*, F: 28.3*	Amerti Reservoir, Ethiopia	Hailu (2013)
F: 37.5, M: 24.5	Fincha Reservoir, Ethiopia	Degefu et al. (2012)
M: 34.0, F: 42.0	Lake Naivasha, Kenya	Oyugi et al. (2011)
M: 28.7, F: 27.3	Irrigation channels, Australia	Brown et al. (2003)
M: 30.7 (584 g, 1.1 yrs), F: 32.8 (688 g, 2.7 yrs)	Barmah Lake and Hut Lake, Australia	Brown et al. (2005)
M: 16 * (95 g), F: 17* (100 g)	Lake Ziway, Ethiopia	Abera et al. (2015)
11.0* (both sex)	River Guadalquivir, Spain	Fernandez–Delgdo (1990)

*Fork length.

Common carp are able to adapt their reproductive strategies with local environmental conditions. Therefore, spawning seasons of common carp are generally longer in tropical and sub–tropical regions compared to temperate regions. In temperate regions, they normally spawn in spring or early summer (Table 7). For example, in the UK, spawning period of carp is between May and June. In tropical and sub–tropical regions, common carp may spawn almost across the entire year if sufficient food is available under suitable water temperatures. For example, carp spawn throughout the year in south Asian countries like Bangladesh and India, with two spawning peaks in January–March and July–August. Hailu (2013) observed year round spawning of common carp in Amerti Reservoir, Ethiopia at 18.9 to 23.1°C. Common carp were observed to extend their spawning from September to April (8 months) in northern New Zealand (Tempero et al. 2006). Sometimes they exhibit more than one reproductive cycle per year in tropical and sub–tropical regions. For example, Guha and Mukherjee (1991) reported two clear reproductive cycles of common carp per year in west Bengal, India. In temperate regions, common carp generally spawn once in a year.

Table 7. Spawning season of common carp at various habitats, globally

Spawning season	Habitat	Reference
Throughout the year	Amerti Reservoir, Ethiopia	Hailu (2013)
Throughout the year	Pond, India	Singh and Singh (1980)
March-June	Fincha Reservoir, Ethiopia	Degefu et al. (2012)
April-October	Pond, Iran	Shirali et al. (2011)
May–July	Apa Dam Lake, Turkey	Mert et al. (2008)
July-August	Almus Dam lake, Turkey	Karatas and Sezer (2005)
April–June	River Cayumapu, Chile	Prochelle and Campos (1985)
April–June	Guadalquivir river, Spain	Fernández–Delgado (1990)
February– May (peak)	Lake Ziway, Ethiopia	Abera et al. (2015)
September-April	Northern New Zealand	Tempero et al. (2006)
Mid-September to Mid-May	River Murray, Australia	Smith and Walker (2004)
September–March	Victoria, Australia	Sivakumaran et al. (2003)

Carp typically spawn in weedy shallow areas (about 0.5–1.0 m deep) in natural environments such as lakes and rivers. They prefer to spawn during flooding periods when the level of water is rising. Some scientists believe that common carp, particularly Asian strains start to spawn when the ion concentration in the water decreases abruptly at the beginning of the rainy season. In lakes, common carp generally live in deep water but they move to vegetated shallow areas for breeding. Balon (1995) reported that common carp of the Volga and Danube rivers spawn when water temperature reaches 18°C and large schools of this fish enter recently flooded grass flats within the inundation areas. Chizinski et al. (2016) observed that the adult common carp migrate to Lake Susan and Lake Riley wetlands for spawning over relatively short time periods that last just a few days between April and June, and whose specific timing varies but always occurs after water temperature rose to 10ºC and usually coincided with rain. Adult fish return from wetlands to their original habitats after spawning during summer (Bajer and Sorensen 2010). The young fish stay in wetlands for up to two years (Bajer et al. 2015).

Common carp are a highly fecund fish. This fecundity varies largely on their age, size, health, habitats and the number of times a carp has produced eggs. Their fecundity may vary between 100,000 to 250,000 oocytes per kg body weight (Table 8). A typical 45 cm long female can produce from 0.3 to 1 million mature oocytes over a breeding season (Froese and Pauly 2002). Almost similar information was also provided by Sivakumaran et al. (2003), who stated that a ripe female of 5 kg weight may produce up to 1 million mature oocytes. During breeding, male and female carp spawn by swimming side by side. The male carp are more active than the female, and they generally nudge the female with their fins and head to stimulate spawning. However, finding a mate during spawning is not difficult in natural environments as the ratio of male to female for all sizes is 1:1 in natural (Table 9).

Table 8. Fecundity of common carp in various habitats, globally

Facundity	Size of fish	Habitat	Reference
15,734–65,642	18.5–31.9 cm	Pond, Sri Lanka	Nathanael et al. (1998)
49,070–240,100	30.8–46.0 cm	Victoria Reservoir, Sri Lanka	Nathanael et al. (1998)
29,760±6,540	15.0 kg	Apa Dam Lake, Turkey	Mert et al. (2008)
34,684±3,738	2.2 kg	Apa Dam Lake, Turkey	Mert et al. (2008)
9,927–104,884	1 year old	India (pond)	Dobryal et al. (1990)
36,955–318,584 (177 786/kg fish)	–	Amerti Reservoir, Ethiopia	Hailu (2013)
917,000 (120,000/kg fish)	5.4 kg	River Cayumapu, Chile	Prochelle and Campos (1985)
75,645–356,743	0.6–2.3 kg	Lake Ziway, Ethiopia	Abera et al. (2015)
29,800–771,000 (19,300–216,000/kg fish)	–	Newzeland	Tempero et al. (2006)
120,000–154,0000 (163,000/kg fish)	–	Australia	Sivakumaran et al. (2003)
0.22 million/kg fish	–	Mississippi Basin, U.S.A.	Lubinski et al. (1986)
106,765±18,209	–	Lake Naivasha, Kenya	Oyugi et al. (2011)
375,000	–	Aral Sea, Kazakhstan	Nikolsky (1963)
190,778	–	River Guadalquivir, Spain	Fernandez–Delgdo (1990)

Table 9. Reported sex ratio of common carp in various habitats, globally

Sex ratio	Habitat	Reference
1:1	Apa Dam Lake, Turkey	Mert et al. (2008)
1:1	Amerti Reservoir, Ethiopia	Hailu (2013)
1:1	Lake Naivasha, Kenya	Oyugi et al. (2011)
1.1	Barmah Lake and Hut Lake, Australia	Brown et al. (2005)
1:1	River Guadalquivir, Spain	Fernandez–Delgado (1990)

BEHAVIOR

Study on common carp behaviour in natural environments is difficult due to high turbidity and, in consequence, low visibility. Therefore, bbehavioral studies on common carp through direct observation have largely been restricted under natural conditions particularly in lakes, reservoirs and rivers. Nevertheless, direct observation can provide important information on grazing and swimming behaviour and inter and intra–specific social interactions. These can provide more insight on feeding niches occupied by various species in presence of common carp in natural ecosystems. A very limited number of studies on common carp behavior (e.g., Rahman et al. 2008b; Rahman et al. 2008c; Rahman and Meyer 2009; Rahman et al. 2010) were undertaken under semi–natural conditions and therefore, knowledge regarding their behaviors under semi-intensive pond aquaculture is extremely scarce.

Rahman et al. (2008a) observed the behavior of common carp in tanks in which semi-natural conditions were simulated and which had a large glass wall allowing to observe various behaviors including inter and intra–specific social interactions. They stocked common carp with various combinations of water column dweller fish rohu (*Labeo rohita*) and studied the behaviors of both fishes under different species combination and natural/supplemental food situations. They concluded that the common carp is a benthic feeder as it grazed 63–68% of its total grazing time on the bottom. Common carp did not show any intra- or inter–specific aggressive behavior but the behavior of other species (rohu) was significantly influenced by the presence of common carp. However, both common carp density and supplemental feeding significantly influenced inter and intra-specific social interactions, and the time spent for grazing natural food by common carp. Common carp grazed during both day and night but primarily by day (Rahman et al. 2008c; Rahman and Meyer 2009).

As mentioned earlier, common carp is an omnivorous fish that primarily feeds on benthic macroinvertebrates (chironomids, tubificids) and zooplankton, but the bulk of its diet consists of detritus (Rahman et al. 2009). When common carp had access to both their preferred prey of benthic macroinvertebrates and zooplankton, they grazed on the bottom, whereas fish with access to only zooplankton grazed in the water column. The dependency on zooplankton shifts the common carp's feeding niche from the benthic zone to the water column. In this case, common carp spends the majority of its grazing and swimming time in the water column (Rahman and Meyer 2009; Rahman et al. 2010). Common carp increased their grazing time when food availability was very low in the environment (Rahman and Meyer 2009). However, the behavior of common carp is consistent with classical optimal foraging theory, whereby they selected their preferred prey (benthic macroinvertebrates) when available but

switched to less preferred prey (zooplankton) when the preferred prey were not avail able (Rahman and Meyer 2009; Rahman et al. 2010). Behavioral observations are particularly very useful to clarify species interactions and feeding ecology in polyculture ponds and can also provide some insight into such interactions in their natural environments.

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Chapter 94

ASSESSING THE EFFECTIVENESS OF CONTROLLED REPRODUCTION OF THE COMMON CARP FROM 16 BREEDING LINES

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ABSTRACT

The effectiveness of reproduction of the common carp (*Cyprinus carpio* L.) from sixteen breeding lines (four Polish lines: 2,3,6, K; five Hungarian lines: 7,8,W,0,T; two Lithuanian lines: B and BVP; Israeli line D; French line F; Yugoslavian line J; German line N; Ukrainian line UR) after ovulation stimulated with Ovopel [(D-Ala^{6,} Pro^{9} NEt-mGnRH-a) + metoclopramide] was examined. The following parameters of reproduction effectiveness were investigated: the weight of eggs in grams and expressed as a percentage of the female's body weight, the percentage of fertilized eggs after 12-h incubation, the percentage of living embryos (24 and 36 h), the number of eggs, the number of living embryos 36h and the percentage of spawning females after hormonal stimulation. Considering the traits investigated, it can be concluded that the best reproduction effect was obtained in lines B, BVP and 8, while the least successful breeding was noted in lines N, J and 3. The highest number of living embryos after 36-h incubation was obtained in fish from lines B, T, BVP and UR (757.9; 692.9; 685.5; 656.1 and 655.6 thousand, respectively), and the lowest in lines N, 3, 2 and J (343.7; 404.5; 446.1; 459.3 thousand, respectively). The highest number of living embryos (36h) is to be expected from fish aged 14, 13 and 9 years (976.1; 879.5 and 826.6 thousand, respectively), whereas the lowest was from fish aged 15 and 11 years (259.5 and 279.9 6 thousand).

Keywords: reproduction, controlled condition, ovopel, common carp, reproduction effectiveness, breeding line, ovulation

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INTRODUCTION

Research on the effectiveness, of controlled reproduction of various breeding lines of the common carp (*Cyprinus carpio* L.) after stimulation with natural and synthetic preparations has been conducted at the Institute of Ichthyobiology and Aquaculture in Gołysz (Polish Academy of Sciences) (PAN ZIGR Gołysz) since the 1970s. The scope of the studies conducted in the years 1978-2014 has been described by Brzuska (2006a, 2012a, 2016). This type of research is possible thanks to a genetically varied herd of the common carp developed after decades of genetic studies and husbandry work at the Institute. The herd is comprised of 17 breeding lines, constituting a "living gene bank", unique on the European scale. The breeding lines of carp in Gołysz are of different origins: Hungarian (line 7, line 8, line W, line 0, line T), Polish (line 2, line 3, line 6, line K), Lithuanian (line B, line BVP), Ukrainian (line U_R, fully scaled line Up), French (line F), German (line N), Yugoslavian (line J), and Israeli (line D) (Białowąs et al. 2008).

Being so genetically valuable, this material is the basis for not only genetic, immunological (e.g., Rakus et al. 2003; Jurecka et al. 2009; Napora-Rutkowski et al. 2017) and breeding studies, but also for genetic improvements to the common carp population in Poland and national programs of preserving genetic reserves of the species, which is extremely important for the economy. It needs to be emphasized that several of these lines (line 3, line 6, line W, line 7, line 8, line 0, line F) comprise material for important cross-breeds (Guziur et al. 2003).

Research into the effectiveness of controlled reproduction of different breeding lines of the common carp conducted in Gołysz has made use of various natural preparations to induce ovulation, such as carp pituitary homogenate CPH (e.g., Brzuska 1991, 1997, 2000, 2003, 2006b; Cejko and Brzuska 2015, 2017), bream pituitary homogenate (Brzuska 2017), human chorionic gonadotropin hCG (Brzuska 2017) and pregnant mare serum gonadotropin PMSG (Brzuska and Ryszka 1990). Synthetic preparations, that is GnRH analogues, such as: [D-Tle^6, Pro^{9}NHEt^9] GnRH-a *Lecirelin* (Brzuska 2004, 2006b) and DesGly^{10}[D-Ala^6] GnRH-ethylamide *Kobarelin* (Brzuska 2000) and complex preparations, containing both GnRH analogue and a dopamine receptor antagonist, have been used as well. Complex preparations applied to this species in Gołysz include Ovaprim (Syndel, Canada), containing salmon analogue D-Arg^6,Pro^{9}Net-sGnRH and domperidone (Brzuska and Adamek 1997), and Israeli agent Dagin, which contains a super active GnRH salmon analogue D-Arg^6,Pro^{9}Net-sGnRH and a dopamine receptor antagonist, metoclopramide (Brzuska 2005, 2006c). In other experiments, common carp were also stimulated with Aquaspawn (Spawnrite, Republic of South Africa), which consists of D-Lys^{6}Trp^{7}Tys^8-mGnRH and domperidone (Brzuska 2001a).

The largest number of experiments on common carp of different origin have been conducted at PAN ZIGR Gołysz with the use of spawning agent Ovopel (e.g., Brzuska 2003, 2005, 2006b,c, 2011, 2012b, 2014; Brzuska and Grzywaczewski, 1999; Brzuska and Białowąs 2002; Cejko and Brzuska 2015, 2017). This complex preparation produced in Hungary contains a mammalian analogue GnRH (D-Ala^6, Pro^{9}NEt) mGnRH and metoclopramide (Horváth et al. 1997). It has been used in Gołysz since 1998 to induce ovulation and spermation in the common carp, not only in experimental spawning, but also in commercial reproduction. It should be emphasized that Ovopel has also been used with other

species of fish, not exclusively from the *Cyprinidae* family, yielding satisfactory results in propagation (Kłodzińska and Okoniewski 1998; Brzuska 2001b, 2012c; Kucharczyk et al. 2001; Szmyt et al. 2012).

Most studies conducted at the Gołysz Institute on common carp of different origins aimed to establish whether reproduction effectiveness after ovulation induced with Ovopel differs from the results of treatment with carp pituitary homogenate (CPH) (e.g., Brzuska 2003, 2005, 2010a; Brzuska and Grzywaczewski, 1999; Brzuska and Białowąs 2002; Cejko and Brzuska 2015, 2017). Three studies investigated the effectiveness of reproduction in several breeding lines of *Cyprinus carpio* after ovulation stimulated with Ovopel (Brzuska 2011, 2012b, 2014). However, there has been no comprehensive study that would report breeding results of more than ten lines of common carp available at the Institute after Ovopel-induced stimulation. This study presents the effects of propagation in 16 lines. The full-scaled Ukrainian line (Up) and 5-year-old females could not be included in the analysis due to incomplete data on its controlled reproduction.

Both the current and previous (e.g., Brzuska 1991, 2010b; Cejko 2007) research addresses the key issue of a potential association between the effectiveness of reproduction and the origin and age of females, as well as the spawning agent used. This is of particular importance in view of the fact that the results could be used to decide whether it is profitable to keep old females with a high body mass and use them in spawning. For example, maintaining and breeding such females is labor-intensive and is very costly, because of expensive agents inducing ovulation and spermation.

A crucial practical question that needs to be addressed in long-term studies on the effectiveness of the controlled reproduction of common carp originating from different breeding lines is whether it is possible to accurately predict the effectiveness of reproduction (expressed in the weight of eggs obtained, the percentage of living embryos, the total number of eggs and the number of living embryos) on the basis of easily measurable parameters (age and body weight of females, the weight of eggs obtained). Research questions concerning regression predictions of traits affecting the effectiveness of common carp reproduction have been the subject of previous studies (e.g., Brzuska 1997, 2011, 2012b, 2014; Brzuska and Grzywaczewski 1999).

The aim of the present study was to assess the effectiveness of controlled reproduction in 16 breeding lines of the common carp of different origin, to establish whether the age of females significantly determines the investigated effectiveness parameters and to make regression predictions of the weight of eggs (g), the percentage of living embryos after 36h incubation, the total number of eggs and the number of living embryos (36h).

MATERIAL AND METHODS

The data for statistical analysis was obtained from artificial spawning of the common carp conducted at PAN ZIGR Gołysz, in 12 breeding seasons (1998, 1999, 2001, 2002, 2004, 2005, 2008, 2009, 2010, 2011, 2012, 2013). The 323 females used for reproduction were 4 and 6-16 years old and weighed 3.40-15.30 kg. The females came from 16 breeding lines (2, 3, 6, K, 7, 8, W, 0, T, B, BVP, D, F, J, N, UR). The agent used for stimulating ovulation was the Hungarian preparation Ovopel [(D-Ala^6, Pro^{9}NEt-mGnRH-a) + metoclopramide] (Unic-

trade, Hungary), applied in two doses (1/5 pelletkg^{-1}, and after 12 h 1 pelletkg^{-1}; Horváth et al. 1997). The eggs were obtained from each female by stripping, and were fertilized separately with pooled milt of 3-4 males from the same line as the female. Hormonal stimulation was also conducted on males, which were treated with Ovopel applied in a single dose of 1 pellet kg^{-1}. The fertilized eggs were treated in accordance with the Institute's procedures (Cejko 2007, Cejko and Brzuska 2017). Eggs from every female were incubated in a separate 7-L Weiss glass in water whose temperature was 21 ± 1°C. The percentage of fertilized eggs was calculated after 12 h incubation, and the percentage of living embryos after 24- and 36-h incubation.

The parameters of reproduction effectiveness investigated for each of the breeding lines were: the weight of eggs (in grams and as % of female BW), the percentage of fertilized eggs (after 12-h incubation), the percentage of living embryos (after 24 and 36 h), the total number of eggs and the number of living embryos after 36 h incubation. The number of eggs and the number of living embryos (36 h) were calculated for the mean egg weight attributed to each female body weight class following a five-class categorization (Cejko and Brzuska 2015). The mean egg weight for class I (> 3 ≤ 5 kg) was 1.27 mg; for class II (> 5 ≤ 7 kg) 1.29 mg; for class III (> 7 ≤ 9 kg) 1.38 mg; for class IV (> 9 ≤ 11 kg) 1.48 mg and for class V (> 11 kg) 1.56 mg.

The data was verified using the least squares analysis of variance (Harvey 1987), with the breeding line as the main classification factor. The linear model (1) used in the analysis included regression on female body weight and on age of females. The analysis determined the least square constants (LSC) and the least square means (LSM) for the parameters investigated in each breeding line (Tables 1, 2, 3).

$$Y_{ij} = \alpha + a_i + bW_{ij} + dG_{ij} + e_{ij} \quad (1)$$

where:

Y_{ij} – observation j within line i
α – the theoretical general mean with the assumption that $W_{ij} = 0$ and $G_{ij} = 0$
a_i – the effect of the breeding line i i = 1…….16
b – regression on the age of the female
W_{ij} – the age of the female
d – regression on the female body weight
G_{ij} – female body weight
e_{ij} – random error associated with observation j

In order to establish whether the age of females significantly determined the effectiveness of reproduction in the breeding lines of different origin, least squares analysis of variance was performed according to the following linear model (2).

$$Y_{ijk} = \alpha + a_i + w_j + dG_{ijk} + e_{ijk} \quad (2)$$

where:

Y_{ijk} – observation k within line i and female BW class j
α – the theoretical general mean with the assumption that $G_{ijk} = 0$
a_i – the effect of the breeding line i i = 1…….16
w_j – the effect of female age group j j = 1…….12
d – regression on the female body weight
G_{ijk} female body weight
e_{ijk} – random error associated with observation k

The breeding line was introduced into model (2) in order to bind the variability regarding this effect, reduce the error and increase the precision of estimating the effect of the age of females on the traits studied. The least square constants (LSC) and the least square means (LSM) estimated in the analysis of variance conducted according to model (2) are presented in Tables 8, 9, 10. The F-test was used to check the significance of the main classification factors (line and age of females) on the reproduction effectiveness parameters (Tables 1, 2, 3, 8, 9, 10). The significance of differences between mean values calculated for each breeding line and age group were verified for these parameters using the Duncan test with Kramer's modification (Harvey 1987) (Tables 4, 5, 6, 7, 11, 12, 13).

The percentage of ovulating females after hormonal stimulation was also calculated for each breeding line and each female age group (Figures 1, 2). Multiple regression analysis was used in order to predict the weight of eggs (g), the percentage of living embryos after 36h incubation, the total number of eggs and the number of living embryos after 36-h incubation for each breeding line. In the equations where the dependent variable was the weight of eggs in grams, the independent variables were age and body weight of the females. In the equations where the dependent variable was the percentage of living embryos (36 h), the number of eggs or the number of living embryos (36 h), the independent variables were age, body weight of the females and also the weight of eggs (g). For predicting the number of living embryos after 36-h incubation for each female age group regression equations were derived with the independent variable being the body weight of females and weight of eggs in grams. The precision of estimation and adequacy of the constructed equations for a given set of data was characterized with the coefficient of determination (R^2), describing the degree of variance explained by the model. The degree of correlation between the dependent variable and independent variables was estimated with multiple correlation R, describing the proportion between the dependent variable and the combined effect of the other variables. The results of regression predictions are presented in Tables 14, 15, 16.

RESULTS

Female Ovulation after Ovopel Treatment

Eggs were obtained from all females in line 3, K, 0 and BVP, from 94.44% of females in line B and from 90% of females in line T. The lowest percentage of ovulating females was for line J (46.66%) and line 2 (66.67%). In three lines (7, W and N) the percentage of ovulating

females fell within the range of 76.47-79.48%, while for five lines (6, 8, D, F. U_R), the range was 83.87 - 89.47% (Figure 1). Eggs were produced by all females aged 11, 12 and 16 and from 90.78% of the 8-year-old fish. The lowest percentage of ovulating females was observed for fish aged 4, 6 and 15 (68.75%, 75.75% and 77.17%, respectively). For fish aged 10, 13, 7, 14 and 9 the percentage of ovulating females fell within the range of 79.31-84.09% (Figure 2).

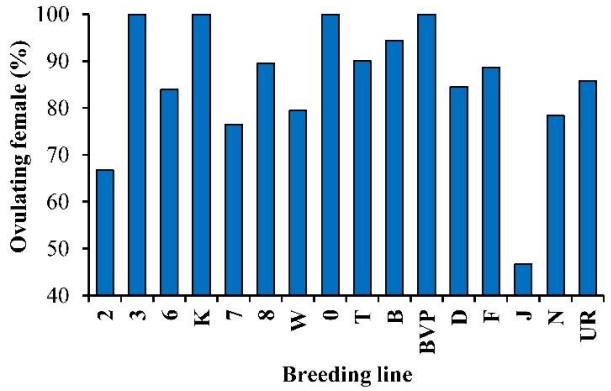

Figure 1. The percentage of ovulating female common carp from 16 breeding lines after Ovopel treatment.

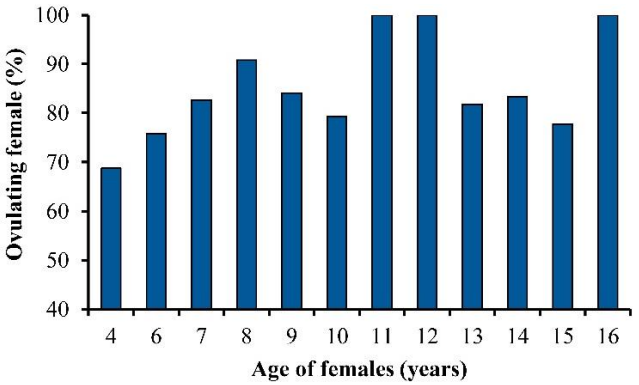

Figure 2. The percentage of ovulating female common carp aged 4 and 6-16 years after Ovopel treatment.

The Effect of the Origin of Females on the Weight, Quality and Total Number of Eggs and the Number of Living Embryos after 36 h Incubation

The results of the analysis of variance and the F test show that the origin of females significantly determined all the traits investigated pertaining to the effectiveness of reproduction (Tables 1, 2, 3). Based on the least squares means that were calculated, it can be concluded that the highest weight of eggs (g) was obtained from females in lines T and B (1363.83 and 1248.73g, respectively). The mean scores showed positive deviation from the overall mean by as much as 347.43 and 232.34g (Table 1). High means (over 1100g) were

also noted for this trait in lines 6, F, BVP and U_R (Table 1). The lowest LSM scores for the weight of eggs (g) were obtained for lines N, 3 and 2 (664.48, 765.24 and 773.25g, respectively), with deviations from the overall mean of -351.91, -251.15 and – 243.13g, respectively (Table 1).

The mean scores calculated for the weight of eggs expressed as the percentage of the body weight of females in the lines studied showed the lowest weight of eggs for females in lines N, 2 and 3 (8.77%, 9.70% and 10.45%; Table 1), and the highest weight for females in lines T, B, 7 and F (18.29%, 15.26%, 14.48% and 14.37%; Table 1). The least squares means calculated for lines 6, F, D and U_R also showed positive deviation from the overall mean. However, the scores were lower than those for lines T, B, 7 and F (Table 1).

The percentage of fertilized eggs was the highest for eggs obtained from females from lines B and BVP (97.03 and 96.17%). The means calculated for this trait showed positive deviation from the overall mean by as much as ~5% (Table 1). An exceptionally high percentage, over 90%, was also noted for lines 2, 3, K, 8, 0 and J. The lowest mean scores for the percentage of fertilized eggs were observed in lines T, D and N (81.87, 85.06 and 86.11%), while the means for lines 6, 7, U_R, F and W also showed a negative deviation from the overall mean (Table 1).

Table 1. Constants (LSC) and least-squares means (LSM) estimated for the weight of eggs (in grams and as % of female BW) and fertilized eggs (12 h) in 16 breeding lines of common carp and results of F-test

Classification factor	Investigated traits								
	Weight of eggs (g)			Weight of eggs % of female body weight			Fertilized eggs after 12 h incubation (%)		
	α = 1016.39			α = 12.97			α = 91.20		
	LSC	LSM	F	LSC	LSM	F	LSC	LSM	F
Breeding line			b			b			b
2	- 243.13	773.2		- 3.27	9.70		1.83	93.03	
3	- 251.15	765.2		- 2.52	10.45		2.42	93.62	
6	122.20	1138.6		1.28	14.26		- 1.36	89.83	
K	0.50	1016.9		- 0.54	12.43		0.50	91.70	
7	64.07	1080.4		1.50	14.48		- 2.01	89.18	
8	57.83	1074.2		0.72	13.70		2.99	94,19	
W	- 16.44	999.9		0.97	12.00		- 0.37	90.82	
0	- 140.66	875.7		- 1.77	11.20		3.45	94.65	
T	347.43	1363.8		5.31	18.29		- 9.32	81.87	
B	232.34	1248.7		2.28	15.26		5.83	97.03	
BVP	110.29	1126.6		1.12	14.10		4.97	96.17	
D	44.89	1061.3		0.30	13.28		- 6.13	85.06	
F	114.69	1131.1		1.39	14.37		- 1.10	90.09	
J	- 196.13	820.2		- 1.64	11.33		3.81	95.01	
N	- 351.91	664.5		- 4.20	8.77		- 4.78	86.41	
UR	105.12	1121.5		0.98	13.96		- 0.74	90.45	
Regression on female BW on female age									
		25.07	b		- 0.30	-		0.31	-
		45.71	a		0.38	-		-0.38	-

b, $P \leq 0.01$; a, $P \leq 0.05$; α, theoretical general mean

The highest least squares means for the percentage of living embryos after 24-h incubation were noted for lines B and BVP and lines 3 and 8 (93.79 and 92.54%; 91.47 and 90.93%; Table 2). During the next 12 h of incubation, the high percentage of living embryos continued in three lines: B, BVP and 8 (Table 2). Importantly, there was a significant decrease in the quality of eggs obtained from females from line 3 between the 24 h and 36 h hour of incubation (the percentage of living embryos after 24 h was 91.47, and after 36-h only 82.32). The lowest percentage of living embryos after 24- and 36-h incubation was noted for lines T, D and N (73.33 and 72.79%; 82.37 and 78.02%; 82.73 and 80.83%; Table 2).

Table 2. Constants (LSC) and least-squares means (LSM) estimated for percentage of viable embryos after 24 h and 36 h of incubation in 16 breeding lines of common carp and results of F-test

Classification factor	Investigated traits					
	Percentage of living embryos					
	after 24 h incubation			after 36 h incubation		
	α = 87.02			α = 84.11		
	LSC	LSM	F	LSC	LSM	F
Line			b			a
2	0.30	87.32		1.64	85.76	
3	4.45	91.47		- 1,79	82.32	
6	- 2.13	84.88		- 1.89	82.22	
K	0.88	87.90		0.89	85.01	
7	- 2.83	84.19		- 2.24	81.87	
8	3.90	90.93		3.92	88.04	
W	1.55	88.57		0.69	84.81	
0	2.71	89.74		1.67	85.79	
T	- 13.69	73.33		- 11.32	72.79	
B	6.76	93.79		6.14	90.26	
BVP	5.52	92.54		7.89	92.01	
D	- 4.65	82.37		- 6.09	78.02	
F	- 2.02	86.99		1.31	85.43	
J	0.01	87.03		- 0.70	83.41	
N	- 4.29	82.73		- 3.18	80.93	
UR	1.52	88.54		3.06	87.18	
Regression on female BW on female age						
		0.46	-		0.46	-
		0.53	-		- 0.19	-

b, $P \leq 0.01$; a, $P \leq 0.05$; α, theoretical general mean

The analysis of the least squares means for the total number of eggs obtained from females in the lines studied has demonstrated that the highest number of eggs was produced by fish in lines T (909,200) and B (832,400). The means for these lines deviated from the overall mean by +231,600 and +154,800 (Table 3). Mean scores higher than 700,000 were calculated for seven lines (6, 7, 8, BVP, D, F and UR; Table 3). The lowest mean number of eggs was noted in lines N, 3, 2, J and 0 (442,900; 510,200; 515,500; 546,800 and 583,800), and the mean calculated for line N deviated from the overall mean by more than 200,000 (Table 3).

The highest number of living embryos after 36-h incubation was produced by fish from lines B, T and BVP (757,900; 692,900 and 685,500, respectively). The means for this trait deviated from the overall mean by + 182,000, + 117,000 and + 109,600 in the respective lines. A high number of living embryos (36 h) was also observed in lines 6, 8, F and UR (638,000; 630,600; 656,100 and 655,600 (Table 3). The lowest number of living embryos (36 h) (343,700), was noted in line N, and the mean deviated from the overall mean by as much as - 232,100. A relatively low mean score for the trait was also observed in lines 3, 2 and J (404,500; 446,100 and 459,300; Table 3).

Table 3. Constants (LSC) and least-squares means (LSM) estimated for total number of eggs and number of living embryos (36 h) in 16 breeding lines of common carp and results of F-test

Classification factor	Investigated traits					
	Number of eggs (in thousands)			Number of living embryos (in thousands) after 36 h		
	α = 87.02			α = 84.11		
	LSC	LSM	F	LSC	LSM	F
Line			b			b
2	- 162.10	515.50		- 129.60	446.10	
3	- 167.40	510.20		- 171.30	404.5	
6	81.40	759.00		62.20	638.00	
K	0.33	670.90		11.60	587.40	
7	42.70	720.30		23.20	599.1	
8	38.50	716.10		54.80	630.60	
W	- 10.90	666.60		62.40	582.0	
0	- 93.70	583.80		- 74.50	501.2	
T	231.60	909.20		117.00	692.90	
B	154.80	832.40		182.00	757.9	
BVP	73.50	751.10		109.60	685.50	
D	29.90	707.50		- 2.9	572.80	
F	76.40	754.00		80.20	656.10	
J	- 130.70	546.80		- 116.4	459.30	
N	- 234.60	442.9		- 232.10	343.70	
UR	70.00	747.60		79.80	655.60	
Regression on female BW		61.60	b		55.80	b
on female age		30.40	-		23.20	-

b, $P \leq 0.01$; a, $P \leq 0.05$; α, theoretical general mean

Regression on the age of females included in the model was significant only for the weight of eggs (g), while regression on the body weight of females was significant for the weight of eggs (g), for the number of eggs and the number of living embryos after 36 h incubation (Tables 1, 2, 3). The result of the Duncan test, showing the significance of differences between the mean values calculated for the traits studied within the breeding lines, are presented in Tables 4, 5, 6, 7.

Table 4. The results of the Duncan test with Kramer's modification showing the significance of differences between mean values calculated for weight of spawn in grams (above diagonal) and for weight of spawn as % female's body weight (under diagonal) within 16 breeding lines of common carp

lines	N	2	3	6	7	8	B	D	F	J	0	W	K	UR	T	BVP
N	x	-	-	b	b	b	b	b	b	-	-	a	a	a	b	a
2	-	x	a	a	a	a	b	a	b	-	-	-	a	a	b	a
3	-	-	x	b	a	a	b	a	b	-	-	-	-	a	b	a
6	b	b	a	x	-	-	-	-	-	a	a	-	-	-	-	-
7	b	b	b	-	x	-	-	-	-	a	a	-	-	-	a	-
8	b	a	a	-	-	x	a	-	-	a	a	-	-	-	a	-
B	b	b	b	-	-	-	x	-	-	b	b	a	-	-	-	-
D	a	a	-	-	-	-	-	x	-	a	a	-	-	-	a	-
F	b	b	b	-	-	-	-	-	x	a	a	-	-	-	-	-
J	-	-	-	a	a	-	a	-	a	x	-	-	-	a	b	a
0	-	-	-	a	a	-	b	-	a	-	x	-	-	a	b	a
W	a	-	-	a	a	-	a	-	a	-	-	x	-	-	a	-
K	a	-	-	-	-	-	a	-	-	-	-	-	x	-	a	-
UR	b	a	a	-	-	-	-	-	-	-	-	-	-	x	-	-
T	b	b	b	a	a	a	a	a	a	b	b	-	b	a	x	-
BVP	b	a	a	-	-	-	-	-	-	-	-	-	-	-	a	x

b, $P \leq 0.01$; a, $P \leq 0.05$

Table 5. The results of the Duncan test with Kramer's modification showing the significance of differences between mean values calculated for percentage of fertilized eggs after 12 h of incubation (above diagonal) and for percentage of living embryos after 24 h of incubation (under diagonal) within 16 breeding lines of common carp

lines	N	2	3	6	7	8	B	D	F	J	0	W	K	UR	T	BVP
N	x	a	a	-	-	a	b	-	-	a	a	-	-	-	-	a
2	-	x	-	-	-	-	-	a	-	-	-	-	-	-	b	-
3	a	-	x	-	-	-	-	b	-	-	-	-	-	-	b	-
6	-	-	a	x	-	a	a	-	-	a	a	-	-	-	a	a
7	-	-	a	-	x	a	b	a	-	a	a	-	-	-	a	a
8	a	-	-	a	a	x	-	b	a	-	-	-	-	-	b	-
B	a	-	-	a	a	-	x	b	b	-	-	a	a	a	b	-
D	-	-	a	-	-	a	b	x	a	b	b	a	a	a	-	b
F	-	-	-	-	-	-	a	-	x	a	a	-	-	-	a	b
J	-	-	-	-	-	-	a	-	-	x	-	-	-	-	b	-
0	a	-	-	a	a	-	-	a	-	-	x	-	-	-	b	-
W	-	-	-	-	-	-	a	a	-	-	-	x	-	-	a	-
K	-	-	-	-	-	-	-	-	-	-	-	-	x	-	a	-
UR	-	-	-	-	-	-	-	-	-	-	-	-	-	x	a	b
T	a	b	b	a	a	b	b	a	b	b	b	b	b	-	x	b
BVP	b	-	-	a	a	-	-	a	-	-	-	-	-	-	b	x

b, $P \leq 0.01$; a, $P \leq 0.05$

Table 6. The results of the Duncan test with Kramer's modification showing the significance of differences between mean values calculated for percentage of living embryos after 36 h of incubation (above diagonal) within 16 breeding lines of common carp

lines	N	2	3	6	7	8	B	D	F	J	0	W	K	UR	T	BVP
N	x	-	-	-	-	a	a	-	-	-	-	-	-	-	a	b
2		x	-	-	-	-	-	a	-	-	-	-	-	-	a	-
3			x	-	-	-	a	-	-	-	-	-	-	-	a	a
6				x	-	a	a	-	-	-	-	-	-	-	a	a
7					x	a	a	-	-	-	-	-	-	-	a	a
8						x	-	a	-	-	-	-	-	-	a	-
B							x	b	-	a	-	a	-	-	b	-
D								x	a	-	a	a	a	a	-	b
F									x	-	-	-	-	-	a	-
J										x	-	-	-	-	a	a
0											x	-	-	-	a	-
W												x	-	-	a	-
K													x	-	a	-
UR														x	a	-
T															x	b
BVP																x

b, $P \leq 0.01$; a, $P \leq 0.05$

Table 7. The results of the Duncan test with Kramer's modification showing the significance of differences between mean values calculated for total number of eggs (above diagonal) and for number of living embryos after 36 h incubation (under diagonal) within 16 breeding lines of common carp

lines	N	2	3	6	7	8	B	D	F	J	0	W	K	UR	T	BVP
N	x	a	-	b	b	b	b	b	b	a	b	b	b	b	b	b
2	a	x	-	b	b	b	b	b	b	a	a	a	a	b	b	b
3	a	-	x	b	b	b	b	b	b	-	-	b	a	b	b	b
6	b	b	b	x	-	-	a	a	-	b	b	b	a	-	b	-
7	b	b	b	-	x	-	b	-	-	b	b	a	a	-	b	-
8	b	b	b	-	-	x	b	a	a	b	b	a	-	-	b	-
B	b	b	b	b	b	b	x	b	a	b	b	b	b	b	-	a
D	b	b	b	b	-	a	b	x	-	b	b	-	a	-	b	-
F	b	b	b	-	a	-	b	b	x	b	b	b	b	-	b	-
J	b	-	a	b	b	b	b	b	b	x	-	b	b	b	b	b
0	b	a	b	b	b	b	b	a	b	a	x	a	b	b	b	b
W	b	a	b	a	-	a	b	-	b	b	a	x	-	b	b	a
K	b	b	b	-	-	-	b	-	b	b	b	-	x	b	b	b
UR	b	b	b	-	a	b	b	b	-	b	b	b	a	x	b	-
T	b	b	b	-	b	a	a	b	-	b	b	b	b	a	x	b
BVP	b	b	b	a	b	a	b	b	-	b	b	b	b	-	-	x

b, $P \leq 0.01$; a, $P \leq 0.05$

The Effect of Female Age on the Weight, Quality and Total Number of Eggs and the Number of Living Embryos after 36 h Incubation

The age of females significantly determined all the parameters investigated pertaining to the effectiveness of reproduction, except for the percentage of living embryos after 36-h incubation (Tables 8, 9, 10). The least squares means calculated for the weight of eggs expressed both in grams and the % of female BW showed the highest weight of eggs for females aged 14, 13, 9 and 10 years (1563.28g and 18.95%; 1412.35g and 16.53%; 1302.46g and 16.56%; 1078.94g and 13.79%) and the lowest weight of eggs for fish aged 15, 11 and 16 years (502.29g and 6.99%; 708.80g and 9.03%; 763.85g and 10.74% (Table 8).

Table 8. Constants (LSC) and least-squares means (LSM) estimated for the weight of eggs (in grams and as % of female BW) and fertilized eggs (12 h) for 12 female age classes of common carp and results of F-test

Classification factor	Investigated traits								
	Weight of eggs (g)			Weight of eggs % of female body weight			Fertilized eggs (%) after 12 h incubation		
	α = 975.40			α = 12.26			α = 92.01		
	LSC	LSM	F	LSC	LSM	F	LSC	LSM	F
age of females (years)			b			b			a
4	- 122.80	852.6		- 2.31	9.95		3.71	95.72	
6	- 151.96	823.4		- 1.64	10.62		4.68	96.69	
7	- 8.24	967.1		0.11	12.37		- 0.42	91.58	
8	- 25.80	949.6		- 0.09	12.17		0.40	92.41	
9	327.06	1302.4		4.30	16.56		1.87	93.88	
10	103.53	1078.9		1.53	13.79		- 3.53	88.47	
11	- 266.61	708.8		- 3.23	9.03		- 10.17	81.83	
12	- 195.37	780.0		- 2.85	9.40		6.03	98.05	
13	436.95	1412.3		4.27	16.53		4.08	96.09	
14	587.88	1563.3		6.69	18.95		1.73	93.74	
15	- 473.11	502.3		- 5.27	6.99		- 3.37	88.63	
16	- 211.55	763.8		- 1.51	10.74		- 5.03	86.97	
Regression on female BW		79.2	b		- 0.45	b		0.50	-

b, $P \leq 0.01$; a, $P \leq 0.05$; α, theoretical general mean

The lowest means for the percentage of fertilized eggs and the percentage of living embryos after 24-h incubation were noted in the 11 years of age group (81.83% and 71.16%). A relatively low percentage of living embryos (24h) was observed for females aged 15 and 16 years (78.86% and 79.16%, respectively) and the highest for those aged 12, 6, 4, and 13 years (95.91%, 94.79%, 94.57% and 94.46%) (Table 9). As shown by the least squares means estimated for the age groups, the quality of eggs expressed in the percentage of living embryos after 36-h incubation was the highest for females aged 12 and 13 (93.29% and 92.27%, respectively), and the lowest for those aged 11 and 15 years (71.00% and 78.86%). (Table 9).

The highest number of eggs and the highest number of living embryos (36 h) was noted for females aged 14, 13 and 9 years (1092,200 and 976,100; 975,000 and 879,500; 946,300 and 826,600; Table 10). Within the twelve age groups, the least squares mean scores for the number of living embryos (36 h) fell within the range of 505,100-664,300 in as many as six

groups (4, 6, 7, 8, 10 and 12 years; Table 10). The lowest mean scores calculated for the total weight of eggs and the number of living embryos (36h) were noted for 15 and 11-year-old females (355,200 and 259,500; 495,900 and 297,900; Table 10).

Table 9. Constants (LSC) and least-squares means (LSM) estimated for percentage of viable embryos after 24 h and 36 h of incubation for 12 female age classes of common carp and results of F-test

Classification factor	Investigated traits					
	Percentage of living embryos					
	after 24 h incubation			after 36 h incubation		
	α = 87.55			α = 85.64		
	LSC	LSM	F	LSC	LSM	F
Age of females			a			-
4	7.01	94.57		5.41	91.06	
6	7.23	94.79		5.41	91.06	
7	0.87	88.43		0.72	86.37	
8	1.10	88.66		- 0.87	84.77	
9	1.56	89.11		0.42	86.06	
10	- 2.55	84.99		- 3.84	81.80	
11	- 16.38	71.16		- 13.64	71.00	
12	8.35	95.91		7.64	93.29	
13	6.90	94.46		6.62	92.27	
14	2.96	90.52		4.63	90.28	
15	- 8.69	78.86		- 6.75	78.86	
16	- 8.38	79.16		- 5.18	80.46	
Regression on female BW		0.83	-		0.75	-

b, $P \leq 0.01$; a, $P \leq 0.05$; α, theoretical general mean

Table 10. Constants (LSC) and least-squares means (LSM) estimated for total numbers of living embryos (36 h) for 12 female age classes of common carp and results of F-test

Classification factor	Investigated traits					
	Number of eggs (in thousands)			Number of living embryos (in thousands)		
	α = 695.80			α = 592.40		
	LSC	LSM	F	LSC	LSM	F
age of females			b			b
4	- 89.40	606.30		- 61.60	530.80	
6	- 85.10	610.60		- 32.50	559.90	
7	4.80	700.60		17.90	610.40	
8	- 19.40	676.30		- 19.10	573.30	
9	250.50	946.30		234.10	826.60	
10	98.40	794.20		71.80	664.30	
11	- 19.90	495.90		- 294.40	297.90	
12	- 148.30	547.40		- 87.30	505.10	
13	279.20	975.00		287.00	879.50	
14	396.40	1092.20		383.60	976.10	
15	- 340.50	355.20		- 332.90	259.50	
16	- 146.50	549.20		- 166.60	425.80	
Regression on female BW		36.10	a		36.9	a

b, $P \leq 0.01$; a, $P \leq 0.05$; α, theoretical general mean

Regression on the body weight of females included in model 2 was statistically significant for the weight of eggs expressed in grams and as % of females BW, and for the number of eggs and the number of living embryos after 36-h incubation (Tables 8, 9, 10). The results of the Duncan test, showing the significance of differences between mean values calculated for the investigated traits within female age groups, are presented in Tables 11, 12, 13.

Table 11. The results of the Duncan test with Kramer's modification showing the significance of differences between mean values calculated for weight of spawn in grams (under diagonal) and for weight of spawn as % female's body weight (above diagonal) within 12 age groups of common carp

Age of female (years)	4	6	7	8	9	10	11	12	13	14	15	16
4	x	-	-	-	a	a	-	-	b	b	-	-
6	-	x	-	-	b	b	-	-	b	b	-	-
7	-	-	x	-	b	-	-	-	b	b	b	-
8	-	-	-	x	b	a	-	-	b	b	b	a
9	b	b	a	b	x	b	b	b	-	-	b	b
10	a	b	-	a	b	x	a	a	a	a	b	-
11	-	-	-	-	b	a	x	-	b	b	-	-
12	-	-	-	-	a	-	-	x	b	b	-	-
13	b	b	b	b	-	b	b	b	x	-	b	b
14	b	b	b	b	-	b	b	b	-	x	b	b
15	a	a	b	b	b	b	-	a	b	b	x	-
16	-	-	-	-	b	a	-	a	b	b	a	x

b, $P \leq 0.01$; a, $P \leq 0.05$

Table 12. The results of the Duncan test with Kramer's modification showing the significance of differences between mean values calculated for percentage of fertilized eggs after 12h of incubation (above diagonal) and for percentage of living embryos after 24h of incubation (under diagonal) within 12 age groups of common carp

Age of female (years)	4	6	7	8	9	10	11	12	13	14	15	16
4	x	-	-	-	-	a	b	-	-	-	-	a
6	-	x	a	a	-	b	b	-	-	-	a	b
7	-	a	x	-	-	-	a	a	-	-	-	-
8	-	a	-	x	-	a	b	-	-	-	-	-
9	-	-	-	-	x	b	b	-	-	-	-	-
10	a	b	-	-	-	x	a	a	a	a	-	-
11	b	b	b	b	b	b	x	b	b	a	a	-
12	-	-	-	-	-	b	b	x	-	-	a	a
13	-	-	-	-	-	a	b	-	x	-	-	a
14	-	-	-	-	-	-	b	-	-	x	-	-
15	b	b	a	a	a	-	-	a	b	a	x	-
16	b	b	-	-	-	-	-	b	a	-	-	x

b, $P \leq 0.01$; a, $P \leq 0.05$

Table 13. The results of the Duncan test with Kramer's modification showing the significance of differences between mean values calculated for total number of eggs (under diagonal) and for number of living embryos after 36 h of incubation (above diagonal) within 12 age groups of common carp

Age of female (years)	4	6	7	8	9	10	11	12	13	14	15	16
4	x	-	b	-	b	b	b	-	b	b	b	b
6	-	x	b	-	b	b	b	-	b	b	b	b
7	-	-	x	b	b	b	b	b	b	b	b	b
8	-	-	-	x	b	b	b	b	b	b	b	b
9	b	b	b	b	x	b	b	b	a	b	b	b
10	a	b	-	a	b	x	b	b	b	b	b	b
11	-	-	-	-	b	a	x	b	b	b	a	b
12	-	-	-	-	b	a	-	x	b	b	b	b
13	b	b	b	b	-	a	b	b	x	a	b	b
14	b	b	b	b	-	b	b	b	-	x	b	b
15	a	a	b	b	b	b	-	-	b	b	x	b
16	-	-	-	-	b	a	-	-	b	b	-	x

b, $P \leq 0.01$; a, $P \leq 0.05$

Regression Predictions

Table 14 shows multiple regression equations for the weight of eggs (g) in the 16 breeding lines and values of the coefficient of determination (R^2). The adequacy of the equations to the existing conditions of individual variability and also to environmental and genetic factors indicates that predicting the weight of eggs (g) only on the basis of the age and female body weight cannot be considered satisfactory. By comparing the R^2 values for the lines studied, it can be seen that in comparison with other lines, the coefficient of determination was relatively high for lines D, T and W (0.63, 0.61 and 0.60). The highest R^2 was noted in line UR (0.93) (Table 14).

Table 14. Regression predictions for weight of eggs in grams within 16 breeding lines of common carp. R^2 – Coefficient of determination

Breeding lines	Equations of multiple regression	R^2
2	$Y_1 = -1709.32+383.78_{x1} – 72.30_{x2}$	0.38
3	$Y_1 = 542.86 -19.23_{x1} + 48.12_{x2}$	0.13
6	$Y_1 = 281.06 – 2.11_{x1} +103.53_{x2}$	0.39
K	$Y_1 = -857.49 +151.10_{x1} +81.36_{x2}$	0.53
7	$Y_1 = 0.4883 + 117.04_{x1} +5.13_{x2}$	0.02
8	$Y_1 = 59.56 + 68.33_{x1} + 44.12_{x2}$	0.05
0	$Y_1 = 3278.44 - 314_{x1} +71.48_{x2}$	0.22
W	$Y_1 = -215.02 - 3.32_{x1} + 144.17_{x2}$	0.60
T	$Y_1 = 521.58 – 151.67_{x1} +22.47_{x2}$	0.61
B	$Y_1 = 3613.64 – 283.36_{x1} +46.91_{x2}$	0.18
BVP	$Y_1 = -1695.07 + 245.33_{x1} +119.98_{x2}$	0.31
D	$Y_1 = -1657.44 + 346.80_{x1} +11.78_{x2}$	0.63
F	$Y_1 = -281.76 + 47.48_{x1} + 120,81_{x2}$	0.37
J	$Y_1 = 1128.45 – 48.96_{x1} + 28.12_{x2}$	0.32
N	$Y_1 = -68.56 + 66.12_{x1} + 27.86_{x2}$	0.03
UR	$Y_1 = -12680 + 1175_{x1} + 246.60_{x2}$	0.93

Dependent variable: weight of eggs in grams (Y_1); Independent variables: body weight of females (x_1), age of females (x_2).

Table 15. Regression predictions for percentage of living embryos (36 h), number of eggs and number of living embryos (36h) within 16 breeding lines of common carp. R^2 – Coefficient of determination

Breeding lines	Equations of multiple regression	R^2
2	$Y_2 = 78.41 + 1.64_{x1} - 0.96_{x2} + 0.0043_{x3}$	0.19
	$Y_3 = -441801 + 183555_{x1} + 37300_{x2} + 53756_{x3}$	0.98
	$Y_4 = -512967 + 39207_{x1} + 26480_{x2} + 46454_{x3}$	0.97
3	$Y_2 = 101.92 + 2.44_{x1} – 1.58_{x2} - 0.039_{x3}$	0.56
	$Y_3 = -398990 - 21883_{x1} + 81289_{x2} + 45740_{x3}$	0.93
	$Y_4 = -132048 + 10371_{x1} + 24284_{x2} + 23706_{x3}$	0.77
6	$Y_2 = 69.32 + 1.96_{x1} – 1.54_{x2} + 0.007_{x3}$	0.23
	$Y_3 = -94431 + 24691_{x1} + 74747_{x2} + 61493_{x3}$	0.94
	$Y_4 = -929184 + 35409_{x1} + 57262_{x2} + 54127_{x3}$	0.91
K	$Y_2 = 75.83 – 0.24_{x1} + 1.91_{x2} - 0.003_{x3}$	0.03
	$Y_3 = -75472 + 22974_{x1} + 67324_{x2} + 60081_{x3}$	0.96
	$Y_4 = -710383 + 20935_{x1} + 65080_{x2} + 52433_{x3}$	0.96
7	$Y_2 = -47.23 + 12.43_{x1} + 2.296_{x2} + 0.019_{x3}$	0.37
	$Y_3 = -671985 + 73124_{x1} + 72129_{x2} + 55180_{x3}$	0.93
	$Y_4 = -129024 + 80401_{x1} + 69762_{x2} + 44926_{x3}$	0.83
8	$Y_2 = 116.34 – 2.36_{x1} – 0.78_{x2} - 0.01_{x3}$	0.16
	$Y_3 = -565832 - 6870_{x1} + 76579_{x2} + 53655_{x3}$	0.99
	$Y_4 = -303137 - 23395_{x1} + 64537_{x2} + 46193_{x3}$	0.89
0	$Y_2 = 80.46 – 0.37_{x1} + 1.67_{x2} - 0.0039_{x3}$	0.08
	$Y_3 = -517416 + 8917_{x1} + 57653_{x2} + 49540_{x3}$	0.97
	$Y_4 = -577795 + 22540_{x1} + 51628_{x2} + 40258_{x3}$	0.84
W	$Y_2 = 89.13 - 0.03_{x1} - 0.80_{x2} + 0.007_{x3}$	0.53
	$Y_3 = -481675 + 5351_{x1} + 59722_{x2} + 52547_{x3}$	0.97
	$Y_4 = -466052 + 12959_{x1} + 44056_{x2} + 50296_{x3}$	0.96
T	$Y_2 = 119.24 – 5.55_{x1} – 5.37_{x2} + 0.027_{x3}$	0.46
	$Y_3 = -294811 - 60385_{x1} + 127063_{x2} + 34134_{x3}$	0.99
	$Y_4 = 85131 - 143520_{x1} + 118856_{x2} + 37173_{x3}$	0.68
B	$Y_2 = 114.12 – 2.77_{x1} + 0.70_{x2} - 0.002_{x3}$	0.45
	$Y_3 = -849206 - 10317_{x1} + 84487_{x2} + 72044_{x3}$	0.93
	$Y_4 = 85131 - 143520_{x1} + 118856_{x2} + 37173_{x3}$	0.68
BVP	$Y_2 = 268.23 – 33.908_{x1} + 10.05_{x2} - 0.0032_{x3}$	0.95
	$Y_3 = 119208 + 9078_{x1} - 27438_{x2} + 763_{x3}$	0.99
	$Y_4 = 864558 - 151133_{x1} + 34065_{x2} + 668_{x3}$	0.99
D	$Y_2 = 116.91 – 2.15_{x1} – 6.27_{x2} + 0.02_{x3}$	0.31
	$Y_3 = -590537 + 52050_{x1} + 57125_{x2} + 31767_{x3}$	0.98
	$Y_4 = -500117 - 58341_{x1} + 20374_{x2} + 31537_{x3}$	0.82
F	$Y_2 = 116.36 – 1.59_{x1} – 5.99_{x2} + 0.02_{x3}$	0.66
	$Y_3 = -480507 - 10413_{x1} + 89406_{x2} + 41290_{x3}$	0.96
	$Y_4 = -434617 - 12138_{x1} + 72727_{x2} + 43003_{x3}$	0.93
J	$Y_2 = 37.22 + 2.82_{x1} - 3.67_{x2} + 0.05_{x3}$	0.02
	$Y_3 = -876772 + 20888_{x1} + 53598_{x2} + 68086_{x3}$	0.96
	$Y_4 = -1326032 + 36805_{x1} + 54555_{x2} + 86361_{x3}$	0.92
N	$Y_2 = 139.13 – 12.16_{x1} + 6.36_{x2} – 0.01_{x3}$	0.81
	$Y_3 = 421808 - 7290_{x1} + 55680_{x2} + 57746_{x3}$	0.99
	$Y_4 = 68870 - 77805_{x1} + 68820_{x2} + 45873_{x3}$	0.97
UR	$Y_2 = -73916 + 76.87_{x1} + 1526_{x2} - 0.01_{x3}$	0.95
	$Y_3 = -974162 + 98372_{x1} + 17740_{x2} + 571_{x3}$	0.96
	$Y_4 = -6327374 + 594513_{x1} + 124679_{x2} + 45.00_{x3}$	0.99

Dependent variables: percentage of living embryos 36 h (Y_2), number of eggs (Y_3), number of living embryos 36 h (Y_4); Independent variables: age of females (x_1), body weight of females (x_2), weight of eggs in grams (x_3).

Table 15 shows multiple regression equations, where the percentage of living embryos (36 h), the number of eggs and the number of living embryos (36 h) constituted the dependent variables, while the independent variables were: age, body weight of the females and the

weight of eggs (g). Predictions for the percentage of living embryos after 36-h incubation made on the basis of the age and body weight of females and the weight of eggs obtained (g) were most precise for lines U_R, BVP and N (0.95; 0.95 and 0.81, respectively; Table 15). The coefficient of determination (R^2values fell within the range of 0.45 - 0.66 in five lines (3, W, T, B and F). The prediction rate for the number of eggs can be considered satisfactory for all the breeding lines. The R^2 for the respective lines was high, falling within the range of 0.93 - 0.99 (Table 15). The reliability of prediction for the number of living embryos after 36-h incubation was satisfactory for all the breeding lines and the highest R^2 values were noted for lines U_R, BVP, 2, N, K and W (0.99; 0.99; 0.97; 0.96, and 0.96), and the lowest for lines T and 3 (0.68 and 0.77) (Table 15).

Table 16 shows multiple regression equations for the twelve age groups of females, where the dependent variable was the number of living embryos after 36 h incubation, while the independent variables were the body weight of females and the weight of eggs in grams. Taking into consideration the coefficient of determination, it can be concluded that the reliability of prediction of the number of living embryos (36 h) was very high for as many as nine age groups, that is, females aged 4, 7, 8, 9, 10, 12, 13, 14 and 15. The prediction was not reliable for 11-year-old females only ($R^2 = 0.05$).

Table 16. Regression predictions for number of living embryos after 36 h incubation in 12 age groups of common carp. R^2- coefficient of determination

Age of females (years)	Equations of multiple regression	R^2
4	$Y_1 = -2145 - 2455.2_{x1} + 772.8_{x2}$	0.99
6	$Y_1 = 22606 - 3700.4_{x1} + 650.9_{x2}$	0.82
7	$Y_1 = 112923 - 29830.3_{x1} + 764.8_{x2}$	0.92
8	$Y_1 = 56066 - 7602.2_{x1} + 619.5_{x2}$	0.90
9	$Y_1 = 232900 - 25514.7_{x1} + 614.8_{x2}$	0.90
10	$Y_1 = 67974 - 5682.1_{x1} + 590.9_{x2}$	0.89
11	$Y_1 = 536058 - 10477.2_{x1} - 29.5_{x2}$	0.05
12	$Y_1 = 129807 - 24172.3_{x1} + 720.9_{x2}$	0.99
13	$Y_1 = 147622 - 7048.7_{x1} + 543.9_{x2}$	0.95
14	$Y_1 = 670961 - 54737.6_{x1} + 561.6_{x2}$	0.98
15	$Y_1 = 60864 - 22102.2_{x1} + 806.7_{x2}$	0.90
16	$Y_1 = -7553 - 1401.7_{x1} + 595.1_{x2}$	0.62

Dependent variable: number of living embryos after 36 h incubation (Y_1);
Independent variables: body weight of females (x_1), weight of eggs in grams (x_2)

SUMMARY

On the basis of the results presented it can be summarized that considering the rate of ovulating females and the least squares means estimated for the traits investigated, the best breeding effects were obtained for two Lithuanian breeding lines, that is, line B and BVP, and the Hungarian line 8.

The number of living embryos (36 h) estimated for line B was higher than the number of living embryos (36 h) calculated for the other lines studied and the reliability of prediction of this trait can be considered high.

Regarding line BVP, eggs were obtained from all females and the quality of the eggs after 36 h incubation was the highest. Moreover, in the Hungarian line 8, the percentage of ovulating females and the number of living embryos (36 h) was high. The reliability of prediction of both the number of living embryos (36 h) and the number of eggs can be considered high.

The Hungarian line T was characterized by the highest mean weight of eggs and the highest number of eggs obtained. However, attention should be paid to the fact that the percentage of fertilized eggs and living embryos (24 and 36 h) estimated for line T was significantly lower in comparison with the other lines studied. The lowest percentage of ovulating females was noted for the Yugoslavian line J and Polish line 2. It cannot remain unnoticed that the very high quality of eggs for line J 12 h after incubation was drastically lower in the subsequent incubation stages.

The percentage of ovulating females in Polish line 3 and the mean weight of eggs were relatively low, while the percentage of living embryos was significantly lower after 36-h incubation in comparison with the percentage of living embryos calculated after 24 h. The reliability of prediction for percentage of living embryos (36 h) can be considered not very high for line 3. The females of the German line N produced eggs of the lowest mean weight, and thus these eggs should be considered to be of low quality. The number of living embryos for this line was also the lowest and the prediction rate for these parameters was very high.

Low quality of eggs (24 and 36 h) was also noted for the Israeli line D, however, the mean number of living embryos (36 h) estimated for this line was close to the overall mean. The Ukrainian line UR can be considered highly effective in reproduction due to the significant percentage of ovulating females and the high number of eggs and living embryos.

The highest mean weight of eggs was obtained from females aged 14, 13 and 9 years. The quality of eggs obtained from 9-year old females was lower in comparison with the quality of eggs produced by 13 – and 14 year old fish. The highest number of living embryos (36 h) was noted for 14 – year old females. A relatively high weight of eggs was also obtained from 7- and 8 – year old females, but the percentage of ovulating fish was higher in the 8 – year old group. The study show higher number of living embryos (36 h) for 7 – year old females.

Following the Ovopel treatment all the 11 – and 12 – year old females produced eggs but the mean weight estimated for these age groups deviated negatively from the overall mean. The number of living embryos (36 h) for 11 – year old fish was very low, although the reliability of prediction for this parameter was very low. The 16 – year old females treated with Ovopel ovulated as well, but the number of living embryos (36 h) for this age group was lower than the overall mean. Only the 4 – and 6 – year old group percentage of ovulating females was lower than the 15- year old group. Females from the 15 – year old group produced the lowest number of living embryos (36 h).

CONCLUSION

For economic reasons and breeding effectiveness, artificial reproduction should involve females which produce the highest possible number of offspring. The present study shows that the best female age range for breeding is 13-14 years and 9 years. However, it needs to be

noted that the cost of maintaining fish that age with a large body weight is high. Moreover, ovulation stimulating agents administered per 1 kg of body weight are also expensive. When using 4- and 6-year old females for reproduction, one needs to remember that the share of ovulating fish in the total number of the treated fish will (most likely) not exceed 75%, and the number of living embryos after 36 h incubation will be about 500000. By estimating the effectiveness of reproduction in various breeding lines of the common carp and different age groups of females one may make decisions that will ensure the best possible results of spawning under controlled conditions. Such conditions make it possible to precisely monitor the percentage of ovulating females after hormonal stimulation (one of the most important parameters describing the effectiveness of reproduction), the weight of the eggs obtained and their quality throughout the incubation period.

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Chapter 95

UTILISATION OF NATURAL FOOD RESOURCES BY CARP IN FISH PONDS

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ABSTRACT

Carp (*Cyprinus carpio* L.) in both Central and Eastern Europe and Asia are mainly reared in shallow earth ponds that allow a large proportion of their diet to be provided in-situ via pond zooplankton and zoobenthos. Fish farmers generally consider natural foods superior to artificial diets (especially for younger fish) due to their high digestibility, high water content and rapid growth rate, which ensures high fish densities. Further, such a diet has significant benefits for fish growth and survival and reduces the fish farmer's outlay considerably. However, while the production costs of carp farmed using natural production only are low, stocking densities and yields are also low in comparison to more intensive production systems. Following a period of increasingly intensive management up to the late 1980s, most carp pond farmers now employ a semi-intensive management system characterised by the combination of natural food items (the abundance of which is increased by the addition of animal manure and lime) with supplementary feed that provides the missing nutritional elements, prolongs the growing season and increases growth rates and yield-per-unit volume. In this chapter, we briefly examine the role of zooplankton and zoobenthos in carp diet and nutrition, summarise present semi-intensive management systems and the role of manure and lime in promoting zoobenthos and zooplankton availability, and assess the impact of supplementary feeding on natural food uptake by carp. Finally, we highlight a number of suggestions for better balancing the uptake of natural and supplementary feed resources, thereby reducing overall costs, increasing production intensity and efficiency and reducing environmental impact.

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Keywords: carp pond management, C*yprinus carpio*, dietary preference, fish diet, pond productivity, semi-intensive management, supplementary feeding

INTRODUCTION

The common carp (*Cyprinus carpio*) is an omnivorous, benthivorous cyprinid fish that has been cultivated by humans in Europe and Asia for hundreds of years as a source of food, not least as it is highly adaptable in relation to habitat and diet (Soltani et al. 2010; Manjappa et al. 2011; Rahman 2015a). Indeed, carp are now the third most widely cultivated freshwater fish species in the world (Table 1; FAO 2013), with production levels in some Central and Eastern European countries now representing over 80% of total fish production (Woynarovich et al. 2010; Anton-Pardo et al. 2014). To this day, carp in both Central and Eastern Europe and Asia are mainly reared in shallow, extensively or semi-intensively managed earth ponds, with the advantage that a large proportion of their food can be provided in the form of naturally occurring dietary items such as zooplankton and zoobenthos (Woynarovich et al. 2010; Adámek et al. 2012). As such, carp pond farming is usually seen as an efficient, stabilising and environmentally friendly form of farming (Szucs et al. 2015). Indeed, its ability to effectively utilise natural food resources and its low impact on the surrounding environment makes carp an ideal candidate for organic farming (Adámek et al. 2015). However, the ever growing need to increase production levels and efficiency has led to changes in pond management aimed at lengthening the carp growing season and increasing stock levels. As such, most farms now use a system that tries to balance use of autochthonous (natural) and allochthonous (imported) food sources.

Table 1. World production of the top ten aquacultural fish species in 2015 (data based on statistics from FAO 2017b)

Species	Production		Value	
	tonnes	%	10^3 USD	%
Grass carp *Ctenopharyngodon idella*	5 822 869	7.32	7 462 316	4.73
Silver carp *Hypophthalmichthys molitrix*	5 822 869	7.32	6 776 963	4.29
Common carp *Cyprinus carpio*	4 328 083	5.44	5 905 279	3.74
Nile tilapia *Oreochromis niloticus*	3 930 579	4.94	6 017 377	3.81
Bighead carp *Hypophthalmichthys nobilis*	3 402 870	4.27	4 373 102	2.77
Catla *Catla catla*	2 764 944	3.47	4 813 647	3.05
Salmon *Salmo salar*	2 381 576	2.99	11 945 146	7.56
Rohu *Labeo rohita*	1 785 900	2.24	3 034 446	1.92
Milkfish *Chanos chanos*	1 115 095	1.40	1 663 531	1.05
Wuchang bream *Megalobrana ambilycephala*	796 830	1.00	1 314 770	0.83
World total	79 599 902		157 919 520	

Here, we provide a short overview of the role natural food resources play in the diet of carp, how carp affect zooplankton and zoobenthos communities and how modern pond management methods seek to increase pond productivity by increasing food supply (e.g., by use of fertilisers and supplementary feeds). In general, we concentrate on examples from Central Europe (and especially the Czech Republic); however, both the management methods used and the resulting dietary preferences are generally applicable elsewhere.

THE CHANGING ROLE OF NATURAL FOOD RESOURCES IN CENTRAL EUROPEAN CARP FARMING

A Short History

Fishponds of up to several hundreds of hectares have been constructed in Central Europe for the rearing of mainly common carp since at least the 11th century (Pechar et al. 2002). Most were (and still are) no more than 1-2 m deep and were constructed by damming marshes, swamps or small river floodplains (Pokorný and Hauser 2002). In what is now the Czech Republic (and elsewhere in Central and Eastern Europe), a particularly intense period of building took place at the end of the 15th and beginning of the 16th centuries, during which the methods employed in carp aquaculture were greatly improved (Hoffmann 1996). In particular, a more systematic approach was employed, with carp categorised and separated into individual age groups (Hartman et al. 2015). Following a period of decline, resurgence in pond building took place during the 19th century when a number of management practices aimed at increasing productivity were introduced, e.g., summer drainage, supplementary feeding, liming and fertilisation (Šusta 1898). This was followed by a second period of intensification in the second half of the 20th century when more intensive pond management measures were introduced, including high levels of supplementary feeding (mainly cereals), use of solid and liquid manure, fertilising with inorganic fertilisers and intensive liming (Figure 1). All such measures aim to increase the production of natural food resources (zooplankton/zoobenthos) in the pond in order to increase the biomass of carp (and other fish) that could be harvested. Many duck farms were also established on ponds at this time in order to diversify production (Pokorný et al. 2015). This era of highly intensive management reached its peak in the 1980s, at which time most ponds in Central Europe were highly eutrophic (Pechar 2000; Vsetickova et al. 2012), being subject to extreme algal blooms and disease outbreaks, including botulism, which has been linked to duck production (IUCN 1997; Babinszky 2009). Aside from an excess of nutrients in the water, high densities of fish stock meant that larger *Daphnia* sp. became overgrazed and smaller zooplankton species, which are unable to check phytoplankton growth (especially cyanobacteria), proliferated (Potužák et al. 2007).

Since then, pond farming has shifted to a more semi-intensive management system, with the majority of food based on natural zooplankton and zoobenthos production supplemented by processed or artificial feeds. Today, use of organic and inorganic fertilisers is controlled to prevent the eutrophication and algal blooms, increased turbidity and low oxygen concentrations associated with high nutrient concentrations, while lime is mainly used to counter the negative effects of fertilisation (Adámek et al. 2012; FAO 2017c). Central and

Eastern European and Central Asian semi-intensive carp ponds generally have relatively high stocking densities, with fish yields generally ranging between several hundred to several thousand kilograms per hectare (Kestemont 1995). The ponds are usually harvested over a three- to four-year production cycle utilising three to four main fishpond types: (a) an optional pre-nursery pond for production of advanced fry (C_a, 0.3–1.5 g), (b) nursery ponds for production of one-year carp (C_1, 20–70 g), (c) on-growing ponds for production of two-year carp (C_2, 100–350 g) and (d) marketing ponds for production of three- to four-year marketable fish (C_3, 600–1750 g; C_4, >2 kg) (Horváth et al. 1992). As the majority of marketable fish are not sold during or directly after the pond harvest, the fish will be kept in storage ponds until transported or sold (Stibranyiová and Adámek 1998).

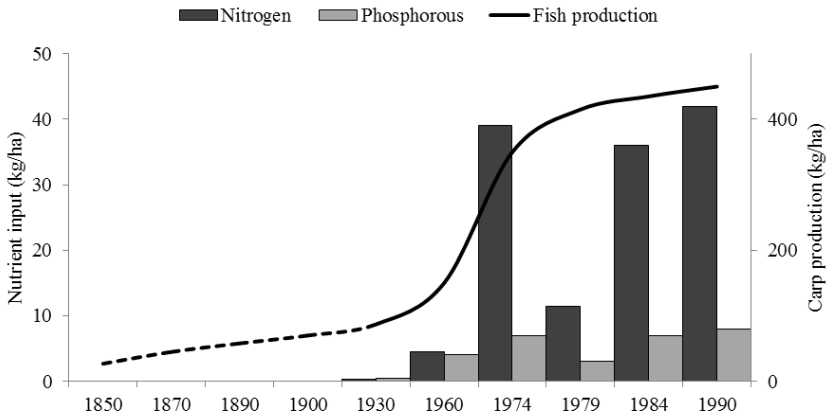

Figure 1. Relationship between fish pond production and input of nutrients in Czech fish ponds between 1850 and 1990 (adapted from Potužák et al. 2007).

NATURAL FOOD RESOURCES IN CARP DIET

The Role of Natural Food Resources in Carp Diet

Carp larvae and fry feed mainly on zooplankton (e.g., rotifers, copepod nauplii); however, they switch to larger prey (e.g., copepods, cladocerans) as they grow (Osse et al. 1997; Chakrabarti and Sharma 1998; Nunn et al. 2010; Woynarovich et al. 2010; Dulić et al. 2011; Anton-Pardo and Adámek 2015; Füllner 2015). As adults, carp switch to a mainly benthic feeding style, their large, accordion-like mouths and barbels (one pair each on the upper and lower lips) enabling them to dig, sift and filter bottom muds and sediments for food (Froese and Pauly 2011). The size at which this shift occurs will vary in relation to a range of factors, including the density and ratio of both food types (Anton-Pardo and Adámek 2015). Osse et al. (1997) suggested that carp shifted from planktivorous to benthivorous feeding at around 8-18 mm total body length. Studies on functional morphological traits, however, suggest a somewhat later shift, the carp's mouth position shifting from 'terminal' to downward projecting (reflecting the shift to benthic feeding) at around 20-25 mm (Vilizzi and Walker 1999). Crustaceans remain the dominant dietary item until around 100-150 mm (Khan

2003; Britton et al. 2007), though some studies have noted the change to a primarily benthic diet as early as 30-50 mm (Kloskowski 2011).

From the juvenile stage onward, carp are able to utilise a wide range of natural food types, though animal prey usually represents 75% or more of the prey taken (Michel and Oberdorff 1995). Prey types most often taken include plankton, crustaceans and insects (including larvae and pupae), especially chironomids and other benthic invertebrates (Michel and Oberdorff 1995; Colautti and Remes Lenicov 2001; Khan 2003; Rahman et al. 2008; Anton-Pardo et al. 2014). In addition, they will also take the softer parts of aquatic plants and their seeds, along with fish eggs and larvae and even smaller fish or crayfish larvae (Hinojosa-Garro and Zambrano 2004; Weber and Brown 2011; FAO 2017a). Zooplankton may persist as an important part of the adult diet, however, with the percentage taken varying between 0% and 90+% (Adámek et al. 2003; Khan 2003; Rahman et al. 2006; Marković et al. 2009; Woynarovich et al. 2010; Kloskowski 2011; Anton-Pardo and Adámek 2015), depending on food availability and spatial and temporal variation (Garcia-Berthou 2001; Saikia and Das 2009; Rahman et al. 2010). As the branchial sieve of the adult carp is incapable of retaining organisms smaller than 0.25 mm (Sibbing et al. 1986; Colautti and Remes Lenicov 2001; Dulić et al. 2011), large cladocerans tend to be the main zooplankton ingested. As carp are visual predators, such large zooplankton are easier to see and catch, not least as they are more highly pigmented than copepods and have a slower escape speed (Drenner et al. 1978; Dulić et al. 2011).

In general, the proportion of different prey classes in the diet will vary through the seasons, with zooplankton more important in spring and early summer, molluscs and copepods in winter, and chironomids from the end of spring to autumn (Michel and Oberdorff 1995; Bauer and Schlott 2004; Kloskowski 2011). Such shifts in feeding preference will usually be matched by a shift in feeding niche from the benthic zone to the water column and back (Rahman et al. 2010; Rahman 2015b). Overall, common carp are flexible and opportunistic feeders that can switch between preferred and alternative dietary items, depending on availability (Anton-Pardo et al. 2014; Hoole et al. 2001).

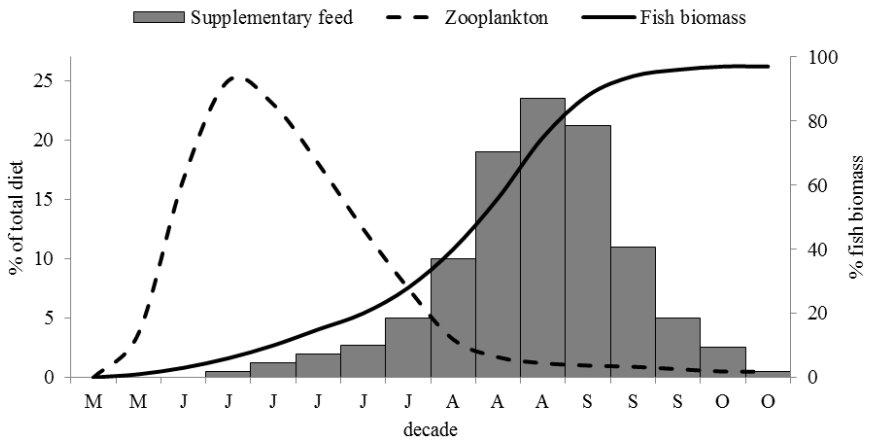

Figure 2. Schematic depiction of fingerling biomass development during the first growing season in a typical Central European fishpond, showing the changing importance of natural and supplementary food over time. X-axis = ten day period (decade) during the growing season; M = May, O = October (adapted from Füllner 2015).

Theoretically, carp can be farmed using the natural production of the pond only (e.g., zooplankton, benthos and aquatic plant material); however, while this type of pond production is characterised by low production costs it is also limited to low stocking densities and low yields (Stanković et al. 2011), not least as carp growth is highly dependent on the seasonal availability of zooplankton/benthos. In most temperate regions, for example, while peak production of zooplankton and zoobenthos occurs in spring, water temperatures are optimal for carp growth around mid-summer, when natural food levels have started to drop (Dulić 2007; Dulić et al. 2009; Füllner 2015; see Figure 2). Further, while natural dietary items are an important source of lipid and protein in carp diet, they are poor in carbohydrates, containing just 3 to 4.8% dry matter (Mitra et al. 2007). For these reasons, supplementary (mainly cereals) or commercial feeds (along with fertilisers) are commonly used in carp aquaculture (Kibria et al. 1997; Woynarovich et al. 2010).

Natural Food Resources and Carp Nutrition

As stated above, carp larvae and fry feed mainly on zooplankton but switch to larger prey and zoobenthos as they grow. Zooplankton may remain an important dietary component well into adulthood; however, this depends on their relative abundance and season. Albrecht and Breitsprecher (1969), who undertook a large-scale review of literature concerning fish food in ponds, provided average composition values as follows (% dry matter in parentheses; see Table 2 for a more detailed breakdown by prey class): water 85.8%, protein 7.4% (52.1), carbohydrate 3.8% (27.3), lipids 1.1% (7.7) and ash 1.1% (7.7). The nutritional value of zooplankton and zoobenthos varies greatly, however, depending on the group analysed and environment factors such as water temperature (Anton-Pardo and Adámek 2015). While there have been fewer studies on the nutritional content of zoobenthos, values tend to be similar to those for zooplankton (Tables 2 & 3), especially regarding the two species most often taken by carp, chironomids and daphniids (Michel and Oberdorff 1995; Colautti and Remes Lenicov 2001; Khan 2003; Rahman et al. 2008; Epler et al. 2010; Anton-Pardo et al. 2014). Again, lipid and amino acid content vary both seasonally and with developmental stage, especially in copepods (Kibria et al. 1999; Brucet et al. 2005). Protein content can reach more than 50% in some genera (e.g., Daphnia, Cyclops), though content in rotifers is usually somewhat less at between 25-50% dry wt. (Conceicao et al. 2010; Jeeja et al. 2011; Yin et al. 2013). Protein levels in chironomids are also estimated at around 50% and lipid content at 10% (Habib et al. 1997). For most cladocerans and copepods, total amino acid content generally lies above 50% (Kibria et al. 1997; Mitra et al. 2007). Bogut et al. (2007) estimated protein content in *Chironomus plumosus* at 55% dry wt. (7% wet wt.), mineral content at 8% dry wt. (1% wet wt.), crude fat at 1% wet wt., nitrogen-free extractive substances at 28% dry wt. and water content at 88%. For *Daphnia magna*, Bogut et al. (2010) estimated protein levels at 39% wet wt. (1% dry wt.), raw fat at 5% dry wt., fibre at 4% dry wt and water content at 97%. Aside from methionine and phenylalanine, levels of essential amino acids are found in "adequate" quantities for carp (Bogut et al. 2010).

Unlike protein and amino acid levels, which are generally genetically determined, lipid content tends to reflect levels in the fish's diet (DeMott and Muller-Navarra 1997). Fatty acids are essential for fish larval growth and development and a deficiency in juvenile and adult fish has been linked with poor fecundity, fertilisation rates and viability (Rainuzzo et al.

1997), along with fatty liver degeneration, myocarditis, low erythrocyte haemoglobin concentrations, changes in cell membrane permeability, fast breathing, poor food utilisation, lowered growth rates and increased susceptibility to stress (Bogut 1996). Carp, in particular, require omega-3 fatty acids (Watanabe et al. 1975; Csengeri 1993), their deficiency being reflected in a lowered growth rate and synthesis and accumulation of non-essential omega-9 fatty acids in muscle tissue. In general, zooplankton tend to have a high proportion of highly unsaturated fatty acids (HUFA), such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and total conjugated linoleic acids (CLAs), all omega-3 fatty acids. It has been estimated that carp require between 0.5 and 1.0% HUFA and polyunsaturated fatty acids (PUFA) for optimal growth (Bogut 1995; Robinson 1984; Takeuchi 1996). While fatty acid content will vary with species, temperature and season (Kibria et al. 1999; Persson and Vrede 2006; Mitra et al. 2007); lipid content in zooplankton tends to be around 10% dry wt., usually with more unsaturated than saturated fats (Kibria et al. 1997; Bogut et al. 2010; Lei et al. 2013). In *D. magna*, for example, Bogut et al. (2010) recorded 19% saturated fatty acids and 66% unsaturated fats, with 27% of unsaturated fats comprising omega-3 fatty acids. Lipid levels in rotifers tend to be relatively high at approx. 20% dry wt., though they tend to be low in HUFAs (Yin et al. 2013). Copepods, on the other hand, have only moderate levels (predominantly unsaturated DHA), while cladocerans have higher proportions of monounsaturated fatty acids (MFA) and EPA (Persson and Vreded 2006; Guo et al. 2008; Smyntek et al. 2008; Mraz et al. 2012). (For a full description of the nutritional requirements of carp, see FAO 2017a and references therein).

Table 2. Nutrient composition of dominant carp prey items (mean + range; adapted from Albrecht and Breitsprecher 1969)

	Water % fresh wt	Crude protein % dry wt	Crude fat % dry wt	NFE* % dry wt	Crude ash % wt
Chironomids	85.8 (79.0-92.7)	52.1 (52.6-9.6)	7.7 (3.6-11.3)	26.7 (16.6-34.1)	7.7 (5.3-8.0)
Daphnia	90.6 (87.8-93.8)	43.6 (14.3-8.1)	9.6 (2.9-21.3)	23.4 (10.9-44.1)	17.0 (10.4-28.9)
Copepods	90.0 (87.9-93.8)	42.0 (25.1-0.2)	33.0 (11.7-54.9)	20.0 (10.9-31.7)	6.0 (4.1-7.3)
Tubificides	83.0 (77.8-87.2)	51.8 (46.1-6.6)	17.6 (6.3-19.8)	20.6 (14.6-28.2)	5.9 (4.0-9.0)
Ephemerids	80.5 (74.9-87.2)	60.5 (44.0-1.6)	15.9 (5.1-26.3)	15.4 (8.2-23.2)	8.7 (5.1-10.2)
Caddis larvae	76.7 (70.4-87.3)	56.7 (48.5-8.6)	17.2 (3.5-12.1)	19.7 (12.7-31.5)	6.4 (3.5-12.1)

*Nitrogen free extracts (carbohydrates consisting almost entirely of indigestible chitin).

Table 3. Nutrient composition of benthic carp prey items (adapted from Epler et al. 2010)

	Dry matter	Crude protein	Crude fat	NFE*	Crude ash
% fresh matter	13.9 – 14.9	6.6 – 7.5	0.8 – 1.0	2.0 – 4.2	3.2 – 3.6
% dry matter	100	44.0 – 53.8	5.7 – 6.4	14.5 – 28.2	21.4 – 25.9

*Nitrogen free extracts (almost entirely indigestible chitin).Benefits of Utilising Natural Food Resources

The nutritional value (as human food) of carp is highly dependent on the quality and composition of the fish's diet. Fish farmers generally consider natural foods superior to artificial and supplementary diets (especially for younger fish) due to their high digestibility (particularly proteins); high water content (85-95%); soft structure (allowing deformation soon after digestion) and motility, which attracts fish to the food ensuring high take-up (Bogut et al. 2007. Indeed, there is considerable evidence that a natural diet has significant benefits for fish growth and survival (Anton-Pardo and Adámek 2015). The content of dry matter in zooplankton, for example, is relatively low at around 10%. As such, they are easier to break down than dry feed and are more palatable for larvae and juveniles (Lie et al. 1997; Kolkovski 2001; Mitra et al. 2007; Conceicao et al. 2010). Further, it is thought that exogenous enzymes in natural live foods play a fundamental role in the breakdown of organisms in the larval digestive tract as the larvae themselves naturally have low levels of digestive enzymes (Govoni et al. 1986). A number of studies have noted increased growth rates (and weight gain) in carp as the amount of available zooplankton increases (Jana and Chakrabarti 1990; Chakrabarti and Jana 1992). Further, both yield and survival rate, especially in carp larvae and fry, have been shown to increase as the proportion of zooplankton in the diet increases (Chakrabarti and Sharma 1998; Sharma and Chakrabarti 1999; Markovic et al. 2009). More indirectly, live prey remain active (e.g., edible and available) if uneaten, while supplementary and artificial feeds (which may contain up to 10x more dry matter than natural food) fall to the pond bottom and contribute to nutrient enrichment and eutrophication (Breukelaar et al. 1994; Conceicao et al. 2010; Anton-Pardo and Adámek 2015; see also Torres and Adámek 2013 and references therein as regards remobilisation of nutrients through bioturbation caused by carp feeding). Finally, use of natural foods reduces the fish farmer's costs considerably and the rapid growth rate of zooplankton ensures high densities shortly after the pond is filled (Kibria et al. 1997; Lubzens et al. 2001).

SUMMARISING CARP IMPACT ON ZOOPLANKTON AND ZOOBENTHOS ASSEMBLAGES UNDER EXTENSIVE CONDITIONS

As a result of their feeding habits, carp can have a significant impact on the quantitative and qualitative composition of extensive pond zooplankton and zoobenthos assemblages. As outlined earlier, for example, the removal of large cladocerans by planktivorous carp can result in cyanobacterial blooms as smaller zooplankton species, which are unable to check phytoplankton growth (especially cyanobacteria), then proliferate unchecked (Potužák et al. 2007). Further, Tátrai et al. (1998) demonstrated that carp tend to avoid eating smaller zooplankton following a decline in cladoceran biomass; instead, they switch to intensive feeding on zoobenthos. Tátrai et al. (1997) were also able to show that the intensity at which zoobenthos (mainly chironomids and *Chaoborus*) are reduced by benthivorous feeding cyprinids (including carp) was significantly correlated with pond stock biomass. By digesting these benthic macroinvertebrates, carp also transfer nutrients from the benthic zone to the pelagic zone, thus enhancing internal nutrient loading (Andersson et al. 1988). Season, age and size (correlated with age) have all been shown to influence the composition of zooplankton and zoobenthos taken in the diet (Nieoczym and Kloskowski 2014). While

copepods and small bodied cladocerans tend to be least abundant in the presence of small carp (0+), for example, large cladoceran grazers appear to be most suppressed in ponds with medium sized fish (1+). On the other hand, the density of small dipteran (mainly chironomid) larvae tends not to differ across fish sizes, though larger larvae are usually more abundant in ponds stocked with small carp, as larger carp (2+) are known to penetrate deeper into the substrate than smaller fish (Panek 1987).

Surprisingly, after summarising current knowledge on the impact of carp on aquatic ecosystems based on 129 laboratory and "natural" field experiments, Vilizzi et al. (2015) concluded that there was no clear evidence for a decrease in zooplankton. The majority of impacts on benthic invertebrates were negative (decreased abundance), however, both under laboratory and field conditions. An absence of any direct impact on zooplankton community structure was also noted by Lougheed et al. (1998), though total zooplankton biomass was reduced by increased turbidity and nutrient loading. Other studies have noted a positive (increased abundance) relationship between carp presence and zooplankton biomass (Parkos et al. 2003), and a negative relationship on macroinvertebrate abundance (Parkos et al. 2003), zoobenthos biomass (Wahl et al. 2011) and zoobenthos diversity (Wilcox and Hornbach 1991). This suggests that there is, as yet, no overall unifying pattern to the impacts of carp on the natural food community. In general, however, under extensive conditions, benthic foraging will tend to impact phytoplankton abundance and diversity through bottom-up processes, such as increased nutrient concentrations and turbidity, while zooplankton and benthic invertebrates will be affected by top-down processes, such as predation and reduced foraging efficiency (Weber and Brown 2009).

The impacts of carp on natural food populations will be somewhat different under semi-intensive and intensive pond management, with quantitative and, especially, qualitative factors largely influenced through the addition of allochthonous foods and fertilisers that artificially raise productivity and extend the growing season. The impacts of modern semi-intensive management techniques on the zooplankton and zoobenthos community are discussed in more detail below.

PRESENT POND MANAGEMENT PRACTICE AND PRODUCTION OF NATURAL FOOD

In most parts of Central and Eastern Europe and Central Asia, carp are now farmed using a semi-intensive, three-stage polyculture approach. Briefly, following artificial spawning, larvae are grown on in special ponds that have previously been kept dry to remove pathogens, surviving fish and predatory insects. About 15-30 days are required for feeding larvae to grow into 'advanced fry', and a further 45-85 days to reach the 'fingerling' stage (FAO 2017a). In the past, pesticides/insecticides were usually added at this point to prevent development of crustaceans (cladocerans and copepods); thereby promoting development of rotifers, the preferred food of larval carp (Adámek et al. 2014). As use of pesticides has now been banned in most countries, rotifer development may be promoted by rapid inundation and immediate manuring. The addition of animal manure (or artificial fertiliser) ensures sufficient food (mainly phytoplankton) for the successful development of zooplankton, especially during the later stages of carp rearing (Li et al. 1996; Jana and Chakrabarti 1997; Kaur and Ansal 2010;

Füllner 2015). The addition of straw or hay bundles will further increase the numbers of small zooplankton such as ciliates, infusoria and rotifers. Liming may also take place at this stage to control water pH and disinfect the pond bottom (Füllner 2015). In most cases, the pond will have first been refilled gradually to around 50-60 cm. After stocking the larvae, the pond will then be fully filled over a further five to ten days. This also helps ensure a high initial concentration of rotifers. A number of studies have shown that the ideal sustainable zooplankton density at the point of larval stocking ranges from 8.5 to 40 rotifers ml^{-1} (Valdenberg et al. 2006) or 20 to 40 mg L^{-1} biomass (Li et al., 1996). After one summer, the fingerlings are stocked in growing or rearing ponds, with 25-50 gram fingerlings grown on to table size over a three-year cycle and 100 gram fingerlings over a two-year cycle (Füllner 2015). The size of table fish varies from country to country but generally varies between 1 and 3 kg. The two-step process tends to be more common, with fish stocked at a higher density in the first step and grown on to 0.25–0.50 kg, while in the second step the fish reach between 1–3 kg. Both steps last around 120–170 days (FAO 2017a). The rearing ponds will also have been kept dry prior to stocking, in order to improve substrate structure and remove pathogens and surviving fish, following which unwanted vegetation is removed and organic manure spread on the surface, adding essential mineral nutrients and carbon that are generally lacking in fish ponds. The most important of the minerals essential for fish pond primary production are nitrogen (N) and phosphorus (P). In general, the recommended total quantity of soluble N and P available in ponds should be around 100 to 200 kg/ha/season, with an ideal proportion of N to P of around 6:1 (Pócsi 1982; see also Figure 1). Over winter, the carp will be left in the rearing pond until they are harvested in spring, moved to special overwintering ponds or harvested in autumn and moved to a different rearing pond where they will be kept over the next season. Finally, carp ready for sale (usually at 3-4 years) may then be moved to storage ponds fed with clean water to ready them for the table.

Generally speaking, both fish and zooplankton production are improved when multiple fish species differing in feeding style (and other biological features) are farmed together in a balanced manner (polyculture). Not only are the natural food resources utilised more efficiently but intensive consumption can actually stimulate zooplankton production further (Rahman 2015). Furthermore, as monocultures utilise natural fish foods less effectively than polycultures, such systems also tend to be highly dependent on the addition of supplementary feeds (e.g., grain) and fertilisers, unless stocked at very low densities (Woynarovich et al. 2010; Rahman 2015). When planktivorous fish are kept together with common carp, for example, ponds generally require between 20 and 40% less fertiliser to maintain adequate natural food levels (Rahman 2015).

USE OF MANURE AND LIME FOR INCREASING NATURAL FOOD AVAILABILITY

Farmyard manure, together with the bacteria that develop on its surface, serves as an ideal protein-rich food for zooplankton as they are able to consume the organic materials either directly or through a very short food chain. Spreading manure on the pond bottom prior to inundation can save time, manpower and costs. Manure will often be added to the pond after inundation, however, as well as through the production season, thereby ensuring a more

effective result. The manure is normally applied as a relatively large first treatment, followed by repeated smaller doses at regular intervals (e.g., daily, weekly, biweekly or monthly), thus ensuring that the pond ecosystem is not overloaded and all organisms are kept in an active production phase for as long as possible (Woynarovich 1963; Woynarovich et al. 2010). Growth of natural food resources can also be supported through the addition of inorganic fertilisers, which, while lacking carbon (essential for photosynthesis), provide essential minerals through an alternative route. One advantage of inorganic fertilisers is that the contents can be standardised, unlike farmyard manures whose contents vary depending on how the animals were kept. It should be noted here that the use of fertilisers and farmyard manures is now strictly limited in many countries due to eutrophication and algal blooms in ponds and receiving waterbodies caused by high nutrient loading and the proliferation of bacteria and pathogens (Kestemont 1995). As a result, supplementary feeding is now the main tool used for intensifying fish (and supporting zooplankton) production in most Central European countries.

The addition of lime is an important part of modern pond management. Prior to filling, lime helps disinfect the pond bottom and, after filling, helps stabilise ecological processes and control phytoplankton blooms. As most fish do best in water with a pH of between 7.0 and 8.5, for example, lime is usually added to raise the pond's pH (McLarney 1984). Lime also reduces extremes in daily pH fluctuations and supports chemical processes such as decomposition and mineralisation of organic materials (Ribianszky and Woynarovich 1962; McLarney 1984; Woynarovich et al. 2010).

Zooplankton enter the pond either externally, along with feed water (e.g., allochthonous), or internally, after hatching from viable resting eggs (ephippia) deposited in pond sediments. Many zooplankton are able to survive dry periods by forming resistant stages that remain in diapause in the sediment and hatch when conditions improve, e.g., when the pond is refilled with water (Hairston et al. 1995). Disturbance of the sediment, e.g., by carp feeding, can also promote zooplankton development by releasing ephippia, which hatch once they reach more suitable aerobic conditions in the surface layers (Hairston et al. 1995). Both temperature and light are also important factors affecting when zooplankton eggs hatch and when peak biomass of zooplankton becomes available (Li et al. 1996). To ensure optimal growth rate in carp, it is important that fish are stocked during the peak period of natural food abundance in the spring (Valdenberg et al. 2006; see Figure 2).

SUPPLEMENTARY FEEDING AND ITS IMPACT ON NATURAL FOOD UTILISATION

Semi-intensive carp production is characterised by use of natural foods produced within the pond supplemented with additional feed that provides missing nutritional elements (e.g., dry matter, minerals, carbohydrates), prolongs the growing season, increases growth rate and increases yield-per-unit volume. In addition to their direct influence on fish growth (Billard 1999), supplemental feeds also benefit fish indirectly by providing nutrients that stimulate production of zooplankton and benthos (Hepher et al. 1989; Milstein 1999).

In general, as carp grow, use of supplementary feeds increases, with the result that carp often significantly reduce their intake of natural foods (Rahman et al. 2008b, 2010). As stated

previously, however, most fish farmers now recognise the benefits of naturally produced food in carp diet and much effort is now spent on balancing the input of allochthonous and autochthonous food sources and on improving the formulation of such feeds. Most commercial feeds now aim to mimic the composition of natural food, for example, with around 25-50% protein (including essential amino acids), 10-15% carbohydrates and 12-15% lipids (Jirásek and Mareš 2001a,b). Generally speaking, fish farmers now aim to achieve around 70–75% of carp growth through consumption of natural foods such as zooplankton and zoobenthos, and around 25-30% from supplementary feeding with (mainly) raw whole cereals such as wheat or barley (Adámek et al. 2012). More protein-rich artificial feeds may be provided near the end of the production season, before winter sets in (Woynarovich et al. 2010; FAO 2017a).

In recent years, significant progress has been made in fish nutrition through the use of pelleted and extruded feeds, their higher digestibility resulting in a lower feed conversion ratio and a reduction in excessive nutrients released into the pond system (Hardy and Barrows 2000). Such feeds (especially pellets) have been shown to provide an increase in weight gain (Cirić et al. 2013, 2015), improve meat quality (Zivić et al. 2014) and increase the abundance of zooplankton (especially Cladocera and Copepoda) and benthic macroinvertebrates while lowering the abundance of cyanobacteria (Cirić et al. 2013 2015). Surprisingly, Hlaváč et al. (2015, 2016) observed no difference in zooplankton abundance between ponds with and without supplementary feeding or between different feed types, though they suggested this may have been due to increased turbidity from bottom-feeding adult carp inhibiting zooplankton ingestion of phytoplankton and masking any potential change. On the other hand, some studies have documented an effect on zooplankton composition when wheat, corn and barley are used as supplementary feed, with rotifers (e.g., *Keratella*), a species of no importance in adult carp diet, often coming to dominate the community (e.g., Adámek et al. 2004). While some copepods are known to predate on rotifers, *Keratella* sp. are resistant to such predation (Plassmann et al. 1997); hence, their dominance was most likely the result of high carp predation pressure on cladocerans, which are superior competitors than rotifers (Gilbert 1988; Christoffersen et al. 1993). According to Másílko (2014), the density, biomass and species diversity of zooplankton and zoobenthos were richer when carp were fed a mixture of mechanically treated and raw cereals.

In Central and Eastern Europe, supplementary feed is usually spread from a barge moving along a linear route, three to six days a week. The actual amount of food delivered will vary depending on water temperature and oxygen concentration, fish size and periodic analysis of zooplankton quality and quantity accompanied by *in vivo* evaluation of carp gut content (Faina 1983; Adámek et al. 2014). In late summer and autumn, as food requirements increase and availability of natural food begins to decline (see Figure 2), the amount of supplementary food provided will increase and may be spread over a wider area (FAO 2017a). While supplementary feeding has been in use for some time now, there is a surprising lack of information available on how carp actually respond to this feeding style. Anecdotal information, for example, suggests that carp quickly learn where food is easily available (e.g., at repeatedly used supplemental feeding sites) and reduce their ranges accordingly. Two recent studies (Adámek et al. 2016; Jurajda et al. 2016) have confirmed that concentrated and repeated supplementary feeding at one main site does indeed result in heavy aggregations of carp.

While activity patterns revealed by telemetry indicated high individual variability, the general pattern of activity was very similar across fish, with carp utilising the whole pond area (regardless of size or age) but with the greatest proportion of readings for almost all fish centred on the supplementary feeding area during the feeding period (Figure 3). Those fish excluded from such sites (e.g., through poorer competitive ability) relied more heavily on natural foods and, as a result, showed lowered growth rates and fat content, resulting in poorer individual 'quality' and uneven stock size composition (Jurajda et al. 2016). There was also an increased likelihood of water quality issues near the feeding area as turbidity was increased through heavy bottom stirring and overcrowding, potentially leading to eutrophication, pH and oxygen peaks/troughs and reduced photosynthesis. Feeding sites also showed significantly lower dissolved oxygen values, which dropped below the physiological optimum for carp (3 mgl^{-1} O_2) on hot summer days shortly after feed application (Adámek et al. 2016).

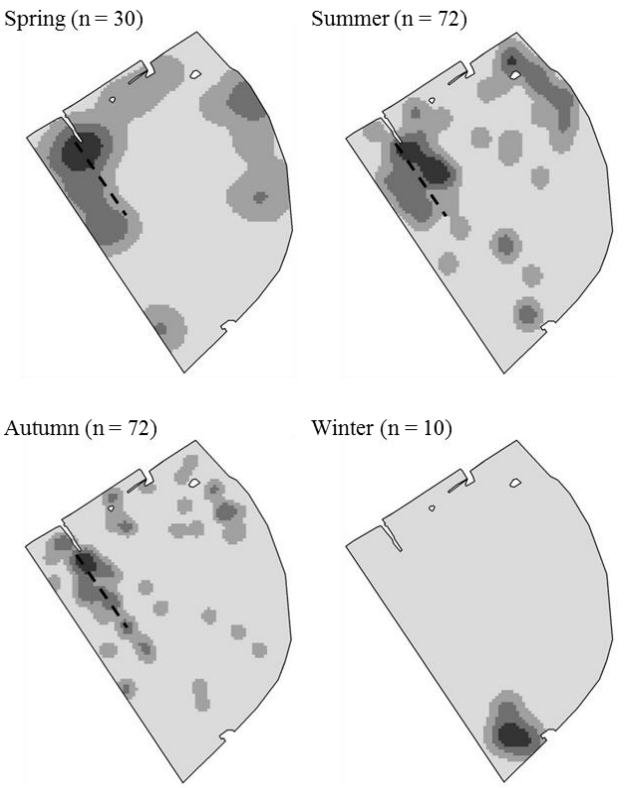

Figure 3. Concentration of carp activity (telemetry with kernel analysis; 10 carp; numbers in parentheses represent positive readings; dark areas represent concentrated activity) in a Czech semi-intensively managed pond. The dashed line indicates the main supplementary feeding route. Note there was no supplementary feeding in winter; all winter readings are from deeper water under the outlet (adapted from Jurajda et al. 2016).

Overall, zooplankton appeared to be underutilised, with biovolume varying only slightly between feeding and non-feeding sites. Macrozoobenthos density and biomass, on the other hand, were significantly lower at feeding sites; presumably as carp concentrating at such sites

were ingesting cereal and macrozoobenthos from the bottom without distinguishing between them. The only exception to this pattern was in June, when there was no significant difference between feeding and non-feeding sites due to a considerable increase in chironomid numbers prior to emergence (Matěna 1990). In general, carp activity was more concentrated at the feeding area in spring and more evenly spread over the pond in summer and autumn, when feed tended to be spread more widely (Adámek et al. 2016; FAO 2017a).

Diet analysis showed that grain represented between 69 and 99% of ingested food at the feeding sites, with no grain found in fish from the non-feeding sites. Interestingly, a similar trend was observed after four days with no supplemental feeding (September), with grain still making up 36% of the diet in carp collected close to feeding sites and no grain found in fish at non-feeding sites, suggest that there was still an excess of grain available to carp. Natural food, represented mainly by zooplankton, always made up a greater proportion of the diet at non-feeding sites (61–53%) during those periods when supplemental food was being supplied, though fish at the feeding sites still included between 11 and 29% zooplankton in the diet. In general, fish at the non-feeding sites also had a wider diet (e.g., benthos, etc.), bringing the proportion of natural foods up to 57–82% overall at non-feeding sites. When grain was spread more evenly (summer/autumn), however, zooplankton consumption dropped dramatically at both feeding and non-feeding sites, not exceeding 0.5% by proportion in either area. After a period of no supplemental feeding, the proportion of zooplankton remained relatively stable at non-feeding sites but rose considerably at feeding sites (up to 29%). During the summer and autumn, grain comprised around 93% of the diet at both feeding and non-feeding sites, indicating a clear preference for grain over natural foods when available and when competitive exclusion was reduced (Adámek et al. 2016; Jurajda et al. 2016; Table 4).

Table 4. Diet composition of carp sampled by electrofishing from supplemental and natural feeding areas in a Czech semi-intensive fishpond. SFA = supplemental feeding area, NFA = natural feeding area (Table adapted from Adámek et al. 2016)

	July 2014		August 2014		September 2014	October 2014	
	NFA	SFA	NFA	SFA	NFA	SFA	SFA
Erpobdella sp.					+		
Bithynia tentaculata					1.18		
Plumatella repens	19.46				3.26		
Zooplankton	60.99	11.27	0.08	0.41	52.56	29.19	1.72
Chironomus plumosus	0.17	0.69				0.8	0.05
Orthocladiinae g.sp	1.22						
Σ natural food	81.84	11.96	0.08	0.41	57.00	29.99	1.77
Plant debris					2.56	0.34	
Detritus	18.16	19.39	6.29	0.57	40.45	34.04	1.78
Sand		0.13					
Σ emergency food	18.16	19.52	6.29	0.57	43.01	34.38	1.78
Cereals (feed)	0.00	68.52	93.63	99.02	0.00	35.63	96.45
	Regular feeding at SFA only		Feeding spread over SFA and NFA		After 4 days of no feeding	Regular feeding at SFA only	

While the two studies reported above (Adámek et al. 2016; Jurajda et al. 2016) examined a series of semi-intensive ponds in the Czech Republic, the dietary patterns and impacts reported are likely to be common to all carp ponds managed in a similar manner elsewhere. In general, these studies showed that concentrated and repeated supplementary feeding with grain or artificial feed in one small area results in aggregations of carp, potentially leading to uneven population growth, body condition and flesh quality, uneven utilisation of natural food resources and reduced water quality from turbidity, increased nutrient input and localised reductions in dissolved oxygen.

As the financial sustainability of fish farming depends mainly on market prices and production efficiency (Bosma and Verdegem 2011), future sustainability requires a reduction in costs, along with an increase in production intensity and efficiency, without harming the aquatic environment. Up until now, it has generally been assumed that up to 75% of carp growth under semi-intensive conditions is provided by natural food items (Adámek et al. 2012), and carp farmers have tended to base their management decisions on such figures. The studies outlined above, however, indicate that this is not necessarily true, at least not for all carp in the pond. As a means of ensuring the future sustainability of carp pond farming, therefore, Adámek et al. (2016) and Jurajda et al. (2016) suggested that both water quality and carp quality could be improved by spreading less supplemental feed over a greater area at wider time intervals. This would ensure full utilisation of supplementary feed (reducing economic costs and increasing water quality) and a more homogenous spread of fish over the pond, thereby ensuring more efficient use of natural food (further reducing the amount of supplemental feed needed), reduced turbidity from concentrated feeding (further improving water quality) and more homogenous size/weight distribution and improved meat quality.

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Chapter 96

ZOOPLANKTON ECOLOGY IN COMMON CARP PONDS

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ABSTRACT

Common carp (*Cyprinus carpio* L.) aquaculture is performed almost exclusively in earth ponds to benefit from the natural resources for fish growth and development. Since zooplankton organisms are important
components in these freshwater aquatic habitats, the aim of this chapter is to present a review about the relevance of zooplankton and its interactions with cultured carp and the carp pond environment. Zooplankton constitute one of the major protein sources in carp diet with varying importance depending on the availability (density, biomass, composition and size structure), fish age, season and environmental variables. On the other hand, as carp are size-selective predators, they can strongly modify composition and structure of zooplankton communities, impeding the presence of vulnerable taxa which leads to increasing abundance of zooplankton taxa that can avoid predation. The strength of the predation pressure is also related to pond management measures, depending on parameters such as fish density, supplementary feeding or fertilization (manuring). In addition, zooplankton community dynamics vary along the whole growing season as a result of carp body size and total biomass increase. Zooplankton and its resting stages may also be affected by bioturbation caused by carp searching for benthic food organisms in pond sediments. Finally, the position of zooplankton organisms in intermediate levels of pond food chain predestines them to act as good indicators of water

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quality, especially with respect to eutrophication processes that are becoming steadily more common in carp ponds.

Keywords: common carp, stocking density, copepods, cladocerans, freshwater habitats, natural resources, supplementary feeding, water quality

INTRODUCTION

Common carp (*Cyprinus carpio* L.) aquaculture is performed almost exclusively in earth ponds to benefit from the natural resources for fish growth and development (Figure 1). It is a traditional aquaculture widely extended in Central and Eastern Europe and Asia.

Zooplankton are important inhabitants of carp ponds. They play a key role in aquatic ecosystems, holding the central position between primary producers and higher trophic levels. They have an essential function in transferring carbon and energy from phytoplankton to fish, contributing to the recycling of nutrients through food chains in freshwater and marine water bodies (Attayde and Hansson 1999; Harris et al. 2000). Due to a high dispersal capacity, through wind, rain, birds and even reaching long distances (e.g., 1000 km) through humans (Bilton et al. 2001; De Meester et al. 2002), zooplankton can readily colonize a wide variety of habitats. Therefore, zooplankton communities are found in most freshwater bodies, ranging in size, productivity, type of fish community and geographical location (Dodson 1992; Hessen et al. 2006).

Compared to marine populations, freshwater zooplankton is less diverse in terms of phylogeny. Freshwater zooplankton comprises organisms with limited swimming ability and ranging from 0.2 to 2 mm (meso-zooplankton; Harris et al. 2000). The main taxa included are from the phyllum Rotifera, and the sub-phyllum Crustacea (sub-class Copepoda and infra-order Cladocera). Most species are filter-feeders, feeding on small algae, bacteria and detritus, but some copepods are either selective feeders, selecting small algae or bacteria, or predators, preying upon small rotifers and cladocerans, or even other copepod species (Fryer 1957b). Cladocerans and rotifers reproduce through cyclical parthenogenesis (Gliwicz 2004): after a period of propagation through parthenogenesis, a type of successful asexual reproduction mode (Decaestecker et al. 2009), they can reproduce sexually when females produce fertilized eggs (Harris et al. 2000). This allows these species to reach high-density values in a short time through parthenogenesis; but also to restore genetic variation through sexual reproduction (De Meester et al. 2006). On the contrary, copepods only have sexual reproduction (Gyllström and Hansson 2004). During their life cycle, many species of zooplankton have a period of diapause, or a state of dormancy by producing dormant stages that provide survival when unfavorable external conditions arise, e.g., drought, low temperature and oxygen, predation, food limitation etc. (Brendonck and De Meester 2003). In fish ponds, which are usually dried for longer periods, this diapause period is especially relevant, since it will allow the rapid colonization of the pond once it is filled again the next growing season. Apart from this autochthonous source of zooplankton, most of the ponds are filled with water coming from other ponds or other water sources, with the subsequent associated organisms (allochthonous source). The combination of these two sources of zooplankton populations results in the rapid development of zooplankton assemblages in carp ponds, and influences the abundance, biomass and zooplankton species composition. Thus, it

is recommended to monitor inlet water variables to ensure that it has the appropriate parameters for zooplankton development, since certain conditions, such as high conductivity and low hardness that can arise in water coming from deep tube wells, can result in a significant decrease in the size of the zooplankton community (Dulić et al. 2014).

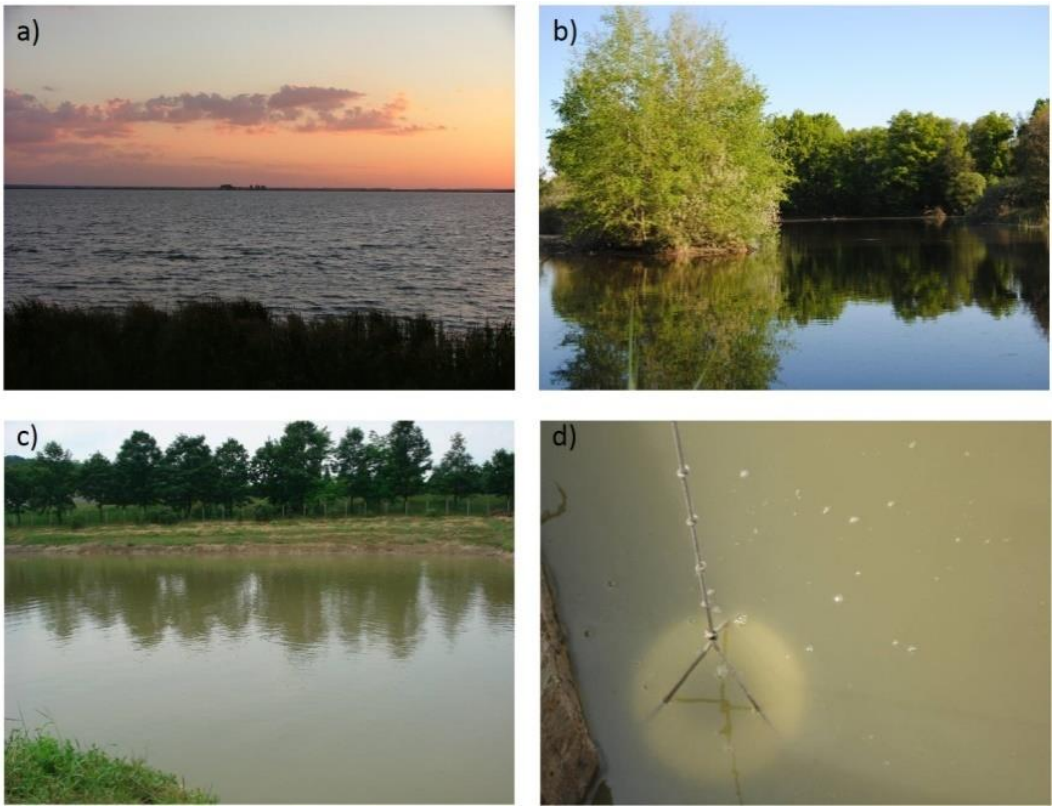

Figure 1. Pictures of a variety of carp ponds: a) pond Belo jezero at Ečka fish farm, Serbia (photo by Z. Dulić) b) pond Horak in Třebon, Czech Republic (photo by M. Anton-Pardo); c) Experimental carp pond at faculty of Agriculture School Estate, University of Belgrade (photo by Z. Dulić); d) Secchi disk measuring water transparency in a carp pond at faculty of Agriculture School Estate, University of Belgrade (photo by Z. Dulić).

The aim of this chapter is to provide a review about the ecology of zooplankton in common carp ponds. Firstly, the interaction of zooplankton with carp stock will be reported, highlighting the importance of zooplankton as natural food for carp growth, but also how carp stock and pond management influence zooplankton ecology. Other interactions such as predation of copepods upon carp fry or parasitic interactions will be also described. Secondly, the interaction of zooplankton with the environment in carp ponds will be reviewed: how zooplankton can modify the ecosystem variables, and how the variation in environmental variables affects them.

INTERACTIONS ZOOPLANKTON-CARP

Zooplankton as Food for Carp

Common carp is considered omnivorous, with a high proportion of animal food in its diet (Michel and Oberdorff 1975). Many studies point out that adult carp feed on benthic organisms, mainly chironomids (Michel and Oberdorff 1975; Rahman 2008a), but at different life stages, zooplankton of different size may be an important part of the diet of carp, depending on their availability in a given fish pond (Adámek et al. 2003; Rahman et al. 2006; Kloskowski 2011; Anton-Pardo et al. 2014).

In carp larvae, the yolk sac consumption period is relatively short (3-5 days), so the beginning of feeding on external sources of nutrients is much earlier compared to other fish species. This is usually related to the diameter of fish eggs, so larger eggs determine a bigger and longer lasting yolk sac that provides fish larvae with endogenous nutrients up to three weeks after hatching (Lavens and Sergeoloos 1996). Additionally, the dimension of the eggs also reflects the mouth gape size at first feeding of larvae. As in most teleost fish, common carp larvae are quite small (around 5 mm in length after hatching; Jones and Houde 1981), and have a small mouth gape (0.3 – 0.4 mm; Dabrowski et al. 1983) preventing them from ingesting large prey. Thus, they can feed only on the smallest zooplankton organisms, such as rotifers and copepod nauplii (Nunn et al. 2012). The development of the gill rakers (size and interraker spaces) is usually related to the increase of the mouth gape, so they gradually modify the retention of prey of certain size. The gill raker slits in early carp larvae are less than 0.21 mm, while in older carp they are at a minimum of around 0.78 mm (Dabrowski and Bardega 1984). This increased gill raker-slit size enables adult carp to retain zooplankton organisms such as large cladocerans, with body size length around 0.500 mm, mainly *Daphnia* species, while smaller forms pass through the raker slits (Dabrowski and Bardega 1984).

Different authors (Osse et al. 1997; Vilizzi and Walker 1999 and references therein; Nunn et al. 2007) investigated the onset of carp feeding on bottom fauna and reported ranges from 8 to 25 mm total body length. This shift in feeding preference is significantly related to changes in the position of the mouth from terminal in larvae to downwardly projected in older carp (Vilizzi 1998). This is also understood to be a transitional period from larval to juvenile carp. However, the preference towards different prey in carp juveniles and adults depends largely on their availability in the environment and other variables, such as the use of different microhabitats for feeding or physiological changes (Nunn et al. 2012; Anton-Pardo et al. 2014). For instance, in new fish ponds with poorly developed sediment, zoobenthos is scarcely present, thereby carp feed mainly on microcrustaceans (Dulić et al. 2011).

Regarding nutritional content, for early larvae of *Cyprinus carpio*, which are characterized by an underdeveloped digestive system, zooplankton play an important role as a source of exogenous enzymes, improving the digestion of complex molecules in larval gut (Delbare and Dhert 1996; Kolkovski 2001). Several authors (Dabrowski and Glogowski 1977; Lauff and Hofer 1984; Pedersen et al. 1987; Munilla-Moran et al. 1990) investigated digestion processes in larvae of different fish species and found between 40 and 80% of enzymatic activity was provided by live food organisms. Additionally, zooplankton

organisms have gut neuropeptides and nutritional 'growth' factors, which also improve fish digestion (Kolkovski 2001).

Zooplankton is also a valuable source of amino acids, especially for early life stages of common carp, but it is also a significant provider of highly unsaturated fatty acids (HUFA) that are pivotal for the survival and growth of larvae (Olsen 1999; Sargent et al. 1999; Brett et al. 2009; Anton-Pardo and Adamek 2015). Some authors reported that the essential and non-essential amino acid content of zooplankton can meet the general requirements of farmed fish (Delbare and Dhert 1996; Kibria et al. 1999; Ovie and Ovie 2006; Mitra et al. 2007). Mitra et al. (2007) found that methionine was only slightly lower in mixed zooplankton from earth carp ponds compared to the amino acid requirements of common carp (NRC 1993). Furthermore, since amino acid content is species specific (Guisande 2006), and, generally, not influenced by food (Mitra et al. 2007), feeding on a greater variety of zooplankton species could provide a more balanced protein nourishment for fish in ponds.

In contrast to amino acids, lipid content in zooplankton and fish reflects the lipid profile of their diet (Sekino et al. 1997; Steffens and Wirth 2007; Bauer and Schlott 2009; Burns et al. 2011). For zooplankton, the accumulation of highly unsaturated fatty acids is strongly influenced by both, the quality of phytoplankton ingested and the taxonomic group. Several studies have found that cladocerans are rich in eicosapentaenoic acid (EPA) and arachidonic acid (ARA), while copepods have a higher content of docosahexaenoic acid (DHA) (Persson and Vrede 2006; Smyntek et al. 2008; Burns et al. 2011). Additionally, both zooplankton groups accumulate a similar level of α-linolenic acid (ALA; Smyntek et al. 2008; Kainz et al. 2009) which is significant for carp nutrition since they can convert ALA to n-3 HUFA's (Tocher 2003). Trbović et al. (2017) reported that at the end of the growing season in earth ponds, when zooplankton increased its proportion in the feed bulk, there was a strong positive influence on the level of n-3 HUFA in the dorsal muscle of farmed carp. Thus, even a small increase in natural food availability compared to supplemental feed can significantly result in improved carp meat quality.

Influence of Pond Management on Zooplankton Ecology

Fish Stock Density and Biomass

The density and composition of fish stock is a crucial factor influencing the quantitative and, especially, the qualitative composition of zooplankton assemblages in aquatic environments (Jepessen et al. 2011), including carp ponds. Composition of the pond zooplankton community is highly influenced by fish predation pressure, affecting not only its biomass (Figure 2), but also the density and species composition (Figure 3). At the beginning of the growing season when carp food intake is limited by water temperature, larger species of cladocerans, such as Daphnia magna, D. pulicaria or *Simocephalus vetulus*, are abundant (Potužák 2009). Cladocerans of the genus Daphnia are exceptionally efficient in reducing food resources (phytoplankton and bacterioplankton) and out-competing smaller zooplankton species (Brooks and Dodson 1965). Generally, adult common carp reduce the abundance of large-bodied zooplankton either directly (Lewkowicz and Zurek 1991; Rahman et al. 2006) or indirectly through increased nutrient load and increased turbidity which inhibits phytoplankton development and its ingestion by filter-feeders (Lougheed 1997). In contrast, smaller planktivorous carp commonly reduce large-bodied zooplankton density directly

through predation (Chumchal et al. 2005; Weber and Brown 2009; Carey and Wahl 2010). Fott et al. (1980) documented the changes in the development of zooplankton assemblages in a carp pond during two years. The first year, though the carp stock was abundant (800 – 1000 individuals ha-1), its biomass was low (~ 100 kg ha-1) and large daphniids prevailed. In the second year, carp stock density declined due to natural mortality (associated with winter temperatures), but its biomass was higher due to growth. As a consequence, the strong predation pressure led to the elimination of large crustaceans and its replacement by smaller zooplankton taxa (*Daphnia galeata*, *Ceriodaphnia spp.*, *Bosmina spp.*, Copepoda and Rotifera). Exceptionally, Parkos et al. (2003) recorded higher values of zooplankton biomass in experimental enclosures with carp compared to fishless control. However, the carp used for the experiments were between 2 and 2.5 kg, and thus too big to provide effective top-down control of zooplankton development.

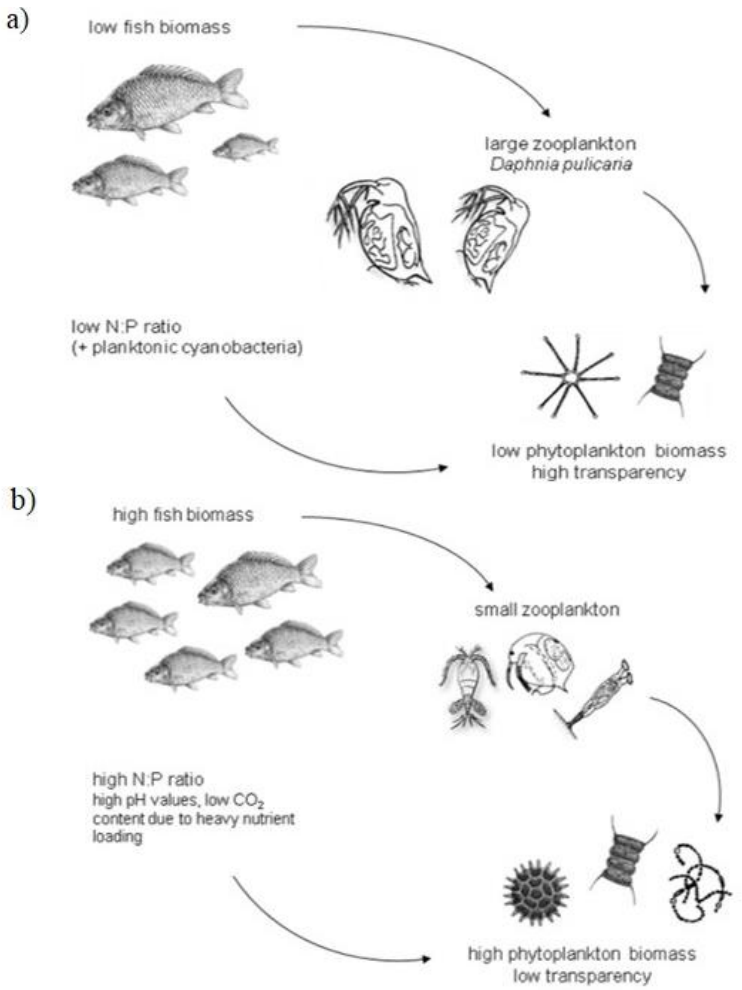

Figure 2. Main features of development of a pond ecosystem with high (a) and low (b) fish stock predation pressure (Adámek et al. 2014). Note: small zooplankton < 2 mm; large zooplankton – cladocerans > 2 mm).

The crucial role of common carp in pond ecosystem functioning was proved by experimental three-year exclusion of carp from a hypertrophic pond (Pechar et al. 2017). In general, common carp exclusion resulted in the dominance of large filtering zooplankton (genus Daphnia) keeping the pond ecosystem in a "clear water" state. In the second growing season, a rapid onset of submerged macrophytes (mainly *Ceratophyllum demersum*) was observed. However, pond stability was threatened by oxygen deficiencies, and especially, by the invasion of the omnivorous r-strategist topmouth gudgeon (*Pseudorasbora parva*), which led to zooplankton disappearance, cyanobacterial blooms of *Anabaena sp.*, and macrophytes disappearance. The experiment clearly demonstrated how vulnerable the carp pond ecosystem can be, if stocking is not appropriately managed.

However, in lower trophic states, which are generally very rare in carp ponds, the response of zooplankton community to fish density is quite different, as shown by Christoffersen et al. (1993). In fishless enclosures, total zooplankton density was significantly higher, except for rotifer density, which was lower, probably as a combined effect of mechanical interference with Daphnia and food competition (Gilbert 1988), but also as a result of copepod predation (Plassman et al. 1997). In contrast, cyanobacteria (Microcystis, Aphanizomenon and Anabaena species) bloomed in enclosures with fish.

Increased biomass of planktonic cyanobacteria is often a consequence of increased fish predation pressure on cladocerans, impacting nutrient concentrations and zooplankton food resources ("edible" phytoplankton) and food selectivity (Gragnani et al. 1999; Gasiunaite and Olenina 1998). Cyanobacterial water blooms, however, may enter also into the carp pond trophic chains with negative impacts on zooplankters, such as cladocerans, as shown by Hietala et al. (1997), Lauren-Maatta et al. (1997) or Claska and Gilbert (1998). Nevertheless, Nandini and Rao (1998) have demonstrated the tolerance of cladocerans to high Microcystis biomass resulting from genetically increased resistance to cyanotoxins and increased digestibility of Microcystis.

Despite all these documented results, a review of 119 studies by Vilizzi et al. (2015) revealed inconsistent evidence for impacts of carp on zooplankton, showing the high variability on zooplankton dynamics in particular.

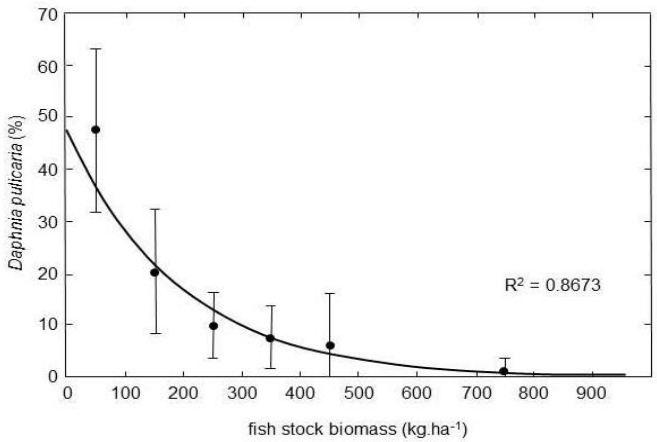

Figure 3. Relationship between percentage representation of Daphnia pulicaria in zooplankton and a stock biomass in a carp pond (adapted from Potužák, 2009).

As the changes in zooplankton community structure and biomass invoked by carp grazing pressure are very relevant, the analyses of zooplankton quality and quantity is necessary for the implementation of appropriate management steps in supplementary feeding on carp pond. The in vivo evaluation of carp gut content is often used for the support of proper subsequent decision (Faina 1983).

Increased ingestion of zooplankton by carp can also be associated with limited benthic food resources, since carp have been shown to shift their feeding niche from the bottom to the water column and feed principally on zooplankton in the absence of benthic macroinvertebrates (Rahman et al. 2010).

In general for zooplankton diversity, the highest values are found in ponds with a low to medium level of intensification (Přikryl 1996). In such ponds, sufficient extent of aquatic vegetation is often maintained and there is also a sufficient food on offer for filtering zooplankton. Due to high species richness and diversity of littoral zooplankton, reflecting the development and extent of submersed and emerged vegetation, community structure in littoral areas is often a better indicator of the biological value of a pond than the structure of pelagic zooplankton.

To summarize the available knowledge about common carp–zooplankton interactions, it is evident that the response of pond zooplankton assemblages is essentially influenced by carp density. This is seemingly in a certain contradiction with the conclusions of Vilizzi (2015); however, his conclusions (see above) have been acquired by the analysis of the results from both natural (mostly) and pond farming conditions. Thus, the effect of carp stock is poor and unpredictable under natural conditions and low stocking densities, while strong and apparent effects are noticeable under pond farming conditions (e.g., Potužak et al. 2007, Potužak 2009).

Supplementary Feeding

Although basic fish nutrition in earth ponds remains based on natural food resources, supplementary feeding is also commonly provided, with the aim of supplementing nourishment to farmed fish. The density of carp stock can be doubled if supplementary feed is provided (Rahman 2015). Regular supplementary feeding in designated feeding places often reduces feeding activities of carp, so they concentrate on feeding places, which reduces the use of natural food resources including zooplankton, across the entire area of the pond (Adámek et al. 2014, 2016; Jurajda et al. 2015). Several studies confirmed that the sufficient amount of artificial feed in pond environment shifts the feeding behavior of carp (Rahman et al. 2006, 2008b, 2010) reducing the predation pressure on the biggest zooplankton. A recent study by Ćirić et al. (2015) reported a significant increase in the abundance of large-sized cladocerans and a decrease in the density of phytoplankton in ponds receiving high protein pelleted feed stocked with intermediate densities of common carp. However, the trend towards the intensification of semi-intensive carp production through favoring supplementary feeding regimes have negative implication on the exploitation of natural food - zooplankton by older carp (Anton-Pardo and Adámek 2015).

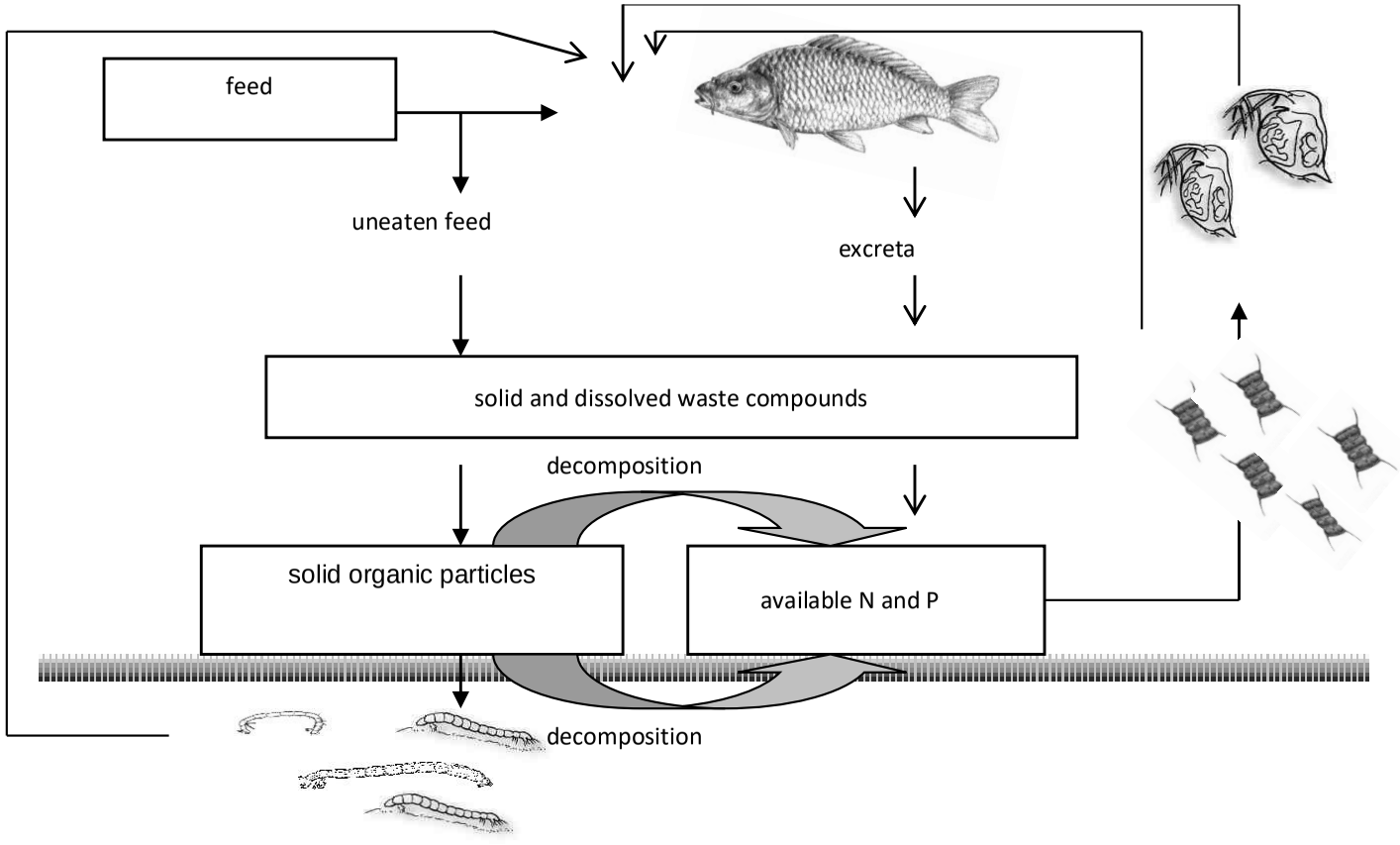

Figure 4. The fate of feeds applied in a carp pond with semi-intensive fish farming (adapted from Rahman 2006).

From the hydrobiological point of view, supplementary feeding also represents a certain nutrient loading to the pond ecosystem (mainly phosphorus to consider the needs of the carp individuals; Figure 4, Adámek et al. 1997). Therefore, zooplankton abundance may be also increased by nutrient input leading to a rise in phytoplankton growth (Moriarty 1997; Milstein et al. 2002). It is not clear, however, whether this impact is stronger than the effects of fish predation on zooplankton (Schindler 1992; Khan et al. 2003). Hlaváč et al. (2016) reported that the addition of nutrients through supplementary feeding may stimulate algal growth, though this response may have been masked by high zooplankton predation pressure or by increasing turbidity caused by carp benthic feeding.

Fertilization

The objective of pond fertilization is to supply macro- and micro-nutrients necessary for natural primary production, and thus, increase invertebrate development. Strong influence of artificial fertilization on zooplankton in ponds may, however, confound cascade effects (Havens 1993; Khan et al. 2003). As a result, the undesirable high trophic level is manifested mainly in the summer period when cyanobacteria often dominate phytoplankton. This results in instability and low efficiency of productive processes due to poor transfer of nutrients and energy in pond food chain, since cyanobacteria dominance represents a source of primary production which cannot be effectively used in higher trophic levels. As a consequence of inappropriate phytoplankton food resources on one side, and heavy predation pressure by fish on large species on the other side, zooplankton usually have an unsuitable structure (dominated by small species) as carp food. Nutrient input can also benefit the so-called microbial loop, since organic substances produced in the system are decomposed by bacteria, which are consumed by live heterotrophic flagellates and ciliates consumable by large cladocerans. Nevertheless, it causes a considerable increase in overall heterotrophic activity and risks of oxygen deficits (Pechar et al. 2002; Adámek et al. 2014).

Zooplankton Succession in Carp Ponds

The successional patterns of zooplankton populations in different habitats are influenced by a combination of abiotic (temperature, oxygen concentration, nutrients etc.) and biotic factors (competition and predation; Sommer 1989). In artificial water bodies with a considerable level of management, such as earth carp ponds, zooplankton community is shaped by several factors such as stocking density, age of carp, water quality, pond management and supplementary feeding (Sommer et al. 1986).

As size-selective predators, carp significantly modify zooplankton communities in these habitats. The most vulnerable taxa to predation are large cladocerans such as individuals of the genera *Daphnia* or *Simocephalus*, that are more conspicuous, especially females carrying eggs (Gliwicz 1994; Gliwicz 2004) or those with ephippia (cladoceran resting eggs) due to the intense pigmentation (Lazzaro 1997). These larger taxa develop at the beginning of the growing season, when water temperatures are still low and predation pressure by carp is not high. When temperature rises, a rapid depletion of large herbivorous cladocerans in nursery and on-growing ponds (for one- and two-year-old carp, respectively) releases the competition for zooplankton food resources (phytoplankton), leading to the domination of small-sized

cladocerans, e.g., *Bosmina* (Figure 5a), *Ceriodaphnia*, and *Daphnia longispina* and *D. galeata*, a variety of rotifers, small cyclopoid copepods and their larval stages -nauplii and copepodites- in particular (Figure 5b). The eutrophic conditions in carp ponds favor the midsummer shift towards blooms of large and inedible phytoplankton, mostly cyanobacteria, and unstable levels of oxygen concentration and pH (Potužak et al. 2007).

With knowledge of size and species composition of zooplankton community, it is thus easy to estimate fish stock density, its health status and/or composition. So occurrence of large planktonic filtrators (*Daphnia magna*, *D. pulicaria*, *D. pulex*, *D. longispina*), together with high water transparency, indicates weak predation pressure by carp stock due to low biomass, high mortality, incidence of diseases, or poor environmental conditions. On the other hand, it is possible to predict a high stock biomass or mass occurrence of small cyprinid fish, from great occurrence of small zooplankton (nauplii, small cladocerans and rotifers) together with low water transparency, which it usually occurs in ponds at the end of the growing season (Potužák 2007; Adámek et al. 2014).

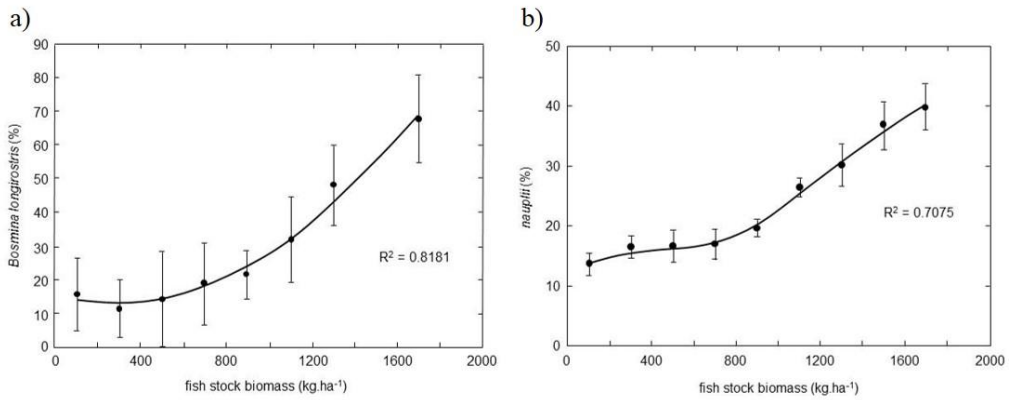

Figure 5. Regression between fish stock biomass and the percentage of the cladoceran *Bosmina longirostris* (a) and copepod nauplii (b) (Potužák, 2009).

In carp nursery ponds, natural succession of zooplankton is usually manipulated in order to achieve the desirable development with regard to their size and density. The early larvae are vulnerable to predators and diseases present in the pond environment (Section 2.4). Additionally, small carp larvae can ingest only the smallest zooplankton organisms, such as rotifers, at the beginning of feeding. Different pond management practices as fertilization and specific zooplankton inoculation are used to couple fry feeding needs and zooplankton size (Ludwig 1999; Morris and Mischke 1999). The right timing between the larval developmental stages and zooplankton of matching size is crucial for the success of the rearing process (Valdenberg et al. 2006; Antón-Pardo and Adámek 2015). The best results, in terms of good larval growth and development, are obtained by the gradual succession of zooplankton starting with rotifers and copepod nauplii, followed by small cladocerans and copepods, and larger cladocerans, mainly daphnids, thereafter. A carefully monitored fertilization regime will provide enough nutrients for phytoplankton development and it will increase zooplankton densities (Jana and Chakrabarti 1997; Kaur and Ansal 2010). However, the abundance and composition of zooplankton emerging from both pond sources, internal (dormant stages in the

sediment) and external (water input), during the pond inundation cycle (filling of the ponds with water), are quite unpredictable. A common practice among fish farmers, to ensure a large production of rotifers in nursery ponds and the elimination or reduction of crustacean competitors for food resources (cladocerans and copepods) and potential predators of carp larvae, is the application of agricultural insecticides a week before stocking the larvae (Woynarovich et al. 2010). However, application of pesticides and other chemicals should follow the regulations for each country and it should be applied in the correct dosages and time, to avoid undesirable side-effects upon non-target aquatic organisms. Specific zooplankton inoculation or reseeding can also be applied if the density of required zooplankton is not obtained by the above-mentioned measures (Billard 1999).

Other Interactions

Predation of Copepods on Carp Juveniles

One of the negative effects of zooplankton on common carp populations is represented by predation on fish larvae. Many studies point out the predatory behavior of adult copepods belonging to certain genera, such as *Eudiaptomus*, *Mesocyclops*, *Acanthocyclops*, *Cyclops* or *Diacyclops* (Fryer 1957a; Brandl 2005). These are widespread genera commonly found in freshwaters, and specifically, in carp ponds (Dulić et al. 2009; Adámek et al. 2014). A few number of studies report predation of copepods upon age-0 fish of several species (Hartig and Jude 1984; Cooper 1996; Brandl 1998; Fregadolli 2003), with copepods attacking prey much larger than the attacker itself (up to 15 times larger; Cooper 1996). The damage caused to the fish is more significant when copepod densities are high (Fabian 1960; Frimpong and Lochmann 2005). In addition, copepod attacks are a factor of stress for fish fry, which can negatively affect larval growth (Fregadolli 2003). This could be one of the causes of serious mortality in fish early stages, since in experimental studies, a mortality of fry caused by copepods between 25 and 50% has been found (Fabian 1960; Brandl 1998; Fregadolli 2003).

Regarding common carp, there are just a few studies reporting copepod predation on larvae. In an experiment with the cyclopoid copepod *Acanthocyclops robustus* and 10 individuals of 1-day-old common carp, an average of 2.2 individuals were dead in 5 days (Piasecki 2000). This same study reports that cyclopoids were able to prey upon larvae up to six days. Additionally, the presence of alternative preys influenced the larval mortality: in the presence of *Daphnia magna* and rotifers, the predation effect on carp larvae was lower (average reduction of 1.7 larvae in 5 days; Piasecki 2000). Therefore, predatory copepods may strongly affect the survival of cultured common carp, especially in spawning and nursery ponds, despite that the density of rotifer and small cladoceran (as alternative prey) could be high in these ponds.

Management strategies to avoid high copepod predation rates on small carp larvae include the pond bottom drying, disking and liming; monitoring the species composition and abundance of zooplankton in the input water; and stocking of fish prior to the development of large zooplankton populations (Fregadolli 2003; Piasecki et al. 2004).

Zooplankton as Parasites and Disease Vector

Freshwater copepod species of the genus *Lernaea*, *Lamproglena* (Lernaeidae, Cyclopida) and the families Lernaeopodidae (Siphonosto-matoida) and Ergasilidae (Poecilostomatoida) are known fish ectoparasites, and some of these species can be found in common carp (Hudson and Bowen 2002; Piasecki et al. 2004; Gutiérrez-Galindo and Lacasa-Millán 2005; Boane et al. 2008; Davydov et al. 2011). Parasitic cyclopoids show modifications of the second antennae to facilitate the attachment on fish gills, skin and/or fins (Tedla and Fernando 1969).

Lernaeid copepods are cosmopolitan and they occur in all the continents (Piasecki et al. 2004). They are common ectoparasites, which cause lethargy, but they can also penetrate into fish tissues, resulting in heavy mortality and morbidity, especially in young fish, with subsequent economic losses (Hemaprasanth et al. 2008). The occurrence of these parasites can be high in common carp in different parts of the world (Gutierrez-Galindo and Lacasa-Millan 2005; Davydov et al. 2011). Nevertheless, Hemaprasanth et al. (2011) studied the susceptibility of common carp fingerlings to this parasite and they concluded that in monoculture, they were completely resistant, while in polyculture, all the individuals were infected. However, 25-35 days post infection parasites started to disintegrate and the fish were completely recovered.

Some copepods can also act as intermediate hosts of fish parasites, which penetrate into fish organisms via their food (Cooper 1996, Piasecki et al. 2004). However, the infection rate is usually very low: between 0.01 and 1.0% (Marcogliese 1995). On the contrary, cladocerans rarely are intermediate host for parasites (Marcogliese 1995).

Some of the parasite taxa using copepods (Cyclopidae and Diaptomidae) as intermediate hosts, can cause strong damage in common carp, such as the tapeworm of the genus *Bothriocephalus*, which can cause an inflammation of digestive tract, a reduction in feeding rates and growth, and even mortality in carps (e.g., Brandt et al. 1981). Other examples are the tapeworm *Ligula intestinalis* and the nematode species which cause philometrosis (*Philometra* spp. and *Philometroides* spp.; Piasecki et al. 2004; Moravec and Cervinka 2005)

Finally, possible negative impacts of zooplankton may also include the transmission of viral diseases to fish, although studies on this subject are scarce (Overstreet et al. 2009), and to our knowledge, absent in common carp.

INTERACTIONS ZOOPLANKTON-ENVIRONMENT

Impact of Zooplankton on Pond Ecosystem Health

Zooplankton can themselves modify the environmental conditions in the ponds, which can then lead to negative effects on carp growth. These effects would be detrimental when zooplankton density is extremely high, so their impacts are amplified.

For example, oxygen consumption by zooplankton increases at higher temperatures and with organism sizes (Blazka et al. 1982). The oxygen availability also decreases in eutrophic waters, which is the case for many carp ponds (Lampert and Sommer 2007). These facts, together with lower solubility of oxygen at higher temperatures and increased oxygen

consumption in high fish densities, could have consequences in oxygen availability in fish ponds, especially in the summer months (Blazka et al. 1982).

The excretion of nutrients by zooplankton can also modify the nutrient balance in fish ponds (Andersson et al. 1998). Ammonium and phosphorus excretion is positively correlated with temperature and negatively with body weight (Ejsmont-Karabin 1983; Gulati et al. 1989). In the case of fish ponds, the contribution to nutrient regeneration by zooplankton can be considerable, especially with high temperatures and when the community is dominated by small size organisms (Gulati et al. 1989). However, an excess in ammonium excretion could result in toxicity to common carp fry and adults, especially when pH is high, so the concentration of toxic ammonia (NH_3) increases. Despite this, common carp does not seem to be highly sensitive to this pollutant and it has physiological mechanisms to avoid its toxicity (Hasan and Macintosh 1986; Liew et al. 2013).

Zooplankton Organisms and Pond Water Quality

As ecosystem engineers, carp can widely modify the environmental characteristics of the aquatic habitats where they live (Matsuzaki et al. 2009). The strength of this influence depends on fish density and biomass (carp biomass above 50 kg ha-1 can produce impacts on water quality; Parkos et al. 2003; Mehner et al. 2004), but also on fish age, since carp larvae and fry are planktivorous organisms with a minor effect on nutrient concentration (Weber and Brown 2009). Bioturbation (Section 3.3) and carp excretion are other activities that release a considerable amount of nutrients, sediment, detritus and other organic matter to the water column. The increase in nutrients and the rise of chlorophyll concentration leads many carp ponds to eutrophic and hypertrophic states (Roozen et al. 2007; Rahman et al. 2008c; Matsuzaki et al. 2009).

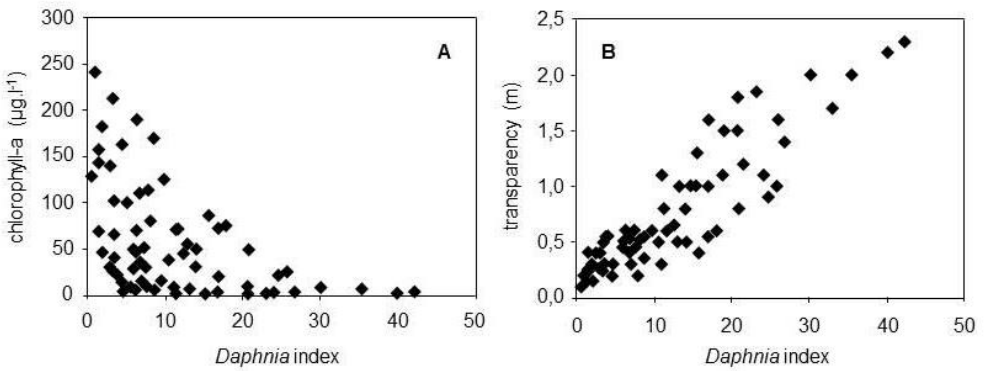

Figure 6. Relationship between the *Daphnia* index and chlorophyll concentration (A) and transparency (B) (from Potužák, 2009).

Traditionally, some fish species have been used to control the eutrophication and to prevent the appearance of toxic algae, such as tilapia or silver carp (Jancula et al. 2008; Ke et al. 2009). Recently, the use of zooplankton organisms for such purposes has been also proposed, since the capacity of zooplankton to filter great amounts of phytoplankton and

bacteria has been widely demonstrated when organism densities are adequate (Fukushima et al. 1999; Nandini et al. 2004). Integrated plankton-fish systems have been applied using recycling aquaculture systems where the wastes of fish tanks are used to grow algae for zooplankton consumption, which in turn, is used for rearing fish (van Rijn 1996, Gilles et al. 2013).

Zooplankton density is a good estimator of the pond productivity, since good production results are obtained when zooplankton and zoobenthos remain high throughout the whole production cycle (Woynarovich et al. 2010). Some studies use zooplankton community to assess water quality in carp ponds, using different metrics: e.g., species richness, zooplankton abundance, taxonomic composition, ecological functioning, diversity index or saprobic index (Dulić et al. 2009; Dulić et al. 2010).

In general, zooplankton abundance is positively correlated with increasing eutrophication, because the increase in abundance is mainly caused by the rise of rotifers and small zooplankton taxa (Haberman and Haldna 2014; Azevedo et al. 2015). Rotifers and copepod nauplii are normally less affected by algal blooms (common in nutrient-rich habitats) than large crustaceans, since they seem to be less susceptible to the toxicity of some cyanobacteria (Gilbert 1990; Ceirans 2007). Additionally, larger zooplankton taxa are more sensitive to water pollution, such as pesticides or heavy metals (Fleeger et al. 2003), so their absence in aquatic habitats may be used as indicator of water quality deterioration. Therefore, large cladocerans are one of the most widely group used as indicator of changes in water variables (Jeppesen et al. 2011). Suppressed abundances or absences of cladocerans in fish ponds have often been the result of increased pond eutrophication (Potužák et al. 2007), along with the gradual increase of fish stock in the last decades (Komarkova et al. 1986; Pechar 1995). These modifications involve changes in zooplankton community composition and abundance, increasing the abundance of cladoceran competitors. Thus, with a relative abundance of *Daphnia* spp. below 40%, the average body length of zooplankton decreases, caused by the dominance of rotifers, copepods and small cladocerans as Bosmina (Pechar 1995). In relation to algal community, when less efficient filter-feeding zooplankters dominate, the frequency of large colonial forms of algae and the trophic level of the ponds increase (Sipauba-Tavares et al. 2011). In contrast, when the abundance of *Daphnia* species is high, their filter-feeding efficiency results in the control of phytoplankton development, thereby increasing water transparency (Pechar 1995). Based on this fact, Potužák (2009) defined the so-called *Daphnia* index that expresses the relationship between the *Daphnia* size and its relative percentage in relation with total zooplankton, so it also defines their filtering capacity, largely dependent on their size. The *Daphnia* index demonstrates well the ability of zooplankton to control development of pond phytoplankton (Figure 6), with the exception of situations in hypertrophic ponds with high biomass of non-consumable cyanobacteria. Additionally, in some situations in fish ponds, large *Daphnia* can be recorded even with high fish stocks. This fact could be explained by the decreased predation pressure upon zooplankton when high protein supplementary feeding is used in the fish ponds (Rahman et al. 2006; Rahman et al. 2008b; Ćirić et al. 2015), together with a reduction in the visibility of carp in turbid waters (Potužák et al. 2007, 2008).

Influence of Bioturbation

Bioturbation is a common process in earth carp ponds. It is a result of the burrowing activity of benthivorous fish and/or different sediment-dwelling invertebrates that physically disturb and mix the sediments (Brönmark and Hansson 2005; Matsuzaki et al. 2007 and references therein; Adámek and Maršálek, 2013). Bioturbation initiates resuspension of sediments as well as aeration of the pond sediment, whereas it increases the turbidity of fish ponds and releases a substantial amount of nutrients and particulate organic material accumulated in the sediments to the overlaying water. To a lesser degree, resuspension can be promoted by wind induced wave actions in shallow waters (Avnimelech et al. 1999). Increased level of nutrients (particularly phosphorus and nitrogen) in the water can positively affect the zooplankton community through bottom-up processes via enhanced production of phytoplankton (Rahman 2015). However, nutrient overload and high turbidity of water can either interfere with large zooplankton filtering activity leading to the increase of small-bodied zooplankton (Kirk 1991), or can decrease the overall phytoplankton production due to the lack of light (Billard 1999).

In recently created earth carp ponds, zooplankton (active and resting stages) are mostly introduced from the adjacent water bodies that are used as a water source for the ponds. In older fishponds, a part of the zooplankton community comes from the internally formed dormant egg depositions in the sediment, usually up to a few centimeters (2 – 10 cm) thick. Some studies showed that fishponds that have been used for a long time could have more than 1000 resting eggs cm^{-2} of pond sediment (Li et al. 1996). In all groups of zooplankton, e.g., rotifers, cladocerans and copepods, an active egg bank exists, with egg densities in the range of 103 – 105 per m^2 of sediment (Brendonck and De Meester 2003). Deposition of dormant eggs happens regularly in natural water bodies that dry out periodically as it is the case of most carp ponds (Geiger 1983; Hairston 1996; Hairston et al. 2000; Brendonck and De Meester 2003; Gyllström and Hansson 2004; Ning and Nielsen 2011). Disturbance of the egg bank in the sediment caused by bioturbation can either bring the eggs towards the surface or can bury them deeper into the sediment (Gyllström and Hansson 2004).

To a lesser extent, benthic fauna can also contribute to the resuspension of particulate matter from the bottom (Adámek and Maršálek 2013), since in older fishponds the sediment layer is usually thick and supports a rich benthic fauna (mainly chironomid larvae, e.g., *Chironomus plumosus* and oligochaets as *Tubifex* sp. and *Limnodrilus* sp.; Dulić et al. 2011; Adámek et al. 2014).

Apart from bioturbation, pond management measures such as disking (harrowing), applied during pond drying in winter prior to filling with water in spring, can contribute to the translocation of zooplankton resting eggs either upward to the surface or deeper into the sediments (Dulić et al. 2014). If not buried deeper than 10 cm, viable dormant eggs of rotifers, cladocerans and copepods will hatch in spring during the fishpond inundation cycle, when triggered by suitable conditions, usually appropriate temperature and photoperiod (Hairston et al. 2000; Brendonck and De Meester 2003).

CONCLUSION

Zooplankton assemblages constitute an important component of aquatic ecosystems, and in particular, of carp ponds. Their interactions with the other inhabitants of these habitats, especially with carp, make zooplankton an essential factor not only for fish performance, but also to help keeping carp pond ecosystems stable and healthy.

Finding the right balance between the availability of zooplankton and the addition of supplemental feed is the biggest challenge in current carp pond aquaculture. Regular monitoring of the zooplankton community in the pond is a good tool for the adjustment of the amount of added feed. This can improve the exploitation of natural resources and at the same time, it can reduce the cost of supplemental feed. Additionally, the daily distribution of supplemental feed that is not restricted to fixed areas in carp ponds can shift the feeding habits of carp resulting in increased search for zooplankton present in other microhabitats of the pond. Adjusting the quantity of external feed provided to carp needs will impose a decrease in nutrient input in the carp ponds. This will lead to a lower trophic level in these habitats, improving the water quality, and ultimately, converting these aquatic ecosystems into biodiversity reservoirs for aquatic and other associated organisms.

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Chapter 97

HEAVY METALS AND MICROELEMENTS CONTENT IN COMMON CARP

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ABSTRACT

Over the last few decades there has been a growing interest in determining metals levels in the aquatic environment. Estimating heavy metals and microelements in fish is very important, particularly commonly consumed fish like common carp (*Cyprinus carpio* L.). A study was conducted to assess the level of bioaccumulation of heavy metals and microelements, and to analyze their distribution among different common carp organs from various locations. Bioaccumulation of Al, As, B, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sr, and Zn in common carp at four different localities along the Serbian stretch of the Tisa River revealed that their bioaccumulation occurs in different concentrations in various fish organs. Bioaccumulation of heavy metals and microelements are significantly different among various organs with the accumulation of heavy metals and microelements in gills and liver being higher than in the brain. It is noted that the average bioaccumulation was fairly uniform at all sites, with no significant variability for bioaccumulation across sampling locations. The difference among fish species from common carp, silver bream, sterlet and northern pike in relation to content of heavy metals and microelements was significantly different in common carp compared to silver bream, sterlet and northern pike. Based on these results it can be concluded that common carp have an important role in the biomonitoring of heavy metals and microelements levels in river ecosystems.

Keywords: common carp, heavy metals, microelements, rivers, biomonitoring

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INTRODUCTION

The contamination of aquatic ecosystems with metals has seriously increased worldwide attention into this issue (Karadede and Unlu 2000). Over the last few decades there has been a growing interest in determining metals levels in the aquatic environment.

Fish are one of the best bioindicators for estimating metals pollution and risk potential for human consumption in freshwater systems. They are considered an excellent biological marker of the increasing concentration of heavy metals and microelements in aquatic environments because they are constantly exposed to contaminants and occupy various trophic levels in food chains. Fish are often at the top of the food chain and may accumulate large amounts of metals. Also, given they are exposed immediately to any pollution event they represent an obvious sign that environmental impacts have occurred. They can be used to monitor the concentrations of the polluting substances accumulated over time (Simonović 2001).

Metals enter fish both in food particles and water via the gills and skin. Once in the body, they move into the blood stream and are carried to either a storage point or to the liver where they are transformed or stored. As a result of different mechanisms of adsorption, regulation, storage and excretion of metals, there are differences in the concentration of heavy metals and microelements in certain organs (Rao and Padmaja 2000; Storelli et al. 2006). Variations in the bioaccumulation of metals in various fish tissues are conditioned by a large number of factors: nutrition type, trophic status, source of each specific metal, remoteness of organisms from the contamination sources, presence of other ions (Giesy and Weiner 1997), food access (Chen and Folt 2000), occurrence of metallothionein and other proteins responsible for detoxification in fish tissues (Deb and Fukushima, 1999). Water temperature, transport of metals through the membrane, rate of fish metabolic activity, different mechanisms of adsorption, regulation, depositing and excretion of metals, species, age and size of fish, exposure time (Idodo Umeh 2002), as well as position and organ function (Nussey 2000) are also factors from which conditioned variations in the bioaccumulation of metals in various fish tissues. Numerous studies have analyzed metal accumulation in the muscles since it is the main part of a fish consumed by humans. Has-Schön et al. (2006) claimed that fish muscles are not a good indicator of total contamination, and that it is necessary to include metabolically active organs such as the liver and gills where the accumulations are significantly higher than in muscle tissue due to the existence of metal-binding proteins (Ploetz et al. 2007; Uysal et al. 2009). However, gills and liver are a much better contamination indicators than fish muscle. Gills are the primary organ by which the metals are adopted from the water, thus they reflect the concentration of metals in the water in which the fish live (Heath 1987). On the other hand, the ability of the liver to accumulate metals is a result of the activities of the metallothionein, a protein which bonds metals and reduces their toxicity (Ekpo et al. 2008; Višnjić Jeftić et al. 2010). The liver is the main site of accumulation, biotransformation and excretion of pollutants in fish (Shinn et al. 2009).

It is very hard to compare the concentration of metals in the same tissue among different species due to species-specific differences in the bioaccumulation patterns of metals. Such differences are a result of highly species-specific membrane permeability and enzyme systems along with differences in fish nutrition, habitat type, fish mobility and other features connected with fish behavior. According to past research, filter-feeding fish species that filter

particles in surface waters and benthivorous fish that feed particularly on mollusks and crustaceans which are known to accumulate high levels of heavy metals in their body accumulate the highest concentrations of heavy metals (Chen et al. 2000; Folt et al. 2002).

This paper assess the level of bioaccumulation of heavy metals and microelements and analyzes their distribution among different common carp (*Cyprinus carpio* L.) organs in different spatial locations. These results were presented to explain whether there are targeted bioaccumulation organs for individual metals and whether heavy metal and microelements concentrations vary across sites using common carp as a bioindicator. Passive biomonitoring was analyzed and autochthonous organisms were collected as bio-monitors from certain sites.

MATERIALS AND METHODS

Study Area and Sample Collection

The Tisa River in Serbia was chosen as a study area. The basin of the Tisa River is one of the largest natural river systems in Southeastern Europe, and is located almost exactly in the geographical centre of Europe. The river originates in the Zakarpatian Mountains in western Ukraine and flows into the Danube near Slankamen in Serbia. With respect to its length of 966 km, the Tisa forms the largest tributary of the Danube River. Anthropogenic activities asociated with communal, industrial and agricultural uses have resulted in permanent pollution of the Tisa River.

This study was performed at four sites on the Tisa River in Serbia. The first fish sampling site was on the border with Hungary where the Tisa River enters the territory of Serbia at the 153rd km of the river flow. The second and third sampling points were situated at 58th km and 72nd km of the river flow. The forth sampling point was at 3rd km of the river flow near confluence of the Tisa River at the Danube River (Figure 1). All marks refer to the upstream distance from the confluence with the Danube River.

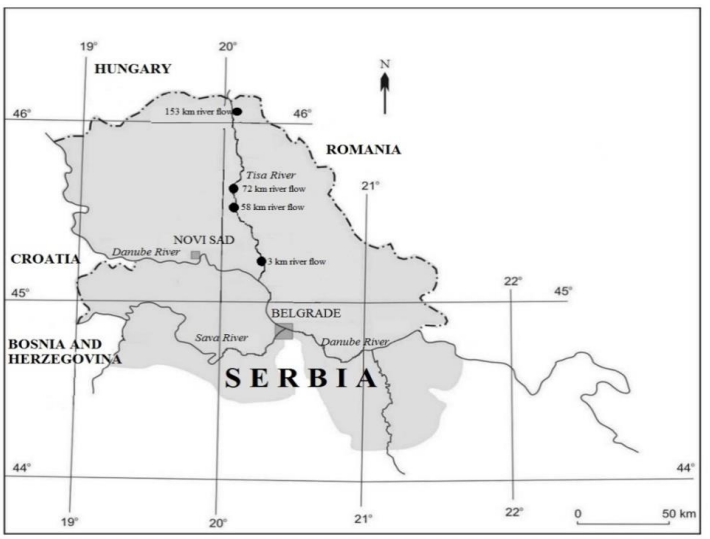

Figure 1. Map showing different sampling sites (●) in the Tisa river.

A total of 40 fish were caught by professional fishermen during October 2010. On the day of sampling, fish were transported to the laboratory of Faculty of Environmental Protection, Educons University and stored at -20°C. Prior to dissection, fish were thawed at room temperature and their total body (TL), standard (SL) and fork (FL) length, height (H), and total wet weight were measured. The average values and standard deviation for body weight (g), TL (mm), FL (mm) and SL (mm), H (mm) and age are given in the Table 1.

Dissection was performed on a polypropylene base with stainless steel scissors, scalpels and forceps. Gills, liver, and brain were taken from each individual. Dissected organs were packed in plastic bags and stored at -18°C until chemical analysis. The same fish organs from the same site were merged into a composite sample of 10 individuals.

Table 1. The mean (± standard deviation) body weight (g), total length (TL, mm), fork length (FL, mm) and standard length (SL, mm), height (mm) and age (years) of common carp

	Body weight	TL	FL	SL	H	N	Age
3 km river flow	284±90.5 (153-431)	274.5±30.9 (223-325)	250.4±28.3 (204-303)	224.9±25.6 (181-268)	78.6±5.9 (68-84)	10	3-4
58 km river flow	311.2±142.3 (116-525)	282.9±53.4 (196-364)	256.6±49.3 (183-330)	235.9±45.6 (167-304)	89.7±18.8 (54-124)	10	3-5
72 km river flow	449.1±155.4 (204-790)	304.1±39.8 (230-370)	282.5±38.4 (210-344)	256.9±34.6 (190-310)	104.7±11.3 (90-132)	10	3-5
153 km river flow	313.7±100.0 (193-539)	278.5±30.4 (250-358)	256.4±31.8 (223-332)	235.8±30.5 (205-308)	91.1± 9.6 (78-110)	10	2-4

N, number of common carp.

Sample Preparation

All samples' portions in amount of about 1 g were digested in a microwave digester (ETHOS 1, Advanced Microwave Digestion System, MILESTONE, Italy) using 8 ml of 65% HNO_3 and 1 ml of 30% H_2O_2 (Carlo Erba, Italy) of analytical reagent grade. The samples were allowed to ramp to 200 °C for 15 min, digest at 200°C for 20 min, and cool down for 10 min. After cooling to a room temperature, digested samples were diluted with distilled water to a total volume of 25 ml. All the plastics and glassware were washed in nitric acid for 15 min and rinsed with deionized water before use. High purity argon was used as inert gas. The analysis was performed by inductively-coupled plasma optical spectrometry (ICP/OES, Thermo Scientific iCAP 6500 Duo Instrument, Thermo Fisher Scientific, Cambridge, UK), and comprised assessment of concentrations of 15 heavy metals and microelements (Al, As, B, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sr and Zn). All element concentrations were expressed in $\mu g\ g^{-1}$.

Statistical Analysis

In order to investigate significant differences between the sample means, one–way ANOVA with post hoc test analysis based on the Scheffe and Games-Howell test was

applied. Data showed mostly normal distribution or close to normal distribution and therefore no transformations were done for statistical analyses. Statistical confidence was set at $\alpha = 0.05$. All data were analysed using software SPSS version 15.0 (SPSS Inc. Chicago, USA).

RESULTS AND DISCUSSION

The contents of heavy metals and microelements in common carp were determined in the liver, gills and brain. The mean values and standard deviations of heavy metals and microelements (expressed in μg g^{-1} wet mass) measured in different fish organs from different locations are given in the Tables 2 and 3. Based on the results, Al, Fe and Zn were the elements that were most accumulated in the common carp.

Table 2. The mean (± standard deviation) heavy metals and microelements (μg g-1 wet mass) in various organ of common carp

	organ	Al	As	B	Cd	Co	Cr	Cu
153 km	Liver	6.470± 0.058	0.300± 0.017	0.050± 0.018	0.730± 0.008	0.070± 0.018	0.110± 0.003	11.430± 0.084
	Gills	116.730± 0.201	0.130± 0.058	0.250± 0.028	0.030± 0.006	n.d.	0.340± 0.008	2.050± 0.051
	Brain	0.180± 0.007	0.060± 0.015	0.230± 0.031	0.010± 0.004	0.020± 0.016	0.240± 0.019	1.020± 0.024
3 km	Liver	45.560± 0.063	0.120± 0.008	n.d.	1.250± 0.014	0.030± 0.016	0.220± 0.008	11.200± 0.103
	Gills	97.840± 0.563	0.100± 0.021	0.410± 0.051	0.040± 0.003	n.d.	0.410± 0.019	3.120± 0.045
	Brain	0.270± 0.017	0.0330± 0.016	0.040± 0.021	0.020± 0.002	0.020± 0.027	0.170± 0.007	2.350± 0.047
72 km	Liver	5.260± 0.016	0.090± 0.024	n.d.	0.380± 0.006	0.020± 0.027	0.100± 0.010	10.560± 0.036
	Gills	189.210± 0.282	0.100± 0.025	0.280± 0.026	0.030± 0.003	n.d.	0.540± 0.013	1.330± 0.030
	Brain	0.220± 0.013	0.040± 0.038	n.d.	0.010± 0.006	0.010± 0.013	0.200± 0.021	0.770± 0.014
58 km	Liver	12.700± 0.058	0.040± 0.039	n.d.	0.750± 0.004	0.030± 0.015	0.120± 0.015	30.120± 0.113
	Gills	62.500± 0.043	0.040± 0.031	n.d.	0.040± 0.005	n.d.	0.350± 0.006	6.840± 0.015
	Brain	0.270± 0.017	n.d.	n.d.	0.020± 0.007	0.020± 0.015	0.150± 0.015	8.900± 0.023

Target Organs for Bioaccumulation of Heavy Metals and Microelements in Common Carp

In total the average values for the content of heavy metals and microelements in the wet mass of particular fish organs across the four sites were as follows: Al (116.57 μg g^{-1}), B (0.31 μg g^{-1}), Cr (0.41 μg g^{-1}), Mn (6.27 μg g^{-1}), Ni (0.13 μg g^{-1}) and Sr (14.08 μg g^{-1}) accumulated mostly in the gills; As (0.14 μg g^{-1}), Cd (0.78 μg g^{-1}), Co (0.04 μg g^{-1}), Cu (15.83 μg g^{-1}) and Pb (1.01 μg g^{-1}) in the liver; Fe (144.88 μg g^{-1}; 162.54 μg g^{-1}) and Zn

(155.01 μg g^{-1}; 135.91 μg g^{-1}) in both liver and gills, while Se accumulated most in brain (0.40 μg g^{-1}) (Tables 2 and 3). The average values of Hg were about the same in all fish organs (Table 3). The obtained results showed that the bioaccumulations of heavy metals and microelements in the carps' liver (22.49 μg g^{-1}) and gills (29.36 μg g^{-1}) were much higher than their bioaccumulation in the brain (5.83 μg g^{-1}) (Figure 2). The difference in bioaccumulation between particular carp organs was significant ($F_{(2, 11)} = 21.477$, $p < 0.0001$) and the Games-Howell post hoc test denoted gills and liver as the organs where the bioaccumulation was significantly higher than in the brain.

Table 3. The mean (± standard deviation) of heavy metals and microelements (μg g^{-1} wet mass) in various organ of common carp

		Fe	Hg	Mn	Ni	Pb	Se	Sr	Zn
153 km	Liver	193.660± 0.673	0.010± 0.012	2.170± 0.007	0.030± 0.014	n.d.	0.350± 0.176	0.300± 0.1140	181.670± 0.533
	Gills	154.940± 0.350	n.d.	5.320± 0.007	0.100± 0.013	0.530± 0.021	n.d.	15.100± 0.051	168.440± 0.514
	Brain	35.1900± 0.152	n.d.	0.330± 0.001	0.020± 0.016	0.200± 0.024	0.290± 0.148	1.380± 0.003	19.370± 0.1118
3 km	Liver	179.560± 1.136	n.d.	2.230± 0.014	0.080± 0.022	0.900± 0.028	0.220± 0.206	0.320± 0.004	173.680± 0.455
	Gills	150.87± 1.376	n.d.	4.400± 0.035	0.130± 0.008	1.360± 0.037	n.d.	10.760± 0.050	126.370± 0.447
	Brain	33.910± 0.246	n.d.	0.290± 0.001	0.020± 0.017	n.d.	0.140± 0.014	2.840± 0.021	18.050± 0.114
72 km	Liver	76.000± 0.522	0.010± 0.017	1.890± 0.010	0.010± 0.011	n.d.	0.580± 0.087	0.170± 0.102	114.210± 0.190
	Gills	212.250± 1.019	n.d.	9.190± 0.039	0.210± 0.013	0.590± 0.020	n.d.	13.030± 0.065	113.220± 0.578
	Brain	39.440± 0.200	n.d.	0.280± 0.001	0.010± 0.004	n.d.	0.440± 0.228	3.320± 0.020	12.680± 0.074
58 km	Liver	130.280± 1.338	n.d.	1.530± 0.013	0.020± 0.002	1.110± 0.030	0.030± 0.128	0.190± 0.001	150.490± 0.669
	Gills	132.090± 0.621	n.d.	6.150± 0.024	0.070± 0.012	0.490± 0.028	n.d.	17.430± 0.079	135.610± 0.098
	Brain	137.300± 0.509	0.050± 0.011	1.440± 0.002	n.d.	0.320± 0.019	0.720± 0.180	0.110± 0.001	26.190± 0.703

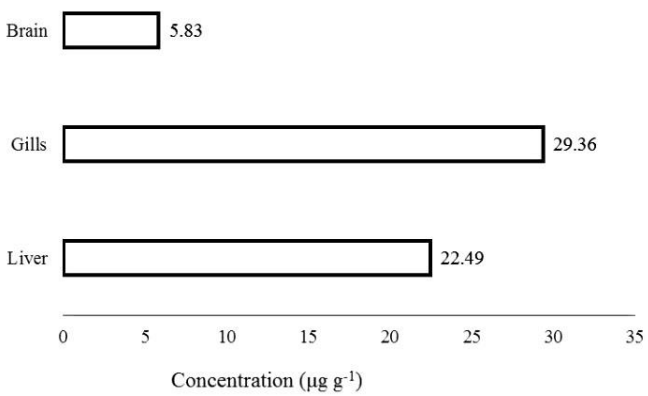

Figure 2. Mean heavy metals and microelements concentrations (μg g^{-1}) in different organs of common carp.

These studies showed that the liver and gills accumulate heavy metals and microelements more compared to the brain. This result is in agreement with Vinodhini and Narayanan (2008), Khaled (2009) and Jarić et al. (2011). Based on these results, the fish organs were a factor which remarkably impacted the bioaccumulation of heavy metals and microelements (Dural et al. 2007; Yılmaz et al., 2007). Accordingly, bioaccumulation in the liver was the greatest for As, Cd, Co, Cu and Pb, while in gills the bioaccumulation was the greatest for Al, B, Cr, Mn, Ni and Sr, and in both liver and gills bioaccumulation was the greatest for Fe and Zn. Higher concentrations of Cu in the liver, Al and Sr in the gills, Fe and Zn in liver and gills were expected, considering that the liver represented the target organs of Cu (Vapa and Vapa 1997), and gills for Al and Sr bioaccumulation (Schiffman 1961; Handy and Eddy 1989). The greatest concentrations of Fe in the liver and gills were expected as it enters the composition of hemoglobin (Carpene et al., 1994). Liver tissue was assigned as highly active in adopting and depositing heavy metals and microelements (Dural et al. 2007). The ability of the liver to accumulate metals is the result of the activities of metallothionein, a protein which bonds metals and reduces their toxicity (Ekpo et al. 2008; Oguzie and Izevbigie 2009; Višnjić Jeftić et al. 2010). Depending on the type of metal, its concentration, fish species, length of exposure and other factors, metals on one hand can increase or decrease the activity of hepatic enzymes, and on the other hand they can lead to histopathological changes in the liver (Paris-Palacios et al., 2000). In contrast to the liver, the reasons for the high concentration of metals in the gills are the result of both metal complexes presence in the mucus (which cannot be totally removed from the lamellae prior to analysis), and the absorption of metals on the surface of the gills which is conditioned by the accessibility of the protein with which the metal bonds (Palaniappan and Karthikeyan 2009; Dural et al. 2006; Erdogrul and Erbilir 2007).

The present research confirms that the liver is a good indicator of chronic exposure to heavy metals and microelements because it is the site of metal metabolism. Thus, it is the main indicator of the state of the surrounding aquatic ecosystem due to the fact that the concentrations of metals in the liver are often in proportion to those in the environment (Dural et al. 2007). Unlike the liver, gills are the primary organ by which the heavy metals and microelements are adopted from the water thereby reflecting the concentration of metals in the water in which the fish live (Heath 1987). In the gills, the concentrations of metals are especially greater at the beginning of exposure, before the metals reach the other parts of the organism (Rao and Padmaja 2000).

Sampling Sites Variations of Bioaccumulation of Heavy Metals and Microelements in Common Carp

Based on Figure 3 which shows descriptive statistics of the average values of heavy metals and microelements in carp depending on the site, it is noted that the average bioaccumulation is fairly uniform across site. The largest bioaccumulation was at the site "153 km" (20.44 μg g^{-1}), and the smallest at the site "72 km" (17.93 μg g^{-1}). According to the concentrations of each heavy metal and microelement that were found in fish at four different localities of sampling, it seemed that Al, Fe and Zn were the elements that accumulated the most in total average values at all sampling localities in fish, followed by Cu, Mn and Sr that

accumulated slightly less (Tables 2 and 3). There was no significant variability for bioaccumulation across sampling localities ($F_{(3, 11)} = 0.018$, $p > 0.996$).

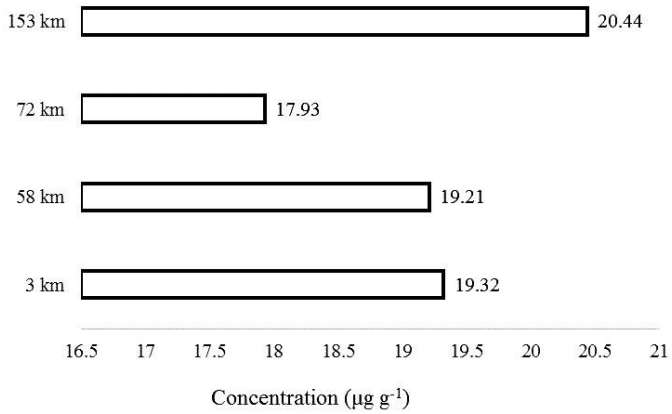

Figure 3. Mean heavy metals and microelements concentrations (μg g^{-1}) in common carp in various sampling sites.

Species-Specific Differences in the Bioaccumulation of Heavy Metals and Microelements

In order to determine the importance of common carp in the biomonitoring of aquatic ecosystem loads with heavy metals and microelements, the average content of heavy metals and microelements in the common carp were compared to the average values in the silver bream (*A.brama*), starlet (*A. ruthenus*) and northern pike (*E. lucius*) from the same river. Based on the distribution of heavy metals and microelements concentrations in different fish species for each of the tested elements, it can be noticed that the average bioaccumulation of heavy metals and microelements is the greatest in common carp (19.22 μg g^{-1}), and the smallest in the silver bream (6.28 μg g^{-1}) (Figure 4).

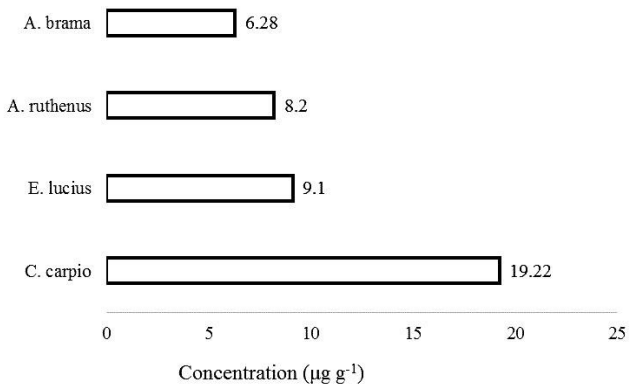

Figure 4. Mean heavy metals and microelements concentrations (μg g^{-1}) in various fishes.

In order to determine the existence of statistically significant differences in the bioaccumulation of heavy metals and microelements in different fish species one-way ANOVA was conducted. The difference among fish species in concern for the content of heavy metals and microelements was significantly different ($F(3, 59) = 9.410$, $p = 0.0001$), with a higher content of heavy metals and microelements is in common carp than silver bream, sterlet and northern pike.

The differences in the bioaccumulation obtained in four fish species can be best explained by differences in their feeding behavior. Northern pike are piscivores, silver bream and starlet are benthivores, while common carp are omnivores. Kidwell et al. (1995) and Voigt (2004) noted that predatory species such as pike accumulate the most Hg. The increased concentrations of Hg can be explained in the way that this is the only metal which shows a certain degree of biomagnification, the concentration increasing from the lowest trophic level of the food chain to the highest one. The nutrition of benthivorous fish such as silver bream and sterlet consists of larvae of various groups of insects (Trichoptera, Chironomidae, Ephemeroptera, Plecoptera and Simulidae), small mollusks, articulate worms (Oligochaeta), crayfish and other invertebrates. Several sources reported that increased concentrations of heavy metals in water and sediment coincide with the increase of their concentrations in water insects, relating to the bioaccumulation of heavy metals by the bottom fauna as the main components of the nutrition of benthivorous fish species (Smith et al. 1996).

Given that different species adopt metals in different ways, the exact relationship between metals in the environment and their presence in the bodies of insects can vary in regard to fish taxa and metals (Chen et al. 2000). Woelfl et al. (2006) point to a small bioavailability of heavy metals, as well as a low factor of bioaccumulation for the larvae of chironomids which are highly abundant in the benthic sediment of the Tisa River in the area investigated here. Duquesne et al. (2000) have determined higher concentrations of Cu and Cd in amphipod shrimp species *P. walker* as a result of their increase in the aquatic environment. Unlike the water insects, it has been confirmed that mollusk tissue is not a good indicator of the state of an ecosystem. A number of authors have noted an inconsistency of results and concluded that it is hard to establish the general trend of bioaccumulation on the part of the mollusks (Timmermans et al. 1989; Elder and Collins 1991; Van Hattum et al. 1991; Gundacker 2000). Laua et al. (1998) confirmed that there was no link between the concentration of metals present in tissues or the mollusk shell and the total metal concentrations in the environment. In the nutrition of omnivores, along with the bottom fauna, plant material can also be an important dietary component. Janković (1983) determined that at the age of 2+ and over common carp starts to feed on macrophytes as well as benthic invertebrates. In contrast to the bottom fauna featuring no general trend of bioaccumulation of heavy metals and microelements, water plants can accumulate significant amounts of metal in their tissues, which can be up to as much as 10^6 times more in relation to their concentrations in water habitats (Kovacs et al., 1984). The statistically significant differences determined for the bioaccumulation of heavy metals and microelements between common carp on one and northern pike, sterlet and silver bream on the other side, can be explained by feeding of common carp on aquatic vegetation that accumulates significant amounts of heavy metals and microelements in comparison to other components occurring in the diet of other fish species discussed here. The age of all common carp we investigated was from 2+ to 4+, which infers

that macrophytes are likely a significant part of their diet, along with the bottom fauna elements.

Ecosystems Variations of Accumulations of Heavy Metals in Common Carp

This study has established that the concentrations of heavy metals and microelements in fish vary from organ to organ, and from fish species to fish species, but the locality within one ecosystem does not affect them. So, in, order to assess the quality of the Tisa River ecosystem, we also included a comparison of the content of heavy metals and microelements in the common carp from different ecosystems in addition to the Tisa River.

The contents of heavy metals and microelements in the liver and gills of the common carp from the Tisa River were compared to the contents of heavy metals and microelements in the liver and gills of common carp from Kaban Dam Reservoir (Varol and Sünbül, 2017), Peyang Lake (Wei et al., 2014), the Danube River (Subotić et al. 2013), Beyşehir Lake (Altındağ and Yiğit 2005), and the Begej River (Škorić et al. 2012) (Table 4).

Table 4. Comparison of heavy metal and microelements content ($\mu g\ g^{-1}$) in common carp from different ecosystems

Metal	Tisa River		Kaban Dam Reservoir	Peyang Lake		Danube River		Bey-şehir Lake	Begej River	
	liver	gills	gills	liver	gills	liver	gills	gills	liver	gills
Al	7.50	116.57	124.8	0.36	-	3.86	14.62	-	9.90	159.43
As	0.14	0.09	-	-	0.31	0.49	0.29	-	-	-
B	0.05	0.31	-	-	-	0.31	0.52	-	-	-
Cd	0.78	0.04	1.29	1.31	0.12	0.28	0.03	0.66	-	-
Co	0.04		-	-	-	-	-	-	-	-
Cr	0.14	0.41	-	2.94	1.97	0.01	0.01	0.16	-	-
Cu	15.83	3.34	0.78	73.8	3.56	33.49	1.90	-	4.90	-
Fe	144.88	162.54	-	-	-	141.44	139.26	-	121.42	276.93
Hg	0.01	-	-	-	0.04	1.63	0.89	3.02	0.61	0.72
Mn	1.96	6.27	-	-	-	2.21	10.55	-	2.80	6.55
Ni	0.04	0.13	-	0.09	1.64	-	-	-	-	-
Pb	1.01	0.74	17.93	0.12	0.70	-	-	0.42	-	-
Se	0.30	-	-	-	-	0.36	0.06	-	-	-
Sr	0.25	14.08	-	-	-	0.18	86.75	-	0.30	34.75
Zn	155.01	135.91	12.33	356	3129	325.37	1186.37	-	143.30	276.29

Comparing the content of heavy metals and microelements in the liver and gills of the common carp from the Tisa River, the followings results are observed. In the liver of the common carp from the Tisa Al were higher compared to the Danube River, but lower compared to the Begej; in the gills they were higher compared to the Danube, but lower than the Kaban Dam Reservoir and the Begej River. As in the liver and gills of the common carp from the Tisa River is lower than from Peyang Lake and the Danube. B in the liver and gills of the common carp from the Tisa River were lower than from the Danube. Cd in the liver and gills of the common carp from the Tisa were lower than from Kaban Dam Reservoir, Peyang Lake and Beyşehir Lake. Cr in the liver of the common carp from the Tisa River were higher compared to the Danube, but lower in relation to the Peyang Lake; in gills they were

higher compared to the Danube River and Beycheher Lake, but lower compared to Peyang Lake. Cu in the liver of common carp from the Tisa River were higher compared to the Begej River, but lower in relation to the Peyang Lake and the Danube, in gills were higher compared to the Kaban Dam Reservoir and the Danube. Fe in the gills of the common carp from the Tisa River were lower compared to the Begej River. Hg in the liver of the common carp from the Tisa were lower than from the Danube and the Begej. Mn in the liver and gills of the common carp from the Tisa were lower in relation to the Danube and the Begej. Ni in the liver and gills of the common carp from the Tisa were lower than from Peyang Lake. Pb in the liver of the common carp from the Tisa were higher than from Peyang Lake, but in the gills they were higher than from Peyang and Beycheher Lake, but lower than from Kaban Dam Reservoir. Sr in the gills of the common carp from the Tisa were lower in relation to the Danube and the Begej. Zn in the liver of the common carp from the Tisa were higher compared to the Begej, but lower in relation to Peyang Lake and the Danube, in gills they were higher than from the Kaban Dam Reservoir, but lower than from the Peyang Lake, the Danube and the Begej.

CONCLUSION

Bioaccumulation of certain heavy metals and microelements in common carp at four different localities along the Serbian stretch of the Tisa River revealed that their bioaccumulation occurs in different concentrations in various fish organs. The analyzed elements were accumulated significantly more in the gills and liver compared to brain. The bioaccumulation of the tested elements was significantly greater in common carp compared to silver bream, sterlet and northern pike, which can be explained by omnivore feeding of common carp in age 2+ and over, with the great proportion of aquatic vegetation (which is known to accumulate significant amounts of heavy metals and microelements) in their diet. Fish species also appeared to be a factor which impacts the bioaccumulation of heavy metals and microelements, i.e., there was a difference between four species due to their interactions at an ecosystem level via trophic position and general habitat use. Locality as a factor revealed no impact on the bioaccumulation of heavy metals and microelements in the Tisa River fishes. Based on the results it can be concluded that *Cyprinus carpio* have important role in the biomonitoring of heavy metals and microelements levels in river ecosystems given they are a major bioaccumulator of heavy metals and microelements.

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Chapter 98

FACTORS AFFECTING THE MEAT QUALITY OF COMMON CARP

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ABSTRACT

Common carp (*Cyprinus carpio* L.) is one of the most important fish species in the world and together with other cyprinid fish species represents an important source of protein and various unsaturated fatty acids. Fish meat has been shown to be an important part of a healthy human diet. This paper reviewed the factors that affect the meat quality of common carp including chemical and fatty acid compositions. It was found that the fat content and fatty acid composition significantly differs among different fish species, even when they belong to the same family. In addition, the same parameters were found to be influenced by different environmental factors, especially by diet. Significant roles of good production technology and appropriate structure of planktonic and benthic organisms, which play a significant role in carp nutrition, meat quality and desirable chemical and fatty acid composition, have been also discussed. Using properly formulated feed mixtures had a positive effect on fish health, production parameters and meat quality. Both animals and vegetable components as ingredients in formulated carp feed and their effects on production performance and meat quality have been reviewed.

Keywords: Cyprinids, chemical composition, fatty acids, culture systems, nutrition

INTRODUCTION

The common carp (*Cyprinus carpio* L.) is one of the most important fish species in the world and together with other cyprinid fish species represents an important source of protein and various unsaturated fatty acids. It is a freshwater fish species which is often reared in

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warm-water fish ponds. It is one of the earliest domesticated fish species which has been cultured for human nutrition. It was reared in China as early as the 500 BC. Today, this species is reared not only in Asia, but throughout the world and is one of the most domesticated fish species. The common carp is a very strong and robust fish species capable of surviving in across a wide array of temperature and water quality. Common carp is abundant across Serbia as well as in many areas worldwide and is a highly important and esteemed fish species. Because of numerous desirable traits, such as high fecundity, good conversion of both natural and high muscle protein content, fast growth-rate, both healthy and tasty meat and resistance to poor environmental conditions and diseases, this fish species is widly domestricated in Europe and Asia. Common is one of the highly preferred fish due to the specific flavors, savory flavors in various recipes, and require a short time for its preparation for cooking. Muscles are the main part of the body of the fish that are used in human nutrition. Common carp is a medium fatty fish and most of its fat stored as adipose tissue in the abdominal wall (Mráz and Pickova 2009).

Meat of common carp is an important nutritional source of protein and monounsaturated fatty acids (MUFA) (Ćirković et al. 2011; Ćirković et al. 2012; Ljubojević et al. 2013a,b,c,d; Ljubojević et al. 2015), and contains n-3 highly unsaturated fatty acids (HUFA), especially eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid (Steffens and Wirth 2007; Ljubojević et al. 2016; Župan et al. 2016), which play an important role in key physiological processes in human health. The fat content and fatty acid composition of cyprinid fish species are influenced by age, season, various environmental factors, culture systems and, especially nutrition.

THE SIGNIFICANCE OF FISH IN THE HUMAN NUTRITION

Consumption of fish meat is encouraged due to high protein content, presence of essential amino acids, minerals and vitamins, and in addition a sufficient amount of polyunsaturated fatty acids (PUFA), such as n-3 PUFA. Fish is the main source of n-3 fatty acids (n-3 HUFA) and it is considered as a healthy food with many positive effects on human health, especially in the prevention of cardiovascular disease (Conor and Conor 2010). PUFA may be important in the reduction of mortality caused by heart diseases (Kris-Ethertonet al. 2002). In addition, n-3 PUFAs decrease the content of low-density lipoproteins (LDL) in human serum, have antiarrhythmic effects, and inhibit lipogenesis in the liver, platelet aggregation and damage of blood vessels. They are very important in the prenatal development of the nervous system (Allen and Harris 2001). Further, the PUFA from n-6 series, and in particular from n-3 series participate in the prevention of diseases of the nervous system, improve learning ability, play an important role in ontogenesis (Arts et al. 2001) and also play anti-inflammatory functions (Dorea 2008). Arachidonic acid (ARA) and DHA are essential for the development and function of the brain and retinas (Lauritzen et al. 2001). The shortage of α-linolenic acid (18:3 n-3, ALA) is responsible for neurological disorders and poor growth (Cundiff et al. 2007). There is strong evidence that dietary docosahexaenoic acid (DHA) lowers the cholesterol level in serum, and other n-3 PUFAs lower triglycerides in the serum, but do not affect serum cholesterol (Balk et al. 2006; Ryan et al. 2009).

Essential polyunsaturated fatty acids such as ALA, EPA and DHA are not synthesized in the human body and effectively synthesized only by aquatic organisms; therefore, humans can receive these essential fatty acids through consumption of marine and freshwater fishes (Jabeen and Chaudhry 2011). There are recommendations from various world organizations related to fish consumption and intake of n-3 fatty acids. The European Food Safety Authority recommends intake values of 250 mg of EPA and DHA per day for healthy people (EFSA 2009), while in the case of people who suffer from some form of heart disease and have elevated triglyceride levels recommends a daily intake of EPA and DHA over 1 and 2 to 4 g, respectively (Kris-Etherton et al. 2002). The recommended optimal n-6/n-3 ratio in food for human consumption, according to numerous studies ranges from 1:1 to 4:1 (Simopoulos 2002) and this ratio is lower than occurs in most fish species. N-6 fatty acids are also essential, but they are present in most feedstuffs. Results from analyses of the most common meat products of domestic animals and poultry have shown that the ratio of n-6/ n-3 fatty acids was in the range of from 6.5 to 43.2 (Sampels et al. 2009). Increased intake of n-6 fatty acids is associated with a global increase in the number of obese people (Strandvik et al. 2008) and inflammatory processes (Calder 2008). Conversely, the intake of n-3 fatty acids decline and numerous scientific publications indicate that this phenomenon is closely related to several health problems (Calder 2008). While fish is a food that is traditionally considered a good source of n-3 fatty acids, measures to improve fat quality of fish are necessary for future human health needs and fish production technologies also need improvement from an economic point of view.

It is very difficult to determine a standard recommended intake of fish when the diversity of various fatty acids composition among various fish species is considered (Ćirković et al. 2012; Ljubojević et al. 2013cd). Further, providing such guidelines is also difficult due to the variation of fatty acid composition in fish. For example, fatty acid composition in common carp can vary significantly depending on their levels and type of nutrition (Mráz and Pickova 2009; Ljubojević et al. 2013a; 2014). When all of the above issues are considered, it can be said that the ability to produce quality fish with a predictable and known content of n-3 HUFA could be very important for producers. There have been some significant studies that covered different culture systems of growing carp and different aspects of its nutrition (Steffens and Wirth 2007; Mráz and Pickova 2009; Ćirković et al. 2011; Ćirković et al. 2012a,b,c; Ljubojević et al. 2016) but there is high uncertainty around them as they have gathered results from highly variable systems ranging from farmed-carp in intensively managed systems (Ljubojević et al. 2015; Župan et al. 2016) to wild-caught in natural river systems (Ljubojević et al. 2013d).

Epidemiological and nutritional studies have shown a link between nutrition and the incidence of heart disease. Therefore, therapy in the form of proper nutrition is considered to be important as a medicinal treatment of cardiovascular disorders. Beneficial effects of fatty acids from fish for human health is highlighted in the text, but it is impossible to explain the beneficial effects of fish meat on the human health only in terms of EPA and DHA, because fish do not only provide fats and fatty acids, but also provides a many other nutritional ingredients, such as proteins. Previous investigations have shown that, in relation to casein, fish protein in the commercial feeds reduce the level of blood cholesterol in laboratory animals (Hosomi et al. 2011). In addition, fish proteins have other beneficial effects such as anti-hypertensive properties. Further, fish proteins also lower low-density lipoprotein (LDL-

C, often known as "bad cholesterol") while increasing high density lipoprotein (HDL-C, often known as "good cholesterol"), and also have a favorable effect against obesity (Boukortt et al. 2004; Oishi and Dohmoto 2009). Many epidemiological and clinical studies have shown that an increase in LDL-C levels, and decrease in HDL-C levels are important factors for the occurrence of cardiovascular diseases (Jacobson et al. 2007). Results of Hosomi et al. (2011) indicate that the fish proteins lower the cholesterol content in the liver and serum by increasing the excretion of cholesterol and bile acid in the faeces as a result of low micellar solubility of cholesterol, and high capacity for binding bile acids.

Content of essential amino acids in fish meat is very high, especially for example tryptophan, an amino acid which is the precursor of serotonin, which is responsible for the state of mind in humans (Gadoth 2008).

FACTORS AFFECTING CHANGES IN THE CHEMICAL AND FATTY ACID COMPOSITION OF MEAT OF COMMON CARP

The chemical composition including fatty acids of common carp is affected by many factors, of which the most important factors are rearing system, diet, genetic factors, gender and sexual maturity, obesity and age of the fish. The aim of this paper is to discuss the effects of these factors on chemical composition including fat quality in common carp.

Chemical Composition of the Fillets of Various Fish Species

The major differences are established in the content of fat in muscle tissue between different fish species, as well as differences in the fatty acid composition (Fontagné-Dicharry et al. 2010). Ćirković et al. (2012) performed evaluation of meat quality of different fish species reared in polyculture and compared productivity and meat quality of carp (*Cyprinus carpio* L.), silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), catfish (*Silurus glanis*), zander (*Stizostedion lucioperca*) and tench (*Tinca tinca*), which were grown in polyculture on natural food in ponds fertilized with livestock manure. In the above mentioned investigation fish were grown in earthen ponds with a surface area of 1 ha and average depth of 1 m in the polyculture on natural food. The fish pond was stocked in April 2011 and harvested in October 2011. Samples of two-year-old carp, two-year-old silver carp, grass carp, catfish and zander and three-year-old tench were taken during the harvesting. The stocked tench was older in comparison with the other species to avoid the possibility that catfish and zander eat up two-year-old tench. Fish species were reared under variable natural atmospheric conditions and fed natural food. The production of natural food was based on the natural production of benthic and planktonic organisms that were increased by application of agrotechnical measures such as drying of fishponds during winter, soil treatment, fertilization and adding lime. Livestock manure (2000 kg/ha) was applied to the bottom of empty ponds and later bi-weekly over the water surface (4000 kg/ha during growing season). Agricultural limestone was applied to the bottom of empty ponds and over the water surface and fish ponds were aerated. After fish removal, they were weiged, and muscle tissue samples from each breed were taken for further analysis. It was found that the production parameters,

chemical composition and quantity of n-3 fatty acids varied widely among different species, even when they belonged to the same family as the majority of the examined fish belonging to the family *Cyprinidae* (Table 1, 2, 3).

Table 1. Production parameters of fish species reared in polyculture (Ćirković et al. 2012)

Variables	Common carp	Silver carp	Grass carp	Wels Catfish	Zander	Tench
Individual/ha	1000	100	50	30	20	100
Weight at stocking (g)	250	50	130	50	30	100
Weight at harvesting (g)	910	1450	500	1320	400	200
Survival (%)	70	80	75	90	60	40

Values are presented as means (n = 60).

Table 2. Proximate composition of fish reared in polyculture (Ćirković et al. 2012)

Variable	Common carp	Silver carp	Grass carp	Wels catfish	Zander	Tench
Moisture (%)	80.36	76.93	78.03	78.76	79.32	82.41
Protein (%)	16.21	18.1	15.76	18.16	19.26	15.74
Fat (%)	2.42	3.82	5.24	2.19	0.38	0.79
Ash (%)	1.02	1.15	0.98	0.88	1.03	1.06
Cholesterol (mg/100 g)	55.81	62.32	60.06	34.34	42.91	35.87

Table 3. The main fatty acid groups in fish reared in polyculture (Ćirković et al. 2012)

Fatty acid (%)	Common carp	Silver carp	Grass carp	Wels catfish	Zander	Tench
SFA	27.15	33.05	32.84	24.97	30.79	27.34
MUFA	28.79	37.04	36.03	35.61	30.05	28.18
PUFA	44.08	29.95	31.23	39.44	39.19	44.50
n-6	22.96	9.68	9.53	12.20	17.78	17.29
n-3	21.12	20.26	21.69	27.24	21.41	27.21
n-3/n-6	0.92	2.09	2.28	2.23	1.21	1.57
n-6/n-3	1.09	0.48	0.44	0.45	0.83	0.64
PUFA/SFA	1.62	0.91	0.95	1.58	1.27	1.63
USFA/SFA	2.68	2.03	2.05	3.01	2.25	2.66
PUFA/MUFA	1.53	0.81	0.87	1.11	1.30	1.58

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; USFA, unsaturated fatty acids; PUFA, polyunsaturated fatty acids series n-3 (n-3 PUFA) and n-6 (n-6 PUFA).

The mean stocking weight was the highest for common carp (250 g) and the lowest in zander (30 g) and harvesting weight was the highest in silver carp (1450 g) and the lowest in tench (200 g), while the survival rate was the highest in catfish (90%) and the lowest in tench (40%). Total stocking density was 274 kg/ha and harvesting density was 820 kg/ha.

The amount of protein was the highest in the fillets of zander (19.3%) and the lowest in grass carp and tench fillets (15.8%). Fat ranged from 0.38% in the muscles of zander to 5.24% in the muscles of grass carp. The total cholesterol content was highly variable, being the highest in the silver carp fillets (62 mg/100 g) and the lowest in catfish (34 mg/100 g). The total amount of saturated fatty acids (SFA) was the highest in silver carp (33%) and the

lowest in catfish (25%). Individiual SFAs were variable by species. Palmitic acid (C16:0) was predominant, whereas lauric acid (C12:0) was the least SFA in samples. The most abundant MUFA was oleic acid (C18:1, n:9), ranging from 22% in silver carp to 18% in tench, followed by palmitoleic acid (C16:1, n:7). The MUFA proportion was the lowest in tench (28%) and greatest in silver carp (37%). Tench had the highest (44%) and silver carp had the lowest (30%) PUFA proportions.

Samples were also highly variable in terms of n-3 and n-6 contents, for instance the n-3/n-6 ratio was the lowest in common carp 0.92, whereas it was the greatest in grass carp (2.28). Similarly, the PUFA:SFA, an indicator of the quality of lipids, was the least favorable in silver carp (0.91) and the most favorable in tench (1.63).

Meat quality of carp is highly variable, and changes depending on age, breeding system, and nutrition. Fat content in carp ranges from 2.3 to 16.8%, protein content lightly varies between 14 and 18% (Vladau et al. 2008; Trbović et al. 2009; Ćirković et al. 2011; Ljubojević et al. 2013ab). Cholesterol content varies by season. Trbović et al. (2009) reported 48.9 mg/100 g fat in one-year-old carp in April and 54.3 mg/100 g fat in the same age samples harvested in June. According to results obtained by Ćirković et al. (2012), cholesterol level was 55.8 mg/100 g fat in two-year-old carp. Cholesterol content of carp varies considerably from 47 to 120 mg/100 g, depending on fish breed and age, husbandry system, and harvest season (Bieniarz et al. 2001; Kopicova and Vavreinova 2007). This variability was very notable in silver carp, grass carp, tench, and catfish (Kopicova and Vavreinova 2007). Fat of terrestrial animals also contain variable amounts of total cholesterol. Williams (2007) reported that cholesterol content in meat of beef, veal, lamb and mutton was 50, 51, 66 and 66 mg/100 g, respectively. Pork meat has been reported to contain 44-98 mg/100 g (Bragagnolo and Rodriguez-Amaya 2002). Further, the average fat content in beef, sheep and pig meat is 3.8-17.3; 6.3-35.0 and 1.6-11.5%, respectively (Vladau et al. 2008).

Organic fertilization with livestock manure is a very cheap and effective method of increasing practically all nutrient components in fish pond ecosystems. Indeed, rearing of fish in polyculture on natural food with fertilization results in satisfactory fish production with a preferrable fatty acid composition as well as enhancing utilization of natural food resources. It could be said that rearing fish in polyculture on natural food with the use of agricultural limestone and livestock manure achieved satisfactory results in terms of the final weight of two-year-old fish and their nutritive composition as presented earlier. It is necessary to take into account the stocking density of fish to grow in pond-polyculture, as well as on the combination of appropriate species for the greatest production outcomes. Furthermore, nutrient composition varies widely among fish breeds. Lipid profiles particularly, appear more strongly variable among fish species than fish feeding habit (i.e., herbivorous, omnivorous or carnivorous).

Ljubojević et al. (2013d) performed a comparative analysis of proximate and fatty acid composition of seven fish species from the Danube River including asp (*Aspius aspius*), common bream (*Abramis brama*), common barbel (*Barbus barbus*), common carp (*Cyprinus carpio*), sterlet (*Acipenser ruthenus*) and northern pike (*Esox lucius*). The chemical composition and quantity of n-3 fatty acids varied largely among fish species. (Table 4, 5).

Average body weight of experimental fishes: asp (*Aspius aspius*), common bream (*Abramis brama*), common barbel (*Barbus barbus*), common carp (*Cyprinus carpio*), sterlet (*Acipenser ruthenus*) and northern pike (*Esox lucius*) was 1220, 1230, 870, 1420, 1320 and 1480 g, respectively. The amount of protein was the highest in the fillets of barbel (18.61%),

without significant difference with protein content in the flesh of pike and asp ($p > 0.01$), and the lowest in common carp fillets (16.69%). Fat ranged from 1.61% in the muscles of pike to 7.78% in the muscles of barbell and there was significant difference in the fat content among all examined fish ($p < 0.01$). Results of ash content in fish flesh also showed statistically significant difference among species, except for common carp and sterlet. The total cholesterol content was highly variable, being the highest in the sterlet fillets (73.59 mg/100 g) and the lowest in asp (36.26 mg/100g) and variation was statistically significant among fishes ($p < 0.01$).

Table 4. Proximate composition of seven freshwater fish species from the Danube (Ljubojević et al. 2013d)

Parameters	Asp	Common bream	Common barbel	Common carp	Sterlet	Northern pike
Water (%)	78.51	78.66	72.39	73.73	75.38	79
Protein (%)	18.07	17.59	18.61	16.69	17.54	18.43
Fat (%)	2.78	3.24	7.78	7.13	5.39	1.61
Ash (%)	1.16	0.80	1.33	0.88	0.93	0.64
Cholesterol (mg/100g)	36.26	41.93	53.12	45.43	73.59	36.37

Table 5. The main groups of fatty acids of seven freshwater fish species from the Danube (Ljubojević et al. 2013d)

Fatty acid, %	Asp	Common bream	Common barbel	Common carp	Sterlet	Northern pike
SFA	27.99	27.27	28.63	27.59	32.67	39.9
MUFA	47.69	56.09	45.27	52.94	45.97	31.66
PUFA	24.60	17.07	26.31	19.7	22.17	28.15
n-6	10.75	6.76	8.65	13.73	5.7	14.11
n-3	13.85	10.31	17.66	5.97	16.47	14.04
n-3/ n-6	1.29	1.53	2.04	0.44	2.9	1
n-6/n-3	0.78	0.66	0.49	2.30	0.35	1.00
PUFA/SFA	0.8	0.63	0.92	0.71	0.68	0.71
USFA/SFA	2.58	2.68	2.5	2.63	2.09	1.5

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; USFA, unsaturated fatty acids, PUFA, polyunsaturated fatty acids from series n-3 (n-3 PUFA) and n-6 (n-6 PUFA).

A lipid analysis in the paper of Ljubojević et al. (2013d) enabled the classification and quantitative determination of 21 fatty acids and besides that the sum of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), n-3 acids, n-6 acids, n-3/n-6 ratio, n-6/n-3 ratio and ratio of PUFA/SFA, as well as ratio of USFA/SFA which represent the indicators of lipid quality (Table 5). The total amount of saturated fatty acids (SFA) was the highest in pike (39.9%) and the lowest in bream (27.27%). Individual SFA were variable by species. Palmitic acid (C16:0) was predominant in all species, whereas arachidic acid (C20:0) was the least SFA in samples. Caprylic acid (C8:0) was detected in fat of asp, common bream, and pike and percentage of these fatty acid was higher in this species with regard to pentadecylic acid (C15:0), margaric acid (C17:0) and arachidic acid (C20:0), while, in barbell, carp and sterlet C8:0 was not detected. The most abundant MUFA was oleic acid (C18:1, n-9), ranging from 17.70% in pike to 32.11% in bream, followed by palmitoleic

acid (C16:1, n-7) and vaccenic acid, C18:1cis-11The MUFA proportion was the lowest in pike (31.66%) and greatest in bream (56.09%). Pike had the highest (28.15%), while bream had the lowest (17.07%) PUFA proportion. Asp, which is also carnivorous like pike had high PUFA content (27.99%). Samples were also highly variable in terms of n-3 and n-6 contents, for instance the n-3/n-6 ratio was the lowest in common carp (0.44), whereas it was the greatest in sterlet (2.9). Similarly, the PUFA/SFA, an indicator of the quality of lipids, was the least favorable in bream (0.63) and the most favorable in barbel (0.92).

The flesh obtained from the experimental fishes by Ljubojević et al. (2013d), was characterized by a variable fat and water content. The same regularity was observed by Żmijewski et al. (2006), Luczynska et al. (2008) and Ćirković et al. (2012), who found a reverse correlation between the fat and water contents, which is common for many fish species. The results of proximate composition of common carp, pike and common bream obtained by Ljubojević et al. (2013d) are quite similar with those of Bud et al. (2008), where fishes were obtained from aquaculture and no data was given about age or body weight of fishes.

It has been reported that under culture conditions, fish muscle contains more lipid than in the wild (Cahu et al. 2004). An interesting fact is that common carp from the free catch had fat content 7.1% (Ljubojević et al. 2013d) which is much higher compared to cultured carp (Ćirković et al. 2012; Ljubojević et al. 2012b). The exception to this is for cultured carp fed with grains with fat content between 10–15% and confirming that proximate composition of common carp is highly dependant on their diet (Steffens and Wirth 2007).

Significant differences in the content of ash, which was the lowest (0.63%) in pike and the highest (1.31%) (Ljubojević et al. 2013d) in barbel may be due to the presence of small bones in fish fillets. Namely, the calcium, which released during bone demineralization, can contribute to a greater mass fraction of ash in the total chemical composition of fish meat.

According to Andrade et al. (1995), the most dominant saturated acids in freshwater fish from south Brazil were palmitic (C16:0) and stearic (C18:0), whereas palmitoleic (C16:1) and oleic (C18:1) acids were the major component among monounsaturated fatty acids. Among saturated and monounsaturated acids in the most fish studied, oleic acid was the highest, followed by palmitic and palmitooleic acid (Ljubojević et al. 2013d).

Almost all freshwater species, with the exception of pike, contained higher amounts of monounsaturated fatty acids (MUFA) than saturated fatty acids (Ljubojević et al. 2013d), in compliance with results found by Luczynska et al. (2008) for bream and pike. The relative contents of n-3 PUFA (10.31-17.66%) was higher than the n-6 polyunsaturated fatty acids containing from 6.76 to 14.11%, except for common carp, which is in a line with results noted by Luczynska et al. (2008) for fresh water fishes.

From a nutrition viewpoint, humans could also consider the relative fatness of fish as for PUFAs expressed per 100 g of fish meat, intake of n-3 PUFAs is larger for fat fish than lean fish (Cahu et al. 2004; Lichtenstein et al. 2006). Wood et al. (2008) have suggested that ratio of PUFA/SFA should be above 0.4 and according that all examined fish species have had favorable (from 0.63 to 0.92) PUFA/SFA ratio. The n-6/n-3 ratio in all examined fishes (Ljubojević et al. 2013d) was in the optimal range of 2/1–4/1 for human health as suggested by Simopoulos (2002).

The knowledge about cholesterol content in food is important, especially in fish meat, because consumption of fish is currently increasing based on the recommendations for

healthy nutrition. Cholesterol content in female and male carp fillets was in the range of 69.4 -77.6 mg/100g (Komprda et al. 2003). Trbović et al. (2009) reported that the amount of total cholesterol was 48.9 mg/100 g in one-year-old carp in April and 54.3 mg/100 g in the same age samples harvested in June. In the study implemented by Ćirković et al. (2012), cholesterol level was 55.8 mg/100g in two-year-old carp. Cholesterol content in carp muscle tissue can vary considerably from 38 to 120 mg/100 g, depending on fish breed and age, husbandry system, and harvest season (Bieniarz et al. 2001; Ćirković et al. 2011). Kopicova and Vavreinova (2007) detected the content of total cholesterol in common carp, sterlet, northern pike and asp and it amounted 47, 61, 86 and 45 mg/100g, respectively. The pike used in the study conducted by Kandemir (2012) had an average weight of 1457 g and the amount of cholesterol found in tissue was 146.4 mg/100g. Ćirković et al. (2012) found differences among six freshwater fishes with respect to the amount of cholesterol detected in muscle tissue was significant and detected the level of total cholesterol rangomg from 34.34 mg/100g in catfish to 62.32 mg/100g in silver carp. Research conducted by Moreira et al. (2001) on the cholesterol content of many freshwater fish species showed that the values ranged between 40.99 and 52.79 mg/100g. According to Luzia et al. (2003) the amount of total cholesterol in freshwater fish is lower in comparison with marine fish. The daily intake of cholesterol is currently recommended not to exceed 300 mg (James and Ralph 2000). It can be argued that mentioned freshwater fish in the study conducted by Ljubojević et al. (2013d) are well-favored sources of cholesterol.

Fish should be included in human diets for at least three reasons: as a general source of nutritional components; as low fat, high protein food; and as source of polyunsaturated fatty acids (Ljubojević et al. 2013d). At the same time, the recommended ratio of polyunsaturated fatty acids (PUFA) to saturated fatty acids (PUFA/SFA) should be increased to above 0.4 (Wood et al. 2008). Since some meats of terrestrial farmed animals naturally have a PUFA/SFA ratio of around 0.1 (Wood et al. 2008), meat has been implicated in causing the imbalanced fatty acid intake of today's consumers. Fish lipids are particularly rich of polyunsaturated fatty acids (PUFA) that are only slowly synthesized in humans which is the major difference between meat of fish and meat of farmed terrestrial animals (Vladau et al. 2008). The n-3/n-6 ratio was found to be generally lower in cultivated than in wild fish (Orban et al. 2003). That is mainly due to nutrition and lowered level of fish meal as a protein source and its completely or partial replacement with cheaper protein sources. The recommended ratio of n-6/n-3 should be less than 4 (Scollan et al. 2006) and this ratio in some meats of terrestrial animals is higher than this (Wood et al. 2008). However, it is difficult to rank the examined fishes from best to worst from the point of view of human nutritive value. The fat content in fish tissue contributes to its organoleptic properties, texture and flavour. Tissue which is rich in fat is juicy, while lean tissue is dry and perceived as thickly fibrous (Żmijewski et al. 2006). On the other hand, there are certain groups of people who require meat with minimal fat and cholesterol content.

The importance of the results obtained by Ljubojević et al. (2013d) lies in the fact that there were no data on meat quality of freshwater fish species from the Danube River in the Serbia region, therefore it can be valuable information to ecologists, environmentalists, nutritionists, food scientist and other scientists. The meat of warm water fish from the Danube River represents a valuable source of healthy nutrition for consumers. Chemical and fatty acid composition varied between different species and among the same species. All examined

species have had PUFA/SFA ratio higher than 0.4, and n–6/n–3 ratio was lower than 4 which are the prescribed values recommended from WHO/FAO organization. The potential of exploiting presently insufficiently used freshwater species for developing high-protein foods for market and for introducing as a new species in aquaculture, underscore the need for reliable analytical data.

Ljubojević et al. (2013c) examined the economically most important fish species which are presented on the market for the Republic of Serbia, whereby the attention was not devoted only to differences in the meat quality of studied species caused by the influence of fish species, but also the differences that arise as a consequence of different growing systems and the use of different nutrients and completed feed for carp. The objective of the above study was to assess the chemical and fatty acid compositions of common carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), grass carp (*Ctenopharyngodon idella*), catfish (*Silurus glanis*) and zander (*Stizostedion lucioperca*) which were collected from retail stores in the area of Novi Sad. The samples were taken on 19th December (St. Nicolaus day), when the fish consumption is greatest in the Republic of Serbia and approximately half of the annual sales of fish is reached on that day. Eight samples of each fish species were taken from different retail stores (each sample of fish per species was taken from a different shop) and stored at –18°C till the next day when the analyses were performed. The weight within each fish species (marketable fish) was almost the same (average weight of common carp, silver carp, bighead carp, grass carp, Wels catfish, and zander were 2200, 2740, 2850, 1790, 2610, and 420 g, respectively). All fish were two years old (the end of growing season). All sampled fish were farm-raised (from different fish farms) in the semi-intensive systems of rearing, which is the main type of fish production in Serbia. In addition to natural food, cereals or extruded fish feed were supplemented depending on the feeding technology of the fishpond facilities. The results of the chemical composition determination are shown in Table 6. It was observed that fat content varies most in common carp (6.3–15%), bighead carp (3–10%), silver carp (4–8%), and at the least in zander (1.5–2.2%). Protein content also varied but less than fat content, and the variation was also the greatest in common carp (14.1–16.9%) and the lowest in silver carp (17.6–18.6%). Variation within the same species was noted in the amount of total cholesterol, which was the highest in common carp (43.2–65 mg/100 g). Interspecific differences of chemical composition were noticeable. Significant variations in the distribution of various fatty acids were noted between and within species. Fatty acid compositions, which are shown in Table 7, were ranged widely especially in the fillets of common carp, thus C16:0 was in the range of 17.3–33.4%. The amount of saturated fatty acids was notably constant in all examined species being around 30%, with palmitic acid being the dominant saturated fatty acid, no significant differences between species were observed ($p>0.01$). The amount of C18:1cis was in a wide range in common carp fillets and the lowest value was observed in silver carp fillets (12.5%). The amount of C22:6n-3 varied between the species and within the same species, and it was the lowest in the fillets of common carp and most favorable in the fillets of zander. The greatest deviation of total SFA was observed in common carp and in the contents of MUFAs and PUFAs in catfish and zander. The lowest n-3/n-6 ratio was found in one sample of common carp (0.12), and the greatest one in one sample of bighead carp (3.7). This ratio also varied between the species, the lowest value having been observed in common carp and the highest in the fillets of bighead carp, with no statistically significant differences between the silver and bighead carps.

Table 6. Chemical composition of seven fish species obtained from retail stores from Serbia (Ljubojević et al. 2013c)

Parameters	Common carp	Silver carp	Bighead carp	Grass carp	Wels catfish	Zander
Moisture (%)	73.16	74.68	74.48	76.17	77.74	77.89
Protein (%)	15.64	18.01	18.03	14.73	17.34	19.27
Fat (%)	10.07	6.1	6.29	8.02	3.96	1.8
Ash (%)	1.14	1.21	1.2	1.07	0.96	1.04
Cholesterol (mg/100g)	56.38	65.38	65.2	65.29	33.14	42.34

Values are means (n = 8).

Regarding chemical (Table 6) and fatty acid compositions (Table 7) of muscle tissue in the paper of Ljubojević et al. (2013c), it was expected that some major differences would be found within the same species and among different species in percentages of the nutrients monitored between the tested fish species in the present experiment because the fish came from the different environments, different species were included, and they were fed with different diets.

Table 7. The main groups of fatty acid composition of seven fish species obtained from retail stores from Serbia (Ljubojević et al. 2013c)

Fatty acid (%)	Common carp	Silver carp	Bighead carp	Grass carp	Wels catfish	Zander
SFA	33.55	34.57	32.82	28.72	30.22	28.6
MUFA	46.3	36.36	33.48	50.6	41.43	37,92
PUFA	20.1	29.46	33.73	20.72	28.39	33.67
∑ n-6	13.48	9.78	9.31	13.26	11.18	12.86
∑ n-3	6.61	19.68	24.54	7.46	17.21	20.81
n-3/n-6	0.48	2.04	2.68	0.68	1.52	1.62
n-6/n-3	2.78	0.49	0.38	2.22	0.69	0.62
PUFA/SFA	0.66	0.86	1.04	0.72	0.96	1.17

Values are means (n = 8). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; USFA, unsaturated fatty acids, PUFA, polyunsaturated fatty acids from series n-3 (n-3 PUFA) and n-6 (n-6 PUFA).

High average values in the fat content observed in the fillets of Cyprinids, especially in those of common carp, showed that the energy-protein ratio was not balanced in the diet of these fishes in a number of ponds. Fauconneau et al. (1995) and Romvári et al. (2002) also reported that lipid content of common carp fillets show high variance (1–13%) in commercial sized fish depending mainly on their previous nutrition.

The total cholesterol content in the carps fillets in the paper of Ljubojević et al. (2013c) is in agreement with the data of Bieniarz et al. (2001) and Kopicova and Vavreinova (2007) who reported slightly higher cholesterol content in the most analyzed fish species (49–92 mg/100 g) in comparison with terrestrial animal meats (45–84 mg/100 g) (Piironen et al. 2002) and it was lower for catfish and zander (33 and 42 mg/100 g, respectively), which is in agreement with our previous results (Ćirković et al. 2012). The total cholesterol content in the animal tissues can be influenced by the composition of the diet (Komprda et al. 2003). That could be the explanation for the variations in cholesterol content because the examined fishes came from different ponds and thus fed with different diet.

The obtained results by Ljubojević et al. (2013c) regarding to the fatty acid composition are in agreement with previous reported results referred to studied fish species (Jankowska et al. 2004; Celik et al. 2005; Steffens and Wirth 2007; Ćirković et al. 2012). The chemical composition including the fatty acid profiles varies widely among different fish species as previously noted by Ćirković et al. (2012).

The reason for the least desirable composition of fatty acid profile in the lipids of common carp can be accounted for by the type of food dominating in the diet. The traditional approach to the rearing of common carp in the Republic of Serbia is based on foods naturally occurring in ponds (zooplankton, benthos). The energy-producing component of their diet is supplemented with untreated cereals (corn and wheat). The feed rich in saccharides leads to an increase in the percentage of oleic acid (C18:1n-9) in the body lipids of the fish. At the same time, a decrease occurs in the percentage of n-3 PUFA (Fajmonová et al. 2003; Buchtová et al. 2007). According to the research conducted by Buchtová et al. (2010) and Ćirković et al. (2012), the carp grown on natural food had a high content of both n-6 and n-3 fatty acids. Ljubojević et al. (2013a) observed that PUFA/SFA ratio was the most favorable in the carp fed completed feed mixtures and the least in that fed with maize and wheat. All species of the warm water fish analyzed contained significant quantities of the n-6 series, particularly C18:2 and C20:4. The presence of these and the other PUFA emphasizes the potential of freshwater fish for use on special low fat diets. Wood et al. (2008) have suggested that the ratio of PUFA/SFA should be above 0.4, and according to that all the fish species examined revealed a favorable (from 0.66 to 1.17) PUFA/SFA ratio. Scollan et al. (2006) suggested that the n-6/n-3 ratio should not exceed 4 for the prevention of cardiovascular, heart, and certain chronic diseases. All studied species meet this suggestion. However, the European Food Safety Authority recommends the intake values: 2 g of n-3 PUFA ALA per day and 250 mg of EPA and DHA per day (EFSA, 2009). The percentage values of fatty acids were transferred to weight in mg/g of fillets according to Exler et al. (1975). A 200 g of edible portion of fillets of common carp, silver carp, bighead carp, grass carp, Wels catfish, and zander contained 436, 1320, 1774, 412, 612, 420 mg of EPA+DHA, respectively; and 1198, 2160, 2780, 1076, 954, 524 mg of n-3 PUFA, respectively.

The elementary prerequisite for the sustainable carp and other fresh water fishes production, with favorable chemical and fatty acid compositions, should be seen in the development of better feeding procedures. Completed formulated feed mixtures are necessary in modern fish farming because they improve the growth performance and chemical and fatty acid compositions in fish (Ljubojević et al. 2013a).

Effects of Diet on the Chemical Composition of Common Carp

Steffens and Wirth (2007) determined the impact of basic breeding systems and therefore the nutrition on survival rate, the yield per unit of area, chemical composition, the amount of total cholesterol and fatty acid composition of common carp, which was confirmed by Ljubojević et al. (2013a) in an experiment on two-year-old common carp (Table 8). Fish were grown in similar ponds and subjected to 1 of 3 feeding systems: only natural food (extensive system), supplemental grain (semi-intensive system), and extruded formula consisting of soybean, sunflower kernel, wheat flour, corn and brewery yeast (semi-intensive system). The

experimental carp were measured biweekly in order to adjust the daily feed rate that was 3% of the total fish mass. Fish were hand-fed twice daily at 8:00 and 15:00 h. The fishponds were stocked in March and harvested in October. All fish were reared under variable natural atmospheric conditions. Natural production in each pond was increased by application of agrotechnical measures such as drying of fish ponds during winter, soil treatment, fertilization and adding lime as described by Ćirković et al. (2012). The same methods of cultivation and fertilization were applied in each experimental units. The aeration of the fish ponds was continuously secured by using one aerator per pond. The water flow was about 3.5 l s^{-1}, that provided that there were no adverse effects of carbon dioxide and ammonia on the carp. Feeding extruded formula doubled production per hectare of pond surface area, compared with feeding supplemental grain and almost thrice compared with feeding only natural food. Chemical composition, fatty acid composition as well as the n-3/n-6 ratio varied widely by the diet (Table 8, 9). Carp fed extruded formula yielded the most preferable the unsaturated fatty acid: saturated fatty acids and polyunsaturated fatty acids: saturated fatty acids ratios. The conclusion was that properly processed plant nutrients can be important and a good source of protein for common carp and lead to improvements of production parameters in the pond, and the quality of the fish meat as a final product for human consumption. The harvesting weight, survival, harvesting density, and specific growth rate were the greatest in the semi-intensive system with addition of extruded feed mixture and lowest in the extensive system. At the end of the rearing period in October the average final live weight of carp in the group that had been fed on natural food was 462.58 g. Final live weight of carp in the group that had been given grains supplementary feeding was 754.08 g and in the group that had been formula feed feeding was 1188.75 g. Total harvesting density was 994.5 kg/ha in the extensive, 1696.68 kg/ha in the semi-intensive and 2734.12 kg/ha in the intensive system. Feeding extruded formula increased production per hectare of pond surface area almost doubled compared with feeding supplemental grains and almost thrice compared with feeding only natural food.

Supplementary feeding with grains leads to improved growth performance in common carp, and especially feeding with extruded formula. Survival rate in carp that ingested only natural food was lower than that of carp fed grains, as well as those fed formulated feed. In general, in all groups the survival rate was satisfactory and it was within the range considered normal for carp pond production in the Republic of Serbia. Additional feeding with grains almost doubled average final body weight, while carp that received extruded formula showed three fold higher final body weight than carp fed only natural food and that lead to doubled harvesting density in the semi-intensive system and threefold higher harvesting density in the intensive system compared with the extensive system. Consequently, all growth parameters were the highest in the intensive system and lowest in the extensive system. These factsct justified the use of supplemental feed in the rearing of carp and it represents a major opportunity to increase production in carp ponds. The growth parameters and total production of the carp were quite affected by that of the diets. Using adequately prepared extruded formula can further improve growth performance and yield of fish per unit of area. Administered extruded feed results in good growth and feed conversion. Higher temperature influenced the significantly higher growth rate of common carp in region of Republic of Serbia compared with Central Europe (Steffens et al. 2005). Besides the direct effect of using feed containing high protein indirect effect was achieved through nitrogen and phosphorus, which are released during digestion of formulated feed and increased development of natural

food in the pond (Marković and Ćirković 2008). The positive effect is certainly significant in maintaining a better quality of water environment. Carp reared in intensive systems had the greatest protein and moderate lipid, ash, and cholesterol contents. Expectedly, rearing in the extensive system reduced total lipid and cholesterol contents.

In the literature, depending on age, rearing system, and feed, fat content varies from 23 to 168 g kg^{-1}DM and protein content varies from 140 to 180 g kg^{-1} in carp (Vladau et al. 2008; Trbović et al. 2009; Ćirković et al. 2010). In the present trial nutrient composition was highly dependant on diet. Supplementary feeding with grains leads to enlarged amounts of crude lipid in fish meat and it was doubled higher compared to supplementary feeding with extruded formula and three-fold higher compared to carp which ingested only natural food. The fillets obtained from the experimental fish were characterized by a varied content of fat and water. The same pattern was observed by Ćirković et al. (2012). The varied content of fat was compensated by the content of water, which is in agreement with the results obtained by Żmijewski et al. (2006), who found a reverse correlation between the fat and water contents, which is common for many fish species. Crude protein level was the highest in the fillets of carp from an intensive system, while there were no significant difference in the amount of protein in fillets of carp from extensive and semi-intensive systems. Cholesterol content in fish meat is not correlated with fat content (Piironen et al. 2002). Data about influence of diet and rearing systems on cholesterol content in carp are limited. However, it is known that cholesterol content in lipids of carp varies considerably, within the range of 470 - 1200 mg kg^{-1} (Bieniarz et al. 2001; Kopicova and Vavreinova 2007). Total cholesterol content in the research of Ljubojević et al. (2012a) was the highest in semi-intensive system and the lowest in extensive system, but in all groups was favorable and within the previously mentioned (Bieniarz et al. 2001; Kopicova and Vavreinova 2007). This great variability could be related to harvest season and age as well as rearing system. The present results confirms that proximate composition of common carp highly depends of diet (Steffens and Wirth 2007). The fat content in fish meat contributes to its juiciness, tastefulness and texture, as well as organoleptic properties. Lipid content in fillets from the extensive system was very low and such lean tissue is dry and perceived as thickly fibrous. On the other hand, there are certain groups of people who require meat with minimal fat and cholesterol content.

Table 8. Proximate analysis results of common carp reared in different culture systems (Ljubojević et al. 2013a)

Variable	Extensive (Only natural Food)	Semi-intensive (Grain mixture: 80% Corn + 20% Wheat)	Semi-intensive (Extruded feed mixture)
Moisture (gkg^{-1})	814.9	764	783.5
Crude protein (gkg^{-1})	154.8	155.9	171.7
Crude lipid (gkg^{-1})	20.7	68.5	31.9
Crude ash (gkg^{-1})	09.6	11.6	10.3
Cholesterol ($mgkg^{-1}$)	379.4	578	513.1

Rearing in the extensive system resulted in the greatest total SFA level in meat of carp, particularly of palmitic and stearic acids. In the semi-intensive system with addition of cereals, MUFA was the greatest, predominantly of oleic acid. Carp that ingested only natural food had higher n-3 fatty acids in the muscle than carp that received supplemental wheat or

extruded formula. However, common carp reared in the intensive culture system had higher n-6 fatty acids than carp reared other two culture systems. Thus, the total amount of PUFA was higher in muscle triacylglycerol of carp fed with extruded formulated feed compared to carp fed only on natural food. This is reflection of dietary fat being transferred to body tissues. The n-3/n-6 ratio of the fish muscle was the highest in carp fed only on natural food, followed by carp fed extruded formula and the lowest value was observed in carp that received supplemental wheat. The highest level of n-3 fatty acids was found in the muscle of carp that received only natural food and the lowest in carp fed supplemental grains. In two year-old-carp fed extruded formula was observed the best ratio USFA/SFA, PUFA/SFA, the highest content of PUFA, the lowest content of SFA compared with other two groups. Lipids of carp in semi-intensive production with addition of extruded feed contained less MUFA (45%) than carp from the semi-intensive production with addition of cereals (64%).

The preference for a feed rich in saccharides leads to an increase in the percentage of the oleic acid in body lipids of the fish, which is produced in the organism by desaturation and elongation of SFA (Buchtova et al. 2007). At the same time, proportion of PUFA n-3 decreases (Fajmonova et al. 2003; Buchtova et al. 2007). Supplementary feeding with grains leads to reduced amounts of essential fatty acids in fish meat and this is due to the lower proportion of natural food in the diet of the carp which received additional grains. The two fatty acids 18:2n-6 and 18:3n-3 are precusors for synthesis of n-6 and n-3 PUFAs, recpectively (Sargent et al. 2002). According to Ljubojević et al. (2013a) carp that received extruded formula showed high values of n-6 fatty acids in their muscle, based on the high content of linoleic acid in the diets (Table 9). However, although grain mixture contained slightly higher amount of linoleic acid than formulated feed, percentage of this fatty acid was lower than in carp fed formulated feed, as well as than in carp which ingested only natural food, but the absolute content of linoleic acid was 2-3 folds greater in muscle of carp fed grains compared with muscle of carp fed only natural food. In general, in all groups the content of the n-6 fatty acids was higher than the content of n-3 fatty acids. The fatty acid composition of the carp muscle triacylglycerols was quite affected by that of the diets. Diets containing soybean or corn were characterized by high linoleic acid content. High contents of n-6 fatty acids in the grain based and extruded formulated (soybean meal, sunflower cernel, wheat flower and corn) diet resulted in high levels of these fatty acids in the carp meat.

The fatty acid composition of common carp reflects, to a large extent, that of the diet. The n-3/n-6 ratio varies between 0.8 and 2.4 (Steffens et al. 2005). There are reports indicating this ratio is about 0.5 (Kopicova and Vavreinova 2007; Ćirković et al. 2010), even less, about 0.2 (Trbović et al. 2009; Ljubojević et al. 2011). Ackman (2000) reported EPA and DHA acid concentrations in farmed carp as low as 0.35. In the study conducted by Ljubojević et al. (2013a), feeds did not contain highly USFAs. Freshwater fish possess the bioconversion capacity to elongate and desaturate C18 PUFA to n-3 and n-6 fatty acids such as arachidonic acid, EPA and DHA (Bell et al. 1997). Ćirković et al. (2011) have found that breeding system and especialy nutrition affected the fatty acid composition of fish. According to Buchtova et al. (2010) and Ćirković et al. (2012), common carp which was grown exclusively using natural food available in the fish pond had a high content of the n-6 and n-3 fatty acids, and Ćirković et al. (2011) noted that the PUFA/SFA ratio was the most favorable in common carp fed completed feed mixtures, and the least favorable in common carp which

receiced grains as supplementary feed. The USFA/SFA ratio was also the most favorable in common carp which received completed feed mixtures.

Table 9. The main group of fatty acids of common carp reared in different culture systems (Ljubojević et al. 2013a)

Fatty Acids (% of the total Lipid)	Extensive (Only Natural Food)	Semi-intensive (Grain Mixture: 80% Corn + 20% Wheat)	Semi-intensive (Extruded feed mixture)
SFA	28.82	24.19	22.4
MUFA	43.49	64.31	45.12
PUFA	27.69	11.53	32.48
n-6	17.93	10.24	26.01
n-3	9.75	1.29	6.48
n-3/n-6	0.54	0.13	0.25
n-6/n-3	1.84[c]	7.99	4.02
PUFA/SFA	0.64	0.18	0.72
UFA/SFA	0.96	0.48	1.45
PUFA/MUFA	2.47[c]	3.13	3.46

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; USFA, unsaturated fatty acids, PUFA – polyunsaturated fatty acids from series n-3 (n-3 PUFA) and n-6 (n-6 PUFA).

Ljubojević et al. (2013a) found that the ratio of n-3/n-6 in common carps varied by feed and/or rearing system. Using adequately processed plant meals as replacement protein sources can further improve productivity and nutritive value of carp, as reflected by n-6 polyunsaturated fatty acids, especially linoleic and arachidonic acids and favorable content of total cholesterol.

Effects of Good Production Technology and Appropriate Structure of Planktonic and Benthic Organisms on Chemical and Fatty Acid Composition of Common Carp

The fatty acid composition of completed feed mixture can change the fatty acid composition of various tissues, including muscle tissue of fish (Pickova and Morkore 2007; Kjaer et al. 2008; Todorčević et al. 2009; Ljubojević et al. 2013a; 2014), and changes in fatty acid composition of tissue will continue to affect many metabolic processes related to the synthesis and catabolism of fatty acid and lipid storage (Tocher 2003). The cost of fishmeal (FM) and fish oil (FO) on the global market increases continuously, rendering their sustainability as questionable components in fish feed (Tacon and Metian 2008). Neither FM nor FO is produced in many countries; the import thereof raises production expenses and poses a serious problem for the fish farming industry. Thus alternative feed components are necessary to meet these requirements. Natural food of common carp is mainly based on zooplankton, zoobenthos and detritus (Adámek et al. 2003). Plankton and benthos contain high levels of n-3 polyunsaturated fatty acids (n-3 PUFA), including EPA and DHA (Mráz et al. 2012; Ljubojević et al. 2014). Therefore, good preparation of fish ponds, and appropriate technology during the growing cycle are necessary for the proper quantities, as well as favorable structure of natural food, which is of great importance regarding improving the fatty

acid composition of common carp carp and other cyprinid species. Grains are commonly used as a supplementary feed for carp. Since the grains are rich in carbohydrates and have a very low level of n-3 fatty acids, the meat of carp fed with grains contains a high percentage of oleic acid and low level of n-3 HUFA (Ljubojević et al. 2013a).

Analysis of the natural food disclosed that it was rich in EPA and DHA, with a high n-3/n-6 ratio which was higher than 3 (Ljubojević et al. 2014). In the total lipid of natural food samples the proportion of SFA, MUFA and PUFA ranged from 27.6 to 28.4%, 26.1–27.4, and 45.3–46.8% of total identified fatty acids (FA), respectively. The content of total SFA as well as of palmitic acid, which was the most prevalent SFA in all groups, did not vary considerably among the experimental units. OA was the most prevalent MUFA in all units. Among n-6 PUFA, the most prevalent was arachidonic acid (AA), and relatively high percentages of LA and ALA were also detected.

Effects of Formulated Feed Mixtures on Fish Health, Production Parameters and Meat Quality

Due to the intensification of cyprinid fish species production, industrially produced feed mixtures have become an essential factor in successful rearing of these species. Fish meal and fish oil represent a good source of protein and fat in feed for fish, but because of the growing volume of production and higher consumption and consequently reduced availability and a drastic increase in the price of these components, there is growing interest for the use of components of vegetable origin as a complete or partial replacement for fish meal and fish oil in fish feed. Replacement of fish meal and fish oil with components of vegetable origin in fish feed can have negative effects on meat quality of fish, including cyprinid fish species, since it is known that fish meal is a good source of amino acids, and fish oil is good source of n-3 HUFA, which is not the case for vegetable oils, which contain a high percentage of 18C n-3 fatty acids, while the vegetable oils are usually devoid of n-3 HUFA, and thus may lead to deterioration of the fatty acid composition of fish meat.

The use of components of vegetable origin and their effect on the production performance, as well as on meat quality parameters of produced fish was the aim of research conducted by Ljubojević et al. (2015), where the experiment was carried out on carp in cage rearing system. For the purpose of this trial, triplicate groups of common carp (*Cyprinus carpio* L.), average weight of 400g were distributed in twelve cages of 125 m3, while stocking density was 1000 individuals per cage. The cages, which are normaly used for commercial production of common carp were placed on Tikveš Lake (Republic of Macedonia). Prior to the feeding trial, the fish were aclimatized and fed with commercial extruded feeds (FSH "Komponenta," Ćuprija, Republic of Serbia) according to the recommendations of the manufacturer. The trial lasted 75 days, from April to June 2013. Four isolipidic extruded diets were formulated (FSH Komponenta, Ćuprija, Serbia) to provide 33% of protein [high protein (HP) diets] or 28% of protein [low protein (LP) diets]. Ingredients used for the basic diet were: soybean meal, sunflower meal with 33% of protein, corn, wheat, yeast and mineral and vitamin mix. 6% of fish oil (FO diet) or rapeseed oil (RO diet) was added in a vacuum coater to the basic formulated feed. Thus, four different diets were obtained: ROHP-rapeseed oil-high protein, FOHP-fish oil-high protein; ROLP-rapeseed oil-

low protein and FOLP- fish oil-low protein. Daily feed amount was given in three sessions at 8.00, 13.00, and 17.00 h; by automatic feeders.

The FOHP diet contained 29.7% saturated fatty acids (SFA) of which approximately two third (19.4%) was palmitic acid and 35.5% MUFA, whereas the content of OA was 28.5%. The FOHP diet contained 14.3% n-6 PUFA, predominantly LA (12.4%) and 20.5% n-3 PUFA, with 5.8% EPA and 11.2% DHA. Lowering protein content resulted in decreased level of SFA, which was 25.3% in the FOLP diet and in slightly increased content of MUFA (39.8%), mainly due to increased content of OA (35.6%). Inclusion of RO in high protein diet result in decreased content of palmitic, eicosanoic, AA, EPA and DHA (approximately for 7.5%; 0.1%; 0.9%; 4.5% and 8.7%, respectively). The n-3/n-6 ratio decreased from 1.4 and 0.9 in diets containing FO (with high and low protein level, respectively) to 0.5 and 0.2 in diets containing RO (with high and low protein level, respectively). The total content of MUFA was the highest in the ROLP diet, mainly due to high content of OA, which is the most abundant fatty acid in RO, but also in corn which was used to replace FM. The content of total MUFA was approximately 1.8 fold higher in diets which contained RO with the same content of protein, and 1.2 fold higher in the FOLP in comparison with the FOHP diet. The total content of n-6 PUFA was increased 1.3 fold in the diets with RO, mainly due to increased content of LA (from 12.2-14% in the diets with FO to 20.2-28.7% in the diets with RO).

There were no significant differences in initial mean weights of the fish. Following 75 days of trial, the mean weight was between 1400 g in FOHP and 1416 g in ROHP group. No statistically significant effect of oil source or protein level or interactions of these factors were observed in final body weight, although the highest body weight was observed in the ROHP group. Moreover, no significant effects of abovementioned factors or their interaction were identified in growth parameters (SGR and WG). Feed conversion ratio (FCR) was satisfactory for all treatments and ranged from 1.58 to 1.67. Two-way ANOVA showed no significant effect of oil source, dietary protein level or their interaction on FCR.

No significant effects of treatments were observed in the content of crude protein and ash in common carp fillets. Moisture content was significantly lower in carp fillets from groups which received diets with lower protein content, which was accompanied by higher content of fat in muscle tissue (Table 10). Oil source showed no significant effect on proximate composition of carp fillets, but resulted in significant changes in fillet fatty acid compositions (Table 11).

The proportions of the analyzed fatty acid groups were significantly affected by the diet used. Oil source and protein level significantly affected muscle fatty acid composition. However, significant interactions between oil source and protein level were observed only for a few fatty acids. It was observed that content of OA increased 1.5-fold, total MUFA 1.5-fold, LA increased 1.7-fold, total n-6 PUFA 1.4-fold and ALA 1.9-fold in flesh of common carp from group ROHP compared to flesh of carp from FOHP group. On the other hand, the content of saturated fatty acids (SFA) decreased 1.2-fold, arachidonic acid (ARA) 1.4-fold, EPA 2.8-fold, DHA 1.9-fold and n-3/n-6 ratio 1.4-fold in flesh of carp from ROHP group compared to FOHP group. The content of EPA in muscle tissue decreased more than twofold, while decrease in DHA content was moderate. It is interesting that content of DHA was higher in muscle tissue of carp fed diets supplemented with RO compared to the RO diets. Decreased level of protein in diets, i.e., replacement of FM with corn resulted in increasing content of margarinic acid (17:0), stearic acid (18:0), OA and total MUFA content and in

decreasing of content of ALA, EPA, DHA, total PUFA, total n-3 PUFA, as well as n-3/n-6 in muscle tissue of carp.

Table 10. Proximate composition (% of wet weight) in fillets of common carp fed the four experimental diets for 75 days (Ljubojević et al. 2015)

Variable (%)	FOHP	ROHP	FOLP	ROLP	Two-way ANOVA p		
					Oil	Protein	Interaction
Moisture	78	78.1	74	74.1	0.93	p<0.001	0.98
Crude protein	16.8	16.9	16.6	16.5	0.93	0.18	0.67
Crude lipid	4	4	8.2	8.1	0.86	p<0.001	1
Crude ash	1.1	1.1	1.2	1.2	0.88	0.32	0.88

All values are mean (n = 3); FOHP, fish oil-high protein group; ROHP, rapeseed oil-high protein group; FOLP, fish oil-low protein group; ROLP, rapeseed oil-low protein group.

Table 11. The main groups of fatty acids (% of the sum of all fatty acids) in fillets of common carp fed the four experimental diets for 75 days (Ljubojević et al. 2015)

FA%	FOHP	ROHP	FOLP	ROLP	Two-way ANOVA p		
					Oil	Protein	Interaction
SFA	23.7	19	24	20.1	0.01	0.65	0.81
MUFA	22.7	34.1	26.9	40.1	p<0.01	0.009	0.57
PUFA	36.3	42.8	31.4	33	0.11	0.01	0.31
n3	21.9	22.3	14.6	10.8	0.1	p<0.01	0.05
n6	14.4	20.5	16.8	22.2	0.004	0.2	0.82
n3/n6	1.5	1.1	0.9	0.5	p<0.01	p<0.01	0.68
n6/n3	0.7	0.9	1.1	2.1	p<0.01	p<0.01	p<0.01
PUFA/SFA	1.5	2.3	1.3	1.7	0.01	0.04	0.29

All values are mean (n = 3); SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-3 PUFA, polyunsaturated fatty acids from the n-3 family; n-6 PUFA, polyunsaturated fatty acids from the n-6 family. FOHP, fish oil-high protein group; ROHP, rapeseed oil-high protein group; FOLP, fish oil-low protein group; ROLP, rapeseed oil-low protein group.

Several studies have shown that total replacement of FO with vegetable oils, including RO in diets of cyprinid fish species, has no negative effects on fish growth and growth parameters (Turchini et al. 2007; Kukačka et al. 2009; Zakęś et al. 2010). Results obtained by Ljubojević et al. (2015) are in agreement with the previous studies and showed no negative effect of total inclusion of RO instead of FO on production parameters and growth of common carp reared in cages under natural conditions.

Ng et al. (2003) stated that the weight gain in the African sharptooth catfish *Clarias gariepinus* was considerably higher when fed with the addition of palm oil than with fish oil and assumed that a high proportion of n-3 PUFA in feed supplemented with fish oil is responsible for reducing the growth rate in this species. The high level of n-3 PUFA in feed is also associated with retardation of growth of tilapia (Ng et al. 2001) and grass carp (Du et al. 2008). Radunz-Neto et al. (1994) reported that very low content of n-3 fatty acids in feed for the larvae of common carp (peanut oil as a source of fat) did not significantly affect the growth parameters compared to the group which received a diet containing cod liver oil. Fingerlings of common carp which were fed diets supplemented with 10% corn oil, soybean oil, rapeseed oil or fish oil showed no significant differences in the growth parameters and

feed conversion ratio (Steffens et al. 1995). The high survival rate (> 95%) was observed in all experimental groups which were included in the study (Ljubojević et al. 2015), with no significant difference between the experimental treatment, indicating that the experimental feed mixtures did not have negative effects on the health of carp during the trial. Moreover, according to Keshavanath et al. (2002), carp growth in low protein diets compared to high protein diets was not significantly affected, which is in line with the results obtained by Ljubojević et al. (2015). Cho et al. (2001) noted that weight gain of common carp decreased linearly with increasing protein level in feed. In the trial conducted by Satpathy et al. (2003) the same growth parameters were observed in Indian carp (*Cyprinus catla*) fed diets contained 35% or 40% of crude protein. This phenomenon may be related to the optimization of protein level which is used for growth, while the fat or carbohydrate in feed satisfies energy needs (Caballero et al. 2002). The optimal ratio of protein and energy is a key factor for the efficient utilization of protein.

The optimal ratio of protein and energy is a key factor for the effective utilization of protein. The carbon atoms of glucose from feed can be efficiently used by carp as an energy substrate, since replacement of animal proteins with high levels of carbohydrates in feed does not affect growth performance growth. Increasing the amount of digestible starch also leads to an increase in the fat deposition, indicating that most of the carbon atoms of glucose from feed are included in the process of lipogenesis. In addition, increasing the amount of digestible starch significantly improves the retention of proteins which highlights the fact that the increasing of utilization of glucose for energy production leads to the reduction of protein wastage. If proteins are present in greater quantities than optimal in feed it leads to their utilization for energy production, which explains the results obtained by Capilla et al. (2004) that the retention of protein was lower in the group of carp fed with feed rich in proteins compared with the group fed with feed where the fish meal was completely replaced with maize and containing the lower protein content. It should be noted that in the study conducted by (Ljubojević et al. 2015), as well as in previously research, lipids in the experimental diets were not just from the added oil, lipids were also from the used vegetable meal and from fishmeal (fat content originating from the added oil in research Ljubojević et al. (2015) was 73.5% of total fats). Keshavanath et al. (2002) observed no changes in production parameters of carp when total amount of FM in the diet was replaced by corn (diet with 19% protein). It must be taken into account that, in the above mentioned experiment, tanks with fertilized bottoms were used, which corresponds to the production of carp in earthen fish ponds. The authors noted a protein sparing effect with the use of ingredients rich in carbohydrates, and this effect was pronounced when protein level was sub-optimal, compared to optimal protein level in diets for common carp fingerlings. A protein-sparing effect was confirmed in the study conducted by Ljubojević et al. (2015), and similar results regarding production performance were found when diets with 33% and 28% protein were used. Experiments conducted to date indicate that, when nutritional needs for essential fatty acids for omnivore species such as common carp, but also herbivores, such as tilapia and grass carp are fulfilled, the use vegetable oils in feed for these species does not affect growth performance or the feed efficiency. In addition, the need for fatty acids with 20 and 22 carbon atoms (C20 and C22 HUFA) can be met from their precursors in feed.

According to previous research (mainly on salmon and trout) results showed that both factors of protein levels and oil sources have an influence on the chemical composition of the examined fish (Solberg 2004; Torstensen et al. 2004; Ruyter et al. 2006; Trbović et al. 2012).

Nutritional requirements of carp for proteins vary from 25% to 35% (Hossain et al. 1997) and are highly dependent on the age of the fish. Steffens (1996) indicates that the increase in energy content in feed is a possible strategy for saving protein and limiting the production of ammonia in fish ponds. Ljubojević et al. (2015) noted that moisture content was significantly lower in carp fillets from groups which received diets with lower protein content, which was accompanied by higher content of fat in muscle tissue. Thus, an increased level of carbohydrates resulted in increasing deposition of fat, both around visceral organs and in muscle tissue. Similar findings were noticed after the inclusion of carbohydrate-rich grains, in the feed of common carp in a semi-intensive system (Vasha et al. 2007; Ljubojević et al. 2013a). A negative correlation between the fat and water contents in the flesh of the carp was also previously shown (Ljubojević et al. 2013a). The research conducted by Keshavanath et al. (2002) also showed that chemical composition of carp flesh was influenced by the experimental diets, especially contents of fat and ash. They observed progressive increase in fat contents in muscle tissue with increase of carbohydrate content in diets, while the protein content was constant in all dietary treatments. Ljubojević et al. (2015) also indicate that the retention of the protein in the muscle tissue was achieved when the level of dietary protein decrease, which further indicates that the protein sparing effect, was achieved. The content of SFA in common carp fillets was higher than in the RO and LP diets tested, and in the fillets of the carp fed diets supplemented with RO and with lower protein content it increased sufficiently, so that it was comparable to the values noted in the FO and HP groups. Accordingly, the quantitative and qualitative content of SFA in the experimental diets supplemented with RO was sufficient to maintain this group of fatty acids at a level comparable to values noted in the fish from group FO. This is a noteworthy finding since SFA in muscle tissue represent an important component of polar lipids. In several studies conducted on marine fish species during feeding with feed with addition of vegetable oil was found lower levels of SFA in muscle tissue compared to fish fed diets with addition of fish oil (Izquierdo et al. 2003, Montero et al. 2005). This was also confirmed by Molnár et al. (2006), during feeding trial in perch fingerlings with the addition of linseed oil in feed. Fish feeding with feed which comprise oils of vegetable origin which are rich in the C18 fatty acid (OA, LA, ALA), leads to an increase in the content of these fatty acids in tissues (Mourente et al. 2005). It seems that common carp utilize OA well as a source of metabolic energy (Ljubojević et al. 2015). Lowered levels of this FA in the muscle tissue, regardless of the dietary treatment, might mark highly active mitochondrial enzymes oxidizing FA (Torstensen et al. 2000). This can be related to the fact that mitochondrial β-oxidation is of specific importance to muscle tissues (Nanton et al. 2003).

Fatty acid composition of carp meat is heavily influenced by the fatty acid composition of carp feed (Steffens and Wirth 2007; Ljubojević et al. 2013a) and there is a linear correlation between individual fatty acids in the total fat in the tissue and their concentration in lipids from feed according to research conducted by Ljubojević et al. (2014). Oil source and protein level significantly affected muscle fatty acid composition. However, significant interactions between oil source and protein level were observed only for a few fatty acids. It was observed that content of OA increased, as well as total MUFA, LA, total n-6 PUFA and ALA in flesh of common carp from group ROHP compared to flesh of carp from FOHP group. On the other hand, the content of saturated fatty acids (SFA) decreased, as well as arachidonic acid (ARA), EPA, DHA and n-3/n-6 ratio in flesh of carp from ROHP group compared to FOHP

group. The content of EPA in muscle tissue decreased more than twofold, while decrease in DHA content was moderate. It is interesting that content of DHA was higher in muscle tissue of carp fed diets supplemented with RO compared to the RO diets. Decreased level of protein in diets, i.e., replacement of FM with corn resulted in increasing content of margarinic acid (17:0), stearic acid (18:0), OA and total MUFA content and in decreasing of content of ALA, EPA, DHA, total PUFA, total n-3 PUFA, as well as n-3/n-6 in muscle tissue of carp. These data showed that when specific fatty acids are in abundance in the diets that they are selectively utilized for energy production via β-oxidation, and for desaturation and elongation (Bell et al. 2003). In contrast, when FA, and especially n-3 HUFA, are limited in the diet they are retained or deposited in the muscle or in other tissues. Along with the selective deposition and retention of n-3 HUFA, moderate reduction of EPA and DHA may also be affected, albeit to a lesser extent, by desaturation and elongation of alpha-linolenic acid occurring in the liver which can be enhanced by including vegetable oils of vegetable in fish feed (Tocher et al. 2003). On the other hand, EPA is likely to be used and catabolized to generate energy and to a lesser extent, will be deposited in the biomembranes than DHA (Morais et al. 2011). It has been shown that feed containing vegetable oil leads to improved retention of n-3 fatty acids in salmon and in this way makes reserves that will be used in case of need (Bendiksen et al. 2003). In addition, it is known that the presence of vegetable oils activated fatty acid desaturation and elongation in vitro (Tocher et al. 2000; Bell et al. 2001). In study conducted by Ljubojević et al. (2015) trends in levels of docosapentanoic acid (22:5n-3, DPA) and DHA in carp fillets relative to their respective dietary contents (DPA and DHA increased in fillets) suggested the bioconversion of EPA to DHA. A similar trend of the selective deposition was seen in n-6 FA in which improved retention was seen in AA.

The intermediates, gamma-linolenic acid (18:3n-6), eicosadienoic (20:2n-6), dihomo-γ-linolenic (20:3n-6) and eiocosatrienoic (20:3n-3) acid were detected in carp fillets in RO groups. Since vegetable oils are deprived of these fatty acids and they are part of the biosynthetic pathways of n-6 and n-3 HUFA, this result features adaptive attempts to moderate HUFA deficiencies. When the content of ALA or LA in the diets was increased, eicosatrienoic acid 20:3n-3 or eicosadienoic acid 20:2n-6 content in muscle tissue was also increased. A similar phenomenon was observed in Atlantic salmon (Tocher et al. 2002). Ghioni et al. (2002) have shown that the ingested stearidonic acid 18: 4n-3 rapidly converted in eicosatrienoic acid 20: 4n-3, and that both of above mentioned fatty acid is further subjected to the process of elongation and desaturation to EPA and 22: 5n-3, but not the 22: 6n-3 in cell culture of salmonids. The high level of LNA and LA in feed mixtures supplemented with rapeseed oil and the content of n-3 HUFA from fishmeal appears to meet the needs for essential fatty acids of common carp in research conducted by Ljubojević et al. (2015). This is probably derived from the ability of common carp to selectively retain DHA and bioconvert LNA to EPA and DHA; EPA to DHA, and LA to AA. Therefore, results obtained by Ljubojević et al. (2015) indicate that the common carp have high tolerance to diets that differ significantly in fatty acid composition. It is also noteworthy that the main consequence of higher DHA and lower LA contents in the fillets of the common carp was the increased value of the n-3/n-6 ratio compared to the levels of this ratio in the diets. However, in the case of the RO and LP groups, value was significantly lower than that in the groups FO and HP. Despite the deficit of C20 and C22 fatty acids in fish feed supplemented with rapeseed oil, a low content of these fatty acids was detected in meat of goldfish (Pozernick and Wiegand 1997), and this phenomenon is explained by the ability of freshwater fish to

produce HUFA from LA and ALA (Buzzi et al. 1996). It is known that MUFA are produced by desaturation of SFA or by synthesis in the body from food rich in energy (Csengeri 1996; Henderson 1996), in a same way as other vertebrates by desaturation of the present compounds by using the Δ-9 desaturase or could be produced from external sources and stored directly into adipocytes.

Deposition of EPA and DHA in the muscle tissue of common carp indicating a high potential for the use of rapeseed oil as an ingredient in feed for carp and further research during the whole growing season is necessary, in order to avoid the possibility that the results obtained in a relatively short period of time show no real degree of change in fatty acid composition to the end of the growing cycle. The biggest problem is the high cost of this type of trial. Despite an increase in total world consumption of fish meal and fish oil in aquaculture, the average share of fishmeal and fish oil in feed for particular species is in steadily decline, as is the case with crabs (from 28% to 20%), marine fish species (from 50% to 32%), salmon (from 45% to 30%) and carp (from 10% to 5%) (Tacon and Metian, 2008). The great number of previous studies, which more than two decades is dealing with this problem, certainly contributed to the above mentioned fact, but there is still a need to reduce consumption of these ingredients in order to preserve natural resources and to ensure economically sustainability of fish production.

Effects of Age, Sex and Genetic Factors on the Chemical Composition of Fish Meat

Age

Results related to meat quality of carp are vary among authors, with differences mostly caused by analysis of different-aged fish. Ljubojević et al. (2011) analyzed the meat quality of one-year-, two-year- and three-year-old common carp and found that age significantly affects the chemical composition of meat, mostly with reduced water content and increased fat content in meat with increasing age, but to a small extent on the fatty acid composition of the fish. The established n-3/n-6 in different categories were in the range from 0.1 to 0.16, and the most favorable ratio was observed in one-year-old carp and the least favorable in three-year-old carp. Results obtained by Ljubojević et al. (2011) were consistent with studies conducted by Trbović et al. (2009) on the yearling carp, and the ratio was lower than the results obtained by Ćirković et al. (2012) for two-year-old carp which were fed with natural food. Fat and moisture content increased with age, while amount of protein and ash decreased in older common carp. It was found that the age of the fish had a much smaller effect on the fatty acid composition of fish meat compared to effect on the chemical composition of fish meat. Thus, no significant effect of age on n-3/n-6 ratio was observed. Percentage shares of EPA and DHA slightly decreased with age, as well as PUFA/SFA ratio which is an indicator of the quality of fat, PUFA content decreased with age while MUFA content increased.

The fat content was the most favorable in meat of two-year-old carp, while the fat content in meat of three-year-old carp was slightly above 10%, and more than 10% of fat in carp meat could lead to a negative sensory properties of meat. The two year production cycle could be a good way to produce carp with optimal chemical composition of meat.

Low content of n-3 fatty acids in all three age categories is due to nutrition, which is applied to the pond from which the samples were taken, where grains are added as additional feed. The consequence of this type of production and great amount of grains in nutrition is reflected in a large percentage of oleic acid in the fat from all three examined age groups (over 50% of total fatty acids). The meat quality of carp could be improved by introducing completed feed mixtures and improving agro-technical measures, in order to increase production of natural food in the pond.

Fauconneau et al. (1995) found that the increase in body weight of carp is stimulated by increased feed intake, regardless of the age of fish and that is closely associated with an increase in fat content, both in the flesh and in muscle tissue of fish. This fact is very important in breeding of carp, because high energy level in feed for stimulation growth and shortening production time affects the increase in fat content, while the protein content in the meat remains constant (Kaushik 1995).

Genetic Factors

It has been shown that the fat content in the muscle highly heritable traits in common carp and that there is a relatively high positive genetic correlation between body size (body length and body weight) and fat content in muscle tissue (Kocour et al. 2007). On the other hand, Buchtova et al. (2007) did not note the impact of hybrids on the fatty acid composition in experiment with four different hybrids of common carp. It is obvious that there is a need for further research when it comes to genetic factors.

Sex and Sexual Maturity

Buchtova et al. (2010) in their research on four hybrids of common carp found only small differences in the fat composition between males and females, which are probably caused by different fat content in muscle tissue. Fajmonova et al. (2003) did not note the difference between the sexes when it comes to fat content and fatty acid composition in three-year-old common carp.

CONCLUSION

Common carp is one of the most important fish species worldwide and represents an important nutritional source of unsaturated fatty acids. Its meat has been shown to play an important role in human health. It was found that the fat content and fatty acid composition significantly differs in different fish species, even when they belong to the same family. In addition, the same parameters were found to be influenced by different environmental factors, good production technology and appropriate structure of planktonic and benthic organisms, especially by diet. Using formulated feed mixtures had a positive effect on fish health, production parameters and meat quality.

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Chapter 99

DISEASES OF COMMON CARP AND THEIR CONTROL MEASURES

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ABSTRACT

Common carp (*Cyprinus carpio* L.) contribute significantly to global freshwater fish production but it may also play a significant role in the expansion of various parasitic fish diseases around the globe as many parasites are dispersed locally and internationally due to common carp trade. The production of common carp in natural habitats and in aquaculture farms often become threatened due to various factors including disease outbreaks. Disease increases the management costs as well as decreasing fish production. To prevent and control various diseases, it is important to understand the mechanism of disease outbreak, which generally involves a complex interaction among fish, pathogens and the environment. High stocking density, poor management practises and various environmental problems result in exposure of fishes to stress that often leads to disease. The disease can also be initiated by various biological agents, which can be prevented and controlled by effective management practices. Due to the preference of soft vegetated sediments as habitat, common carp is also susceptible to many pathogens (e.g., viruses, bacteria, fungi, parasites, etc.), which cause various diseases. A severe pathogenic infection may cause high mortality of fish. This paper reviews various diseases of common carp and their control measures.

Keywords: disease, common carp, causative agent, sign, prevention, control measure, viruses, bacteria, fungi, ectoparasites, endoparasites, life cycle, host

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INTRODUCTION

Common carp (*Cyprinus carpio* L.) perhaps represents the one fish species, freshwater or marine that has attracted the most attention from humans across the globe. It is one of the most widely distributed freshwater fish in the world (Piria et al. 2016). In many countries particularly in Central Asia and Europe, common carp is one of the most popular food fish and is commonly cultured in aquaculture ponds applying extensive or semi-intensive methods. In many countries, common carp is considered as the main aquaculture species. Thus, it contributes significantly to global fish production, particularly global freshwater aquaculture production. In some countries, particularly in Australia and North America, common carp is considered as a destructive invasive alien species as it causes major impacts to habitat and ecosystems including affecting many local species. It is one of the most destructive fish, being among the hundred worst invasive alien species (Zambrano et al. 2006; Khan et al. 2016). It is often believed that common carp play a significant role in the ongoing expansion of various parasitic fish diseases around the globe (Oros et al. 2015). Many parasites have been dispersed locally and internationally due to the common carp trade which are then spread to local fish populations. While there are a multitude of factors that can impact on the production of common carp, in both natural and farmed populations, disease is one of the most important factors (Hoffman 1999). Disease increases the management costs of carp farming and also leads to reduced profits due to diminished fish production. The tentative estimated annual global loss of fish production due to disease is $1.05 billion to $9.58 billion (Shinn et al. 2015). To prevent and control various diseases, it is important to understand the mechanism of disease outbreak, which generally involves the complex interaction among fish (host), causative agents of disease (pathogens) and the environment (Snieszko 1974). High stocking density, poor management practices and various environmental drivers result in exposure of fish to stress that often leads to disease (Snieszko 1974; Lavilla 2001). Biological agents (pathogens) particularly viruses, bacteria, fungi and parasites may initiate disease. Their growth and abundance are related to various environmental factors (e.g., temperature, various dissolved gases, pH, and availability of food). However, sound management practices can reduce the growth and abundance of biological agents.

Common carp prefer to live near the soft vegetated sediments, which is generally characterized by poor water quality and high decayed organic matter. Due to this habitat preference, common carp is susceptible to many benthic-associated pathogens, which cause a range of diseases. Commonly encountered diseases of common carp include Saprolegniosis, Branchyo-mycosis, Erythrodermatitis, Columnaris disease, Bacterial gill disease, Mycobacteriosis, Spring viraemia of carp, Carp pox, Koi Herpes Virus Disease, Costiosis, Coccidiosis, Ichthyophthiriosis, Chilodonellosis, Trichodinosis, Myxobolosis, Dactylogyrosis, Gyrodactylosis, Diplostomosis, Phosthodiplostomosis, Sanguinicoliasis, Ligulosis, Bothriocephalosis, Khawiosis, Nematode infestation, Phylometrosis, Fish Leech infestation, Ergasilosis, Lernaeosis and Argulosis. This chapter reviews diseases of common carp and their control measures.

SAPROLEGNIASIS

Saprolegniasis is a fungal disease caused by water molds of the family *Saprolegniaceae*. Many species are responsible for this disease. Most of the species are from the *Saprolegnia, Achyla, Aphanomyces and Dictyuchus* genus. They infect eggs, fry, fingerlings and adult common carp and this disease is, therefore, considered a major problem in common carp aquaculture (Bruno et al. 2010). Saprolegniasis generally occurs more in hatcheries compared to fish farms. The optimum temperature for the causative agents is 15 to 30°C but the range of temperature for their growth is 3-33°C (Aly and El Ashram 2000). Sudden changes in temperature can make fish vulnerable to saprolegniasis, due to increased physiological stress (Bruno et al. 2010; Das et al. 2012). In India, Das et al. (2012) observed severe mortality of fingerlings by this disease during the period of early November 2009 to late January 2010 (winter months). They observed that fishes died within 12-15 days from the onset of infection. The causative agents generally attack an existing injury on the fish and gradually spread to healthy tissue. Presence of dead eggs and poor water quality particularly low water circulation, low dissolved oxygen and high ammonia can influence rapid spreading of the disease (Ruthing 2009).

Causative Agents

Species in the genera of *Saprolegnia* (most common), *Achyla, Aphanomyces and Dictyuchus*. The major etiological agents of saprolegniasis *Saprolegnia parasitic*, *Saprolegnia diclina* and *Achlya hoferi* for freshwater fish and fish eggs.

Disease Signs

White fungal colonies appear on egg surface, body surface, and wounded or ulcerated areas of fish. The first sign of infection is visible red or grey patches of filamentous mycelium. Gradually, the infected area would appear as cotton-like, white to grey growth on the skin, gills, fins and eyes or eggs of fish. An acute infection takes 7-8 days from the beginning of infection (Das et al. 2012). In severe cases, almost the whole body may be covered with fungal growth.

Management and Control

Malachite green at 5 mg/L for one hour or 5% sodium chloride at 1-2 mg/L or combination of 100 mg/L of formalin and 2.5 mg/L of malachite green for one hour are generally used for the infestation of infected eggs, fry, fingerlings and adult fish (Eli et al. 2011). Repeated doses can be used if necessary, although malachite green and formalin do have an acute impact on the aquatic environment. Immunity of fish frequently treated with these chemicals is suppressed (Prost and Spinska 1989). Das et al. (2012) suggested two steps

at the beginning of infection for a faster recovery: dipping fish with 4g salt per liter of water for 2 minutes followed by with 5 ppm potassium permanganate ($KMnO_4$) for 10 minutes.

BRANCHYOMYCOSIS

Branchyomycosis is a fungal disease and also known as gill-rot disease. This disease is found in fish suffering from an environmental stress particularly low pH and dissolved oxygen (Ramaiah 2006). The main sources of infection are the fungal spores carried in the water and detritus on pond bottoms and it therefore, occurs in the environments in which there is an abundance of decaying vegetation (Jhingran and Pullin 1985). It is an acute infection of the gills that may cause high mortality of common carp due to anoxia through obstructing the flow of blood through the gills (Noga 2010). The tubules of fungus grow into the epithelial cells of the gills, causing inflammation and damaged blood vessels around the epithelial cells. The infected gill can be rotten due to decreased blood supply and gill epithelial cell hyperplasia. It usually occurs at temperatures exceeding 20°C (Jhingran and Pullin 1985). The responsible fungus species can grow best between 25° and 32°C but can still grow at temperatures between 14° and 35°C. Fry and small fingerlings are more susceptible to this disease compared to adult fish.

Causative Agent

Branchiomyces sanguinis

Disease Signs

Fish may appear lethargic. Infected gill filaments become brownish with white or gray streaks. Fish suffer from respiratory distress observed by behaviours such as rapid movement of the operculum. Fish exhibit signs consistent with oxygen deprivation and therefore, gulp air at the water surface. Occurrence of secondary bacterial and viral infection is almost common (NACA 1989). Severely infected fish have typical gill-rot lesions, which may be similar to those associated with other gill infections.

Management and Control

Good management practices can create environmental conditions unacceptable for fungal growth and therefore, culture systems should be dried and treated with quicklime (calcium oxide, dose: 150-200 kg/ha) and copper sulfate (2-3 kg/ha) before starting the culture cycle. A therapeutic bath with 5% NaCl (salt) for 5-10 minutes can improve the infection. Copper sulfate and formalin are also suggested for treating infected fish. An antibiotic may also be used if secondary bacterial infection occurs (NACA 1989). As the tissue dies and falls off, the spores are released into the water and transmitted to other fish. Therefore, great care must be

taken to prevent movement of the disease from infected fish to uninfected fish. Infected fish should be reared separately until recovery from the disease.

ERYTHRODERMATITIS

Erythrodermatitis is commonly known as ulcer disease because it causes inflammatory lesions of the skin and by necrosis leading to ulceration. It is a sub-acute to chronic contagious disease of the skin (Pol et al. 1980). This disease is common all over the world including Europe. In 1987, this disease caused high losses of common carp in Greece (Sioutas et al. 1991). In late 1980, this disease occurred with seriously skin ulcerations in West Java, Indonesia (Jhingran and Pullin1985). Besides common carp, other carp species are also susceptible to this disease. The infection often starts at the site of an injury on the skin and base of fins. The infected area become a red inflammatory zone and gradually raised skin lesions form which then ulcerate exposing the underlying muscles. Bacteria probably release some toxic compounds, which could account for the marked inflammation and the tissue necrosis (Pol et al. 1980). Severe occurrence of this disease generally occurs in the spring when water temperature rise and bacterial growth is increased.

Causative Agents

This disease is caused by a variety of widespread and common bacterium species, but particularly, *Aeromonas hydrophila, Aeromonas salmonicida, Pseudomonas* spp. are the most common agents.

Disease Signs

The following signs are common in fish infected by the Erythrodermatitis (NACA 1989).

- Erythema (redness) on the skin and base of fins, often some small spherical nodules on fins
- Hemorrhages and ulcers with jagged rims
- Protruding scales
- Exophthalmia
- Swollen abdomen
- Hemorrhages on gills
- Pinkish fluid in the body cavity
- Secondary *Saprolegnia* infection

Management and Control

Reducing stocking density and stress by applying extensive culture technologies are beneficial to prevent this disease (NACA 1989). A wide range of sulfonamide drugs such as sulfamerazine, sulfaguanadine, sulfadiazine, sulfamethazine and sulfisoxazole can be used to treat this disease. The drug terramycin (antibiotics) can be supplied with feeds at 5-7 g/100 kg fish daily for 7-10 days or intra-peritoneal injection can be applied at 25 mg/kg body weight (Jhingran and Pullin1985).

COLUMNARIS DISEASE

Columnaris disease is considered to be an important bacterial disease and was first reported by Davis (1992) in warm water fishes of the Mississippi River. It is distributed worldwide in various wild and cultured freshwater fish species including common carp (Declercq et al. 2013). Fish infected by this disease may display skin lesions, fin erosion and gill necrosis and for those infected there is a high degree of mortality (Figueiredo et al. 2005; Declercq et al. 2013). A recent experiment indicates that common carp die within 12 h after inoculation of the causative agent of this disease. This disease exhibits a diffuse lesion by affecting all gill arches bilaterally (Declercq et al. 2015). Generally, outbreaks of this disease are associated with poor water quality with a high organic load, high temperature and high fish density (Decostere et al. 1999). According to Jhingran and Pullin (1985), the outbreaks of this disease usually occur only at temperatures above 18 to 20°C.

A clinically healthy fish which has survived from a previous outbreak of columnaris disease may act as an infection source for other fish (Suomalainen et al. 2005; Declercq et al. 2013). For example, a rainbow trout (*Oncorhynchus mykiss*) surviving from a columnaris infection can release up to 5 thousand colony forming units/ml/h of viable bacteria into tank water (Fujihara and Nakatani 1971). However, a dead fish release the agent of the disease at a higher rate compared to living fish (Kunttu et al. 2009; Declercq et al. 2013). It is very hard to eradicate this bacterium from an aquatic environment. The bacteria can survive at a high temperature (25°C) up to 16 days in hard, alkaline water with a high organic load (Fijan 1968; Declercq et al. 2013). It can grow on particulate fish feed and can even survive in sterile river mud (Declercq et al. 2013).

Causative Agent

Flavobacterium columnare (gram-negative bacterium)

Disease Signs

This disease causes serious infections characterized by lesions on the head, back, gill and fins. The lesions may start as small raised whitish plaques, often with a reddish peripheral zone and soon develop into large hemorrhagic ulcers (Jhingran and Pullin 1985).

Management and Control

Both external treatments and oral administration are applied to recover fish from this disease. External treatments are possible when the infection is still superficial. Potassium permanganate, chloramphenicol, copper sulfate, nifurpirinol, nifurprazine and oxolinic acid are used effectively in bath therapies. Oxytetracycline and terramycin are used with feed if the disease is in an advanced stage.

BACTERIAL GILL DISEASE

Bacterial gill disease is caused by a filamentous bacterium, which is highly contagious to fish. It is probably cosmopolitan in its distribution and nearly all freshwater fish are potential hosts. This disease can reside in carrier fish and culture systems. The bacteria can colonize on fish gill tissues in less than one hour after contamination of viable bacterial cell (Ostland et al. 1995). It disturbs gas exchange due to thickening of the epithelial layer. Poor water quality condition (low dissolved oxygen, high unionized ammonia (NH_3) and water turbidity) together with high stocking density may influence acute outbreak of this disease. Acute outbreaks of this disease generally cause a daily high mortality per day. Suitable water temperature for the disease outbreak is generally from 5° to 19°C (Bullock et al. 1994).

Causative Agent

Flavobacterium branciophyla

Disease Signs

Affected fish generally lose appetite and therefore, they become lethargic and are commonly observed gulping near the water surface. They normally gather near the outlet of the culture system. White areas of gill appear as pale and swollen. Necrosis of infected areas may occur but not common. Sometimes opercula become flared. Epithelial hyperplasia may occur due to attachment of large number of filamentous bacteria to the gill.

Management and Control

Good water quality together with low stocking density is very useful to prevent the disease. Treatment with a salt bath (5%) is also helpful to recover fish from this disease. Adding copper sulfate at 0.5 ppm, and potassium permanganate at 2 to 4 ppm to ponds is also successfully used for recovery of fish from this disease.

MYCOBACTERIOSIS

Mycobacteriosis in fish is caused by mycobacteria, which are abundant in the soil, on the surface of plants, on the skin of vertebrates and in human food (Snieszko 1978; Gauthier and Rhodes 2009). It is one of most common chronic diseases worldwide in cultured and wild fishes (Gauthier and Rhodes 2009). However, fish pathogenic mycobacteria differ from those that cause diseases in other animals. Fish pathogenic mycobacteria belong to the genus *Mycobacterium* under the *mycobacteriaceae* family. All fish, particularly teleost fish are susceptible to this disease. The first case of mycobacteriosis was seen in common carp in a pond in France (Snieszko 1978). However, it is infrequently reported as a problem in wild and cultured common carp. Fish can be susceptible to this when they are in poor condition and also have scale loss, skin ulcers, or a history of reproductive problems (Francis-Floyd et al. 2011). Poor water quality with high organic loads and high fish stocking density can exacerbate the mycobacteriosis infection (Francis-Floyd et al. 2011).

Causative Agent

Mycobacterium foptuitum

Disease Signs

The external signs of mycobacteriosis depend on the species of fish affected and the water temperature. Loss of appetite, sunken abdomens, shallow grayish irregular ulcerations, fin rot, deformities of the vertebral column and mandible, exophthalmos, and loss of one or both eyes are external signs in fish infected by this disease (Snieszko 1978).

Management and Control

There is no widely accepted treatment for this disease and, therefore, destroying infected populations are recommended to reduce spreading of this disease (Gauthier and Rhodes 2009). This bacterium is very resistant to standard anti-mycobacterial antibiotics including streptomycin, isoniazid, rifampicin, and ethambutol (Bragg et al. 1990; Gauthier and Rhodes 2009). There is an incomplete understanding around the management of this disease and significant effort should be directed into its research.

SPRING VIRAEMIA OF CARP (SVC)

Spring viraemia of carp (SVC) is an infectious viral disease of common carp of all age groups. It is distributed mainly in temperate countries. This disease is generally not observed in tropical or subtropical climates. Although other cyprinid fish (e.g., grass carp *Ctenopharyngodon idella*, silver carp *Hypophthalmichthys molitrix*, crucian carp *Carassius*,

goldfish *Carassius auratus*, etc.) are also hosts of this virus, the main host is common carp. It is considered as a notifiable disease as it causes significant mortalities in carp species, particularly common carp. SVCV is bullet-shaped RNA virus. The virion measures approximately 80 to 180 nm in length and 60 to 90 nm in diameter (Ahne et al. 2002).

The disease spreads through blood-sucking parasites and faeces of infected fish. Occurrence of this disease with a high mortality rate usually occurs in spring, when water temperature begins to rise after a cold winter. The suitable temperature for spreading SVC is between 10ºC and 17ºC. The virus can remain infective in the water for more than 4 weeks and in mud for about 6 weeks at 4°C to 10°C (Ahne et al. 2002). The first targeted organ of Spring viraemia of carp virus (SVCV) is gills then gradually entering internal organs (liver, kidney, spleen, alimentary tract, etc.) via blood (Ahne et al. 2002). The first observed clinical signs are edematous viscera, peritonitis, enteritis and hemorrhages in different organs after 8 to 11 days of infection and first mortalities appear after 20 days of infection (Ahne et al. 2002). Generally, fish less than one year old are very susceptible to infection with SVCV. Cumulative mortality of young fish infected by the SVC can be up to 90%.

Causative Agent

Rhabdovirus carpio

Disease Signs

In carp, the external signs of SVC include lethargy, darkened body, hemorrhages of the skin, pale gills, decreased respiration rate, exophthalmia, loss of equilibrium, abdominal distension, and fish congregating near inlet or outlet in enclosed systems, and an inflamed or edematous vent. Internal signs include peritonitis, ascites, catarrhal and hemorrhagic enteritis, edematous viscera and petechial hemorrhages of the internal wall of the swim bladder (Negele 1977; Ahne et al. 2002). The blood vessels can be fully necrotic in the final stage of the disease.

Management and Control

Fish, particularly adult common carp are generally protected from this disease if water temperatures are more than 20°C, as at this temperature fish are able to produce protective levels of interferon and antibodies (Ahne et al. 2002). Juvenile fish can be infected at more than 20°C of water temperature. However, elevated water temperature can prevent the outbreaks of SVC. Infected fish should not be transferred to uninfected farms to prevent spreading of SVCV. Fish develop a strong protective immunity against SVCV if they survive from SVC outbreaks. Presently, there is no vaccine against SVC.

Carp Pox

Carp Pox is one of the oldest known fish diseases, which is caused by a herpesvirus infection. This virus is referred as Cyprinus Herpesviris 1 (CyHV-1). It is a contagious disease in overcrowded condition but it does not generally kill fish. Pox-like appearance on the fish body is due to abnormal dermal cell growth and/or neoplasms. This disease is widely distributed in almost all countries of the world and affects usually cyprinids particularly common carp (Haenen et al. 2004; Goodwin 2012). The fish are not distressed if the infection only attacks small areas of the body. However, fish may be distressed if the lesions cover the mouth or gills that impede feeding and/or respiration (Walker and Winton 2010). Fish may susceptible to secondary infections if they become weak due to severe infection.

Causative Agent

Cyprinid herpesvirus 1 (CyHV-1)

Disease Signs

Initially, the carp pox appears as milky or grey-white lesions. The lesions first appear as flat, smooth and raised and can develop all over the external surface including fins of the fish. In severe case, the lesions can be thicker on the body, which may weaken the fish. Secondary infection by bacteria may occur if immunity is suppressed.

Management and Control

Elevated temperature is helpful for recovering the disease and healing the wounds. Fish, previously infected, although recovered are then sources of infection. Therefore, stocking previously infected fish should be avoided.

KOI HERPES VIRUS DISEASE (KHV)

Koi Herpes virus disease (KHV) considered as one of the most important disease of koi and common carp. It is a highly contagious disease that can cause mass mortalities (up to 80 to 100%) in koi and common carp of all ages (Haenen et al. 2004; Ilouze et al. 2006; Michel et al. 2010). This disease cause severe economic losses to the carp culture industry worldwide (Adamek et al. 2014; Boutier et al. 2017). This disease is caused by Cyprinid herpesvirus 3 (CyHV-3), which contains a double-stranded DNA genome. The virus can infect a wide range of species including freshwater mussels and crustaceans but it causes disease only in koi and common carp. This virus can cause disease in hybrids of koi × goldfish, koi × crucian carp and common carp × goldfish with a high mortality rate (Hedrick et al. 2006; Bergmann et al. 2010).

The virus can stay in a latent or carrier state without obvious clinical signs in host fish for some period of time. Movement by such carrier fish via international trade has resulted in the rapid spreading of the disease across the globe (St-Hilaire et al. 2005). Many scientists believe that the worldwide trade in koi and common carp is generally held responsible for the spread of the virus. Piscivorous birds may also be responsible for the rapid spread of this disease (Boutier et al. 2015). Besides koi and common carp, goldfish, tench, vimba, common bream, common roach, European perch, ruffe, gudgeon, rudd, northern pike, Prussian carp, silver carp and grass carp can carry CyHV-3 and transmit it to native carp (Boutier et al. 2015). Although this disease first occurred in koi carp in Europe in 1996, the first severe outbreak of this disease was in koi and common carp farms in Israel in 1998 (Haenen et al. 2004). Most of the European countries have experienced the widespread mass mortality in carp farms. Presently, the disease has been recorded at least 28 different countries including Australia, USA and Canada. Disease occurs generally at water temperatures between 15.5 ºC and 28ºC on common carp and its varieties with an incubation period of 6-21 days depending on water temperature (Hara et al. 2006; Rakus et al. 2013; Haenen et al. 2004). Sometimes fish die within one hour of the first signs appearance (Haenen et al. 2004). Australia is currently considering the introduction of CyHV3 for the biological control of common carp, where they have been implicated in massive degradation of aquatic ecosystems (McColl et al. 2017)

Causative Agent

Cyprinid herpesvirus 3 (CyHV-3)

Disease Signs

The clinical signs of KHV vary among individual fish depending on the severity of infection (Boutier et al. 2015). Behavioral signs include lethargy, fatigue, disorientation, neurological symptoms with erratic swimming, loss of equilibrium, increased gill beat rate expressed by gasping at the water surface and gathering near well-aerated areas (Perelberg et al. 2003; McDermott and Palmeiro 2013; Rakus et al. 2013). The consistent clinical signs are moderate to severe gill necrosis with diffuse discoloration. Other common clinical signs are anorexia, sunken eyes, fin erosion, folding of the dorsal fin, sloughing of scales, superficial hemorrhaging at the base of the fins, pale, hemorrhages and ulcers on the skin associated with increased mucus secretion and also decreased production of mucus in the ulcer areas, leaving the epidermis with a sandpaper-like texture, and accumulation of abdominal fluid and abdominal adhesions (Haenen et al. 2004; McDermott and Palmeiro 2013; Perelberg et al. 2003). Histopathological changes generally occur in the gills, skin, gut, liver, kidneys, heart, spleen and brain of infected fish (Miyazaki et al. 2008; Boutier et al. 2015). However, most severe damage is observed on the skin and fins (Miwa et al., 2014). Hepatocytes are the most affected cell in the liver (Miyazaki et al. 2008). In the kidney, a weak peritubular inflammatory infiltrate accompanied by blood vessel congestion and degeneration of the tubular epithelium in many nephrons is observed (Pikarsky et al. 2004; Boutier et al. 2015). The main susceptible cells are the splenocytes in the spleen. According to Miyazaki et al.

(2008), a large numbers of necrotic splenocytes accompanied by hemorrhages are observed in extreme cases. Focal meningeal and parameningeal inflammation is usually observed in the fish brain (Pikarsky et al. 2004). Pathologists face a diagnosis problem as secondary infections are very common. The reported major secondary parasitic infections for fish with KHV include those caused by *Ichthyobodo* sp., *Trichodina* sp., *Ichthyophthirius* sp., *Dactylogyrus* sp., *Chilodonella cyprini* and monogenean parasites (Perelberg et al. 2003; Haenen et al. 2004). The bacterial infections with *Aeromonas* sp., *Pseudomonas* sp. and *Shewanella putrifaciens* are commonly reported (Haenen et al. 2004).

It is difficult to detect this disease in its latent stage, and therefore various preventive measures are recommended to avoid infection. Ronen et al. (2003) developed a protocol to induce a protective adaptive immune response in common carp. According to their protocol, healthy fingerlings are exposed to the virus by cohabitation with sick fish for 3–5 days at permissive temperature (23°C). Then, the fish are transferred to the non-permissive temperature of 30 degrees for 25–30 days.

Costiasis

Costiasis is a protozoan disease for freshwater fish including common carp (koi) and goldfish. This disease is normally observed in aquaculture ponds. It is also known as ichthyobodoosis, which is caused by two species of Ichthyoboda, *Ichthyobodo necator* and *I. pyrformis*. Both species are obligate parasites with one host cycle. They are characterized as small, bean-shaped, two flagella (stalk), nucleus and contractile vacuoles. They usually attach to the skin, gill and fins of fish with their adhesive disc positioned at the posterior end. Overcrowding of fish, poor water quality and physical injury may cause severe outbreaks of the disease. The suitable temperature for a rapid reproduction of the parasites is between 10°C and 25°C. The parasites cannot survive at water temperature more than 30°C while, they form a cyst like state at water temperatures below 8°C.

Causative Agents

Ichthyobodo necator, I. pyrformis

Disease Signs

The clinical signs include blue-grey films on the skin and gills, red patches on the skin, excess mucous production, respiratory distress, gulping at inlet (areas of high oxygen) and erratic swimming. Fish become weak as they lose appetite. Severely infected fish may die due to oxygen deprivation. Infected fish are usually affected by secondary infection of fungus and bacteria.

Management and Control

Good environmental and nutritional conditions may prevent the disease. Infected fish should be treated rapidly as both *Ichthyobodo necator* and *I. pyrformis* multiply quickly. Water temperature more than 30°C is helpful to control the disease as parasites cannot tolerate this temperature. Bath treatments with salt (3% for 10 minutes) or acriflavine (10 mg/l for 10 hours) or formalin (50 parts per million for two hours) are also useful to recover fish from this disease. However, treatment using a high water temperature is safer than the treatment using chemicals.

Coccidiosis

Common carp fry are often infected with enteritic coccidiosis (Steinhagen et al. 1998). It is caused by protozoan parasites. Infection is most prevalent in common carp fry from July to September (Steinhagen and Hespe 1998). Coccidiosis is a serious disease for carp fry of 1 to 2 months old fry if they are temperature stressed in fall or spring (Steinhagen et al. 1998). According to Novakov et al. (2015), this disease mostly occurs in one year old fingerlings. However, this disease can be widespread in fish of all age classes. Infection of fish occurs via fecal contamination and indirect transmission via tubificid oligochaetes (Steinhagen and Korting 1990; Steinhagen and Hespe 1998). Carp which had recovered from the parasitic infection have been found to be vulnerable to fecal contamination (Steinhagen and Korting 1990). The most common source of contamination is from sediments with high numbers of infective oocysts and infected tubificids (Steinhagen and Hespe 1998; Novakov et al. 2015). This disease primarily occurs in aquaculture ponds but, some severe cases have been reported in natural environments (Novakov et al. 2015).

Causative Agents

Goussia carpelli (enteritis coccidiosis), *Goussia subepithelialis* (nodular coccidiosis)

Disease Signs

Fish lay on pond bottoms as they become very weak due to pathological changes in the intestine. Other signs of infection include hollow eyes, debilitation, thin body but large head, edema of abdominal membranes and intestinal wall, intestinal wall is dark from swollen intestinal mucosa. Sometimes fish discharge yellowish mucus. Moderate mortalities may occur due to this disease.

Management and Control

The most important prophylactic measure is to disinfect the fish pond bottoms by drying followed by liming. Osarzol, furazolidon, amprolium chloride are effective therapeutics agent against *Goussia* sp. According to Novakov et al. (2013), furan compounds provide the best effect in the coccidiosis treatment in common carp.

Ich (White Spot Disease)

Ichthyophthiriasis, commonly known as ICH is caused by a common obligate protozoan parasite *Ichthyophthirius multifiliisn* that infects the external surfaces of freshwater fish particularly skin and gill. Sometimes the parasite may be present only on the gills and not on the skin as the gills are the primary site of infection. The parasite is generally spherical shaped, but they can change shape. They have hair like structures called cilia and move like an amoeba. Mature parasites may reach 0.5 to 1.0 mm diameter and can be seen with necked eyes (Jhingran and Pullin 1985). Although most fish species are susceptible to this parasite, this disease is mostly observed in common carp. This disease is more generally known as one of the most common diseases found in commercial fish farms worldwide (Clark and Dickerson 1997; Davis et al. 2002).

After attaching on to a fish body, the parasite gradually penetrates the mucus layer and epithelium in the gills or skin. At this stage, it is very hard to treat the disease as mucus and host cells cover the parasite (Durborow et al. 1998). Therefore, diagnosis and treatment should be done at the early stage to avoid fish mortalities. The outbreak of this disease generally occurs during spring when water temperature is between 18° and 25°C and cause mortalities of fish in a short time (Davis et al. 2002). Therefore, severe outbreaks are more common in temperate or sub-tropical areas compared to tropical environments. This disease is very infectious particularly in fish farms and hatcheries with a high fish stocking densities. Fish die in cases of severe respiratory problems. Secondary infection of fungi and bacteria are quite common in fish infected with this parasite.

Causative Agent

Ichthyophthirius multifiliis

Disease Signs

White spots generally appeared on the skin and the fins. Fish may rub or scratch on objects or on the pond bottom, presumably because of irritation. Fish often show violent swimming movements. Gill damage, increased respiratory frequency, gasping at the water surface and gathering near well-aerated areas are common signs of this disease.

Management and Control

In many cases, wild fish were observed to be the carrier of this parasite. Therefore, attempts should be taken to prevent entry of wild fish into the aquaculture ponds. Sometimes, river water is the source for filling aquaculture ponds. In this case, fish should be released into the pond at least 3 days after filling the pond with river water as this period of fishless water will kill any juvenile parasites due to their absence of hosts (Durborow et al. 1998). Water temperature more than 30°C is also helpful to stop reproduction and control the parasite. Bath treatments with malachite green (1-2 ppm) plus formalin (167-250 ppm) or acriflavine (10 ppm) or salt (3%) or quinine hydrochloride (0.1 ppm), methyl blue (0.5 ppm) are also useful to recover fish from this disease.

Chilodonellosis

Chilodonellosis cause major mortalities of at least 16 species of wild and farmed freshwater fishes including common carp (Mitra et al. 2013; Gomes et al. 2017). According to Gomes et al. (2017), this disease has been recorded in 14 countries in the world. This disease is caused by at least two species of ciliated protozoan parasites (*Chilodonella hexasticha* and *C. cyprini*) from the genus *Chilodonella*. Common carp is usually affected by *C. cyprini*, which attach on gills and skin of fish and graze on mucus, bacteria and algae available in gills and skin of the common carp (Dopheide et al. 2011). The parasite penetrates fish's epithelial cells to uptake cell contents using their specialized mouth organ named cytostome or cytopharynx (Noga 2010; Gomes et al. 2017). *Chilodonella cyprini* is microscopic (length: 30-70 μm, width: 20-60 μm), almost heart shaped and usually reproduced by binary fission. This parasite has a wide thermal tolerance range, between 4°C and 20°C but the most suitable temperature for reproduction is from 5°C to 10°C (Gomes et al. 2017). Their reproduction is slowed down and stopped at water temperatures from 15°C then rising to 20°C. Therefore, common carp is safe from this disease when they are cultured at high water temperature (20°C or more).

Causative Agent

Chilodonella spp.

Disease Signs

Fish produce excess mucus on the skin, which is presented as cloudy skin. Fish may hang near the surface due to lethargy or may jump out of water. Gills of fish may become pale in color. Fish condition may be reduced due to loss of appetite. Fish scratch or rub on objects present in the water and may gather at well aerated areas. In severe cases, ulcers may appear on the various places on the body due to epithelial cell necrosis and fish deaths can then occur from respiratory distress.

Management and Control

Good water quality along with low fish stocking density is recommended to prevent the disease. Both bath and pond treatments are used to control the *Chilodonella.* Bath treatment with salt 10 ppt for 1 hour or potassium permanganate ($KMnO_4$) 20 ppm for 1 hour, formaldehyde solution 150 ppt for 1 hour or hydrogen peroxide 200 ppm for 30 minutes is effective to control the *Chilodonella.*

Trichodiniasis

Trichodiniasis is one of the important diseases of cultured marine and fresh water fishes including common carp worldwide (Valladao et al. 2016). This disease is caused by several species of protozoan under the genus *Trichodina*, which are peritrichal ciliated obligate parasites but not always associated with disease. The parasites are saucer shaped, microscopic with 40-60 µm diameters and reproduce through binary fission. They generally attach to the external surface of fish particularly skin, gills and fins. The parasites use fish as their home and transportation but feed on bacteria available on the surface of the fish body. Unsuitable water quality (e.g., unsuitable water temperature, pH, carbon dioxide and free ammonia concentrations) and overcrowding influence the severe outbreaks of the disease, which increase stress, reduce immunity of host fish and influence secondary infection of fungi and bacteria (Kugel et al. 1990; Khoshnood and Khoshnood 2014). A severe outbreak of the disease may cause a significant mortality (Maciel et al. 2018).

Causative Agent

Trichodina spp.

Disease Signs

Fish usually become lethargic and exhibit flashing. Appearance of white patches on the skin surface, frayed fins, pale gills, respiratory distress, secretion of excess mucus are common sings of Trichodiniasis. Fish may lose appetite followed by cessation of feeding, gasping near the water surface, and necrosis of epithelial cells followed by skin ulcers can occurred in severe cases.

Management and Control

Maintaining a healthy environment, suitable fish stocking density and good hygienic practices are the best management to prevent the disease. The same medications for treating Ich can be used to control the parasites of Trichodiniasis. A conventional bath treatment using sodium chloride (1%) plus formaldehyde (1ppt) for 15 min is very effective to control

Trichodiniasis. Valladao et al. (2016), observed a similar result of 100% effectiveness in the treatment of trichodiniasis when they compared a mixture of 0.5 ppt formaldehyde + 1% sodium chloride with the conventional treatment.

Myxobolosis

Myxobolosis in common carp is caused by myxozoan parasites, which are distributed in many countries of the world. Of around 500 species under the genus *Myxobolus*, approximately 30 *Myxobolus* species are well described as pathogens of common carp (Molnar 2002; Eiras et al. 2005). However, some other species, which are the pathogen of other cyprinid fish may also be capable of infecting common carp (Dayoub et al. 2007). Various species are generally described based on the morphological characteristics of spores and typical hosts (Molnar 2002). The size and shape of *Myxobolus* spores are highly variable and identification of species therefore is sometimes difficult. In most cases *Myxobolus* species infect gills, kidney and muscles of fish but they can infect all organs including urinary bladder, brain, heart and gut of fish. In some cases, these parasites enter through muscle cells and become scattered all over the body and are often excreted through the gut. In these cases a large number of spores can be found in the intestinal mucus (Molnar 2002). *Myxobolus* species may cause mild to severe hemorrhage in various infected organs of common carp (Kaur and Katoch 2016).

Causative Agent

Myxobolus spp.

Disease Signs

Based on the infected organs, edema, loose scales, exophthalmia, white nodules on gills are common signs of this disease. Sometimes, mild to severe hemorrhage may occur in muscle, liver and kidney. Necrosis in the muscles and liver occur in severe cases.

Management and Control

The followings actions are effective to control Myxobolosis (Wagner 2002).

- Older fish are less susceptible to this disease compared to younger fish and therefore, prevalence rate can be reduced by stocking large fish only.
- Feeding pelleted feed containing 0.1% dicyclohexylamine (Fumagilin) for 3-7 weeks reduces prevalence rate and eliminates morbidity and mortality.
- Calcium Cyanide at 4,000 kg/ha should be applied to infected ponds.

- Chlorine at 5,000 ppm in a 10 minutes exposure or 1,600 ppm in 24 hours exposure for infected ponds.

Dactylogyrosis

Dactylogyrosis in common carp is caused by monogenean parasites from the *Dactylogyrus* genus, which is the largest helminth genus, with more than 900 species (Neary et al. 2012). The parasites are commonly known as gill flukes. *Dactylogyrus* can complete its life cycle within one host and therefore, they can spread quickly and easily. The parasites have two pair of anchors, which are used to attach to the gills of host fish. They have 4 tiny eyespots at the anterior (head) end. The parasites can lay up to 20 eggs per hour if the temperature is suitable. The eggs hatch within 4 days. In a heavy infestation, the parasites can be attached to other parts of the host body. *Dactylogyrus* are serious parasites for wild and cultured freshwater fish including common carp. Fry and fingerlings are more susceptible to the parasites compared to adult fish.

The prevalence of the infection on fish can vary according to season, which influences water temperature oxygen concentrations of water, size of fish host and fish maturity (Ozturk and Altunel 2006; Tekin-Ozan et al. 2008). Different species show different temperature preference and seasonal variations in their infection parameters (Neary et al. 2012). *Dactylogyrus* generally prefer temperatures above 20°C (Jhingran and Pullin 1985). Poor water quality, particularly when contaminated with industrial and urban pollutants generally increase the occurrence of outbreaks of this disease (Borji et al. 2012). Nutritionally weak fish are more easily infected by the *Dactylogyrus* parasites. The presence of parasites may cause weight loss, and reduced fecundity of host fish (Grabda1991). Heavy infection by the parasites can cause high mortalities of fish.

Causative Agents

Dactylogyrus spp.

Disease Signs

Fish swim to water inlets (or areas of high oxygen); proliferation of gill epidermis; flukes are visible on gills at low (40-60) magnification

Management and Control

Before culturing fish, ponds should be prepared by drying and subsequently liming to prevent the disease. Bath or pond treatments can be used to control disease outbreak. Several chemicals are generally used for bath and pond treatments. Bath treatment with 1% Masoten or Neguvon (trichlorphon) for 2-3 minutes or 200-250 ppm formalin for 30 minutes or 0.1-0.2

ppm Bromex (dimethyl 1, 2-dibromo-2, 2-dichloroethyl phosphate) for a long period or 2-5% salt is effective to control the disease (Jhingran and Pullin 1985). Pond treatment with the similar dose (0.1-0.2 ppm) of Bromex is generally used to control the parasite. Bath treatment with salt for a prolonged period should be avoided because common carp die after exposure to 3.5% salt for 40 minutes, to 2.5% for 60 minutes and 1% for 24 hours (Jhingran and Pullin 1985).

Gyrodactylosis

Gyrodactylosis in common carp is also caused by monogenean parasites from the *Gyrodactylus* genus, which has more than 350 species (Halvorsen and Ex Hartvigsen 1989). According to Rubio-Godoy and Garcia-Vasquez (2015), nearly 500 species of *Gyrodactylus* are currently known. The parasites are usually known as skin flukes primarily attach on skin and fins of fish. They may occasionally found on the gills of fish. They have prominent hooks for attaching to the host's skin but they do not have eyespots at their anterior end. Adult parasites give birth to live offspring, which become immediately parasitic. *Gyrodactylus* generally feed on the epidermis of the skin of the host fish. They can complete their life cycle on one host. Various species of *Gyrodactylus* are normally identified based on the size and shape of the opisthaptor (adhesive organ). A high fish stocking density and poor environmental conditions characterized particularly by low dissolved oxygen and high organic matter loads may accelerate the infection of this disease. Under aquaculture conditions, *Gyrodactylus* populations can sometimes increase almost exponentially and overwhelm their fish hosts (Rubio-Godoy and Garcia-Vasquez 2015).

Causative Agents

Gyrodactylus spp.

Disease Signs

Fish may become lethargic but sometimes they swim restlessly. They produce excess mucus and therefore, skin becomes cloudy. Fish rub upon hard material present in the system. Infected areas may become red due to hemorrhage. Extended periods of infection may also lead to secondary infections from fungus and bacteria.

Management and Control

Same for Dactylogyrosis control

Diplostomosis

Diplostomosis is one of the more well-known diseases of freshwater water fish including all varieties of common carp and is reported from more than 125 freshwater species globally (Valtonen et al. 2003; Larsen et al. 2005; Mama and Abdullah 2013). This disease is also recorded in some brackish water fishes and majority of the published information surrounding Diplostomosis is associated with its impacts on rainbow trout. It does not generally occur in indoor aquaculture systems. Diplostomosis is caused by cercariae and metacercariae of species under the genus *Diplostomum.* Metacercariae of *Diplostomum* species attack fish eye lenses and *Diplostomum* species are, therefore also referred to as eye-flukes. In severe cases, the parasites in the lens can cause cataracts and blindness in fish (Shariff et al. 1980; Behrmann-Godel 2015).

Diplostomum species are digenean trematodes that have complex three-host life cycle involved piscivorous birds (definitive host), snails (first-intermediate host) and fish (second-intermediate host). The piscivorous birds release eggs of *Diplostomum* through their faeces. After embryonic development in the egg, a free-swimming miracidium comes out in the water in 21 days (Palmieri et al. 1976). The miracidium penetrate snails and move to the hepatopancreas of snails, in which they reproduce asexually releasing large numbers of cercariae into the water column. An individual snail may release tens of thousands of cercariae per day (Karvonen et al. 2006). The production of cercariae is season-specific, which is strongly influenced by water temperature. Cercariae of *Diplostomum* generally enter small blood vessels through the gills and fins of fish and reach to the fish eyes within 30 minutes (Palmieri et al. 1976; Karvonen et al. 2006). In the lenses of the fish eyes, cercariae become infective metacercariae in 45-120 days. When a fish-eating bird eats the infected fish, the worm becomes adult within the intestine in 3-5 days (Palmieri et al. 1976).

Causative Agents

Diplostomum spp.

Disease Signs

Infection in the eye generally reduces fish vision, food intake and growth are impacted with altered fish behavior, particularly swimming and grazing behavior. Development of inflammation, hemorrhage, exophthalmia and cataracts in eyes are also commonly observed signs of this disease.

Management and Control

Diplostomosis is usually prevented by disrupting the *Diplostomum* their life cycle, which can be achieved by keeping piscivorous birds away from the pond area, or by eradicating the first-intermediate host (snail) in ponds. Drying and subsequent liming improves pond health

and eradicates snail populations. When common carp are cultured in tanks or raceways, increasing water current is also effective to reduce infections (Field and Irwin 1994). Bath treatment with formalin (10 mg/L for 16 hours) or sodium percarbonate (minimum 40 mg/L for 16 hours) is effective in eradicating the parasites (Larsen et al. 2005). Szekely and Molnar (1991) fed droncit (330 mg per kg body mass) for treating metacercariae parasitizing grass carp with l00% efficacy. They also treated infected grass carp and silver carp with l00% efficacy by exposing to a droncit solution (1 mg/L) for 90 hours. However, droncit treatment as applied by Szekely and Molnar (1991) may also be effective to treat infected common carp.

Phosthodiplostomosis

Phosthodiplostomosis are primarily reported in wide range of Cyprinid fish including common carp but it may occur in other fishes particularly fishes in the Perciforme and Channiformes families (Nguyen et al. 2012; Athokpam and Tandon 2014; Ozturk and Ozer 2014). It is caused by metacercariae of species of the *Posthodiplostomum* genus and is distributed globally (Niewiadomska 2002) with around 30 species of *Posthodiplostomum* known to cause the disease. Metacercariae of *P. cuticola* cause numerous tiny black spots (cysts metacercariae) on the skin, muscle, and in the fins of fish (Rolbiecki 2004). Therefore, infection with *P. cuticola* is also called black spot disease, which is often harmful for fish, particularly fry. A mild infection generally causes little harm to affected fish but a strong infection may cause weight loss and several changes in the blood chemistry of host fish (Rolbiecki 2004). Accumulated blood hemoglobin and chromatophore around the matacercaria are decomposed and produce hemomelanin appearing as dark back pigments (Markovic and Krsmanovic 2008).

Posthodiplostomum species are digenean trematodes and therefore, the life cycle of *P. cuticola* is almost similar to *Diplostomum* species, which have a complex three-host life cycle involved piscivorous birds (e.g., herons) as the definitive host, snails as the first-intermediate host and fish as the second-intermediate host. The main difference is observed during metacercarial stage. At this stage, *P. cuticola* form a cyst and reside on the skin, muscle, and in the fins of fish.

Causative Agent

Phosthodiplostomum cuticola

Disease Signs

Black spots generally appear on the skin, muscle, and in the fins. Infected common carp fry may be deformed. Mild infection is generally not very devastating to fish but in a severe case, fish may have increased physiological stress, reduced feed intake and weight loss, necrosis of skin and muscle fibers, increased erythrocyte sedimentation rate and leukocyte count, reduced hemoglobin content and erythrocyte count (Williams and Jones 1994).

Severely infected juvenile fish and fry may experience vertebral column deformation, heavy blood loss, and even death (NACA 1989; Williams and Jones 1994).

Management and Control

Same treatments for Diplostomosis

Sanguinicoliasis

Sanguinicoliasis is a serious disease of cultured common carp caused by digenean blood flukes and has been reported with mass mortalities in many countries of the world (Sommerville and Iqbal 2006). The parasites live and reproduce within the circulatory system of fish, where their presence may result in the obstruction of blood flow (Lee 1990). Most of the flukes are host-specific. The common life cycle of digenean blood flukes is described by Kirk and Lewis (1993) and Bullard and Overstreet (2002). According to these authors, adult flukes deposit thin-shelled, pliable eggs in the circulatory system of the specific fish. Some eggs travel to and lodge in the host's gill, where the embryonic development and hatching usually occur. After hatching, the first free-swimming form of the fluke, the miracidium, emigrates through the gill epithelium and looks for a suitable intermediate host, which can be a snail, bivalve or polychaete. After reaching the intermediate host, the parasite reproduces asexually, producing the second free-swimming cercaria, which leave the intermediate host. Some cercaria die and other infect fish through gills, skin, eyes, fins, or alimentary tracts. Sometimes, the flukes mature, copulate, and releases eggs in the heart or branchial vessels. The cercaria penetrates the fish host through its gill, skin, eye, fin, or alimentary tract and develops through the juvenile stage as a schistosomule before ending up in a specific site in the circulatory system of the host. They often settle in the heart or branchial vessels where the fluke ultimately matures, copulates, and releases eggs. Both lightly and heavily infected fish have poor growth rates and low food utilization (Iqbal and Sommerville 1986).

Causative Agents

Sanguinicola spp.

Disease Signs

Lethargy; swimming in spiral movement; reduced feed intake; fish spend more time at the water surface; exophthalmia on some occasions; gill inflammation

Management and Control

Controlling blood flukes is easier than controlling parasites, which have direct life cycle or intermediate host. Blood flukes can be controlled by eradicating snails and bivalves by applying copper sulfate when there is no fish in the culture system. Before culturing fish, ponds should be dried and subsequently limed which then can destroy eggs and larvae of snails, bivalves and polychaetes from the ponds. Before stocking in ponds, tanks or raceways, fingerlings should be examined carefully for the presence of either blood fluke eggs or adults or other parasites. Some scientists recommend bath treatment with Praziquantel to treat fish infected by blood flukes (NACA 1989).

Ligulosis

Ligulosis in fish is caused by the plerocercoid stage of *Ligula intestinalis*, which is a pseudophyllidean cestode inhabiting the intestine of piscivorous birds. This disease mostly occurs in freshwater cyprinid fish including common carp, which ingest zooplankton. *Ligula intestinalis* is distributed throughout the northern hemisphere particularly Europe and Russia (Dubinina 1980). It has a complex three-host life cycle: a copepod as the first intermediate host, a cyprinid fish as the second intermediate host and a piscivorous bird (e.g., herons, gulls, ducks, cormorants, etc.) as the final host. The ichthyophagous birds discharge unembryonated eggs into water with their faeces. In the water, ciliated free-swimming coracidiums emerge from egg shells after the embryonic development. Coracidiums are eaten by crustacean copepods, in which coracidiums develop into procercoids within 2-3 weeks (Loot et al. 2001; Sohn et al. 2016). When copepodes with procercoids are eaten by the fish, procercoids develop into plerocercoids in the abdominal cavity of the fish. The plerocercoid larvae stay more than 10 months in the fish host (Dubinina 1980; Sohn et al. 2016). The larvae become adult in the intestine of piscivorous birds (Ogambq-Ongoma 1975). Plerocercoids squeeze the internal organs of the fish and cause disruption of their functions. Severely infected fish die due to their stomachs rupturing.

Causative Agent

Ligula intestinalis

Disease Signs

Infected fish usually accumulate in shallow waters and swim on their sides or belly up. Fish stomachs generally become distended. Parasites can be visible in infected fish. Infected fish show abnormal swimming behavior including slow swimming. Fish usually stop eating, lose body weight and are anemic.

Management and Control

The disease can be controlled by targeting the intermediate hosts, particularly controlling copepod populations in ponds. Masoten (25 ppm) or Dipterex (25 ppm) is commonly used to reduce copepod populations in ponds. Drained, wet ponds can be treated with calcium chloride (about 70 kg/ha) or calcium hydroxide (about 2 tons/acre) or calcium hypochlorite to eradicate eggs and larvae of copepods. The life cycle of the parasite can also be disturbed by expelling birds. Infected fish can be treated with Droncit (5 mg/kg), which can be incorporated into feed pellets.

Bothriocephalosis

The parasitic disease, Bothriocephalosis in common carp is caused by a pseudophyllidean tapeworm *Bothriocephalus acheilognathi*, which is one of the most dangerous helminth parasites of cultured common carp (Nie and Hoole 2000; Sofi et al. 2016). The parasite has several synonymous scientific names including *B. fluviatilis, B. gowkongensis, B. opsariichthydis, B. phoxini, B. kivuensis*, *B. aegyptiacus* and *Schyzocotyle fluviatilis*. Besides common carp, this parasite infects approximately 200 species of freshwater fish with most suitability for cyprinid fish (Salgado-Maldonado and Pineda-Lopez 2003; Sofi et al. 2016). The original distribution of this parasite is the East Asia, however, it spread rapidly in many countries in the world via fish trade. The parasite has a simple, short and two-host life cycle. The parasite requires common copepod species (e.g., species fprm *Acantocyclops*, *Macrocyclops*, *Mesocyclops*, *Tropocyclops* and *Diacyclops* genera as intermediate hosts for around two weeks (Korting 1975; Scholz et al. 2012). Eggs come into the water with the faeces of the host fish and mobile coracidia emerge from the eggs after embryonation. Copepods become the host when they consume Coracidia. The parasites complete their life cycle in the fish when fish ingest infected copepods. Water temperature plays an important role in rapid development of all stages of the parasite life cycle. For example, larval development is completed within 21- 23 days at water temperature 28-29°C, but water temperature at15-22°C it takes 1.5-2 months (Scholz et al. 2012). Bothriocephalosis can have pronounced detrimental effects on fish including negative effects on health, growth and production of fish. Sometimes, it causes mass mortality in fish farms particularly in hatchery ponds.

Causative Agent

Bothriocephalus acheilognathi

Disease Signs

Infected fish generally show the following signs.

- Sluggish movement
- Swimming at the surface
- Enlarged abdomen
- Severe damage to the intestinal tract
- Reduced growth
- Death

Management and Control

Bothriocephaliasis can be controlled by drying fish ponds annually or treating drained wet ponds with calcium chloride (about 70 kg/ha) or calcium hydroxide (about 2 tons/acre) or calcium hypochlorite to kill copepods. Insecticides include Masoten (25 ppm) or Dipterex (25 ppm) can be used to reduce copepod populations in ponds (Hoffman 1983). Infected fish can be treated with anthelmintics, such as Chlorinated salicylanalid or Droncit (5 mg/kg) in feed.

Khawiosis

Khawiosis is the intestinal infection of fish including common carp caused by several species of Caryophyllidean tapeworms from the genus *Khawia*. Among several species, only *Khawia japonensis* and *K. sinensis* are observed to be intestinal parasites of common carp and *K. sinensis* has more potential parasitic effects on common carp compared to *K. japonensis*. *K. sinensis* has two synonymous scientific names (*Tsengia neimongkuensis* and *T. xiamenensis*), while *Khawia japonensis* has four synonymous scientific names (*K. iowensis, K. cyprini Caryophyllaeus japonensis, Bothrioscolex japonensis*) (Scholz et al. 2011). *K. sinensis* was described by Hsu (1935) from the vicinity of Beijing, China while *K. japonensis* was originally described by Yamaguti (1934) from Lake Biwa in Japan (Oros et al. 2015). Both parasites dispersed from Asia to many European countries and the USA with common carp and other fish particularly grass carp (Scholz et al. 2011). *K. japonensis* is larger (length: 19.5–24.5 mm; width: maximum 1.2 mm) compared to *K. sinensis* (length: 41–112 mm; width: 1.3–2.3 mm) (Oros et al. 2009).

Khawia need aquatic tubificids (Annelida: Oligochaeta) as their intermediate hosts. Eggs are laid unembryonated into water and the oncosphere infective for intermediate hosts is formed after a few weeks (e.g., 2–4 weeks in the case of *K. sinensis*) of embryonation within the egg shell. Generally, 30-65 days old oncosphere infects tubificds, in which it stays for around 54 days for the procercoid development (infective larvae) (Scholz 1991). Temperature significantly influences oncosphere and procercoid developments. *Khawia* has some negative impacts on the health and production of the common carp (Williams and Jones 1994). In severe case, *Khawia* infection causes mortalities of common carp.

Causative Agents

Khawia sinensis and *K. japonensis*

Disease Signs

Loss of appetite; sluggish movement; serious inflammations of the intestine and destructions intestinal epithelium of the host; anemia as a consequence decreasing erythrocyte number and leukocyte activity; slow growth; worms may protrude from anus.

Management and Control

The disease outbreak can be prevented by eradicating tubificids (intermediate host) by drying and liming the pond. It is very difficult to kill tapeworms in the fish intestine. However, praziquantel, benzimidazoles and niclosamides can be used for oral treatment (Treves-Brown 2000). Sudova et al. (2010) recommended orally administered praziquantel at a dose of 50 mg/kg for an effective control of *K. sinensis* in the common carp intestine.

Nematode Infestation

Nematodes of the genus *Contracaecum* have a worldwide distribution, with their larvae recorded in many marine and freshwater fish species including common carp (Klimpel and Palm 2011; Tavakol et al. 2015). *Contracaecum* has a complicated life cycle, which involves a variety of hosts that are transferred through the food chain. It has four larval stages (L1–L4) and an adult stage (Anderson 2000; Valles-Vega et al. 2017). According to Huizinga (1967), the parasite generally spends their first and second larval stages in the eggs. Copepods ingest the fee-swimming second larval stage after it hatches from the egg into the water. The second larval stage grows without molting in the coelom of copepod. When a fish eats the infected copepod, L2 grows, molts and become L3 in the abdominal cavity of fish. When this fish is eaten by a suitable piscivorous host, L3 stays in the proventriculus where it grows and molts twice to become the adult stage (Valles-Vega et al. 2017).

Causative Agents

Contracaecum spp.

Disease Signs

Contracaecum infected common carp generally show following signs (NACA 1989).

- Weak or thin body
- Loss of blood into body cavity
- Abnormal protrusion of the eyeballs
- Roundworms in heart and body cavity

Management and Control

Contracaecum infection can be prevented by eradicating copepods, which can be done by drying and liming the pond. Masoten (25 ppm) or Dipterex (25 ppm) is also used directly into the ponds to reduce copepod populations in ponds. Calcium chloride (about 70 kg/ha) or calcium hydroxide (about 2 tons/acre) or calcium hypochlorite can be used in the drained wet ponds to eradicate copepods. There is no effective medicine to treat *Contracaecum* infected fish.

Phylometrosis

Phylometrosis is caused by species of the family Philometridae. Many fish species including common carp are susceptible to this disease. Various Philometrid species infect a range of organs (e.g., skin, eyes, swimbladder, gonads, circulatory system, body cavity, etc.) of fish (Moravec and Ali 2013). In common carp, this disease is caused by *Philometroides cyprini,* which has several synonyms such as *Philometra lusii*, *Philometra schikhobalowae*, *Philometra lusiana* and *Philometroides lusiana* (Moravec and Cervinka 2005). *Philometroides cyprini* was originally distributed throughout East Asia, from where it dispersed into Europe in the early 1960s (Moravec and Cervinka 2005). *P. cyprini* may cause mortality of infected common carp particularly young individuals (Moravec and de Buron 2013).

Philometra has a two-host life cycle: copepods as the intermediate host and fish as the final host. Fish release *Philometra* larvae in their faeces or female parasites migrate to the fish skin surface to release larvae. Larvae of the parasites are ingested by copepods, in which the larvae molt several times. The life cycle of the parasite is completed in the fish body when infected copepods are consumed by the fish.

Causative Agent

Philometroides cyprinid

Disease Signs

Fish may show the following signs (NACA 1989).

- Lost balance
- Floating head down
- Reduced feed intake
- Appearance of red nodules on skin and under scales

Management and Control

The disease can be controlled by controlling copepod populations in ponds. 25 ppm masoten or dipterex can be used to reduce copepod populations in ponds. Calcium chloride (about 70 kg/ha) or calcium hydroxide (about 2 tons/acre) or calcium hypochlorite to eradicate eggs and larvae of copepods in the drained wet ponds. Infected fish can be treated with injections of Nilverm or Ditrazin into their body cavity (NACA 1989).

Fish Leech Infestation

Species of the family Piscicolidae are parasites of many fish species living in fresh, brackish, and marine water ecosystems (Williams and Burreson 2006). The common fish leech *Piscicola geometra* is a well-known parasite of common carp. *Limnotrachelobdella sinensis*, another fish leech has been commonly found infesting common carp (Nagasawa and Tanaka 2011). Leeches need to attach to the fish only for their meal but they can survive for long periods without feeding. A single 2-4 cm leech can suck 150 ml fish blood within about 48 hours (Jhingran and Pullin 1985). Leech lay eggs in dark brown oval cocoons, which are usually attached to debris or plants in their habitats. They complete their life cycle within 24 days to several months depending on water temperature. They generally leave the host after sucking blood but the sucking area becomes injured. Leech do not directly cause any disease to fish but their infestation can lead to a secondary infection from fungus and bacteria. Leech infected fish generally rub or scratch the leech off by flashing. Severe leech infections can occur if fish are weak or unhealthy. Besides high fish stocking density, unsuitable temperatures and a high decayed organic matter load usually increase leech populations in the environment.

Causative Agent

Species of the family *Piscicolidae*

Disease Signs

Leech infested fish generally show following signs:

- Fish may rub or scratch the infected area with any hard substrate present in the pond.
- Hyperactive swimming at the water inlet is commonly observed.
- Appearance of ulcers at the various places of the infected fish.
- Fish generally lose weight.

Management and Control

Trichlorfon at 0.5 mg/L (6 hours in tanks and 48 hours in ponds) treatment is very effective to control leech (Morrison and Fox 1993). Formalin (150 mg/L) or sodium chloride (1%) is also an effective leech control.

Ergasilosis

Ergasilosis is caused by the parasites of the genus *Ergasilus*. *Ergasilus* are often called gill lice. They are a host-specific ectoparasite that infect many freshwater fish species including common carp. Male *Ergasilus* are fee-living, while females are parasitic by nature. Female *Ergasilus* attach themselves to the fish gills and feed on blood and tissue of their host (Ojha and Hughes 2001). They cause extensive tissue damage and inflammation of the gill, and reduce oxygen uptake rate of fish. In the natural environment, Ergasilosis usually starts in April and lasts until November. Water temperature plays a very important role in the state of the *Ergasilus* population. Egg development is generally faster at a higher water temperature (Piasecki et al. 2004). Some scientists believe that a high load decomposed organic matter also accelerate the growth of *Ergasilus* populations. A mild infection is not harmful to fishes but a severe infection of *Ergasilus* may cause many detrimental effects on fish health and even cause mortality (Dezfuli 2011).

Causative Agents

Ergasilus spp.

Disease Signs

Small white patches on gills, gill hyperplasia, necrosis of gill tissues are regularly observed signs of Ergasilosis. Restlessly swimming, weight loss, anemia, secondary infection of fungus and bacteria, mortality are frequently observed sings of this disease.

Management and Control

Removing decayed organic material from the pond bottom and sun-drying followed by liming before fish stocking are beneficial to prevent the Ergasilosis. The disease can be treated with a combination of 0.5 ppm copper sulfate and 0.2 ppm ferric sulfate for 6 to 9 days. A bath treatment with potassium permanganate at 10 mg/L for 20-30 minute is effective in killing the *Ergasilus*.

Lernaeosis

Although Lernaeosis is caused by various species of the genus *Lernaea*, this disease is mostly caused by *Lernaea cyprinacea.* Adult *L. cyprinacea* are usually wormlike 9-22 mm long animals. There are about 37 valid *Lernaea* species (Kabata 1979), which are commonly known as anchor worms. The head of the worm is equipped with horns, which are used as an anchor to attach to the host fish. This disease generally occurs in many freshwater fishes including common carp. Lernaeosis with mass mortalities of common carp has been recorded many countries in the world. For example, Fatma (2014) conducted a study on hatchery reared common carp fry and observed 71.7% prevalence of lernaeosis among fry. However, common carp is one of the more preferred hosts for *Lernaea* species (Piasecki et al. 2004). *Lernaea* species are very dangerous parasites for some fish species particularly minnows, a single parasite in a critical location is enough to kill a minnow (Piasecki et al. 2004).

Only the female *Lernaea* has the parasitic activity and feeds on host's body fluid. Male *Lernaea* has no parasitic activity and it leaves the host after the copulation. Various *Lernaea* species have various temperature suitabilities for their reproduction but the majority of species prefer 25 ^{o}C to 28°C for their reproduction. Mild infections by *Lernaea* species may disfigure the fish making them low consumer preference, while a severe infection may kill the fish in short time. Infected fish are generally susceptible to secondary infections of bacteria.

Causative Agents

Lernaea spp.

Disease Signs

Lernaea can be seen on the body surface. Gill damage including epithelial hyperplasia, telangiectasis, and hemorrhage are common signs of this disease. Lethargy, reduced feed intake and damage to fins are frequently observed signs of Lernaeosis. A thick fibrotic capsule around the anchor may appear as the connective tissue of host reacts to the parasite

Management and Control

Bath treatment using the plant extracts carvacrol and cymene mixture (200 ppm) can eliminate 89% lernaea within 48 hours (Fatma 2014). Dipteiex (0.25 ppm active ingredient) can kill copepodite (a stage of their life cycle) within four to six hours. Gammexane (0.2 ppm for 72 hours) or chlorine (1 ppm for 3 days) may give good results to control *Lernaea.* Traditionally salt, potassium permanganate or formalin baths were used to control *Lernaea.*

Argulosis

Fish Argulosis is caused by many species of the genus *Argulus* (Crustacea: Branchiura). *Argulus* are commonly referred to as fish lice. They are obligate ectoparasites and cause frequent problems in fisheries and fish farms worldwide. They are transparent, disc shaped with a dorsoventrally flattened body and are visual to the naked eye. The length of most species is between 5 and 10 mm. *Argulus* infect a wide range of fish species including common carp. In early German literature, *Argulus* was commonly known as carp lice because they used to cause problems in European common carp farming (Taylor et al. 2005). They complete their life cycle including eggs, free-living infective larvae, and juvenile and adult stages on fish (Shimura 1981; Hakalahti-Siren et al. 2008).

Occurrence of argulosis varies by season with peaks of abundance generally during summer and autumn (Moller et al. 2012**)**. Temperature plays an important role in the development of the egg and in the post-hatching development of *Argulus* (Taylor et al. 2005). The suitable temperature for their rapid reproduction is from 20°C to 28°C. Eggs appear to lie dormant at temperatures of less than 10°C. Although *Argulus* have a wide host range, some fish species are observed to be more susceptible to *Argulus* than others. For example, Aalberg et al. (2016) observed the higher prevalence, mean intensity and abundance of *Argulus* sp. in Pike-perche (*Sander lucioperca***)** compared to common carp. *Argulus* generally infect fish from the side or beneath and gradually they move to a preferred site. Gills and skin are most preferred sites for *Argulus* infection. Young fishes are more prone to infection than old ones (Ozturk 2005). Argulosis may lead to secondary infections and other parasitic disease (Lester et al. 2006). It can cause significant morbidity and mortality when fish are heavily infested.

Causative Agents

Argulus spp.

Disease Signs

Argulus can be seen on the body surface and fins of fish. Lethargy, reduced feed intake, abnormal swimming, excessive mucus production, small hemorrhages, fin erosion and anemia are commonly reported signs of this disease. Secondary infection of fungus and bacteria also frequently observed signs of Lernaea infected fish.

Management and Control

Draining of the pond followed by liming can prevent the Argulosis outbreak. Lindane at 0.01 ppm can kill all free-swimming *Argulus* within five hours, while Lindane concentration of 0.013 ppm can kill all attached *Argulus* in the same time period. Bath treatment with salt (500-1000 ppm for 24 hours) or Potassium permanganate (2-5 mg/L for 24 hours) or

Dichlorvos solution (0.2 mg/L for 24 hours) or Ammonium chloride (1.0-1.5% for 15 minutes) and pond treatment with gamrnaxine (0.2 mg/L) are effective to control *Argulus.*

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Chapter 100

BLOOMS CAUSED BY THE DIATOM *CYLINDROTHECA CLOSTERIUM* ALONG THE NORTHERN COAST OF YUCATAN, SOUTHEASTERN GULF OF MEXICO (2001-2014)

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ABSTRACT

Data on abundances of $>10^5$ cells/L of the widely distributed non-toxic benthic-planktonic pennate diatom *Cylindrotheca closterium* as a causative agent of harmful algal blooms (HAB) in the coastal waters of the northern Yucatan Peninsula, with an emphasis on four marinas (Chuburná, Yucalpetén, Telchac and Dzilam) in 2001-2014 (fortnight and monthly monitoring), are presented. The highest abundance of 2.68×10^7 cells/L was recorded in San Crisanto in September 2011. The multivariate statistical analysis Canoco 4.5 applied to the marinas for the 2011-2013 period showed a positive correlation between the cell abundances and nitrites, phosphates, nitrates and urea. During the 2001-2014 period the major proportion of HAB (with $>10^5$ cells/L) caused by *C. closterium* occurred since 2011; only in Chuburná were the highest abundances found in 2008. The

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seasonality of the species remains unclear: in all the marinas HAB were observed during all months. In Dzilam, *C. closterium* was frequently accompanied by the marine-brackish-water potentially toxic dinoflagellate *Prorocentrum minimum*. An increase in frequency of HAB caused by *C. closterium* in Yucatan waters, especially in the marinas, seems to be due to the anthropogenic impact of increased nitrogen. This species has a wide geographical distribution, has been reported from various habitats, has high growth and reproductive rates and has a high potential for predominance in both planktonic and benthic microalgal assemblages. *Cylindrotheca closterium* became one of the dominant species throughout the year, forming almost monospecific blooms as well as those in association with other species.

Keywords: *Cylindrotheca closterium*, eutrophication, Gulf of Mexico, harmful algal blooms, microalgae, monitoring, phytoplankton, Yucatan

INTRODUCTION

Cylindrotheca closterium (Ehrenb.) Reimann et J. C. Lewin is a non-toxic pennate benthic-planktonic widely distributed diatom, observed in the water column, superficial sediments and the lowermost layer of sea ice, distributed in both marine and brackish water in all climatic zones around the world. Underwood & Provot (2000) characterize it as epipelic estuarine, although it is one of the most frequent and abundant microplanktonic species in subtropical North Atlantic waters (Najdek et al., 2005; Halac et al., 2013) and tropical waters of the western Indian Ocean (Sá et al., 2013). Although Jahn & Kusber (2005) presented their solid arguments and re-established the genus *Ceratoneis* Ehrenb., in literature the name *Cylindrotheca closterium* is still in extensive use. In the present study, we keep using the latter, considering *Ceratoneis closterium* Ehrenb. as basionym and *Nitzschia closterium* (Ehrenb.) W. Sm. and *Nitzschiella closterium* (Ehrenb.) Rabenh. as synonyms. Herein, we followed the proposal to conserve the name *Cylindrotheca* against *Ceratoneis* (Prud'homme van Reine, 2011). Recently, based on molecular biological studies, *C. closterium* was proposed as a complex of cryptic species (Li et al., 2007). The results on the preferred salinity ranges by distinct clones of this species obtained previously (Underwood & Provot, 2000) confirmed the physiological and biochemical heterogeneity of *C. closterium*.

Most studies of this species are biochemical and ecophysiological and date back to the 1920s (Jameson et al., 1922; Peach & Drummond, 1924; Leigh-Clare, 1927). Recently, the species has been used as a model in molecular studies of diatoms (Vanormelingen et al., 2013). *Cylindrotheca closterium* is a species with high growth and reproduction rates, from 0.63-0.97 to 2.3 divisions per day in culture (Eppley et al., 1971; Sun et al., 2004; Affan et al., 2009; Kingston, 2009). Sexual reproduction also occurs rapidly, concluding in 24 h (Vanormelingen et al., 2013). As a harmful algal blooming species in Mexican waters, it has been known from the northwestern Pacific (Longhurst et al., 1967; Licea-Durán et al., 1999; Gárate-Lizárraga et al., 2001, 2009, 2016a, 2016b) and the southeastern Gulf of Mexico (Poot-Delgado et al., 2013; Muciño-Márquez et al., 2014). Recently, Gárate-Lizárraga & Esqueda-Escárcega (2016) reported the infestation of the copepod *Paracalanus* sp. by *C. closterium* cells. Blooms of *Cylindrotheca closterium + Akashiwo sanguinea* have been linked to fish, lobster and marine bird mortality along the west coast of Baja California, the northwestern Mexican Pacific (Gárate-Lizárraga et al., 2001). More recently, successive

infectious stages of *C. closterium* were described for the first time during the early development of sea urchins (Magesky et al., 2017).

To present the data on the occurrence of *C. closterium* during monitoring of the coastal waters of the northern Yucatan Peninsula in 2001-2014, with the aim of determining its variability and spatial-temporal abundance in the water column, were the main objectives of this study. To explain these, physical-chemical variables were also analyzed.

MATERIAL AND METHODS

Quantitative phytoplankton samples were taken in the uppermost surface layer of the water column with a 250-ml plastic bottle along the northern Yucatan Peninsula from July 2001 through April 2014 (Figure 1). Beach monitoring of harmful algal blooms (HAB) was performed in 2002-2013 at 18 stations at 20-30 m distance from the beach between Chuburná and Dzilam de Bravo (Dzilam, for short), monthly from October to April (with minor probabilities of HAB occurrence) and fortnightly from May to September (with major probabilities of HAB occurrence). Monitoring of four marinas (Chuburná, Yucalpetén, Telchac and Dzilam de Bravo) was carried out in 2002-2013, fortnightly from May to September in 2002-2013 and monthly from October to April in 2009-2013. All samples were fixed with an acid Lugol solution.

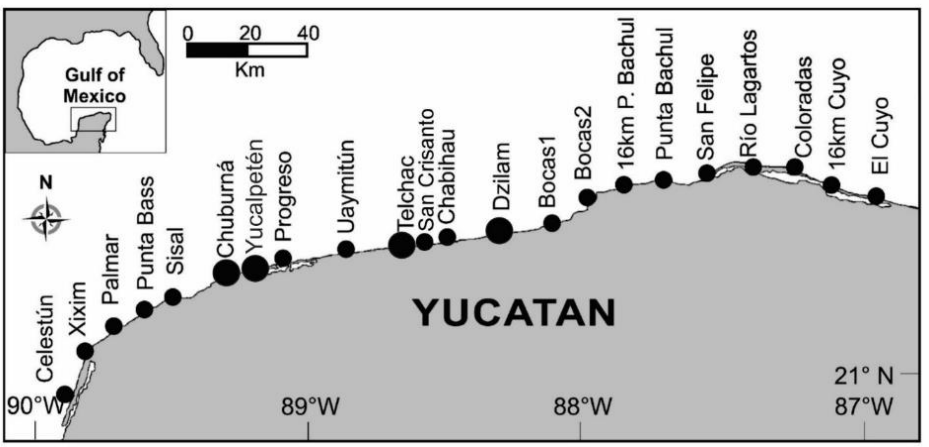

Figure 1. The study zone and sampling sites along the northern Yucatan Peninsula coast (2001-2014), including Chuburná, Yucalpetén, Telchac y Dzilam marinas (2002-2013).

Samples were analyzed using an Olympus CKX 41 or a Carl Zeiss Axiovert 100 inverted miscroscope equipped with phase-contrast objectives, using the Utermöhl technique (Hasle, 1978). Surface water temperature, salinity, turbidity, pH, dissolved oxygen, nitrates, nitrites, ammonium, phosphates, silicates, urea, *in situ* fluorescence and chlorophyll-*a* were determined in the field or in the laboratory. Selected samples were observed in a JEOL JSM-7600F Field Emission scanning electron microscope (SEM) at 5 kV. To correlate the *C. closterium* cell abundance with the physical-chemical variables, multivariate (MVA) and redundancy analyses (RDA) were applied using the Canoco for Windows 4.5 package.

RESULTS

Cell count results are given in Table 1 and Figure 2. For Table 1, only the abundances of $>10^5$ cells/l are shown. Electron micrographs are presented in Plate 1. The RDA applied to the four marinas (2011-2013) demonstrated a positive correlation between *Cylindrotheca closterium* abundance and some physical-chemical variables such as nitrites ($p = 0.03$), phosphates ($p = 0.05$), nitrates ($p = 0.047$) and urea ($p = 0.015$), where "p" is the level of statistical significance. The physical-chemical characteristics associated with the cell abundance of $>10^5$ cells/L were as follows: temperature 21.6-31.2 °C, salinity 16.40-39.80, dissolved oxygen 1.30-11.82 mg/L, dissolved oxygen saturation 18.3-118.5 (178.9) %, nitrates (0.05) 0.84-174.02 (345.82) µM, nitrites 0.01-4.84 (26.68) µM, ammonium 0.35-37.74 µM, phosphates 0.03-8.69 µM, silicates 0.82-115.00 µM, and urea 0.45-12.78 (45.34) µM. The chlorophyll-*a* concentration during HAB usually varied between 0.17 and 26.95 mg/m^3; in only two cases were higher values measured (62.00 and 144.21 mg/m^3) in Dzilam marina. The RDA performed for marinas in 2002-2013 showed $p = 0.05$ for nitrites and $p = 0.03$ for chlorophyll-*a*.

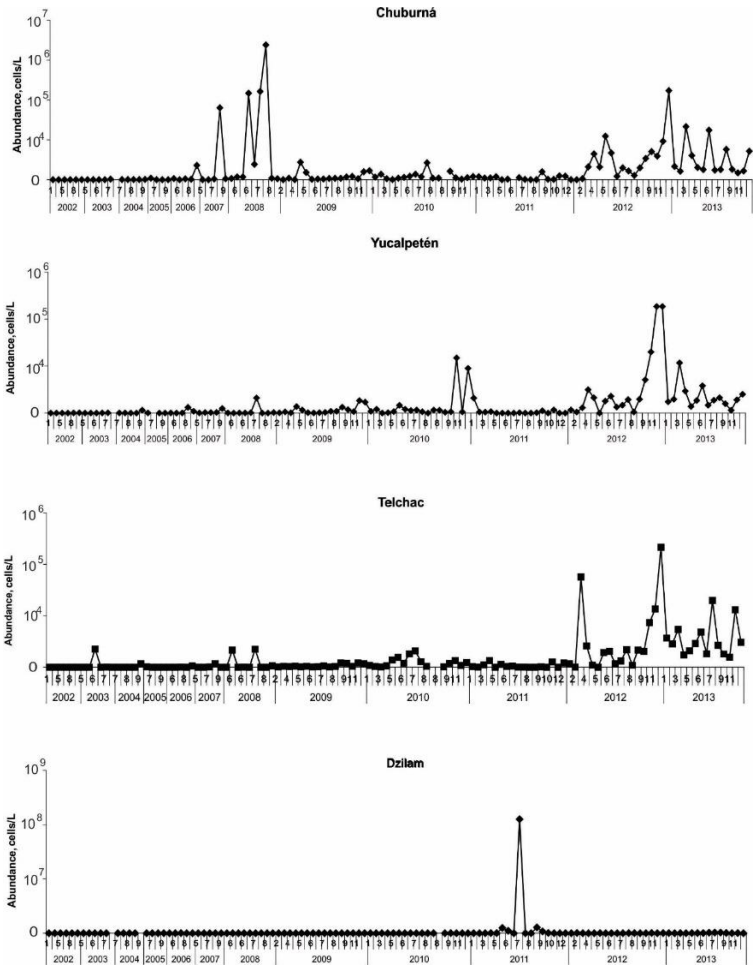

Figure 2. Multi-year dynamics (2002-2013) of *Cylindrotheca closterium* abundance in the four marinas along the northern Yucatan Peninsula coast.

Table 1. Abundances of >10^5 cells/L of Cylindrotheca closterium in coastal waters along the northern Yucatan Peninsula during the 2001-2014 period (beach and marina monitoring, and at 50-1000 m from the coastline). The abundances of >10^6 cells/L are given in bold

Month-year	Locality	Abundance (cells/L)	Month-year	Locality	Abundance (cells/L)
August-01	San Bruno	2.48 x 10^5	June-13	Telchac	2.65 x 10^5
	Uaymitún	1.70 x 10^5		Uaymitun	9.75 x 10^5
	Chicxulub	3.99 x 10^5		Chicxulub	5.45 x 10^5
June-08	Chabihau	**3.00 x 10^6**		Progreso	9.60 x 10^5
	Chicxulub	**1.00 x 10^6**		Yucalpetén	1.34 x 10^5
August-11	Uaymitún	1.93 x 10^5		Chelem	1.54 x 10^5
September-11	Dzilam	**4.58 x 10^6**		Chuburná	1.80 x 10^5
	San Crisanto	**2.68 x 10^7**	July-13	Dzilam	**3.12 x 10^6**
October-11	Dzilam	**1.04 x 10^5**		Santa Clara	2.40 x 10^5
January-12	Santa Clara	1.00 x 10^5		Chabihau	2.45 x 10^5
February-12	Dzilam	**1.08 x 10^6**		San Crisanto	1.04 x 10^5
March-12	Telchac	5.97 x 10^5		Telchac	3.90 x 10^5
April-12	Santa Clara	**1.67 x 10^6**		Uaymitun	2.30 x 10^5
May-12	San Crisanto	**2.11 x 10^6**		Chicxulub	2.95 x 10^5
June-12	Dzilam	**3.24 x 10^6**		Progreso	3.30 x 10^5
July-12	Dzilam	4.20 x 10^5	August-13	Chuburna	1,55 x 10^5
August-12	Santa Clara	**1.88 x 10^6**		Chelem	1.70 x 10^5
September-12	Dzilam	5.06 x 10^5	August-13	Progreso	2.65 x 10^5
October-12	Chuburná	**6.20 x 10^6**		Chicxulub	2.80 x 10^5
November-12	Chicxulub	9.80 x 10^5		Chabihau	1.02 x 10^5
December-12	Chicxulub	9.60 x 10^5		Santa Clara	2.10 x 10^5
January-13	Santa Clara	5.70 x 10^5		Dzilam	1.90 x 10^5
February-13	Chicxulub	9.15 x 10^5	October-13	Chicxulub	1.45 x 10^5
March-13	Dzilam	3.30 x 10^5	October-13	Telchac	1.06 x 10^5
	Chabihau	2.90 x 10^5		Santa Clara	1.95 x 10^5
	San Crisanto	2.60 x 10^5	November-13	Chicxulub	1.54 x 10^5
	Telchac	2.20 x 10^5		Uaymitun	1.65 x 10^5
	Uaymitun	1.90 x 10^5		Telchac	1.40 x 10^5
	Chicxulub	3.10 x 10^5		San Crisanto	1.82 x 10^5
	Progreso	3.80 x 10^5		Chabihau	2.72 x 10^5
	Yucalpetén	2.10 x 10^5		Santa Clara	1.80 x 10^5
	Chelem	1.70 x 10^5	December -13	Chicxulub	2.40 x 10^5
	Chuburná	3.40 x 10^5		San Crisanto	2.97 x 10^5

Table 1. (Continued)

Month-year	Locality	Abundance (cells/L)	Month-year	Locality	Abundance (cells/L)
April-13	Dzilam	1.27×10^5	December -13	Chabihau	3.12×10^5
	Santa Clara	1.68×10^5		Santa Clara	1.87×10^5
	San Crisanto	1.02×10^5	January-14	Telchac	1.29×10^5
	Telchac	1.56×10^5		San Crisanto	$1{,}01 \times 10^5$
	Uaymitun	2.62×10^5		Santa Clara	1.60×10^5
	Chicxulub	9.10×10^5	February-14	Progreso	$\mathbf{1.82 \times 10^6}$
	Yucalpetén	1.22×10^5		Chicxulub	3.99×10^5
	Progreso	1.44×10^5		Telchac	2.09×10^5
	Chuburná	1.88×10^5		San Crisanto	3.49×10^5
May-13	Dzilam	$\mathbf{6.96 \times 10^6}$		Chabihau	3.72×10^5
	Santa Clara	2.40×10^5		Santa Clara	1.20×10^5
	Chabihau	1.40×10^5		Dzilam	2.40×10^5
	San Crisanto	2.80×10^5	March-14	Progreso	1.12×10^5
	Telchac	3.60×10^5		Chicxulub	4.19×10^5
	Uaymitun	2.62×10^5		San Crisanto	1.60×10^5
	Chicxulub	3.90×10^5		Chabihau	1.15×10^5
	Progreso	4.51×10^5		Santa Clara	1.00×10^5
	Chuburná	6.76×10^5	April-14	Telchac	1.46×10^5
June-13	Dzilam	$\mathbf{1.82 \times 10^6}$		San Crisanto	4.95×10^5
	Santa Clara	3.00×10^5			

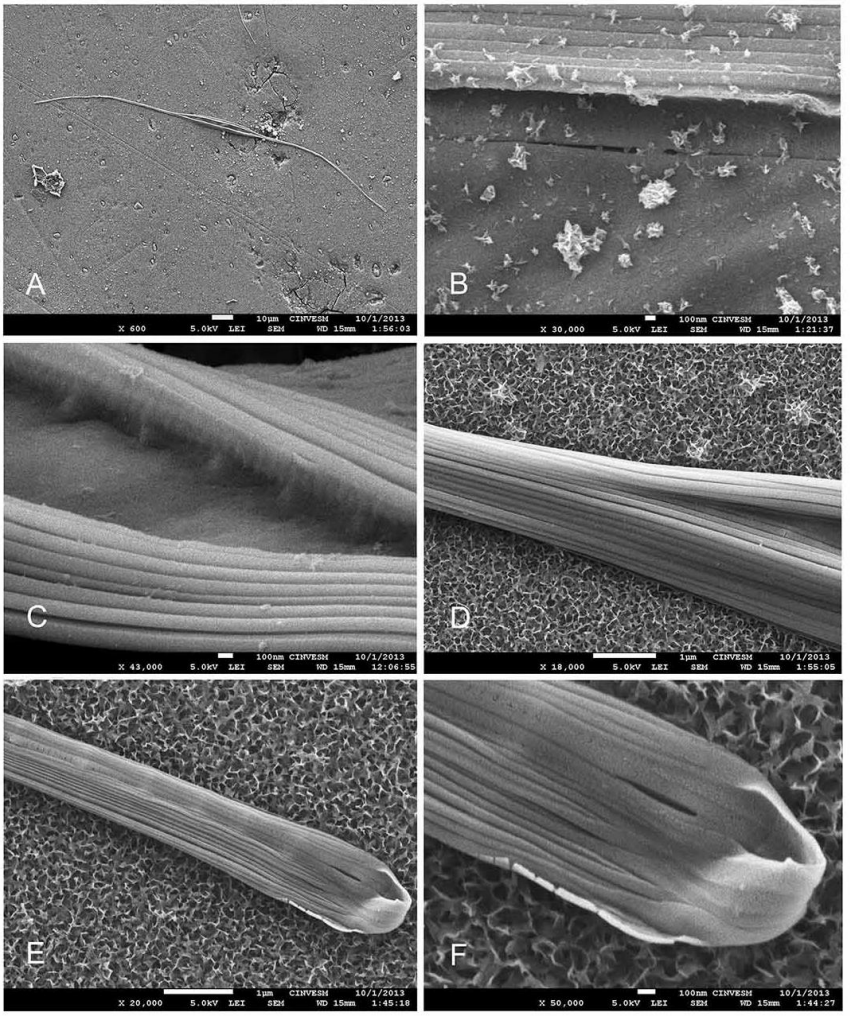

Plate 1. Electron micrographs of *Cylindrotheca closterium*: entire cell (A) and close-ups of a frustule (B-F). Scale bars: 1 µm (D and E), 10 µm (A), and 100 nm (B, C and F).

During the 2001-2014 period most of HAB (>10^5 cells/L of *C. closterium*) caused in coastal waters of the northern Yucatan were observed since 2011 (Table 1). Only in Chuburná marina was the highest cell abundance found in 2008. The seasonality of the species is unclear: in all the monitored marinas HAB were observed throughout the year. In Dzilam marina *C. closterium* was frequently accompanied by the potentially toxic small-sized dinoflagellate *Prorocentrum minimum* (Pavill.) J. Schiller that is also characteristic of both marine and brackish waters.

DISCUSSION

In the samples analyzed with the use of an inverted microscope, it was difficult to distinguish between *Cylindrotheca closterium*, *Nitzschia longissima* (Bréb.) Ralfs in Pritchard

and *N. reversa* W. Smith. Thus, when they occurred together obtaining reliable cell counts for each species was problematic. However, SEM observations confirmed that during pelagic HAB events *C. closterium* was the most abundant.

It is necessary to differentiate between morphologically similar species such as *C. closterium*, *N. longissima*, *N. reversa* and some others such as *Reimerothrix floridensis* A. K. S. K. Prasad (Prasad et al., 2001; Hernández-Almeida & Herrera-Silveira, 2014) and *Fragilaria longifusiformis* (Hains et Sebring) Siver et al. (=*Synedra planktonica* Hains et Sebring), the latter being widely distributed in freshwater continental bodies of North America, Europe (Siver et al., 2006) and South America (Ludwig et al., 2015), as well as the widely distributed marine diatom *Lennoxia faveolata* H. A. Thomsen et K. R. Buck (Thomsen et al., 1993).

CONCLUSION

The results obtained suggest increases in the frequency of HAB events caused by *C. closterium* in the northern Yucatan waters due to anthropogenic impact evidenced by elevated nitrogen content in coastal waters, especially in marinas, the areas where fishing boats are concentrated. This widely distributed species occurs in various habitats, grows and reproduces rapidly, and has a high potential to dominate both planktonic and benthic microalgal assemblages. With the increased anthropogenic influence on the coastal zone of Yucatan, taking advantage of the elevated nitrogen concentration (mainly in the form of nitrites, nitrates and urea), *C. closterium* was one of the dominant species throughout the year, causing almost monospecific HAB events as well as ones in association with other microalgal species. In spite of the high abundances of *C. closterium* found during this study, no marine fauna mortality was observed.

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de las causas, dispersión y consecuencias ambientales de la marea roja en Yucatán" (FOMIX CONACyT-Gobierno del Estado de Yucatán, 2009-2011, responsible: JAHS). Marcia M. Gowing (University of California, Santa Cruz, California, USA) kindly reviewed the English.

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Chapter 101

PHYTOPLANKTON CHLOROPHYLL-α CONCENTRATION ASSOCIATED WITH HYDROGRAPHIC CONDITIONS ON THE CONTINENTAL SHELF IN THE SOUTHERN GULF OF MEXICO

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ABSTRACT

Phytoplankton chlorophyll-*a* data obtained on 27 oceanographic cruises in the southern Gulf of Mexico between 1979 and 2000 were analyzed. Four cruises were selected to report variations of chlorophyll-*a* associated with hydrographic conditions (temperature and salinity) in surface waters, one in April 1983 and three in 1987 in different seasons. The CTD casts were taken from 0 to 150 m depth aboard the R/V "Justo Sierra". Discrete water samples were collected to determine chlorophyll-*a*

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concentration by fluorometry. The observations showed that the autumn was characterized by high chlorophyll-*a* values (0.05 to 6.00 mg m^{-3}), suggesting a real effect of the coastal lagoons and river fronts in late summer – early autumn along with the obvious effect of the Yucatan Current that greatly influences the coastal zone of the southeastern Gulf westward at least to 93ºW. In summer the highest chlorophyll-*a* concentrations (up to 6.50 mg m^{-3}) were observed in the eastern part of the study area. The lowest summer values (0.06 to 0.50 mg m^{-3}) were found in the northeastern region (<0.10 mg m^{-3}). Summer presents a typical stratified condition with most of the sites characterized by strong thermoclines with surface water temperatures from 25.0 to 28.9°C. River runoff contributes to the formation of a surface plume 2-8 m thick that extends seaward. In autumn a mixed water column induced by the effect of the northerlies produces a clear temperature decrease with values from 25.0 to 26.0ºC from the surface to 55 m depth and a thermocline below.

Keywords: Bank of Campeche, chlorophyll, Gulf of Mexico, hydrography, microalgae, phytoplankton, upwelling, Yucatan

INTRODUCTION

Hydrographic conditions in the southern Gulf of Mexico are highly influenced by the local rivers and evapotranspiration processes close to the coast and the presence of mesoscale eddies that can be locally generated (Bay of Campeche cyclone) or detached from the Loop Current. This current was observed to shed more eddies in summer and winter (Chang and Oey, 2012). The occurrence of winter storms between October and February (Hurlburt and Thompson, 1980) is also an important aspect. The Yucatan Current that enters the Gulf of Mexico through the Yucatan Channel causes an intensive upwelling on Cape Catoche, which, in turn, is responsible for high biological production (Bessonov et al., 1971). The current has a mean velocity of 1.5 m s^{-1} near the surface (Candela et al., 2005; Ochoa et al., 2005), reaching a maximum velocity of 2.5 m s^{-1} (Abascal et al., 2003). The core of the Yucatan Current is characterized by velocities of more than 0.6 m s^{-1} at any time, and it is approximately 50 to 100 km wide (Bagan et al., 2005; Athié et al., 2011). The intensity of the Yucatan Current changes the character of the horizontal circulation above the Bank of Campeche and determines the position of cyclonic and anticyclonic gyres in the Gulf of Mexico. In the northern part of the Bank of Campeche, at the shelf margin, a cyclonic gyre was observed during intensification of the Yucatan Current. In spring and autumn both types of circulation are observed. However, the character of circulation at the Bank of Campeche seems to depend, to a greater extent, on interannual fluctuations in the intensity of the Yucatan Current (Bessonov et al., 1971). The main zones of upwellings and downwellings are stable in both time (within a year) and space, which results in low variability in quantitative characteristics of plankton in the Bank of Campeche area (Bogdanov, 1965, 1967; Bogdanov et al., 1968; Bessonov and González, 1971; Nowlin, 1972; Molinari et al., 1978; Merrel et al., 1981; Salas de León and Monreal-Gómez, 1986; Vidal et al., 1992).

In addition, this region is mainly impacted by two rivers, the Coatzacoalcos River and the Grijalva-Usumacinta River System (GURS), that account for approximately one-third of all fluvial discharge into the coastal waters (Tamayo, 1990). Based on chlorophyll-*a* (Chl-*a*) estimations from SeaWiFS (Sea-viewing Wide Field-of-view Sensor) satellite images, the

ecoregion influenced by the rivers expands from 95°W to almost 89°W, covering a wide coastal zone of the states of Veracruz (its eastern part), Tabasco, Campeche and Yucatan (its western part) (Salmerón-García, 2011). There are also two big coastal lagoons in the southern Gulf of Mexico. The Terminos Lagoon is distinguished by its wide communication to the sea through its two inlets and shows Chl-*a* concentrations from 0.30 to 8.20 mg m^{-3} (Day et al., 1982). The Alvarado Lagoon receives a large contribution from the rivers Papaloapan, El Blanco and El Limon, with Chl-*a* concentrations ranging from 3.20 to 10.60 mg m^{-3} (Lozano-Montes, 1993). The geomorphology described above creates a very dynamic coastal ecosystem that produces spatial-temporal hydrographic variations (Czitrom et al., 1986; Schirasago, 1991; Monreal-Gómez and Salas de León, 1992), giving the entire region unique conditions that support the most important commercial fisheries in the Gulf. Its production is highly influenced by a complex system of coastal lagoons and estuaries known for their significant local productivity (Cruz, 1968; Contreras, 1985; Yañez-Arancibia and Sanchez-Gil, 1986; Soto and Escobar-Briones, 1995; Arreguín-Sánchez, 1999). Estuarine outwelling and river runoff account for much of the input of dissolved nutrients discharged into the adjacent coastal waters; thus, the phytoplankton of coastal waters is subjected to both temporal and spatial fluctuations (Licea and Luna, 1999). In addition, winds, coastal currents and upwelling events contribute to the variations. The mesoscale heterogeneity characteristic of marine phytoplankton distribution appears to be a result of the hydrographic variability mentioned above (Duarte et al., 1992).

The Yucatan shelf is highly influenced by the upwelling along Cape Catoche and other areas of the northern Yucatan coast (Bogdanov et al., 1968; Cochrane, 1969; Bessonov et al., 1971; Ruiz and Merino, 1987; Merino, 1997; Salmerón-García, 2011), sometimes interrupted by northerly winds from October to February (Mariño-Tapia et al., 2014; Reyes-Mendoza et al., 2016) due to the synoptic scale of wind variability (Pérez-Santos et al., 2010). Other significant features are the absence of rivers along the coast (Merino and Otero, 1991) and the width of the shelf around the Yucatan Peninsula that extends seaward for over 260 km (Figure 1).

The main objective of this study was to present the data on the spatial-temporal variability of Chl-*a* in surface (down to 150 m depth) waters in relation to hydrographic conditions, with an emphasis on upwelling regions near the Yucatan Peninsula.

MATERIAL AND METHODS

During a long-term oceanographic survey of the southern Gulf of Mexico program (22°30’ to 18°15’N) in the period from 1979 to 2000, 27 oceanographic cruises were conducted. During each cruise, Chl-*a* samples were taken from the water column, providing a total of 5398 data records that were graphed (Figure 1). The top left values ranging from ≥0.5 to 14.00 mg m^{-3} (632 data points) were used for the most important conclusions of this study.

In addition, hydrographic information (water temperature and salinity) obtained on the 14 transects at 57 stations of three cruises in 1987 and another in 1983 was used. In all cases, a series of CTD (conductivity-temperature-depth) hydrocasts was carried out onboard the R/V “Justo Sierra”, utilizing a Neil Brown CTD Mark-IIIB probe coupled to a General Oceanics Rosette sampler with 1.5-liter Niskin bottles. Casts were taken from 0 to 150 m depth,

depending on the site depth. Discrete water samples were obtained from each cast to measure Chl-*a* following the fluorometric method (Yentsch and Menzel, 1963). Samples of 250 ml were filtered immediately using a vacuum system with glass fiber filters of 0.22 μm pore size; 90% acetone was used as the extraction solution within 24 h prior to the analysis in a Turner model 110 fluorometer (Turner Designs, San Jose, CA, USA).

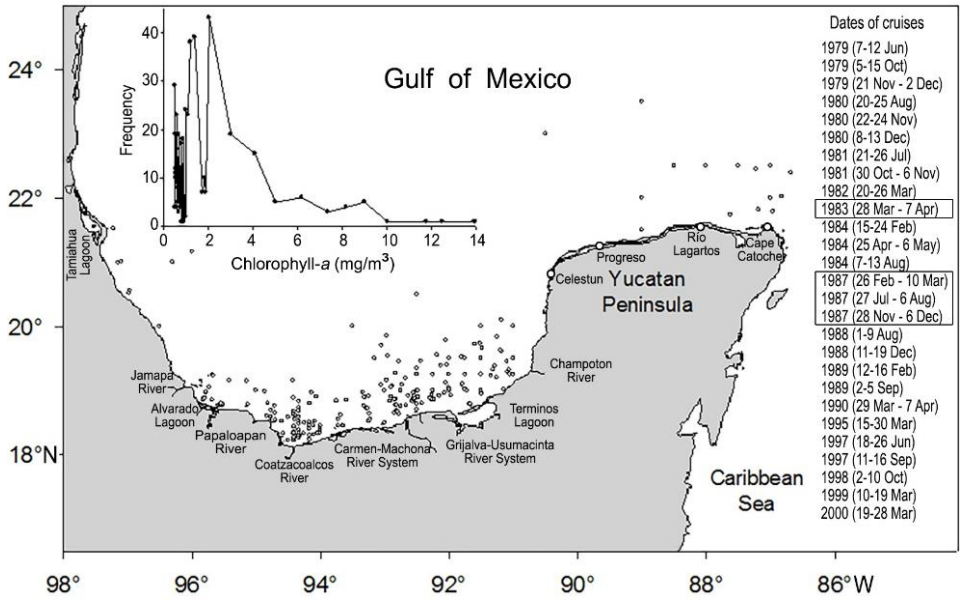

Figure 1. Study area in the southern Gulf of Mexico. The approximate position of stations with chlorophyll-*a* values ≥0.50 mg m^{-3} are indicated by circles. Top left graph shows the frequency of 632 values obtained from 1979 to 2000 during 27 oceanographic cruises (right side). Rectangles delineate the selected cruises referred to in the text.

A set of comparative graphs (Figures 2 and 3) were made by selecting all the stations that had Chl-*a* values of ≥0.50 mg m^{-3} which were then grouped into five regions whose names were taken from the nearest place along the coast where the values were obtained (the Alvarado Lagoon, the Coatzacoalcos River, the GURS, the Terminos Lagoon and the Champoton River), to show regional hydrographic conditions. For this purpose, three cruises of 1987 were selected on the basis of the most complete and representative information obtained. The chosen periods were: winter, summer and autumn (February-March, July-August and November-December, respectively).

SeaWiFS images (SeaStar-Orbview2 satellite) and sea surface temperature (SST) images from the U.S. National Oceanic and Atmospheric Administration (NOAA) series of Advanced Very High Resolution Radiometer (AVHRR-14), both produced by the TeraScan system of SeaSpace Co. (Poway, CA, USA), were obtained in the nearest available periods to field sampling to compare field observations to those obtained by remote sensing (Figures 4 and 5).

To update physical-oceanographic information on the southeastern Gulf of Mexico, for discussion, some hydrographic data obtained during the oceanographic cruises XCAMBO IV (14 September to 2 October 2009; Figure 6) and XCAMBO V (2-20 June 2011, Figures 7 and 8) for PEMEX (Petróleos Mexicanos) were used.

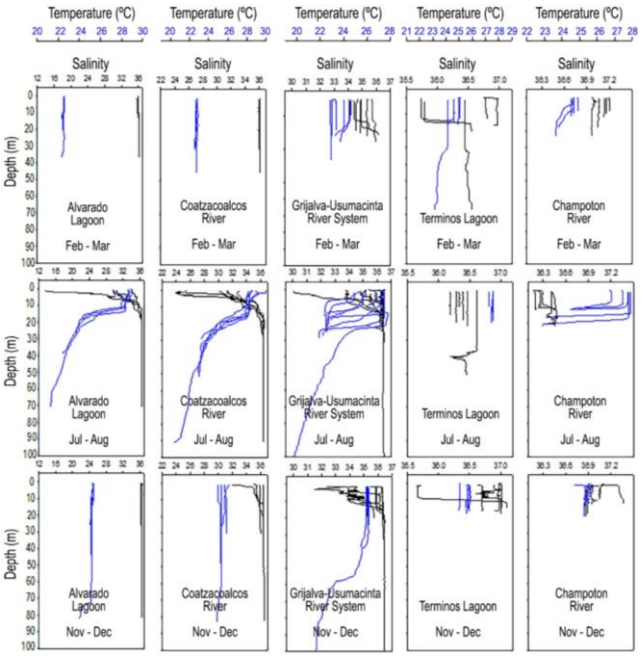

Figure 2. Comparative vertical sections of salinity and temperature (degrees Celsius, in blue) of selected stations from five regions (the Alvarado Lagoon, the Coatzacoalcos River, the Grijalva-Usumacinta River System, the Terminos Lagoon and the Champoton River) with chlorophyll-*a* values ≥0.50 mg m^{-3} during three periods: winter (February-March), summer (July-August) and autumn (November-December).

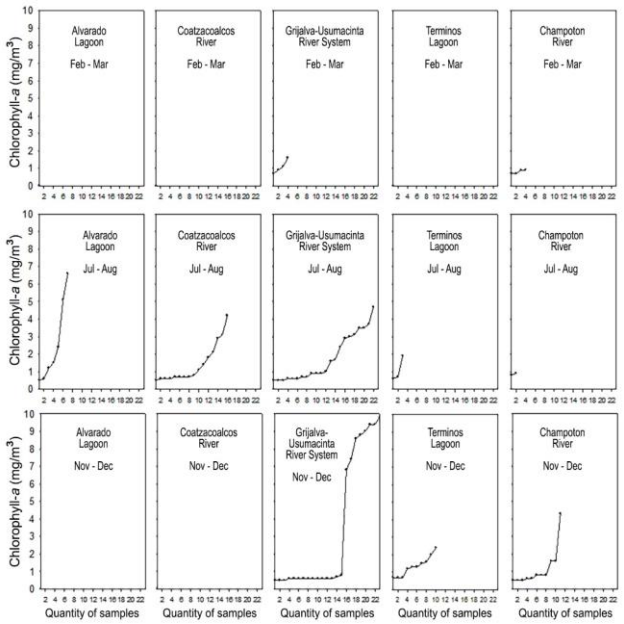

Figure 3. Five comparative regions (the Alvarado Lagoon, the Coatzacoalcos River, the Grijalva-Usumacinta River System, the Terminos Lagoon and the Champoton River) grouped by using stations with chlorophyll-*a* values ≥0.50 mg m^{-3} during at least one of the three periods: winter (February-March), summer (July-August) and autumn (November-December). Empty graphs mean no values ≥0.50 mg m^{-3}.

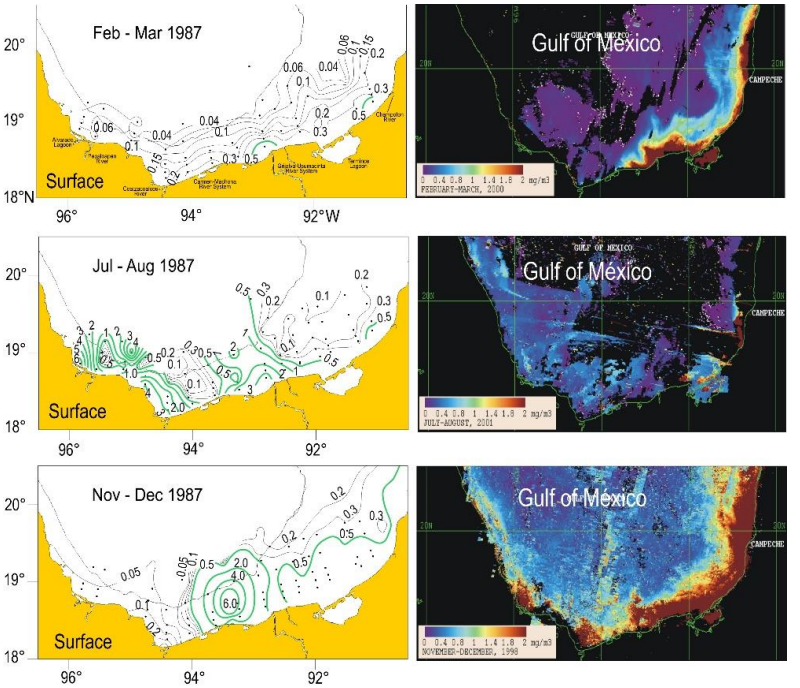

Figure 4. Comparative panels of surface water layer distribution of chlorophyll-*a*. Upper left panel shows winter distribution in February-March; middle left shows summer distribution in July-August; lower left shows autumn distribution in November-December. Green isolines show values ≥0.50 mg m^{-3} (dots indicate the position of sampling stations). Right panels show SeaWiFS images for chlorophyll-*a* in the same periods in different years.

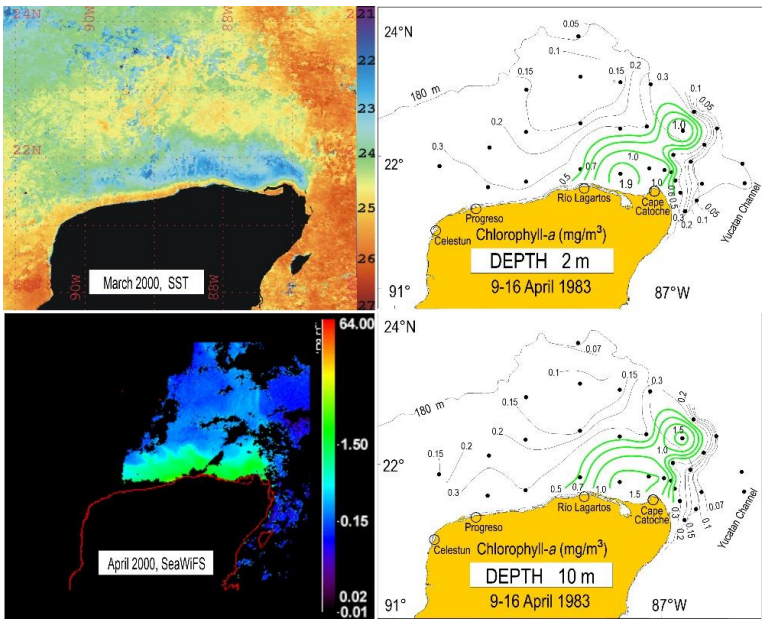

Figure 5. Top left panel shows sea surface temperature (SST) images in March 2000; bottom left panel shows SeaWiFS images in April 2000. Right panels show the distribution of chlorophyll-*a* in June 1983 at the surface (right top) and at the 10 m depth (right bottom). Dots indicate the position of sampling stations. Green isolines show chlorophyll-*a* values ≥0.50 mg m^{-3}.

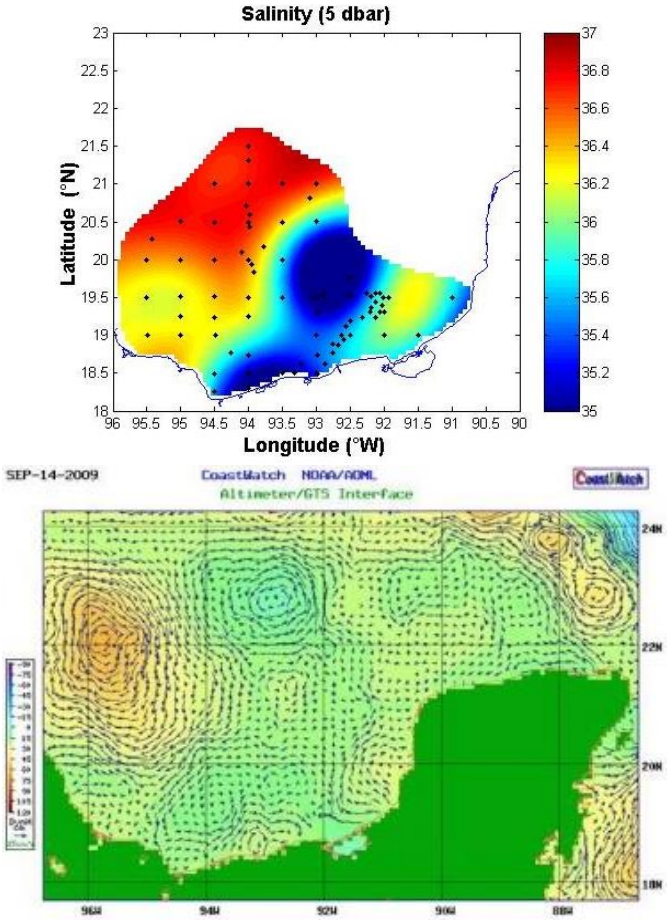

Figure 6. Upper panel: Surface (5 m) salinity in the southeastern Gulf of Mexico in September-October 2009. Lower panel: Circulation for the period obtained from a re-analysis product that includes satellite altimetry and the OCCAM model (http://www.aoml.noaa.gov/phod/dataphod/work/trinanes/INTERFACE/index.html).

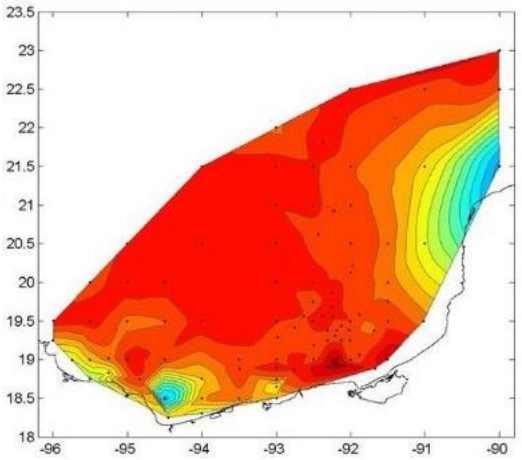

Figure 7. Surface (10 m) water temperature in the southeastern Gulf of Mexico in June 2011, a year when the upwelling was particularly strong.

RESULTS

Regional Hydrographic Conditions

Figure 2 shows a set of comparative vertical sections of salinity and temperature during three cruises (winter, summer and autumn). During winter the water tended to be homogeneous. Two groups of profiles were detected: one with temperatures ranging from approximately 22.5 to 23.5°C that encompassed stations located on the western side of the region (the Alvarado Lagoon, the Coatzacoalcos River and the GURS) and the other with temperatures ranging from 23.7 to 25.0ºC that included the stations located in the eastern region (the Terminos Lagoon and the Champoton River).

Winter was characterized by a vertically mixed water layer from the surface to 50 m depth from the effect of the northern winds that begin in autumn and last through the winter. During this period the local effect of the river plumes on the surface layer of the coastal waters is noticeable. Winter and autumn had nearly the same water column structure; however, summer was the period of maximum influence of river runoff. River plumes of the Coatzacoalcos River and the GURS were detected based on surface salinity over 100 km from the coast. During the rainy season river discharge intensified, thus generating a thin layer of water characterized by low salinity (<30) and temperatures from 27° to 28°C that could be traced almost 100 km off the mouths of the main rivers. Towards the end of the year temperature dropped (<25°C) and salinity fluctuated between 35 and 36; both parameters showed straight vertical profiles on the shelf (Figure 2). The Terminos Lagoon region always showed some turbulence as a consequence of the expelled brackish waters of this lagoon into the adjacent coastal zone.

In summer (Figure 2, middle graphs), a typical stratified condition occurred, with surface waters reaching the maximum temperature (25.0 to 28.9°C) and most stations characterized by strong thermoclines. At stations in the eastern region the top of the thermocline lay between 8 and 30 m, while in the western region it was between 25 and 40 m. During this period the stratified condition produced both vertical and horizontal gradients in both temperature and salinity. A marked seasonal thermocline was present very close to the surface, and its persistence in time was further prolonged by the cold oceanic water (temperature 22.0°C and salinity 36.5) upwelling onto the inner shelf boundary. The river flow contributed to the formation of a surface plume 2-8 m thick that extended seaward. Salinity profiles showed the effect of river runoff and high evaporation rates with values of >36. The section of the Alvarado Lagoon showed a strong vertical gradient in temperature and salinity near the surface and in the layer between the 10 and 20 m depth. Haline structure showed the effect of the Coatzacoalcos River by extending the river plume up to 35 km from the coast and 15 m in depth; below 20 m a homogeneous water mass with salinity of 36 was observed. The region of the GURS showed a thermic structure with almost vertical isotherms, suggesting upwelling of bottom water.

In autumn (Figure 2, bottom graphs) mixing into the water column induced by the effect of the northerlies clearly produced a temperature decrease down to 25-26ºC from the surface to 55 m depth with a residual thermocline below. In this period the highest values of salinity (36.0 to 36.3) were found at the western stations. Similarly, this region appeared vertically

uniform, and the influence of rivers was weak. However, the most important estuarine outwelling of the Terminos Lagoon occurred during this period.

Chlorophyll-*a* Distribution

Chl-*a* concentration of the 5398 data records collected during the period of 1978-2000 ranged between 0.02 and 0.50 mg m^{-3} and can be therefore considered as the most typical concentrations for the southern Gulf of Mexico. In contrast, values of ≥0.50 mg m^{-3} up to 14.00 mg m^{-3} are restricted to some sites along the coast between the Tamiahua Lagoon to the Champoton River and Cape Catoche in the Yucatan Peninsula (Figure 1). These are mainly associated with river and coastal lagoon effects, depending on the local conditions that fluctuate both in time and space as well as in upwelling areas on the Yucatan shelf.

Figure 3 shows a set of five regions where Chl-*a* values were higher than 0.50 mg m^{-3} throughout the year. In summer the highest Chl-*a* concentrations were found toward the west (the GURS, Coatzacoalcos River and Alvarado Lagoon). The highest values were located at seven sites off the Alvarado Lagoon. In contrast, in autumn the pattern changed in the opposite direction; i.e., higher values were found toward the eastern side of the region from the GURS toward the Champoton River (Figure 3). In fact, the GURS produces a hydrographic effect creating a clear division between the western and eastern regions. This separation is indicated by the green isolines in Figure 4 (middle and lower left). In winter Chl-*a* concentrations reached the lowest values, fluctuating between 0.04 to 0.50 mg m^{-3}. Very few values of ≥0.50 mg m^{-3} were found (Figure 3, upper graphs).

SeaWiFS images confirm field observations. Figure 4 (right panel) illustrates the Chl-*a* distribution in the surface layer in the nearest available periods of field sampling. In general, the field data fit well to those obtained by remote sensing in autumn and winter, but not in summer due to the almost permanent cloudy weather in this region. On the other hand, interannual variation could be significant.

The left panels in Figure 5 show satellite images (SST and SeaWiFS) of the Yucatan shelf in which Chl-*a* shows some heterogeneity in its distribution. However, at several sites in the region located north off Cape Catoche, high values between 0.50 and 2.03 mg m^{-3} were found. Intermediate values of about 0.30 mg m^{-3} occurred between Progreso and Río Lagartos along the coast northeast of the shelf. No great difference between the surface and 10 m depth was found.

DISCUSSION

The results of the present study fit well with the patterns of pigments for the southern Gulf of Mexico (Müller-Karger et al., 1991). The use of different cycles of the CZCS (Coastal Zone Color Scanner) allowed us to confirm that Chl-*a* values of ≥0.50 mg m^{-3} along the southern coast of the Gulf are typical for this region throughout the year. On the other hand, some authors have found that the frequent intrusions of the Loop Current bring relatively large amounts of nutrients into the southwestern Gulf of Mexico (Sturges and Evans, 1988; Merino, 1992). This circumstance allows water upwelled onto the shelf to remain trapped

within the euphotic zone for long periods, which could increase the fertilizing potential in this region (Furnas and Smayda, 1987). In the Bank of Campeche area, the influence of upwelling on the phytoplankton is noticeable only in the coastal zone and almost unnoticeable in the oceanic zone (Vinogradova, 1976).

The results confirm that the highest phytoplankton production on the shelf is promoted by the river fronts of the Coatzacoalcos River and the GURS, as well as by the estuarine outwelling through the coastal Terminos and Alvarado lagoons that is dispersed onto the inner shelf boundary. This phenomenon occurs in July-August (Figure 3) when the stratified condition produces not only vertical but also horizontal gradients. The patterns of variability appear to be under the control of seasonal and regional influences. The stations located along the transects of the above-mentioned rivers showed that Chl-*a* was the highest not only in summer but also throughout autumn (Figure 3). The Chl-*a* increase was especially high during summer due to the maximum river discharge. This situation fits well with the Hydraulic State Program of 1988 (Vázquez-Gutiérrez, 1994). Other authors studying the abundance and distribution of benthic organisms in this region have found similar patterns (Soto and Escobar-Briones, 1995) and have reported relatively high Chl-*a* concentrations (3.20 mg m^{-3} in May and 10.60 mg m^{-3} in December) in the Alvarado Lagoon as a consequence of the large quantity of nitrates of agricultural origin (Lozano-Montes, 1993). In the older literature the highest production in the water column, ranging between 50 and 108 mg C m^{-3} day, was measured by the radiocarbon method in mid-April 1965 in the eastern part of the Bank of Campeche, NW off Celestun (Kabanova and López-Baluja, 1970).

After the rainy season it appears that the estuarine plume induced by discharges of the GURS and the Terminos Lagoon system influences the adjacent continental shelf, thus promoting an increase in Chl-*a* concentration. The local effect associated with river plumes and the coastal lagoons contributes to the Chl-*a* increase while its drastic decrease off the coast depends on the fact that nutrients introduced by the river discharge are gradually lost from the plume, and the additional input of few nutrients diminishes. Chl-*a* patches were detected along the limit of the continental shelf, possibly associated with fronts due to the cyclonic circulation patterns already established (Nowlin, 1972; Merrel and Morrison, 1981; Monreal-Gómez and Salas de León, 1985, 1990). However, the magnitude of such influence is yet to be determined.

Recent studies have shown that coastal lagoons and rivers in late summer-early autumn have their maximum runoff that contribute to the formation of surface plumes 2-8 m thick that can extend seaward. Figure 6 (upper panel) shows this effect of the rivers and lagoons of the southern Gulf of Mexico that could be observed from the coast to a latitude of 21^0N during late summer-early autumn of 2009. The offshore movement of the river plume is enhanced by the local circulation shown in Figure 6 (lower panel) that can have a net northward trend along the shelf break, carrying the continental waters towards the offshore region.

In the case of the Yucatan shelf, the existence of the subsurface countercurrent (Merino, 1997) also suggests the close relationship between the countercurrent and the upwelling process in this region, with the expected effects along the eastern Yucatan slope. Data on phytoplankton abundance presented here support this suggestion that the countercurrent may be formed by upwelled water that sinks again as it flows along the slope. The area located between Progreso and Río Lagartos has been characterized as a region suitable for upwellings caused by wind, due to the position of the coast in relation to the direction of winds from the

east (Ruiz-Renteria, 1979). However, the scope and magnitude of such influence is yet to be determined. Nevertheless, the increase in Chl-*a* concentration supports this suggestion. Chl-*a* values decreased toward the adjacent Caribbean Sea with quantities from 0.01 to 0.07 mg m^{-3} that are the typical values reported by other authors for this region. This is in contrast to the coastal zone of the northern Yucatan Peninsula where the Chl-*a* contents are high (average monthly concentrations from 2.10 to 6.96 mg m^{-3}, with the highest values >10 mg m^{-3}), largely due to enhanced nutrient inputs from groundwater (Morales et al., 2010).

The lack of nutrient data impeded a more refined analysis of phytoplankton growth during the surveys; however, considering data by other authors in the study region (El-Sayed et al., 1972; Furnas and Smayda, 1987; Merino, 1997), it is possible to deduce that the phytoplankton abundance (mainly diatoms) primarily reflects the nutrient concentration in the region, especially off Cape Catoche.

The region north of Cape Catoche has been characterized by upwelling phenomena whose maximum intensity occurs during spring and summer (Cochrane, 1969; Bessonov, 1971; Ruiz-Renteria, 1979; Merino, 1997). In this regard, our results fit well with those in the published literature because the highest Chl-*a* values were found there. These data place this region as one of the most productive on the Yucatan shelf, a fact previously mentioned based on high concentrations of dissolved oxygen, seston and plankton biomass (Cruz, 1968; Merino, 1992).

The Yucatan Current can generate a dynamic uplift of the thermocline at Cabo Catoche, inducing an important upwelling long known to enhance productivity in the Yucatan shelf. Recently obtained data have shown that this upwelling is active only during spring and summer. Occasionally, the waters upwelled in the Cabo Catoche region can travel across the entire northern Yucatan shelf, carried by the persistent westward currents influencing the southern Gulf of Mexico. Figure 7 shows the temperature contour map at 10 m depth showing evidence of cold waters (~23ºC) typical for the Cabo Catoche upwelling approaching the Bank of Campeche. In the southern Gulf of Mexico summer presents a typical stratified condition with most of the sites characterized by strong thermoclines with surface water temperatures from 25 to 29°C (Figure 8). In autumn a mixed water column induced by the effect of the northerlies produces a clear temperature decrease with values from 25 to 26ºC.

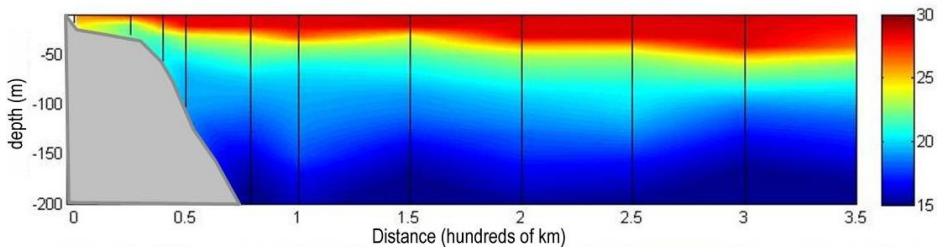

Figure 8. Section along longitude 93ºW in the southern Gulf of Mexico, June 2011.

Intensive growth of the diatoms *Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle, *Skeletonema costatum* (Grev.) Cleve, *Chaetoceros affinis* Lauder, *Chaetoceros messanensis* Castrac., *Pseudosolenia calcar-avis* (Schultze) B. G. Sundström, *Guinardia striata* (Stolterf.) Hasle, *Hemiaulus membranaceus* Cleve and *H. hauckii* Grunow in Van Heurck was found along the Cape Catoche area (Licea et al., 2017). It has been suggested that for supporting

such a high growth rate it is necessary to supply enough nutrients that must originate in the upwelled water. In contrast, several authors report oligotrophic conditions in some sites off the coast (El-Sayed et al., 1972; Merino, 1997), confirming that most of the Yucatan shelf is characterized by oligotrophic conditions. The presence of upwelling in the Cape Catoche area is also supported by SST and SeaWiFS images (Figure 5, left panels) in which both the low surface water temperature and high Chl-*a* concentrations are evident. However, in the coastal zone it is difficult to differentiate between Chl-*a* and CDOM (colored dissolved organic matter; also the so-called yellow substance) due to their identical spectral range of light absorbance; thus the results obtained may be misinterpreted.

The phytoplankton biomass data based on Chl-*a* in the present study may enable optimization of the commercial fisheries in the southern Gulf of Mexico by indicating the high productivity areas. Furthermore, these data present a long-term perspective for comparison between seasons, years and decades.

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Chapter 102

MOLECULAR IDENTIFICATION OF *THUNNUS* SPECIES

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ABSTRACT

The genus *Thunnus* includes eight economically important species. The ability to identify *Thunnus* species is critical in marketing and management because it determines the legitimacy of trading harvested fishes and can prevent incorrect food product labeling that could jeopardize the market value of these tunas. For the sustainable management of tuna, more-effective identification techniques to resolve these issues are essential. Advances in molecular techniques have made genetic identification much more reliable and feasible than traditional approaches. Therefore, several molecular methods which have been evaluated for species identification, phylogenetic analyses, and determining the population structure of *Thunnus* species are reviewed in this article. Those methods for species identification include sequencing, polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP), real-time PCR, and others. Some *Thunnus* phylogenetic studies have also been conducted using allozyme loci, mitochondrial (mt) genes (cytochrome *b*, ATPase, and the control region), and nuclear genes (*ITS1* and 5S ribosomal DNA) sequence analyses. Furthermore, to study population genetics of *Thunnus* species, allozyme loci, mt DNA PCR-RFLP, microsatellite loci, and single-nucleotide polymorphisms (SNPs) are often used as genetic markers to evaluate their population structure.

Keywords: species identification, phylogeny, population structure, sequencing, PCR-RFLP

INTRODUCTION

The genus *Thunnus* contains eight valid species, *Thunnus orientalis* (Temminck and Schlegel 1844), *T. maccoyii* (Castelnau 1872), *T. tonggle* (Bleeker 1851), *T. atlanticus* (Lesson 1831), *T. thynnus* (Linnaeus 1758), *T. albacares* (Bonnaterre 1788), *T. alalunga*

(Bonnaterre 1788), and *T. obesus* (Lowe 1839), with a fossil record extending back to the Middle Eocene, about 40 million years ago (Carrol 1988; Benton 1993).

The accurate determination of species is very important for understanding fishery species distributions, reproductive activities, and migration pathways. Some morphological characters have been systematically described for identifying tuna larvae and juveniles (Matsumoto 1957; Yabe et al. 1966; Ueyanagi 1966; Potthoff and Richards 1970; Matsumoto et al. 1972; Kohno et al. 1982). Nishikawa (1985) and Nishikawa and Ueyanagi (1991; 1992) described melanophore patterns in the larval stage that are the handiest morphological diagnostic characters for distinguishing *T. thynnus*, *T. maccoyii*, and *T. obesus*. However, the use of this character may be limited in other developmental stages. Compared to the larval stage, juveniles of tuna species are even more laborious to identify, because pigment patterns in juveniles become obscured by the gradual development of the general body pigmentation. In addition to pigment patterns, meristic characters in the juvenile stage are almost indistinguishable among *Thunnus* species (Nishikawa and Rimmer 1987).

Adult and young tuna species in fishery markets are commonly used for fillets and canned products. In the fishery industry, adults are customarily cut or fins are excised, which also renders species indistinguishable. However, identifying *Thunnus* species has become extremely important for detecting illegal fishing and trading, especially when external morphological characters are removed or when the fish are filleted or canned making them untraceable (Marko et al. 2004; Wong and Hanner 2008). Furthermore, tuna products have motivated economic deceit involving the mislabeling of fish species, with high-end market fish being replaced with lower-priced species (Hsieh et al. 1995; Rasmussen et al. 2009; Miller and Mariani 2010). Tuna misidentification can result into certain management problems and incorrect quota monitoring occurs. In addition, the conservation sustainability of overexploited tuna species has also been disregarded (Jacquet and Pauly 2008; Wong 2011).

It is extraordinarily difficult to accurately execute morphology-based identification when samples lack key morphological characteristics (Sotelo et al. 1992; Unlusayin et al. 2001; Smith et al. 2008). Although there are numerous protein electrophoretic analyses specifically developed for fish taxonomy (Ochiai et al. 2001; Tepedino et al. 2001), those methods are only available for raw fish samples. For example, Sharp and Pirages (1978) and Dotson and Graves (1984) compared the electrophoretic mobilities of more than ten enzymes from muscle and liver extracts of various tuna species. Their biochemical studies revealed close genetic relationships among species of *Thunnus.* Although allozyme electrophoretic patterns have proven very useful in identifying tuna species, this method requires fresh tissue to produce the best results. When dealing with tuna products, this technique usually becomes inapplicable because thermally labile proteins of fish are denatured by heat (Dooley et al. 2005). Furthermore, protein profiles of closely related fish species may be too similar to distinguish some electrophoretic patterns (Bartlett and Davidson 1991; Smith et al. 1996). In contrast, identifying genetic species using DNA-based techniques is highly reliable in all tissue conditions and life history stages. They should be convenient for fish species identification, phylogenetic assessments, and population genetic studies (Davidson 1998; Bossier 1999; Lockley and Bardsley 2000). Consequently, molecular genetic techniques developed for such studies include sequencing, polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP), real-time PCR, microsatellites, and enzyme-linked immunosorbent assay (ELISA).

REVIEW OF THE MOLECULAR IDENTIFICATION HISTORY OF *THUNNUS* TUNAS SINCE THE 1990S

Traditional identification techniques based on morphological features fall short in terms of the accuracy and resolution of identifying fishes in their early life stages or after having been processed into different forms. Misidentification of fish species often leads to a misinterpretation of their spatial distributions, life history, population dynamics, recruitment rates, survivorship, stock biomass, and the collapse or recovery of localized spawning sites (Vecchione et al. 2000; Ko et al. 2013). After the 1990s, DNA-based identification methods using mitochondrial (mt) and nuclear DNA markers were extensively applied in different research fields such as population genetics, molecular phylogenetics, and seafood authentication (Lockley and Bardsley 2000; Arif and Khan 2009). As for identifying *Thunnus* species, various molecular techniques were adopted including sequencing, RFLP (Cespedes et al. 1998; Hold et al. 2001; Sanjuan and Comesana 2002), microsatellites (Kawaka et al. 2007; Banhos et al. 2008), real-time PCR (Dalmasso et al. 2007; Chuang et al. 2012), and single-nucleotide polymorphisms (SNPs) (Liu et al. 2011; Sun et al. 2014).

THUNNUS SPECIES IDENTIFICATION

Sequencing

There are a number of studies applying nucleotide sequence variations of mt and nuclear DNA for *Thunnus* species identification. Bartlett and Davidson (1991) applied a direct mt cytochrome (Cyt) *b* sequence analysis to identify commercially important *Thunnus* species caught off the east coast of Canada. Portions of nucleotide sequences of the Cyt *b* gene (307 bp) were obtained from *T. thynnus, T. obesus, T. albacares*, and *T. alalunga*. Interspecific variations at this locus were found, *e.g.*, all *T. obesus* revealed a thymine (T) at position 35, but the other three species showed a guanine (G); *T. albacares* contained a cytosine (C) at position 62, while other species presented a T; *T. thynnus* had a C at position 240 where a T was observed in all other tuna; *T. alalunga* stood out among the four species at three sites (68, 89, and 227). More importantly, genetic markers can provide high reliability in identifying these four *Thunnus* species. Sequencing was also used for frozen and canned seafood; a genealogical analysis conducted by Terol et al. (2002) sequenced a 528-bp fragment of the Cyt *b* gene from frozen samples and a 171-bp fragment from canned samples of *T. albacares, T. obesus,* and *Katsuwonus pelamis*. The interspecific and intraspecific nucleotide variations with diagnostic usefulness as well as a high bootstrap value of the analysis were determined.

As detecting trickery and tracing seafood became important for trade controls to meet conservation requirements, identifying *Thunnus* species in trade was difficult due to the forms of various products. Several studies were evaluated to push the efficacy of molecular identification. Bottero et al. (2007) applied diagnostic sites of Cyt *b* with a multiplex primer-extension assay to distinguish four *Thunnus* species (*T. alalunga, T. albacares, T. obesus,* and *T. thynnus*) and *K. pelamis* in raw and canned conditions. Viñas and Tudela (2009) validated the combination of two genetic markers (the mt control region, CR and the nuclear ribosomal DNA first internal transcribed spacer, *ITS1*) to fully distinguish eight *Thunnus* species using

any processed tissue. This methodology also considered the presence of introgression among *T. thynnus*, *T. orientalis*, and *T. alalunga*. A Cyt *c* oxidase subunit I (*COI*) character-based key was developed by Lowenstein et al. (2009; 2010) to identify all *Thunnus* species.

The three bluefin tunas (*T. orientalis*, *T. maccoyii*, and *T. thynnus*) are morphologically similar, which often causes problems for fisheries management and marketing. Tseng et al. (2011) examined intraspecific genetic diversity and interspecific genetic boundaries among these three species by analyzing the full Cyt *b* gene sequences. The phylogenetic tree showed distinct clades with high bootstrapping value support, and the high F_{ST} value indicated significant differentiation among these species, which could be individually distinguished from each other *Thunnus* tuna by the 132nd, 375th, and 1023rd sites of Cyt *b* sequences. A mtDNA sequence database including *T. alalunga*, *T. albacares*, and *T. obesus* was commercialized in South Africa (Cawthorn et al., 2011). The database demonstrated that nearly 98% of fish species examined could be differentiated by their barcode system, and additional CR sequencing was more useful for discriminating the three *Thunnus* species.

Thunnus albacares and *T. obesus* are two of the most economically important tuna species in the world. Their juveniles, especially at sizes of less than 40 cm, are extremely difficult to distinguish, often leading to misidentification and miscalculation of catch estimates. Pedrosa-Gerasmio et al. (2012) applied the mt CR as a marker along with liver morphological phenotypes to differentiate juveniles of yellowfin and bigeye tuna. More recently, Puncher et al. (2015) employed mt *COI* and nuclear *ITS1* to identify larvae of *T. thynnus* (n = 188) collected from three spawning areas in the Mediterranean Sea. Results revealed significantly different accuracies between morphology-based and molecular genetic methods of tuna identification.

PCR-RFLP

The PCR-RFLP method allows very quick, uncomplicated, and low-priced detection of nucleotide mutations within sequences of PCR products. Mutations are discriminated by restriction endonuclease and are recognized by gel electrophoresis followed by staining with ethidium bromide (Ota et al. 2009). Chow and Inoue (1993) analyzed the flanking region between ATPase and Cyt oxidase subunit III genes (ATCO) of eight *Thunnus* species by PCR-RFLP. They found distinguishable interspecific differences among all eight species, except for one Pacific northern bluefin tuna which had the same haplotype as that of an Atlantic bluefin tuna in that study. Takayama et al. (2001) investigated the usefulness of the previous marker developed by Chow and Inoue (1993) on tuna specimens collected from different ocean basins. These markers performed properly in distinguishing all *Thunnus* species and different stocks of northern bluefin and bigeye tunas in spite of their various origins.

PCR-RFLP of the partial Cyt *b* fragment was used to identify six canned tuna species *T. alalunga*, *T. albacares*, *T. thynnus*, *T. obesus*, *Sarda sarda*, and *K. pelamis* collected from the Vigo (Spain) local market (Quinteiro et al. 1998). However, undetected intraspecific variability may exist in each species' gene pool. To confirm the effectiveness of the technique, more work in the future is needed to determine nucleotide substitutions at these target sites. Later, Pardo and Pérez-Villareal (2004) successfully applied a nested primer PCR-RFLP of partial Cyt *b* fragments to authenticate commercially canned tuna (*T. alalunga*,

T. albacares, *T. obesus*, *T. thynnus*, and *K. pelamis*) in oil, pickled, smoked, in brine, spiced, and in sauce. In the Far East, there are four *Thunnus* species: *T. thynnus*, *T. alalunga*, *T. albacares*, and *T. obesus*. Most customers cannot identify species of sliced tuna meat in the market. Therefore, Lin et al. (2005) developed a PCR-RFLP procedure with a portion of the Cyt *b* fragment that could accurately and quickly identify these *Thunnus* species. Lin and Hwang (2007) subsequently developed a PCR-RFLP technique based on a partial Cyt *b* gene to identify *T. thynnus*, *T. alalunga*, *T. obesus*, *T. albacares*, *K. pelamis*, *Euthynnus affinis*, *Auxis thazard*, and *S. orientalis* in canned products. Two sets of primers were designed to amplify the partial Cyt *b* gene followed by analysis of fragment patterns using five restriction enzymes. The method was successfully applied to verify species of commercially canned tuna.

Xu et al. (2016) amplified six mitochondrial genes (Cyt *b*, *COI*, CR, 12S rDNA, 16S rDNA, and ATPase) from *T. alalunga*, *T. albacares*, *T. obesus*, *T. maccoyii*, *T. tonggol*, and their relative species *K. pelamis*, *E. affinis*, *A. thazard*, *A. rochei*, and *S. orientalis*. Results of their PCR-RFLP patterns with two restriction enzymes indicated that ATPase and CR were suitable for sympatric species identification, and the *COI* and CR phylogenetic analyses fully discriminated all tested species in the study.

Real-Time PCR

The real-time PCR technique has gradually become more popular for fish species identification and examination of genetically modified organisms in recent years (Bertoja et al. 2009). It possesses advantages of high sensitivity, high specificity, excellent efficiency, reduced amplicon size, and no post-PCR steps to increase cross-contamination (Rodriguez-Lazaro et al. 2003), but is costly and requires specialized equipment. Lopez and Pardo (2005) developed two specific TaqMan systems using real-time PCR technology to identify *T. alalunga* and *T. albacares*. Terio et al. (2010) also developed a real-time PCR using three different minor groove binder (MGB) probes to rapidly and accurately identify canned *T. albacares*, *T. thynnus*, and *T. alalunga*. The method was advantageous for the quality control of canned tuna, *i.e.*, food traceability and consumer satisfaction. Four species-specific TaqMan probes were designed by Chuang et al. (2012) to identify *T. obesus*, *T. orientalis*, *T. maccoyii*, and *T. albacares*. The entire working time was reduced by half, which makes large-scale examination more feasible.

Liu et al. (2015) developed a duplex real-time quantitative PCR for identifying five tuna species (*T. maccoyii*, *T. obesus*, *T. albacares*, *T. alalunga*, and *K. pelamis*) from processed products. Result showed that only four out of nine commercial specimens were correctly labeled. The 56% incorrectness of labels indicated serious food safety issues of commercial tuna products from China. Processed tuna products usually contain highly degraded DNA and/or PCR inhibitors from additives which often can interfere with the accuracy of molecular methods for tuna identification. Efforts were made to address this issue by Bojolly et al. (2017), who developed a routine TaqMan-based real-time PCR method to distinguish genetically closely related *T. obesus* and *T. albacares* in canned products. The unique character of this method is the design of a specific TaqMan probe for NADH dehydrogenase subunit 2 and Cyt *c* oxidase subunit II genes to identify these two species. In addition, two

comparative quantitative methods were developed using the 12S rRNA gene to quantify the proportion of each species in mixed-tuna canned products.

Other Techniques

Pepe et al. (2010) examined the proteomics of *T. thynnus*, *T. albacares*, and *T. alalunga* for species identification. Proteins from muscle extracts were evaluated by both one- and two-dimensional electrophoresis (2DE) and mass spectrometric techniques. Results of 2DE demonstrated noticeable interspecific differences from the assignment and distribution of electrophoretic spots. Analysis of the 2D gel revealed a protein of approximately 70 kDa in the *T. thynnus* 2DE pattern, which was absent from those of the other two species. Other proteins in the 2DE patterns of *T. albacares* and *T. alalunga* were species-specific.

On the other hand, Santaclara et al. (2015) developed a multiplex PCR-ELISA method for the genetic authentication of three *Thunnus* species and *K. pelamis* in food products of different forms. The remarkable advantage of this proposed method is that the analysis can reliably process high-throughput screening of all kinds of samples in just one working day. In the same year, Abdullah and Rehbein (2015) completed the identification of tunas from Indonesian waters (the Indo-West Pacific and Indian Ocean) using the partial Cyt *b* gene. In addition, the study also examined the differentiation of tuna and other scombrid fishes through amplification of parvalbumin gene introns by single-strand conformation polymorphism (SSCP) and RFLP analyses.

PHYLOGENETIC STUDIES

Allozyme Locus Analysis

Collette (1978) classified tunas into two subgenera of temperate *Thunnus* (*T. alalunga*, *T. obesus*, *T. thynnus*, *T. orientalis*, and *T. maccoyii*) and tropical *Neothunnus* (*T. atlanticus*, *T. tonggol*, and *T. albacares*) by the presence or absence of a central heat exchanger. Although *T. obesus* shares some characters of each subgenus, it is classified as a member of the subgenus *Thunnus*, because its character shows adaptations for living in colder environments (Collette, 1978). Allozyme analysis using 35 loci was first carried out by Elliott and Ward (1995) to explore genetic relationships among eight species of tuna including *T. alalunga*, *T. obesus*, *T. orientalis*, *T. maccoyii*, *T. albacares*, *A. thazard*, *E. affiizii*, and *K. pelamis*. Regrettably, their phylogenetic analyses failed to resolve the branch order among *Thunnus* species.

Gene Sequence Analysis

Finnerty and Block (1995) collected five Atlantic tuna species and explored *Thunnus* systematics using a portion of the Cyt *b* gene. However, results were insufficient to draw conclusions about relationships within the genus *Thunnus*. Partial sequences of both the Cyt *b*

(292 bp) and ATPase (400 bp) genes were first determined for all eight *Thunnus* species (Chow and Kishino, 1995). These molecular data indicated that mtDNA from *T. alalunga* has extensively displaced original mtDNA in the Pacific population of northern bluefin tuna which was later named *T. orientalis*. In the phylogenetic tree, two distinct clades are evident in the genus *Thunnus*: one consists of *T. alalunga* (ALB) and the dominant type of *T. t. orientalis* (PNB), and the other one consists of the rest *Thunnus* species including the rare type of PNB. Results also indicated that *T. alalunga* is highly divergent from all other *Thunnus* tunas, suggesting it was the earliest offshoot from the phylogenetic tree.

Alvarado-Bremer et al. (1997) constructed phylogenetic relationships among tunas from a portion of the mtDNA CR. A Neighbor-joining tree supported monophyletic origins for the temperate subgenus *Thunnus* and the tropical subgenus *Neothunnus*, except for *T. obesus* because it was difficult to place it in a clade in either subgenus. This result is consistent with allozyme data which suggested that *T. obesus* has a greater similarity to *T. albacares* and *T. atlanticus* than to temperate tunas (Sharp and Pirages 1978; Elliott and Ward 1995). Alvarado-Bermer et al. (1997), Ward (1995), and Chow and Kishino (1995) inferred that introgression of *T. alalunga* mtDNA into *T. orientalis* occurred a long time ago, and also suggested that mtDNAs of *T. alalunga* and *T. orientalis* share a common ancestry.

Chow et al. (2006) examined intra- and interspecific nucleotide sequence variations of rDNA *ITS1* among all *Thunnus* species. Their report supported introgression having occurred between species and contradicted the morphological subdivision of the genus into the two subgenera, *Neothunnus* and *Thunnus*. The cladogram constructed from *ITS1* indistinctly resolved phylogenetic relationships among three tropical tunas (*T. albacares*, *T. tonggol*, and *T. atlanticus*). The *ITS1* and mtDNA ATCO sequence data both supported the monophyletic status of the yellowfin tuna group and indicated that these tropical tunas were recently derived taxa; nevertheless, *T. thynnus* and *T. orientalis* shared almost identical *ITS1* sequences, while having distinct mtDNA. These molecular data suggested that intermittent speciation events occurred in species of the genus *Thunnus*. Consequently, relationships among closely related *Thunnus* taxa remain unresolved. Tseng et al. (2012) sequenced full Cyt *b* gene sequences from all eight *Thunnus* species to explore their phylogeny. The genealogical tree contained two explicit clades, the first group consisted of *T. maccoyii*, *T. thynnus*, *T. atlanticus*, *T. albacares*, *T. obesus*, and *T. tonggol*, and the second group included *T. alalunga* and *T. orientalis*, with extremely high bootstrapping (1000 replications) support. The genealogy suggested that both *T. orientalis* and *T. alalunga* are sister species and supported the monophyly of the tropical yellowfin group, but not of the temperate bluefin group. The phylogeny of *Thunnus* species does not fit into the two-subgenus *Thunnus* and *Neothunnus* classification pattern.

Although *T. obesus* can adapt to cold water environments and is currently classified into the bluefin group (Collette 1978), it also shares external morphological features of both groups, and there is still debate as to whether bigeye tuna belongs to the bluefin or yellowfin group. Lee et al. (2018) further examined interspecific differences among *T. obesus*, *T. albacares*, *T. alalunga*, and *T. orientalis* by analyzing karyotypes, whole Cyt *b* genes, and 5S rDNA sequences; moreover, the systematic state of *T. obesus* was also discussed in their report. A phylogenetic analysis of *Thunnus* tunas with 100% bootstrap support showed that *T. orientalis* and *T. alalunga* are sister species. The result of the 5S rDNA analysis also suggested that two different duplicates occurred in *T. obesus*. The larger morphological and

genetic polymorphisms of *T. obesus* may have been caused by a combination of environmental adaptations, introgressions, and a complicated process of speciation.

POPULATION GENETICS STUDIES

Thunnus albacares (Yellowfin Tuna)

Yellowfin tuna are distributed in waters between 35°N and 35°S across the Pacific, Atlantic, and Indian Oceans. Most of them appear in waters near the equator and also move to deeper regions where water temperatures range 15~31°C (Collette and Nauen 1983). Different breeding peaks occur in different regions, *e.g.*, a breeding peak was observed in May to June in the Kuroshio Basin (Yabe and Ueyanagi 1962), while two peaks occurred in April and October in Philippine waters (Yamanaka 1990). Does the yellowfin tuna that is widely distributed throughout the world belong to one panmictic population or consist of different populations?

Scoles and Graves (1993) randomly sampled 20 yellowfin tuna from all specimens at each of five Pacific locations and one Atlantic location, and analyzed their mtDNA using 12 informative restriction endonucleases. The results demonstrated that no genetic differentiation occurred among samples. The null hypothesis that yellowfin tuna in the Pacific Ocean share a common gene pool could not be rejected in that study. Yellowfin tuna maintain sufficient gene flow among areas to prevent the accumulation of significant genetic differences. Ward et al. (1997) examined four polymorphic allozyme loci (*ADA**, *FH**, *GPI-A**, and *GPI-B**) and conducted an mt PCR-RFLP analysis of yellowfin tuna which were sampled from the Atlantic, Indian, and Pacific Oceans. *GPI-A** showed highly significant differentiation. MtDNA differentiation was more limited, but spatial heterogeneity supported the separation of Atlantic, Indian, and Pacific Ocean stocks. After that, the yellowfin tuna stock was found to have very limited heterogeneity in the Western Pacific Ocean using microsatellite markers (Appleyard et al., 2001) which was similar to earlier findings using allozyme and mtDNA markers.

Ely et al. (2005) investigated genetic variations of the partial mtDNA CR from 148 yellowfin tuna collected from the eastern Pacific Ocean, Indian Ocean, Gulf of Mexico, east coast of Florida (USA), and the Ivory Coast (west Africa). Much lower levels of genetic differentiation were discovered among subpopulations of yellowfin tuna. In addition, results of a PCR-RFLP analysis of the ATCO gene indicated low levels of genetic differentiation between yellowfin populations in the Atlantic and Pacific Oceans. Results suggested the occurrence of a very slow genetic drift due to the species' large population size. Similarly, yellowfin tuna populations in the Western Pacific and Western Indian Oceans revealed extensive gene flow between these ocean basins based on non-significant pairwise F_{ST} values (Wu et al. 2010). Since yellowfin tuna are oceanic and highly migratory, they are considered to be one population across the western and central Pacific region (Appleyard et al. 2001; Wu et al. 2010).

On the other hand, yellowfin tuna catch data of the 1990's in the western and central Pacific Ocean (WCPO) showed a slower growth rate along Philippine and Indonesian waters indicating probable population structuring (Langley et al. 2011). Aguila et al. (2015)

established the genetic stock structure of yellowfin tuna in the Philippines in comparison to the species in the Bismarck Sea, Papua New Guinea using nine DNA microsatellite markers. This study concluded that the yellowfin tuna population in the Philippines is independent of the Bismarck Sea population, suggesting there are at least two subpopulations of yellowfin tuna in the WCPO. In addition, Li et al. (2015) assessed polymorphisms of sequence variations in mtDNA *COI* genes and found genetic differentiation among 11 populations of yellowfin tuna sampled from the central Pacific Ocean. Recently Barth et al. (2017) sampled yellowfin tuna from most of its global distribution areas: Rhode Island, USA; Mindelo, Republic of Cabo Verde; Abidjan, Ivory Coast; Cape Town, South Africa; Barka, Oman; Denpasar, Indonesia; Sagami Bay and Okinawa, Japan; and central-eastern Pacific, El Salvador. Whole-genome sequencing was used in concert with a draft genome assembly to resolve the global population structure of yellowfin tuna, and investigate its population demographic history. The conclusions indicated significant differentiation between Atlantic and Indo-Pacific yellowfin tuna populations as well as the possibility of a third divergent yellowfin tuna group in the Arabian Sea. The study further observed evidence of past population expansion as well as asymmetric gene flow from the Indo-Pacific to the Atlantic Ocean.

Thunnus alalunga (Albacore)

The albacore is a highly migratory fish and is mainly distributed in tropical, subtropical, and temperate zones between 50°N and 40°S in the Atlantic Ocean including the Mediterranean Sea, Pacific Ocean, and Indian Ocean. It prefers water temperatures of 13.5~25.2°C, but also can tolerate a much-lower temperature of 9.5°C for a period of time (Collette and Nauen 1983). According to some fishery data, the hooking rates differ between the northern and southern populations, and the catch in the equatorial area is very low (Otsu and Uchida 1959; Beardsley 1969; Nakamura 1969; Suzuki et al. 1977; Murray 1994). Spawning was found to occur in the Indian Ocean between 10°S and 30°S, mainly to the east of Madagascar from October to January (Dhurmeea et al. 2016). Peak spawning of albacore in the Pacific Ocean is generally believed to occur in subtropical waters centered around 20°N and 20°S latitude. It is assumed that the albacore spawns from March through July on grounds located in the western and central Pacific Ocean. There appears to be a distinct spawning ground for albacore in the Mediterranean Sea (Collette and Nauen 1983), and growth rates of Mediterranean and Atlantic albacore are reported to differ (Megalofonou 2000). Furthermore, tag-recovery investigations in 1968 to 1999 also indicated restricted movement of albacore between these ocean basins (Arrizabalaga et al. 2002). According to these information, it is interesting that does the albacore have a significant population structure among different oceans and hemispheres?

Chow and Ushiama (1995) conducted a study using RFLP analysis of the mt ATPase gene of albacore. Highly significant heterogeneity was evident among Atlantic and Pacific samples, but no heterogeneity was observed among North and South Pacific samples, nor among North and South Atlantic samples. The result concluded that gene flow between the Atlantic and Pacific populations of albacore was restricted. Contrarily, Takagi et al. (2001) applied highly polymorphic nuclear microsatellites to detect genetic heterogeneity not only between Atlantic and Pacific samples but also between northern and southern samples within

each ocean. Several molecular analyses detected no or little genetic differentiation between Atlantic and Mediterranean albacore samples (Vinãs et al. 1999; Pujolar et al. 2003; Vinãs et al. 2004); nevertheless, Nakadate et al. (2005) proposed that gene flow between the Atlantic and Mediterranean albacore populations has been significantly restricted. The genetic population structure of albacore was investigated using fragment length variations of the glucose-6-phosphate dehydrogenase (G6PD) gene intron and restriction analysis of the mtDNA D-loop region (267~269 bp). Frequencies of two alleles (L and S) at the G6PD locus significantly differed between samples from the Atlantic and Mediterranean. Phylogenetic analysis using data of the D-loop showed that the emergence of the B type lineage from the ancestral A lineage in the Mediterranean Sea had not yet occurred in the Atlantic Ocean. Furthermore, NOAA (2014) reported that albacore may spawn multiple times in a year, and the spawning grounds appear to be discontinuous between the hemispheres with opposite seasonal peaks.

Thunnus obesus (Bigeye)

Bigeye tuna inhabit tropical and subtropical waters of the Atlantic, Indian and Pacific Oceans, where water temperatures range 13~29°C. They spawn in tropical waters year-round (Kailola et al. 1993). Alvarado-Bremer et al. (1998) used PCR-RFLP with a portion of the mtDNA CR on a total of 248 individuals for population analyses. Frequency distributions of haplotypes indicated that population of bigeye tuna in the Atlantic Ocean was genetically distinct from those in the Indian and Pacific Oceans. These results concluded that bigeye tuna does not have a single global population. Two mtDNA segments were analyzed by PCR-RFLP for a genetic stock study of bigeye tuna. Results clearly indicated that fish migration between the Atlantic and Indian Oceans was severely restricted, but the two populations do intermingle around South Africa (Chow et al. 2000).

Thunnus orientalis (Pacific Bluefin Tuna)

This species is primarily distributed in the North Pacific Ocean, from the Gulf of Alaska to southern California and Baja California and from Sakhalin Island in the southern Sea of Okhotsk to the northern Philippines; the tuna is also found in the South Pacific Ocean around Australia, the Galapagos Islands, the Gulf of Papua, and New Zealand (Collette and Smith 1981; Collette and Nauen 1983; Bayliff 1994; Ward et al. 1995; Smith et al. 2001). Pacific bluefin tuna spawn between Japan and the Philippines from April to June, south of Honshu Island in July, and in the Japan Sea in August (Chen et al. 2006; Tanaka et al. 2006; 2007; del Moral-Simanek and Vaca-Rodriguez 2009; Itoh 2009); larvae are transported northwards towards Japan by the Kuroshio Current, and juveniles are found in waters near Japan. Some young fish migrate east as far as the west coast of North America, and are presumed to return to the western Pacific to breed (Bayliff 1991; 2001). The Pacific bluefin tuna is believed to become sexually mature at about 5 years and to have a maximum lifespan of 25 years (Ueyanagi 1975; Bayliff 1994). Surprisingly little was known about the stock structure and population biology of Pacific bluefin tuna until Tseng et al. (2012) analyzed mtDNA CR and

microsatellite data and showed that Pacific bluefin tuna samples from Taiwanese and New Zealand waters lacked genetic differentiation.

Thunnus thynnus (Atlantic North Bluefin Tuna)

Alvarado-Bremer et al. (1999) sampled 140 individuals of north bluefin tuna from the Mediterranean and Western Atlantic Ocean, and analysis of their mt lineage distribution patterns allowed rejection of the null hypothesis of a global panmictic population. Antoniou et al. (2017) analyzed samples throughout the Mediterranean Sea as well as from the Moroccan coast in the Atlantic Ocean using polymorphic microsatellite loci and genome-wide SNPs which were obtained by a double-digestion restriction-associated DNA sequencing technique. Results showed weak evidence of the genetic structure which suggested the existence of a single panmictic unit. Spatial dynamics of mixed bluefin tuna in the Atlantic Ocean and Mediterranean Sea were examined using informative SNPs by Puncher et al. (2018). The results evidenced a persistent population structure across broad geographic areas, which means extensive mixing in the Atlantic Ocean, particularly in the mid-Atlantic Bight and Gulf of St. Lawrence.

Thunnus maccoyii (Southern Bluefin Tuna)

Southern bluefin tuna are widely distributed in southern hemisphere waters between 30° and 50°S but rarely found in the eastern Pacific. Adults spawn between August and March on a single spawning ground in the Indian Ocean, southeast of Java, Indonesia (Jenkins and Davis 1990; Caton 1991; Chambers et al. 2017). Samples of southern bluefin tuna collected off the coasts of South Africa, Western Australia, South Australia, and Tasmania from 1992 to 1994 were analyzed using six polymorphic allozyme loci (*ADA**, *GDA**, *GPI-A**, *MPI**, *PGDH**, and *PGM-1**, n = 595~733 per locus). In addition, mtDNA variants were constructed using three restriction enzymes to detect polymorphic restriction sites (n = 555). Significant spatial heterogeneity was not detected, nor genetic evidence of heterogeneity found between groups sampled from disparate feeding grounds across the distribution of the species (Grewe et al. 1997). These results are consistent with the null hypothesis of a single stock of southern bluefin tuna within a single spawning area located to the south of Java and off the northwestern coast of Australia.

CONCLUSION

The development of molecular identification techniques has dramatically improved the accuracy of tuna identification under different sample conditions, but none of these methods can yet be considered perfect. All methods reviewed in this article still have various shortcomings that need to be overcome. Sampling of previous studies usually only covers a portion of the known distribution areas of species, and small sample sizes are insufficient to reveal all possible intraspecific variations. Therefore, completeness of sampling is the initial

requirement for all designated methods to have more reliable results. In terms of commercial applications of molecular identification methods, they should have quick turnover times, low costs, and be user friendly. Although most current molecular methods can provide more or less satisfactory accuracy for tuna identification, so far, none of the methods in this review can fulfill all of the above needs. Therefore, developing molecular techniques to meet the requirements of commercial applications in the future is an important goal. Regarding phylogenetic studies of *Thunnus*, *T. orientalis* and *T. alalunga* were confirmed to be sister species, while the evolutionary relationships of other tuna species are still not totally understood yet. In addition to phylogenetic studies, reports focusing on population structures and dynamics of *Thunnus* species remain quite limited. More studies of phylogenetic and population structural dynamics are definitely needed to elucidate these issues. In the future, more researchers need to be involved in examining population structures and dynamics of tunas to achieve the long-term goal of sustainable management.

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Chapter 103

MARINE BACTERIA WITH STRONG SURVIVABILITY TOWARD TRIBUTYLTIN

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ABSTRACT

Tributyltin (TBT) is a toxic compound with broad-spectrum activity toward diverse marine species. The amount of TBT used for preventing the biofouling of aquaculture fishing nets and ships' bottoms was drastically increased by the development of self-polishing copolymer systems. As a result, morphological changes and a reduction in the population of bivalves occurred with exposure to TBT leached into marine environments from marine paints. Now, it has been determined that the compound acts as an endocrine disruptor. After 2008, the use of TBT in marine environments has been severely restricted by The International Convention on the Control of Harmful Anti-fouling Systems on Ships (adopted on October 5, 2001 and effective as of September 17, 2008). Tributyltin is difficult to degrade in seawater. Even after its use was banned in marine environments, the compound remains in the surface of sediments on the seafloor. Therefore, TBT's ecological threat to benthic invertebrates and microbial communities has continued. On the other hand, TBT-tolerant marine bacteria exist, and tolerance toward TBT is closely related to the adsorption capacity of cell walls, indicating that the sublethal damage to the microbial community in TBT-polluted sediment seems to be enhanced by the coexistence of TBT-tolerant marine bacteria accumulating TBT on their cell walls. In order to conserve the microbial ecosystem in sediments, removal of TBT from sediment surface is important. Tributyltin-degrading marine bacteria, such as non-pathogenic *Aeromonas molluscorum* Av27 isolated from an estuarine environment, seem to be useful for the efficient degradation of TBT to less toxic organotin compounds. Practical approaches of bioremediation using TBT-degradable marine bacteria are necessary to prevent human exposure to TBT through the food chain.

Keywords: marine bacteria, survivability, tributyltin, bioremediation

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TRIBUTYLTIN IN A MARINE ENVIRONMENT

Too much tributyltin (TBT) has been used mainly as an anti-fouling agent of marine paints and aquaculture fishing nets. The amount of TBT leaching from marine paints drastically increased with the development of self-polishing copolymer systems (Anti-fouling systems, IMO; Hoch 2001). As a result, morphological changes (Alzieu et al., 1986; Márquez et al., 2017) and a reduction in the population of marine gastropods (Bryan et al., 1986) occurred through exposure to TBT. Even in deep-sea organisms such as fish, crustaceans, and gastropods (Takahashi et al., 1997) and Antarctic marine sediment (Negri et al., 2004), TBT contamination has been confirmed. Near shore of the Antarctic, the efficient detachment of marine paint containing TBT might have occurred during ice breaking by an ice-breaking ship. In recent years, tributyltin has been recognized as an endocrine disruptor in humans (Santos-Silva et al., 2018).

The use of TBT in marine environments has been banned strictly since 2008 by the International Convention, adopted on October 5, 2001, and effective as of September 17, 2008 (International Convention on the Control of Harmful Anti-fouling Systems on Ships, IMO). On the other hand, from the viewpoint of conserving biodiversity, risks of invasion and the establishment of non-native fouling organisms seem to have increased in foreign ports after the enforcement of the International Convention. Balancing the risks from TBT and the invasion of non-indigenous marine fouling organisms is important (Lewis et al., 2004). As one solution for keeping hull surfaces clean for prolonged periods without using toxic chemicals, the application of irradiation-intensity blue LEDs for antifouling has been examined (Mimura and Hirono, 2018).

One property of tributyltin is that it adsorbs to cray-rich sediments in marine environments (pH 8, salinity 32‰) far more than a degrading product such as less toxic dibutyltin, which indicates that adsorption depends on the number of hydrophobic alkyl groups (Hoch, 2003). In such an alkaline pH, TBT seems to be neutralized by hydroxide. Because of the properties of TBT in the water phase, benthic ecosystems are continuously damaged by TBT.

BIODIVERSITY OF TBT-RESISTANT MARINE BACTERIA

Tributyltin-resistant marine bacteria seem to be classified into two groups, although the resistance ability depends on the concentrations of TBT existing externally. In one group, each species has TBT tolerance but does not have degradation ability (Wuertz et al., 1991; Fukagawa et al., 1992; Jude et al., 2004; Mimura et al., 2008a; Mimura et al., 2008b; Mimura et al., 2017). Several marine bacteria became tolerant toward TBT during exposure to approximately 120 μM TBTCl within 4 weeks (Suzuki et al., 1992). Such species adapted to a TBT-polluted environment might be able to be classified into the group "TBT tolerant but with no degradation ability." Marine bacteria possessing "TBT-degrading ability" belong to the other group (Cruz et al., 2007; Abubakar et al., 2015; Hassan, 2018).

Eight "TBT tolerant but with no degradation ability" isolates and three isolates with "TBT-degrading ability" are shown in Table 1. All isolates were to be Gram negative, indicating that the structure of cell wall plays an important role in coping with the inflow of

TBT into the cytoplasm. For example, the cell wall of tributyltin-tolerant *Pseudoalteromonas* sp. TBT1 (Mimura et al., 2008a and 2008b) and *Photobacterium* sp. TKY1 (Mimura, 2017) possessed high adsorption ability to TBT.

Table 1. Marine bacteria possessing "TBT tolerant but with no degradation ability" or "TBT-degrading ability"

Category	Stains	Remarks
TBT tolerant but with no degradation ability	*Pseudomonas putida* (Wuertz et al., 1991)	
	Serratia (Wuertz et al., 1991)	
	Klebsiella (Wuertz et al., 1991)	
	Alteromonas sp. M-1 (Fukagawa et al., 1992)	
	Vibrio sp. (Suzuki et al., 1992)	
	Pseudomonas stutzeri (Jude et al., 2004)	The operon *tbtABM* shows homology with aromaric compound tolerance genes in *P. putida*.
	Pseudoalteromonas sp. TBT1 (Mimura et al., 2008a and 2008b)	Cell wall has high adsorption capacity to TBT.
TBT-degrading ability	*Aeromonas molluscorum* Av27 (*A. veronii* Av27) (Cruz et al., 2007)	TBT is used as a sole carbon source.
	Klebsiella sp. FIRD 2 (Abubakar et al., 2015)	
	Stenotrophomonas chelatiphaga HS2 (Hassan, 2018)	TBT is used as a sole carbon source.

IMPORTANCE OF MARINE BACTERIAL CELL WALLS TO PROTECT AGAINST THE INFLOW OF TBT

Bacterial cell walls play an important role in protecting against the inflow of toxic compounds with hydrophobic properties. In other words, marine bacterial tolerance toward TBT depends on the capacity of cell walls to adsorb TBT. Kubota et al., (2004) quantified the number of Sn elements originating in TBT associated with growing cells of *Pseudoalteromonas* sp. TBT1, which were isolated from a ship's ballast water, as having an atomic density of $10^{19.5}$ cm^{-3} with an accelerator when the cells were grown at an early stationary phase, $10^{8.1}$ Colony-Forming Units (CFU) mL^{-1}, in the presence of 150 μM TBT for 3 days at 30°C in a nutrient medium. A typical particle-induced X-ray emission spectrum provided K_α and K_β lines of Sn with energies of 25.3 and 28.6 keV, respectively, and the yield ratio, Y (K_α)/Y (K_β) = 9 ± 0.9, was consistent with the published value (Mayer and Rimini, 1977).

The number of cell-associated TBT molecules increased exponentially with an increase in the final concentration of TBT added externally up to 3 mM from 3 μM, and the maximum number of TBT adsorbed was $10^{7.6}$ molecules $cell^{-1}$ when resting cells, $10^{9.5}$ CFU mL^{-1}, were exposed to TBT (Mimura et al., 2008a). In the presence of more than 3 mM TBT, the value became saturated. The value was 10 times smaller than that obtained from calculation, i.e.,

the number of Sn originating in TBT adsorbed was $10^{6.5}$ Sn cell^{-1} in the presence of 150 μM of TBT. On the other hand, the maximum value is estimated to be $10^{7.5}$ Sn cell^{-1} ((150×10^{-9} moles mL^{-1}) × (6.0×10^{23} molecules moles^{-1})/($10^{9.5}$ CFU mL^{-1})). The difference was observed for any concentration of TBT added externally. A possible explanation is that a certain number of TBT adsorbed by a single cell might be desorbed to reach a new equilibrium condition during the washing process.

The importance of the function of cell walls to adsorb TBT was confirmed by lysozyme-treated cells (Mimura et al., 2008b). Lysozyme is an enzyme that preferentially hydrolyzes the β-1, 4 glucosidic linkages between *N*-acetylmuramic acid and *N*-acetyl-glucosamine in the mucopeptide cell wall. Resting cells treated with 1% lysozyme for 1 h at 30ºC retained their colony-forming ability. The amount of TBT adsorbed by the cells was reduced by approximately four-fifths as compared to that by native cells. Some kinds of Gram-negative marine bacteria seem to possess cell walls with a unique structure that can function to prevent TBT's interaction with the outer surface of the cell membrane. This function is very important for blocking the inflow of TBT into the cytoplasm.

As another function, the existence of an efflux pump has been reported in TBT-tolerant *Alteromonas* sp. M-1 (Fukagawa et al., 1992; Fukagawa et al., 1993) and *Pseudomonas stutzeri* (Jude et al., 2004). TbtABM in *P. stutzeri*, a multidrug efflux pump, showed the highest homology with the TtgDEF and SrpABC systems involved in aromatic compound tolerance in *P. putida*. It seems possible that highly hydrophobic TBT is pumped out by proteins associated with a hydrophobic membrane.

EXISTENCE OF ENVIRONMENTAL BACTERIA POSSESSING CONGENITAL TBT TOLERANCE

Mimura et al. isolated three strains (Table 2) that could grow on a seawater-based nutrient agar plate containing 100 μM of TBTCl from sediment samples taken in busy Japanese ports (Figure 1), and the TBT tolerance of each isolate was compared with that of two kinds of strains that show high first and secondary similarities to the 16S rDNA sequences (Table 3 and Figure 2 (Mimura et al., 2017). In the experiment, the number of cell-associated TBT that could associate with a single cell could increase by the reduction of the number of resting cells in the mixture while the concentration of TBT was kept constant at 100 μM. As a result, *Photobacterium* sp. TKY1 isolated from Tokyo Bay showed very strong TBT tolerance. Even when the initial number of resting cells was reduced to $10^{4.2}$ CFU mL^{-1} from $10^{9.4}$ CFU mL^{-1}, cells of $10^{3.8}$ CFU mL^{-1} could survive, and the number of cell-associated TBT molecules increased by 5 orders of magnitude (Figure 2A), indicating that the isolate was highly resistant to TBT. The reduction of the number of surviving *P. ganghwense* JCM 12487^{T} and *P. halotolerans* LMG 22194^{T} cells was amplified with the reduction of resting cells initially added. Other isolates, *Halomonas* sp. TKY2 (Figure 2B) and *Photobacterium* sp. NGY1 (Figure 2C) showed sensitivity toward TBT when suspended in a mixture without nutrients, indicating that some kinds of amino acids might be essential for the survival of the isolates, e.g., those amino acids that might be used for the synthesis of stress proteins to cope with TBT toxicity. Type culture *H. alkaliphila* DSM 16354^{T} showed strong

tolerance to TBT. That strain was isolated from the water of a salt pool in Italy (Romano et al., 2006).

Sediments on the seafloor in busy ports, where overseas vessels with huge tonnage arrive with high frequency, have highly contaminated the waters with TBT eluted from their hull bottoms. Sublethal concentrations of TBT existing in the sediment might cause some continuous damage to the local community of marine bacteria. Therefore, it is possible that some marine bacteria species might acquire TBT tolerance by mutation during their prolonged exposure to sublethal concentrations of TBT. However, *H. alkaliphila* DSM 16354^{T} isolated from a non-TBT-polluted salt pool showed strong tolerance toward TBT. Thus, it is possible to think that high resistance toward TBT is not caused by prolonged exposure to sublethal TBT concentrations but is possessed congenitally by a certain strain regardless of exposure to TBT.

Figure 1. Sampling sites of surface sediments. Samples of surface sediments were taken on board at anchorages of Tokyo Bay and Ise Bay, Japan.

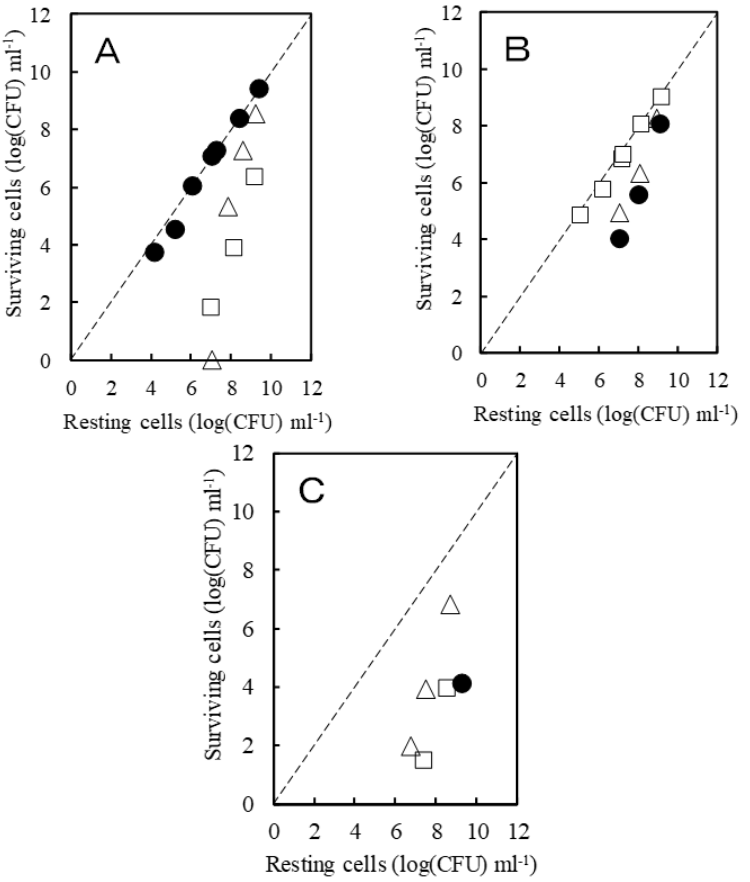

Figure 2. Changes in the survivability of the isolate and the taxonomically similar type strains in relation to the given numbers of resting cells exposed to TBT. Surviving cells were counted by the colony-counting method after exposure to 100 μM of TBT for 1 h. The number of cell-associated TBT molecules varied with the number of resting cells exposed to TBT. Changes in the number of surviving cells of *Photobacterium* sp. TKY1 (closed circles), *P. ganghwense* JCM 12487^T (open triangles), and *P. halotolerans* LMG 22194^T (open triangles) are shown in relation to given numbers of resting cells (Figure 2A). The results obtained from *Halomonas* sp. TKY2 (closed circles), *H. venusta* DSM 4743^T (open triangles), and *H. alkaliphila* DSM 16354^T (open triangles) cells are shown in Figure 2B. For *Photobacterium* sp. NGY1 (closed circles), *P. rosenbergii* LMG 22228^T (open triangles), and *P. damsela* ATCC 33539^T (open triangles), the number of surviving cells is shown in Figure 2C. The dotted line in each figure means perfect survivability, regardless of the initial number of resting cells. The use of the figures is permitted by the Society for Antibacterial and Antifungal Agents, Japan.

Table 2. Sampling sites of sediment surface and isolates possessing "TBT tolerant but with no degradation ability"

Sampling date	Sampling site of sediment	Strain isolated
Sep. 7, 2008	Anchorage in Tokyo Bay, 15 m depth	*Photobacterium* sp. TKY1
Sep. 7, 2008	Anchorage in Tokyo Bay, 15 m depth	*Halomonas* sp. TKY2
Nov. 7, 2008	Anchorage in Ise Bay, 14 m depth	*Photobacterium* sp. NGY1

ESTIMATION OF THE IMPACT OF TBT-RESISTANT MARINE BACTERIA IN TBT-POLLUTED SURFACE SEDIMENT

Estimation of the impact of TBT-tolerant marine bacteria on an indigenous microbial population in surface sediment polluted with a sublethal TBT concentration has been carried out *in vitro* (Table 4). When resting cells of TBT-sensitive *Vibrio natriegens* ATCC 14048^T were exposed for 1 h to 100 μM TBT, as a final concentration, the number of surviving cells was reduced to $10^{6.2\pm0.3}$ CFU mL^{-1}. While resting cells of *V. natriegens* were mixed with those of TBT-resistant *Photobacterium* sp. TKY1, the number of surviving cells of *V. natriegens* was determined to be $10^{4.4\pm0.4}$ CFU mL^{-1}, the value of which decreased 2 orders of magnitude as compared with that when *V. natriegens* was examined alone.

Table 3. List of two type strains with high similarities to the 16S rDNA sequences of each isolate

Strain isolated	Type strains[a]	Origin	References
Photobacterium sp. TKY1	*Photobacterium ganghwense* JCM 12487^T (99.4%)[b]	Seawater	Park et al. (2006)
	Photobacterium halotolerans LMG 22194^T (94.2%)	Saline lake	Rivas et al. (2006)
Halomonas sp. TKY2	*Halomonas venusta* DSM 4743^T (99.6%)	Seawater	Baumann et al. (1972)
	Halomonas alkaliphila DSM 16354^T (99.6%)	Salt pool	Romano et al. (2006)
Photobacterium sp. NGY1	*Photobacterium rosenbergii* LMG 22228^T (98.5%)	Bleached coral	Thompson et al. (2005)
	Photobacterium damsela ATCC 33539^T (95.2%)	Damselfish skin ulcers	Smith et al. (1991)

[a]We selected two kinds of type strains, which showed high first and secondary similarities to the 16S rDNA sequences of each isolate, based on the database of Apron DB-BA Version 4.0 (TechnoSuruga Laboratory Co., Ltd.).

[b]The homologous percentage between the isolate and each type strain is shown in parentheses. The type strain listed in the upper line made a cluster with the isolate on the phylogenetic tree.

Table 4. Changes in the survivability of TBT-sensitive Vibrio natriegens ATCC 14048^T when *Photobacterium* sp. TKY1 possessing "TBT tolerant but with no degradation ability" coexist in the presence of TBTCl

Strains and each number of resting cells (CFU mL^{-1})	Surviving cells in the presence of TBTCl (100 μM)[a]	
	Photobacterium sp. TKY1 (CFU mL^{-1})	*Vibrio natriegens* ATCC14048^T (CFU mL^{-1})
Photobacterium sp. TKY1 ($10^{9.1\pm0.2}$) and *Vibrio natriegens* ATCC 14048^T ($10^{9.4\pm0.2}$)[b]	$10^{9.1\pm0.2}$	$10^{4.4\pm0.4}$
Vibrio natriegens ATCC 14048^T alone ($10^{9.4\pm0.2}$)	—	$10^{6.2\pm0.3}$

[a] Experiments were carried out three times independently, and the data are shown as the averaged value ± standard deviation.

[b] No reduction of colony-forming *V. natriegens* 14048^T cells was observed after being mixed with *Photobacterium* sp. TKY1 cells for 1 h in the absence of TBTCl.

The capacity of cell walls of TBT-tolerant marine bacteria is generally higher than that of TBT-sensitive marine bacteria. This means that the concentration of TBT increases locally with the presence of TBT-tolerant marine bacteria, and TBT toxicity is amplified when TBT-tolerant species exist in the population in TBT-polluted sediment. In order to maintain the

microbial ecosystem, TBT should be removed from surface sediment and degraded, especially TBT in the surface sediment of busy ports.

BIOREMEDIATION OF TBT-CONTAMINATED SEDIMENTS BY TBT-DEGRADABLE MARINE BACTERIA

One of the possible uses of TBT-degradable marine bacteria is to apply the isolates to the bioremediation of TBT-polluted sediment. TBT-degradable *Aeromonas molluscorum* Av27 (Cruz et al., 2007) and *Stenotrophomonas chelatiphaga* HS2 (Hassan, 2018) are good candidates because they can degrade TBT to less toxic dibutyltin and monobutyltin, and they use one to two butyl groups of TBT as a carbon source during the growth. Cruz et al., (2007) compared the degradation ratio of TBT in the autoclaved sediment sample with that in the non-autoclaved sediment sample after inoculation with *A. molluscorum* Av27. The ratio obtained from the autoclaved sample was 2 to 3 times higher than that of the non-autoclaved sample during 150 days of degradation after freshly prepared cells were inoculated, indicating that the degradation activity was reduced by the isolate's competition for survival with the indigenous population. Similar results, showing the difficulties of survival for invasive species in non-indigenous population, have reported. Mimura and Miwa took seawater samples on board at busy ports and the quarantine anchorage in Tokyo Bay (Figure 3) and examined the survivability of non-indigenous *Vibrio* sp. cells (Figure 4). As a result, the survivability of marine *Vibrio* sp. cells was obviously reduced within two weeks under competition for survival with the indigenous population containing living $10^{3.5}$ to $10^{6.1}$ CFU mL^{-1} after the addition of freshly prepared resting cells to give an order of 10^3 to 10^4 CFU mL^{-1} (Mimura and Miwa, 2013). However, *Vibrio* sp. cells added to autoclaved samples could grow up to about 10^2 times after two weeks. Bioremediation of TBT-contaminated sediments by TBT-degradable marine bacteria is better carried out in a bioreactor on land, rather than in situ degradation, after sediment samples polluted with TBT were taken and autoclaved.

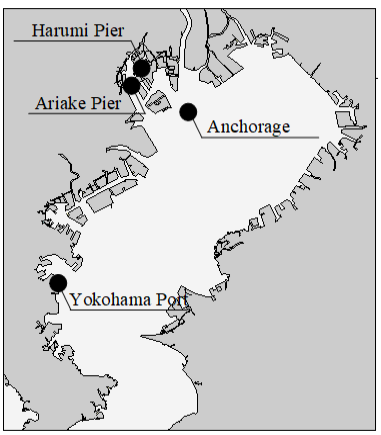

Figure 3. Sampling sites of seawater in Tokyo Bay. Seawater was taken on board to estimate the survivability of a non-indigenous *Vibrio* sp. after invasion against seawater in busy ports.

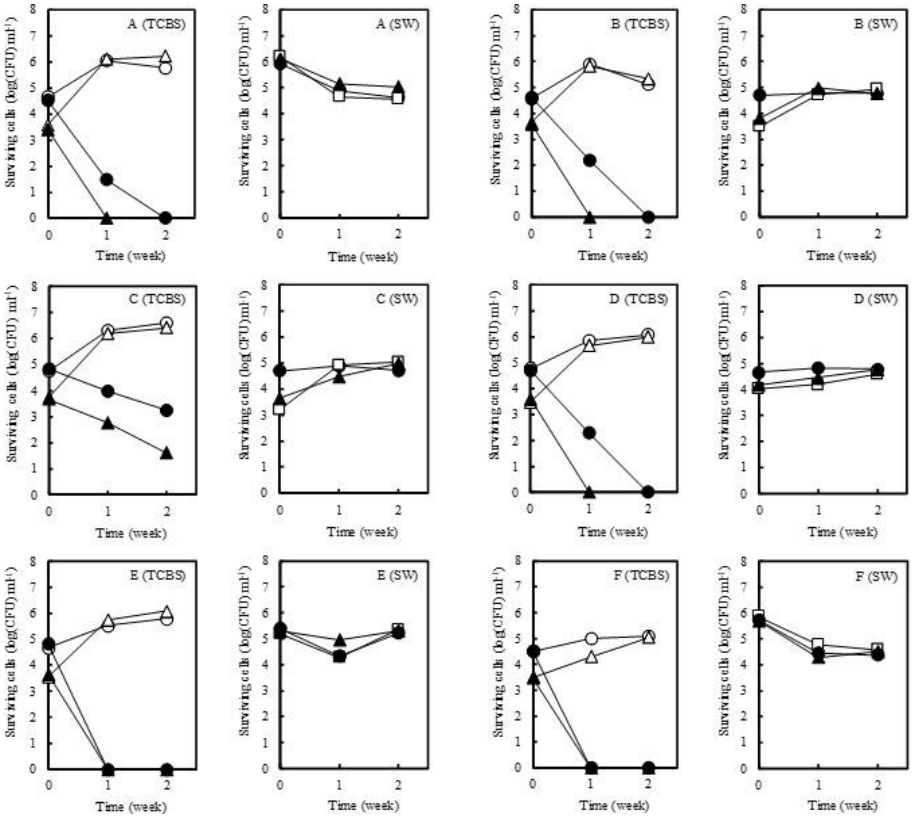

Figure 4. Changes in the number of *Vibrio* sp. cells after their introduction into populations of native marine bacteria. Seawater was taken at Ariake Pier on June 14, 2009 (A), July 20, 2009 (B), and September 19, 2009 (C), Harumi Pier on January 17, 2010 (D), the quarantine anchorage on January 17, 2010 (E), and Yokohama Port on March 10, 2010 (F). Every sampling site is located in Tokyo Bay. Seawater at 5 m depth from the surface of the water body was taken for all the samples, except for sample F. The sediment sample (F) was taken at 10 m depth. Temperatures at the sampling sites were 22, 26, 23, 14, 11, and 12ºC for samples A, B, C, D, E, and F, respectively. *Vibrio* sp. cells freshly prepared were added to seawater in test tubes to give an order of 10^3 (closed triangles) and 10^4 (closed circles) CFU mL^{-1}. Surviving cells were counted using thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates. The survivability of *Vibrio* sp. cells was also examined in the seawater autoclaved as a control (open triangles and open circles) in the left figure. Changes in the numbers of indigenous marine bacterial populations alone are shown in the figure on the right (open squares) using seawater-based nutrient agar plates. In addition, changes in total populations including *Vibrio* sp. cells of an order of 10^3 (closed triangles) and 10^4 CFU mL^{-1} (closed circles) are shown in the figure on the right. "TCBS" and "SW" in parentheses in the left and right figures mean TCBS agar plates and seawater-based nutrient agar plates, respectively. The use of the figures is permitted by the Japan Institute of Marine Engineering.

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BIOGRAPHICAL SKETCH

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Publications from the Last 3 Years:

Mimura, H., Yagi, M., & Yoshida, K. (2017). Environmental impact of tributyltin-resistant marine bacteria in the indigenous microbial population of tributyltin-polluted surface sediments. *Biocontrol Science*, 22, 89–96.

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Chapter 104

THE STRAIT OF HORMUZ: CHARACTERISTICS, CHALLENGES AND OPPORTUNITIES IN THE NORTHERN COASTLINE

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ABSTRACT

The present study describes characteristics, challenges and opportunities in the Strait of Hormuz (Iranian waters) and reviews the present and future conditions of the marine environment in the region. Because of the diversity fauna, the Strait of Hormuz is known as an environmentally unique ecosystem. But, its health is seriously threatened by a number of anthropogenic stressors such as industrialization and urbanization, pollution, overfishing and climate change. We also discuss the potential ecotourism capacity of the Strait of Hormuz and the subsequent benefit to the economy of local communities. A comprehensive management plan is required to promote coastal ecotourism in the region, and unstable approaches would lead to rising anthropogenic pressures.

Keywords: The Strait of Hormuz, living marine resources, anthropogenic stressors, coastal ecotourism

1. GEOGRAPHY AND GEOMORPHOLOGY OF THE STRAIT OF HORMUZ

The Strait of Hormuz, located in southwest Asia, lies between 26˚30́ N and 56˚30́ E, and connects the Persian Gulf to Gulf of Oman and the Indian Ocean (Figure 1). The mean width of the Strait of Hormuz is 56 kilometers (with the narrowest portion at 39 km). The length and maximum depth of the Strait are about 192 kilometers and 120 m. Iran (Islamic Republic of)

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covers the northern shoreline and Oman and United Arab Emirates (UAE) border the southern portion of the Strait (Figure 1). The Strait of Hormuz is one of the most important petroleum transit channels in the world, with nearly 20% of global supply of oil passing through it (Cordesman 2007, Aghajanloo et al. 2013).

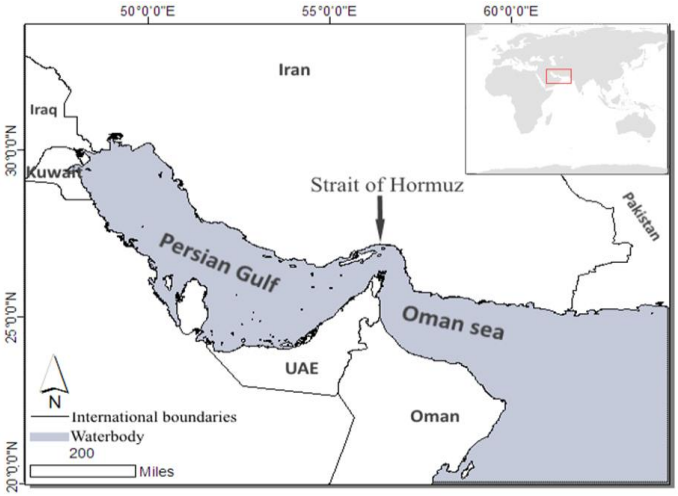


Figure 1. Location of the Strait of Hormuz.

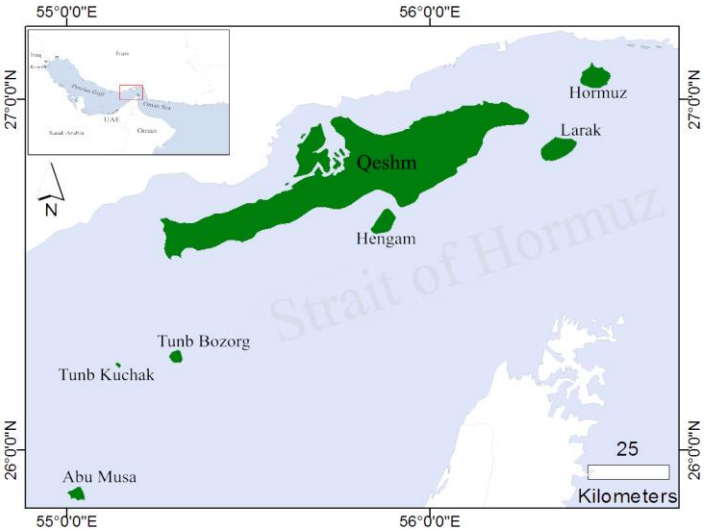


Figure 2. Iranian islands in the Strait of Hormuz.

There are numerous islands in the northern part of the Strait, namely Qeshm, Hormuz, Larak, Hengam, Greater Tunb, Lesser Tunb and Abu Musa, which are strategically important (Figure 2). Qeshm is the largest island with 1491 km^2, 115 km length, and 10-30 km width, is separated from Iranian mainland by the Khoran estuary. Unlike Qeshm, which is mostly flat, Larak is covered by ditches and ridges in particularly in the south and southwestern areas. Hormuz Island is also a salt dome, which has an area of 42 km^2, and is covered by sedimentary rock and layers of volcanic material on its surface. Qeshm Island has an area of

50 km^2, shaped like a truncated cone and is generally calcareous. Abu Musa is close to the equator, and has warmer and more humid weather than other Iranian islands. Greater and Lesser Tunbs, with areas of 11 and 2.5 km^2, are 12 km and lie 20 km from the south of Qeshm. The two small islands are located in the deepest part of the Persian Gulf, as well as within the international marine transportation corridors, and has greater geopolitical and strategic importance (Ramazani 1979).

Previous geological studies indicate that there are two structural regions in the south-west Asia, including Arabian Peninsula and Iranian Plateau. The Strait of Hormuz is the narrowest waterway between these regions. The Iranian Plateau, which the Persian Gulf is a part of it, collided with the north-east portion of the Arabian Peninsula to create the convex-shape in the north of the Strait of Hormuz (Reynolds 1993, Roumina 2015).

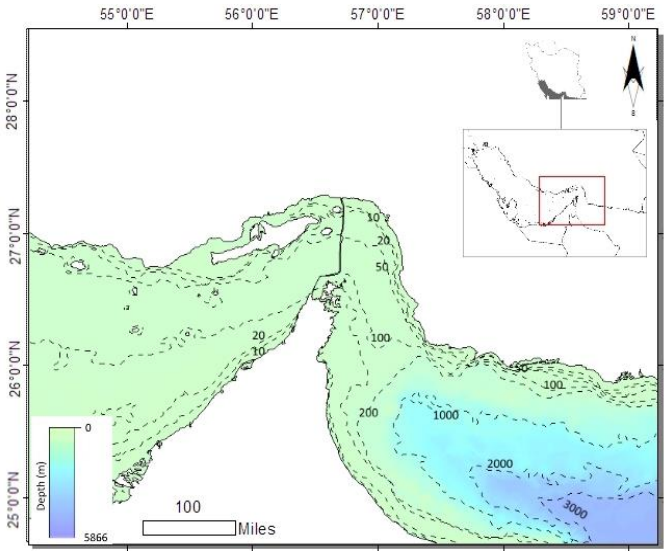


Figure 3. Bathymetric map around the Strait of Hormuz.

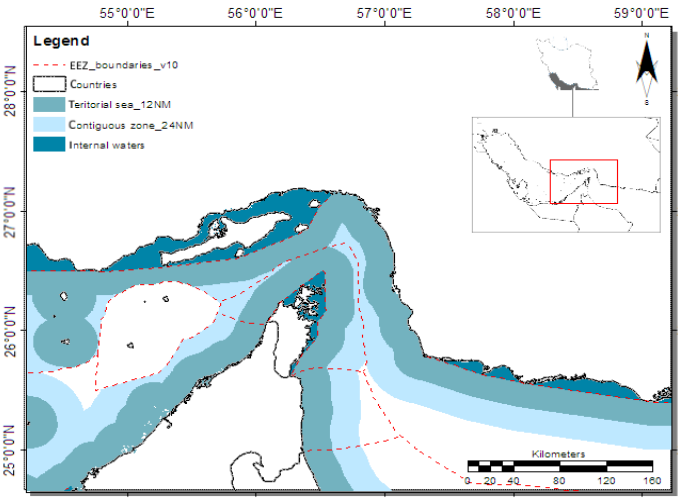


Figure 4. Maritime boundaries around the Strait of Hormuz; Dashed line illustrates economic exclusive zone (EEZ).

Maritime boundaries (based on the United Nations Convention on the Law of the Sea (Albrecht 2014)), in the waters of Strait of Hormuz is shown in Figure 4. Iran's internal waters include the waters surrounding Qeshm, Hengam, Larak, and Hormuz islands, and the territorial waters within 3-12 km of the coastline of the waters surrounding Abu Musa and Greater and Lesser Tunbs, begins at the edges of these islands. Therefore, Iranian territorial waters are wider in this part of the Strait. In the narrowest place of the Strait of Hormuz (Larak and Qeshm islands), a controversial area, Iran and Oman have agreed upon a 12 mile territorial sea.

2. LIVING MARINE RESOURCES

Because of wide variety fauna, the Strait of Hormuz has been known as an environmentally unique ecosystem. Sheppard (1993) argued that species richness in the Persian Gulf and the Strait of Hormuz is mostly related to the high nutrients of the water body. The purpose of this section is twofold. First, to introduce the major living groups and species that are prevalent in the northern part of the Strait of Hormuz, and second, to assess the status of these resources against the probably of future changes.

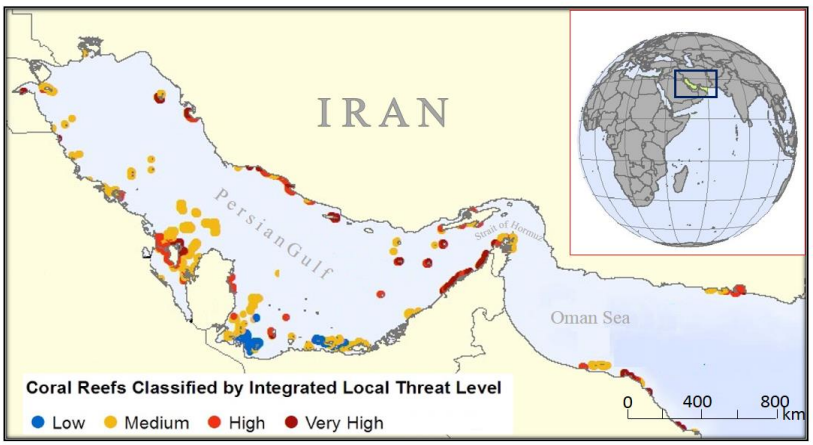
2.1. Coral Reefs

Coral reefs are found in the Pacific Ocean, the Indian Ocean, the Caribbean Sea, the Red Sea, and the Persian Gulf, Florida and southern Japan. Globally, coral reefs cover an estimated 110,000 square miles (284,300 square kilometers). Coral reefs communities throughout the world are experiencing serious declines. Wilkinson (2004) showed that 30% of reefs are already severely damaged and close to 60% might be lost by 2030. Pandolfi et al. (2003) stated that overfishing and pollutions have been causing the decline of coral reefs communities directly and indirectly.

Because of extreme range of salinity and sea temperature and intense low tides in the Persian Gulf, coral reefs live in harsh environmental conditions (Sheppard et al. 2010). In the northern Persian Gulf, along the Iranian coastline, these unique habitats are mostly restricted to offshore islands. Wilson et al. (2002) mentioned that the best reefs in this area are around Khark and Kharku islands, and from the Lavan to Hormuz Islands. There are 27 species belonging to 20 genera and 9 families in the northern part of the Persian Gulf, with *Porites lutea* Milne-Edwards & haime, 1860; *Porites compressa* Dana, 1846 and *Acropora clathrata* (Brook, 1891) being the dominant species (Wilson et al. 2002). Sheppard and Salm (1988) argued that because the ecological conditions of the Persian Gulf are mostly close to the physiological tolerance limits of many species, the coral reefs have relatively low diversity compared to the Gulf of Oman (70 species).

Rezai et al. (2004) reviewed the status of coral reefs in the Persian Gulf, the Gulf of Oman and Arabian Sea during 100 last years. They concluded that reefs were almost certainly healthy at the beginning of the period, dominated by *Acropora* (staghorn) corals to about 4-5 m depth, then by massive corals (*Porites*, faviids) from 5 m to about 10 m. In 1994, substantial nearshore construction, landfilling, and oil and civil development changed coastal

habitat, but corals were mostly affecting coral reef communities. In 2004, because of coral bleaching in 1996-98, many of the reefs declined and did not recover. They also predicted that future temperatures will be unfavorable for coral growth in the area, and the rising stress on nearshore reefs will lead to further destruction. Wilson et al. (2002) also argued that industry (power generation, sea water desalinization, petrochemicals and oil refining, aluminum and steel smelting), shipping and dredging and marine fisheries are the major human activities leading to coral decline in the Persian Gulf and the Gulf of Oman during the last decade. They also asserted petroleum extraction, processing and transport have also added to this decline, particularly in the Strait of Hormuz. Figure 5 shows the classification of coral reefs at different threat levels in the Persian Gulf and Oman Sea. It is obvious that coral reefs from the northern Persian Gulf (along the Iranian coastline), particularly in the Strait of Hormuz, are mostly classified as high or very highly threatened. The reviews above provide evidence to demonstrate that the poor condition of corals reefs in the Strait of Hormuz, and the ineffectiveness of ecosystem management plans at national or regional levels in this area.



Source: World Resources Institute, 2011.

Figure 5. Coral reefs condition in the Persian Gulf and Oman Sea.

2.2. Mangrove Forests

Mangroves are littoral woody plants which exist in tropical and sub-tropical sheltered coastlines. Globally, there are 54-75 species of true mangroves which found only in tidal zones (Saenger et al. 1983). They can grow in conditions of high salinity and temperature, extreme tides, strong winds, muddy and anaerobic soils (Kathiresan and Bingham 2001). Mangrove ecosystems not only reduce coastal erosion but provide also spawning and nursery grounds for aquatic organisms, breeding sites for birds, as well as ecotourism capability. Mangrove branches and leaves are also used to feed domestic animals by local communities. Iranian mangrove forests cover approximately 93 km^2 of the northern Persian Gulf and are one the most biodiverse marine ecosystems in the area, with more than 107 species of fishes, 100 species of sea and shoreline birds, 6 species of shrimp, and 10 species of crabs, 5 species of sea snake and 2 species of sea turtle (Zahed et al. 2010).

Alongi (2002) claimed that approximately one-third of the world's mangrove forests have been lost over the last half century. Walters et al. (2008) also concluded that increasing population in coastal areas and global climate change, particularly sea-level rise, are two important problems facing mangroves worldwide, and mangroves of the Persian Gulf and Oman Sea are no exception. Furthermore, short-term management decisions speed up the destruction of these valuable ecosystems in the region. For example, Azini lagoon a 15,000 ha mangrove area, located in the southwest of Bandar Sirik, is one of the four international lagoons of Hormozgan province. During the recent years, the lagoon's environment has been affected by destructive events such as oil spills. As is shown in Figure 6, the constructed petrol station in vicinity of the mangroves will make irreparable damage in future. Moreover, vessels and boats transition makes water turbulence which has negative impact on the mangrove roots. More description about mangrove forests of the Strait of Hormuz are given in section 5.1.

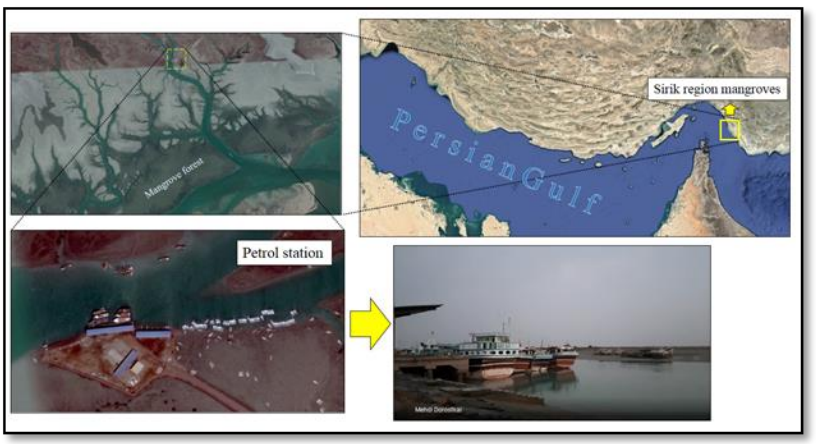


Figure 6. Location of petrol nearing the mangroves of Azini lagoon, Sirik region (satellite images date: September 9, 2016).

2.3. Planktonic Assemblages

Marine phytoplankton, including dinoflagellates; diatoms; blue-green algae; silicoflagellates and coccolithophores, contributes more than 90% of primary productivity in marine waters (Verlencar and Desai 2004). Sheppard et al. (1992) stated that primary productivity in the Persian Gulf is high compared to the Red Sea and Arabian Sea. However, information on planktonic communities in the northern coastal waters of the Persian Gulf is sparse. Research on plankton in Iranian waters of the Persian Gulf has been conducted by Fatemi et al. (2004), Fallahi et al. (2005) Namin et al. (2010), Alamzadeh (2011), Moradi and Kabiri (2012) and Fatemi et al. (2012a). With reference to these, diatoms, with 97 species, is dominant planktonic group in the northern waters of the Persian Gulf. Fatemi et al. (2004) also demonstrated temporal variation of diversity and concentration of diatoms. They observed the highest diversity in winter (with 63 species) and lowest in summer (with 18 species), while the highest and lowest concentrations were in autumn (627067 cell.m^{-3}) and summer (345139 cell.m^{-3}), respectively. Fallahi et al. (2005) proved that planktonic density

has spatial variation in the area, and decreases from the west of the Persian Gulf to the Strait of Hormuz. They also identified 244 species of phytoplankton, of which 124 were diatoms (Bacillariophyceae), 113 Dinophyceae, 5 Cyanophyceae, 1 Chrysophyceae, and 1 Euglenophyta.

The input of excessive nitrogen and phosphorous into near-shore waters, through industrial and urban sewage, is also one of the important anthropogenic pressures on Iranian waters of the Persian Gulf ecosystem, in particularly the Strait of Hormuz, causing eutrophication and planktonic blooms. According to Behzadi et al. (2012) and Rahimi (2013), approximately 700 liters per second of urban and industrial sewage are released to near-shore waters of the Persian Gulf. Whereas, some studies have demonstrated that eutrophication and increasing respiration rates through the excess production of organic carbon in coastal waters, has led to increased acidification (Wallace et al. 2014, Gobler and Baumann 2016).

The marine ichthyotoxic dinoflagellate is mostly responsible for the harmful algal blooms (HABs), commonly referred to as "red tides," in waters of the Strait of Hormuz. Fatemi et al. (2012a) argued that *Noctiluca scintillans*, *Trichodesmium* sp. and *Nitzschia* sp. are the main species that are responsible for HABs in this area. They also noted that fish kills in August 2008 to May 2009 were related to blooms of the toxic dinoflagellate *Cochlodinium polykrikoides*. This event also caused massive damaged to coral reefs, restriction of fishing activities and forcing desalination plants (in Oman and the United Arab Emirates) to cease or modify operations due to clogging of intake filters or the fouling of reverse osmosis membranes (Richlen et al. 2010).

2.4. Bony Fish and Elasmobranches

Fish diversity in the Persian Gulf, considering the Strait of Hormuz and Iranian waters of the Gulf of Oman, includes 908 species belonging to 157 families. This includes 129 families of Teleostei (814 species), 18 families of sharks (60 species), 10 families of rays (34 species). The number of 62 families are mono-species family and 25 families have more than 10 species, such as *Gobiidae* (with 53 species), *Carangidae* (48 species), *Labridae* (41 species), *Blenniidae* (34 species), *Apogonidae* (32 species), *Lutjanidae* (31 species), *Carcarhinidae* (26 species) and *Dasyatidae* (12 species). Nevertheless, fish diversity of the Persian Gulf compared to tropic and subtropics areas in the Indo-Pacific region is most similar to the Red Sea and Bay of Bengal (Owfi 2015).

Fisheries is the most important natural resource, after petroleum, in the Persian Gulf and the Strait of Hormuz. Referring to IFO (2014), total fish landing in Iranian waters of the Persian Gulf and the Strait of Hormuz was 314,121 tons in 2014, with Hormozgan province landing the highest proportion (68%) (Figure 7). Target species in this region include mostly mackerel and various Perciforms such as families of *Carangidae*, *Haemulidae*, *Serranidae*, *Sparidae*, *Stromateidae*, *Lutjanidae*, *Scianidae*, *Mugilidae*, *Clupeidae* and *Trichiuridae*. Herein, a review of the fisheries and scientific research are presented.

Iran is the country with the largest fishery in the Persian Gulf area. Fishing in Iranian waters is both small-scale (using motorized dhows and sambuks, small wooden or fiberglass vessels, intertidal fixed stake net traps etc.) and large-scale (industrial-style trawlers). As shown above, Hormozgan Province which covers the northern part of the Strait of Hormuz,

has the largest fishery of the region. There are now approximately 22,500 and 1,290 fishers employed respectively in the small-scale and large scale fisheries sector in Hormozgan (Daliri et al. 2016). The main types of fishing gear used are gillnets, wire traps (local name: gargoor), intertidal fixed stake net traps (local name: Moshta), hook and lines, beach seines, shrimp trawls, purse seines, Largehead hairtail trawls and some other traditional forms (FAO 2014). Gill-net fishery is the most common fishing method currently undertaken in the area, and drift gillnets are used for large pelagic fisheries such as tuna and tuna-like species. Fixed-gill nets are also used for catching demersal fish species such as *Pampus argenteus* (Euphrasen, 1788), *Pomadasys kaakan* (Cuvier, 1830), *Argyrosomus hololepidotus* (Lacepède, 1801), *Otolithes ruber* (Bloch & Schneider, 1801) and other species.

Some research on gillnet fishery have been conducted in Iranian waters of the Persian Gulf by Parsa (2011), Dastbaz (2011), Moein et al. (2012), Daliri et al. (2017), Hosseini and Kamali (2017) and Haghighatjo et al. (2018). They argued that the gillnets, due to high bycatch and harvesting of undersized target species, are mostly not selective and determining the standard mesh sizes is necessary.

Small-pelagic fish in the Persian Gulf waters are also harvested with purse seines and beach seines. Alaee et al. (2016) discussed that Hormozgan province, had the largest proportion (95%) of small-pelagic fish landing in Iranian waters of the Persian Gulf and Oman Sea. Salarpouri et al. (2008) also stated that waters of Qeshm Island, Bandar Lengeh and Bandar Jask are the main fishing grounds of small-pelagic fishes in Hormozgan, and sardines and anchovy are the most important species.

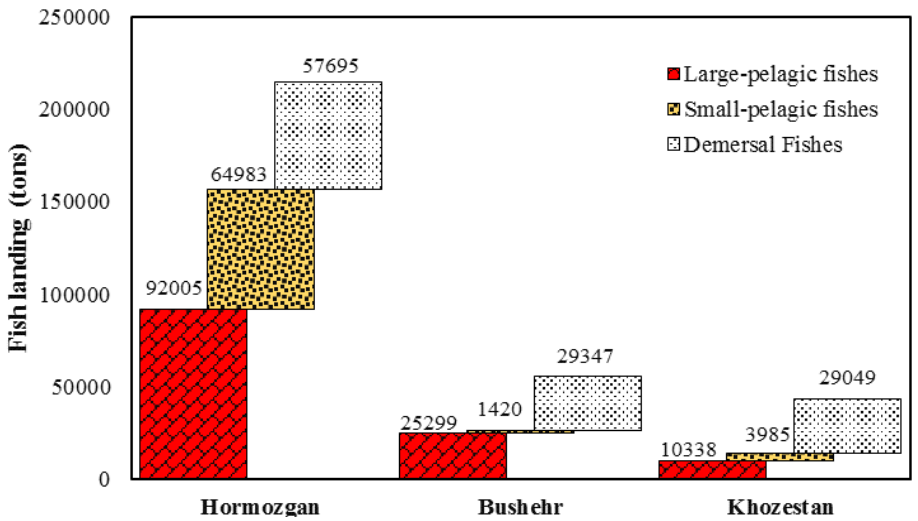


Figure 7. Total fish landing (tons) from Iranian waters of the Persian Gulf and Gulf of Oman. Hormozgan province covers the Strait of Hormuz waters, and Bushehr and Khuzestan provinces cover the central and western parts of Iranian waters of the Persian Gulf.

The intertidal stake traps, known as Moshta, are commonly used to capture littoral fish species in the Persian Gulf, particularly in Hormozgan (Al-Baz et al. 2007). Daliri (2016) reported that Moshta in the Strait of Hormuz waters captured a total of 81 species, including 65 teleost fish, 6 elasmobranchs, 6 crustaceans, 2 cephalopods, 1 sea turtle and 1 echinoderm

species. The catch composition by weight also included 19.66% commercial catch, with a large percentage of the catch consisting of juveniles, and 80.33% discards.

Largehead hairtail trawls and Lanternfish trawls are also used as mid-water trawls in the area. Raeisi et al. (2012) estimated 1941.5 tons of the bycatch produced by the improved trawls (for Largehead hairtail fisheries) in Hormozgan, included 45 species from 31 families. They also reported that catch composition contained 67.75% largehead hairtail, and 32.25 % bycatch (17.81% incidental catch and 14.44% discards). Kiaalvandi et al. (2012) also examined bycatch of Lanternfish trawls in Hormozgan waters and reported that catch composition consisted of 29.64% target species and 70.36% bycatch.

In summary, the fish stocks of Iranian waters of the Persian Gulf are mostly fully exploited or overfished. The available catch data of this area indicates a 21% decrease in demersal fish landings during the recent years, whereas fishing effort has been increasing (Valinassab et al. 2006a, Hosseini et al. 2015).

2.5. Macrobenthos

Because of harsh environmental conditions, biodiversity of microbenthic communities of the Persian Gulf is high, but they have mostly low species richness (Sheppard et al. 2010, Sharifinia et al. 2019). In Iranian waters of the Strait of Hormuz, muddy subtidal habitats support many Macrobenthos species of shrimp and crab. Macrobenthic assemblages of the region have been investigated by some researchers such as Kamrani et al. (2010), Asgari et al. (2012), Fatemi et al. (2012b), Pourjomeh et al. (2014), Taherizadeh and Sharifinia (2015), Naderloo et al. (2015), Amini-Yekta et al. (2017) and Sharifinia (2017). These studies have mostly focused on identifying macrobenthic taxa and the effects of environmental change on their distribution in the region.

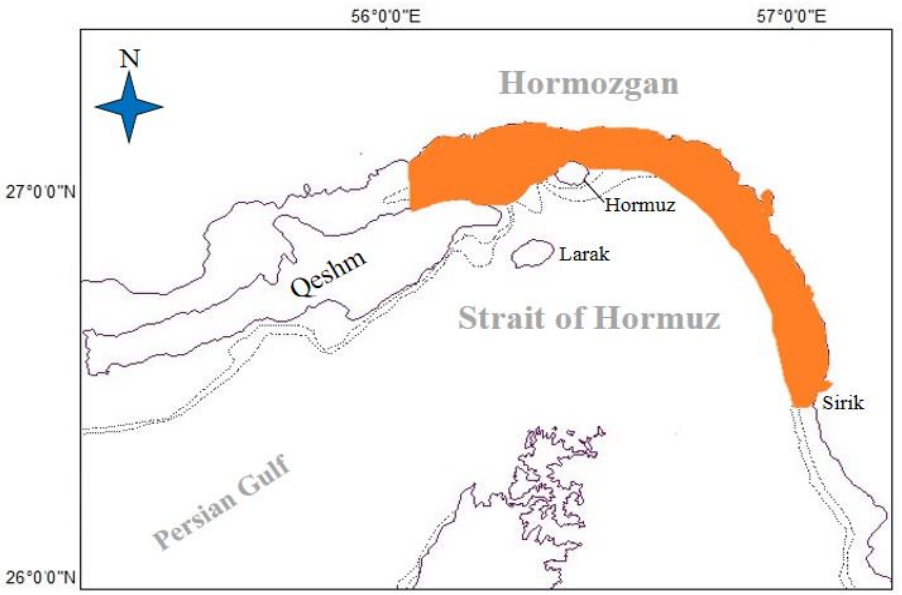


Figure 8. Location of shrimp fishing grounds in the Strait of Hormuz.

Shrimp is the most important commercial Macrobenthos in the northern waters of the Strait of Hormuz, and are captured by bottom trawls. Commercial shrimp species in the region include *Penaeus merguiensis* (De Man, 1888), *Metapenaeus affinis* (Milne-Edwards, 1837), *Penaeus semisulcatus* (de Haan, 1844). Fishing grounds for these species is shown in Figure 8. A high volume of bycatch is the main problem of the shrimp trawl fisheries in the Persian Gulf and the Strait of Hormuz (Valinassab et al. 2006b, Hosseini et al. 2015). Hosseini et al. (2015) reported that bycatch composition of shrimp trawlers in Hormozgan included 52 teleostei species, 23 invertebrates and 6 elasmobranchs. The bycatch-to-shrimp ratio was 6.8:1. They also stated that most of the incidental catches, such as *P. argenteus*, *Parastromateus niger* (Bloch, 1795), *Scomberomorus commerson* (Lacepède, 1800), *Carcharhinus dussumieri* (Müller & Henle, 1839) and *O. ruber*, were caught under the standard size.

3. CHANGING ENVIRONMENTAL CONDITIONS

However, little research has been conducted on changing environmental conditions in the Persian Gulf and the Strait of Hormuz. For example, Jafari et al. (2016) demonstrated a significant rising of sea level between 1995 to 2010 (Figure 9). Data analyses of sea surface temperature (SST) also indicate a steady trend of warming (Figure 10). The change of mean sea level (MSL) as a function of SST in the Strait of Hormuz can be describe as follows (Jafari et al. 2016):

$$MSL = 3.106\, SST + 235 \tag{1}$$

The coefficient of the model explains that changes in SST makes 3.106 times change in MSL. There is seasonal fluctuation of SST in the area, ranging between 20˚ to 34˚c. Inter-annual analysis of SST data also revealed that minimum and maximum temperature occurred in January and August with 19.4˚ and 34˚c (Figure 9).

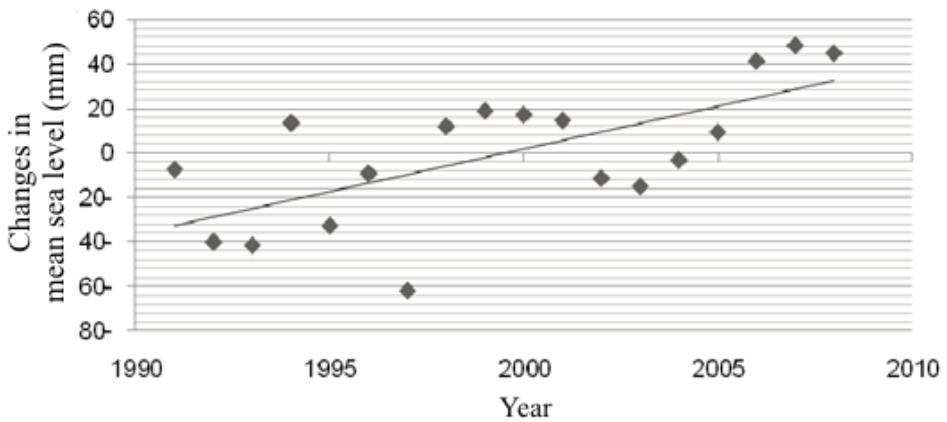


Figure 9. Sea level rising in the Strait of Hormuz (from Jafari et al. (2016)).

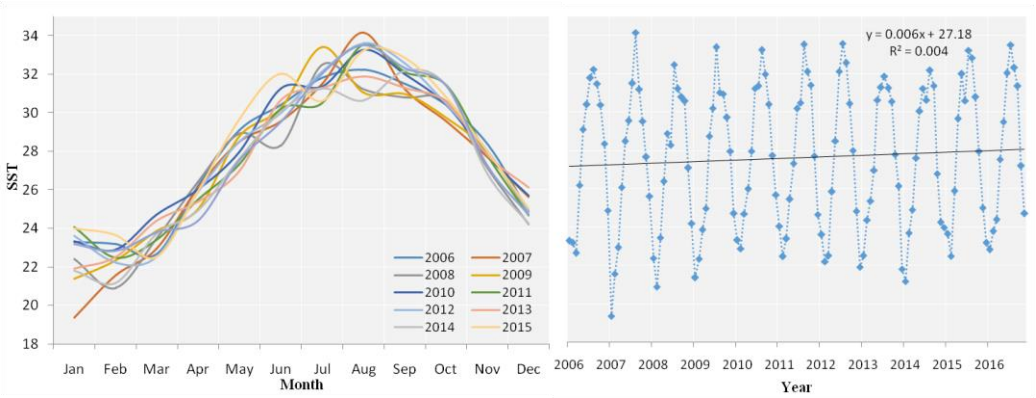


Figure 10. Temporal fluctuation of SST in the Strait of Hormuz.

4. POLLUTION

The Persian Gulf has the largest reserve of oil in the world, transported throughout worldwide through the Strait of Hormuz. Therefore, oil exploration, production, and transport are major contributors to pollution in the Persian Gulf (Khan et al. 2002, Abdul-Wahab et al. 2009). Some literature, such as Naji et al. (2017a), Naji et al. (2017b), Esmaili and Naji (2018) have examined the presence of plastic contamination in marine environment of the Persian Gulf, in particularly the Strait of Hormuz. The plastic pollution, which is steadily rising, is openly threatening marine biota at every level of the food web, from primary producers to higher trophic-level organisms (do Sul and Costa 2014). Naji et al. (2017b) examined microplastic (MPs: <5 mm) contamination in littoral sediments of the Strait of Hormuz and concluded that MPs were exist in 80% of the samples, and fiber particles were the most dominant (88%), followed by films (11.2%) and fragments (0.8%). The sediments with the highest numbers of MPs were from sites in the vicinity of highly populated centers and municipal effluent discharges.

Naji et al. (2017a) also explored abundance of plastic debris and microplastic contaminations in various stations along the beach of the Strait of Hormuz. This area exhibited different levels of industrialization and urbanization, and included a marine protected area (MPA). They found that polyethylene (PE), nylon, and PET (polyethylene terephthalate) were the commonly recovered polymers, and likely sources included beach debris, discarded fishing gear, and urban and industrial outflows. Moreover, trace metals are another type of contamination in the Persian Gulf. Due to the shallow depth and limited circulation the impact of the pollutants on the marine environment are significant (Pourang et al. 2005). Naji et al. (2015) assessed trace metal concentrations (Pb, Ni, Zn, and Fe) and potential ecological risk of them in intertidal surface sediments of a mangrove estuary in the Strait of Hormuz. The concentration of metals (all in μg/g, except for Fe in %) were as follows: 2-9 Pb; 58.9-94.3 Ni; 111.9-185.6 Zn; 1.4- 2.2 Fe. They also found that Zn and Ni were mainly from anthropogenic discharge, while a significant portion of Pb and Fe were likely from natural inputs. Overall, frequent adverse effects were expected for Ni and occasional adverse biological effects were expected for Zn. Likewise, for Pb and Fe rare adverse biological effects were expected. Ghasemi et al. (2018) also investigated

accumulation of trace metals in the sediments of mangrove forests (Hara Protected Area and Azini estuary) in the Strait of Hormuz, and argued that the concentration of selected metals was Pb > Zn > Cu > Cd and in the HPA region and Zn > Cu > Pb > Cd in Azini estuary. Their results also revealed that the HPA region was in the range of moderate ecological risk and the Azini Bay region in the range of acceptable ecological risk. Potential human health risk of trace metals (Cd, Cu, Ni, Pb and Zn) via the consumption of marine fish in the Persian Gulf and the Strait of Hormuz was also assessed by Naji et al. (2016), who showed that metal concentrations were lower than legal limits. Cadmium target hazard quotient values suggested that the threshold to avoid the potential risk for children health is an exposure level lower than 3 meals per week. Hazard index values based on four metals (not including Pb) for the child age class were higher than those of the adult age class, suggesting that children may suffer from a higher health risk.

Figure 11. Example of macroplastic litter items as source of microplastic formation in the Strait of Hormuz (from Naji et al. (2017b)).

5. COASTAL ECOTOURISM POTENTIALS

According to definition of The International Ecotourism Society, ecotourism means "*responsible travel or visitation to natural areas that conserves the environment and improves the well-being of local people*" (Bricker 2017). Ecotourism clearly includes a set of social, environmental and economic principles that contribute to sustainable development. Globally the tourism industry is constantly growing, so that the World Tourism Organization (WTO) statistics indicates an average increase of 10% per year from 1995-2005 (Figure 12). Rakhshani Nasab and Zarabi (2009) argued that the major proportion of this increase is due to ecotourism.

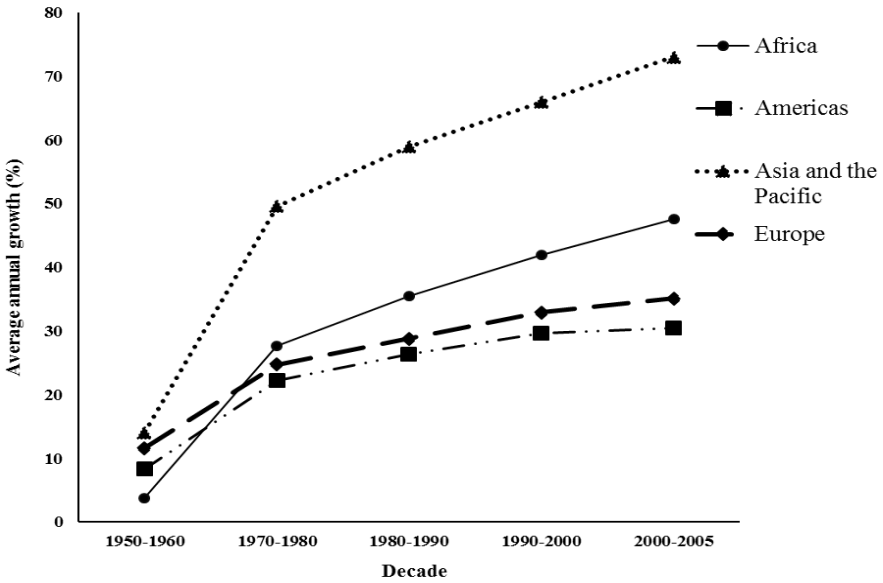


Figure 12. Average annual growth (%) of tourist arrivals during 1950 to 2005 (Vrujci 2005).

Iran (Islamic Republic of), in terms of tourism, ranked 5th among the Middle East countries (Table and Figure 13), whilst has known as the world's 5th largest country in terms of natural diversity (Rakhshani Nasab and Zarabi 2009). Today, all countries are mostly attempting to make maximum use of their tourist attractions, but Iran has not reached its maximum potential in this industry. Because of existence of mangrove forests, high biodiversity and numerous islands, the northern coastline of the Strait of Hormuz has a potential capacity for the development of Iran's tourism industry and the subsequent creation job opportunities for indigenous communities. Therefore, a comprehensive management plan is required to promote coastal ecotourism in this region. Some aspects of coastal ecotourism in Iran's coastline of the Strait of Hormuz are presented as below.

Table 1. International tourist arrivals (million) in the Middle East countries in 2015 (UNWTO Library 2017)

Rank	Country	International tourist Arrivals (million)	Change from 2014 to 2015 (%)
1	Turkey	39.5	- 0.8
2	Saudi Arabia	18.0	- 1.5
3	UAE	14.2	+ 7.6
4	Egypt	9.1	- 5.1
5	Iran	5.2	+ 5.4
6	Jordan	3.8	- 5.7
7	Qatar	2.9	+ 3.7
8	Israel	2.8	- 4.4
9	Oman	1.9	+ 17.8
10	Lebanon	1.5	+ 12.1

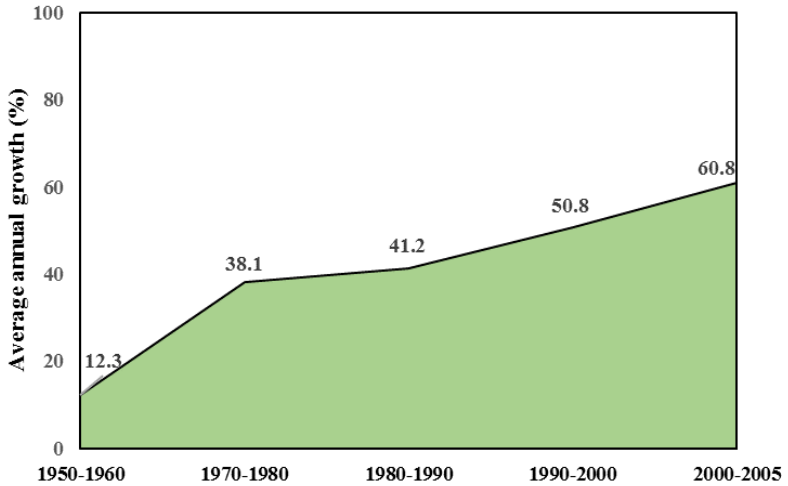


Figure 13. Average annual growth (%) of tourist arrivals in the Middle East countries during the last 55 years (Vrujci 2005).

5.1. Mangrove Forests

As stated above, mangrove forests are the only forests located at the intersection of land and sea in the world's subtropics and tropics (Alongi 2002). These have not only had a significant role in biological and biogeochemical cycles in coastal ecosystems, but also play an important role in regional and national economies. Because mangroves are known as an ecotone environment, they have wide variety of organisms making them a biologically rich habitat (Salam et al. 2000). Mangroves ecotourism throughout the world has been able to generate economic benefits for local communities and promote the countries' tourism industry (Murtini and Kurniawati 2018).

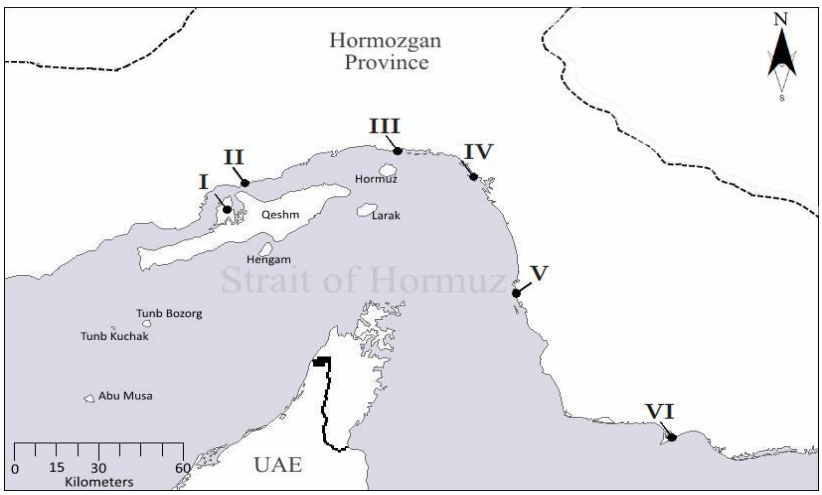
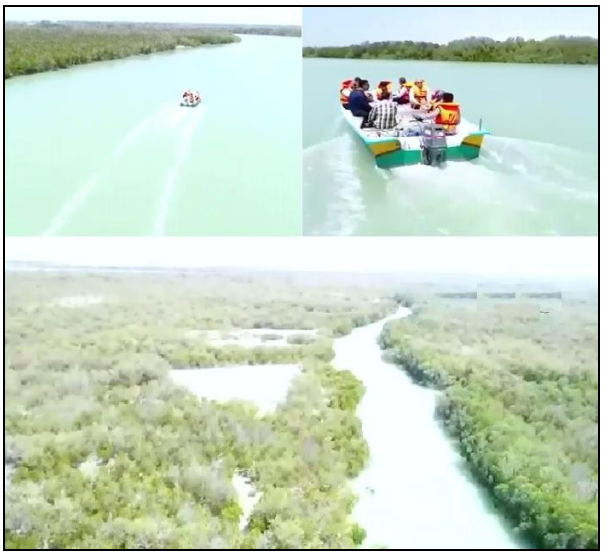


Figure 14. Location of mangroves forests in the northern coastline of the Strait of Hormuz (Zone I: Qeshm Island, called Harra Marine Protected Area; Zone II: Khamir estuaries and mouth of the Mehran River; Zone III: the east of the Bandar Abbas; Zone IV: Tiyab and the Kolahi regions; Zone V: Sirik region and Zone VI: Jask region).

The mangrove forests of coastline of the Strait of Hormuz, along the Iranian coastline (Hormozgan province) are distributed within six zones (Figure 14). Although there are more than 60 species of mangrove in the world, mangroves in this region includes only two species namely *Avicennia marina* (in Persian called Harra) and *Rhizophora macrunata* (in Persian called Chondal) (Danehkar 1996, Zahed et al. 2010). These evergreen forests are one of the most beautiful tourist destinations in the Persian Gulf and Oman Sea. As Zahed et al. (2010) stated, increasing industrialization, changing the environmental conditions as well as unstable political decisions are threatening the status of the mangroves in the region. Therefore, developing ecotourism in a non-destructive fashion could be a solution to mitigate the negative consequences on these unique ecosystems.



Source: Sirik travel agency.

Figure 15. Azini Lagoon visiting in Sirik region.

Source: www.citypedia.ir.

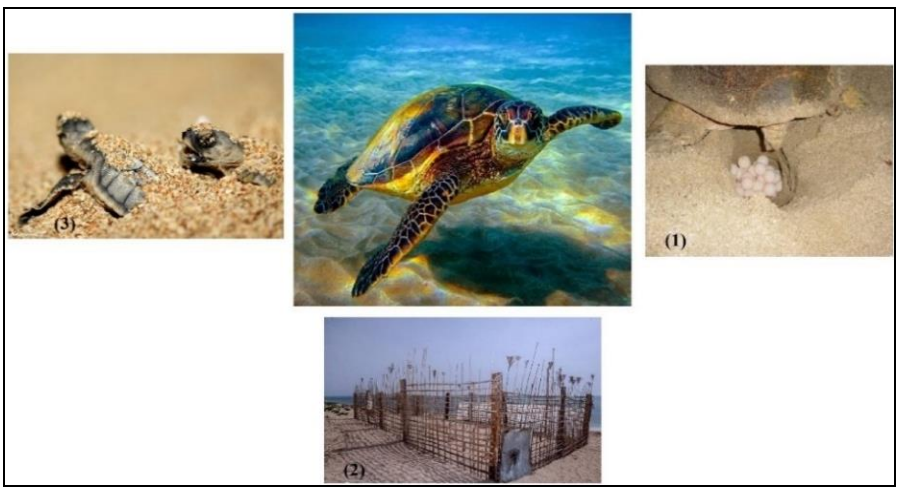
Figure 16. Lagoon birds watching in the mangroves of Qeshm Island.

5.2. Marine Wildlife Watching

Wildlife tourism has been known as a safe way to achieve sustainable economic benefits while supporting wildlife conservation and indigenous people (Higginbottom 2004). Globally marine wildlife watching is increasingly important for the tourism industry. The northern part of the Strait of Hormuz is no exception, and provide good opportunities to observe marine wildlife in their natural environment. Dolphin watching, during mid-autumn to mid-spring, in the coastal waters of Hengam Island is a popular activity for tourists.

Source: www. qeshmgardi.com.

Figure 17. Dolphins watching in Hengam Island.



Source: www.karnaval.ir. (1): Nesting and laying the eggs, (2): Safe sites for protecting the eggs from predators or humans. (3) Hatched Hawksbills.

Figure 18. Hawksbill in the coastline of Shibderaz village, Qeshm Island.

Nesting of Hawksbill turtle, *Eretmochelys imbricata* (Linnaeus, 1766), is another wonderful natural attraction to promote for ecotourism in the Strait of Hormuz (Figure 18). The Hawksbill turtle is a critically endangered (CR) species which has two Atlantic and Pacific subspecies (CITES 2016). The Pacific subspecies lives in the Persian Gulf and comes to its islands beaches, particularly to Qeshm Island, for spawning. The sandy coastline of

Shibderaz village, located in the central southern part of Qeshm Island, is a convenient site for nesting Hawksbill turtles to lay their eggs. Over the past few years, the reproductive behavior of this turtle species has attracted many enthusiasts to Qeshm Island. In addition to diving and water sports, watching the marine ornamental fishes by glass-bottom boat excursions is also one of the exciting recreational activities in this region. The rocky coasts of Qeshm, Hengam and Larak Islands provide natural aquariums, which people can visit and enjoy.

Nevertheless, Iran has not succeeded in utilizing its full potential for ecotourism. However, for developing countries, which mostly have a single-product economy and high unemployment rate, development of the tourism industry is an important key to progress. Coastal ecotourism development in the northern part of the Strait of Hormuz needs more collaboration between the Iranian government and private investors. The government should not only provide proper conditions for the private sector to improve infrastructure (transportation, hotel, restaurant and etc.), but to also develop the economies of local communities (Vahedpour and Jafari 2011).

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Chapter 105

MACROALGAL POLYPHENOLS: ISOLATION, CHARACTERIZATION AND BIOACTIVITY

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ABSTRACT

Marine macroalgae, which are a part of the common diet in the Asian culture and which have beneficial properties for human health, have drawn attention because of the possibility for their application in various branches of industry, due to their bioactive compounds which have shown properties that can be beneficial for human health.

Macroalgae, aside from being a good source of essential amino acids, fatty acids, dietary fiber and minerals, represent an under-exploited source of phenolic compounds known for their high potential in therapeutic and industrial applications. The production of phenolic compounds in marine algae has been involved in protection against stress conditions, such as the production of antioxidant compounds. Polyphenolic compounds such as catechins and flavonols are the most dominant polyphenols in all classes of macroalgae: *Chlorophyta* (green algae), *Rhodophyta* (red algae) and *Phaeophyta* (brown algae). The latter contain a particular type of polyphenols called phlorotannins (polymers of 1,3,5-trihydroxybenzene) with various types of bioactivity. The concentration of polyphenols varies among the classes of macroalgae because of the different chemical composition, geographical occurrence of particular species, seasonal variations and the variation is also present within the different parts of thalli. The extraction of these compounds can be performed by conventional or alternative/modern methods. The modern methods have mostly been used in the last few decades because of their benefits in comparison to the conventional methods. It is necessary to find the most efficient method for obtaining the highest concentration of polyphenols in the extract. Aside from the antioxidant potential of macroalgal polyphenols, they also have anti-inflammatory, antibacterial and anti-allergic properties. They have a high potential for implementation in functional foods as naturally obtained compounds, as well as for replacing available synthetic compounds.

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This chapter will highlight the application of the most active compounds present in marine macroalgae known as polyphenols, as well as their isolation using green extraction methods. Their bioactive properties will be reviewed and their potential for health improvement will be evaluated.

Keywords: marine macroalgae, polyphenols, extraction, bioactivity

1. INTRODUCTION

Due to the fact that 70% of the Earth's surface is covered by oceans, the presence of diverse and complex marine organisms in oceans can potentially be considered as an abundant source of valuable compounds. Marine organisms are considered to be an unexplored source of valuable products, with particular interest devoted to algae, because of their high productivity and taxonomic and chemical diversity. The algal habitat is subjected to various extreme conditions which affect algal chemical composition and lead to the creation of novel and interesting compounds. Consequently, geographical conditions influence algal thalli structure and biochemical composition, which are responsible for the value of algae as a potential source of bioactive compounds (Stengel and Connan, 2015). According to their biochemical metabolic pathways and cellular composition, algae adapt better to external conditions, when compared to terrestrial plants (Mironiuk and Chojnacka, 2018). One of the defense mechanisms is the formation of antioxidant compounds or increased enzyme activity, which are used to deactivate the reactive oxygen species (ROS). The ROS accumulate in macroalgae as a result of environmental stresses (freezing, low temperatures, radiation, desiccation, salinity fluctuations) and can cause damage to the photosynthetic apparatus with the formation of singlet oxygen. The accumulation of antioxidant compounds in the algae contributes to the potential benefits for human health. The antioxidant activities and benefits of consuming terrestrial plants such as vegetables are known, but little information is given for the antioxidant potential of macroalgae. The bioavailability of antioxidant compounds and the intake of macroalgae must be taken into consideration due to a relatively small percentage of the world population consuming seaweed (Cornish and Garbary, 2010). Aside from the environmental variations, the following factors influence the chemical composition and diversity of secondary metabolites found in algae: the species of algae, geographical origin, area of cultivation and seasonal variations (Niemczyk et al., 2018). Compounds such as fatty acids, phenolic compounds, carotenoids, polysaccharides, terpenes and many others are natural metabolites of algae related processes and they represent the products of chemical transformations which occur inside the cells. Biological activity such as antioxidant, anti-inflammatory, immunostimulating and many others types of activities have been examined for these compounds. Due to the presence of biologically active compounds, algae are considered as desirable ingredients in cosmetics, drugs, diet supplements and food (Leska et al., 2018).

Seaweeds have gathered attention among researchers due to their unique composition. Aside from being a good source of carbohydrates, dietary fiber, proteins, vitamins, minerals, fats and polyunsaturated fatty acids, they are a potential source of natural antioxidant compounds such as polyphenols (Gomez-Guzman et al., 2018). In this chapter, we will show the diversity of phenolic compounds which are present in algae, as well as their bioactivity, along with the potential for their technological implementation. The emphasis was on the

extraction of phenolic compounds, using both conventional methods and the most commonly applied novel extraction techniques, such as Supercritical Fluid Extraction (SFE), Subcritical Water Extraction (SWE), Ultrasound-Assisted Extraction (UAE) and Microwave-Assisted Extraction (MAE). The summary of the techniques is followed by an analytical analysis of polyphenols. Various types of polyphenol bioactivity were also reviewed in this chapter.

1.1. The Classes of Marine Algae and Their Bioactive Compounds

Macroalgae are classified into three groups according to their pigment composition; which is related to their sea habitat. Accordingly, green algae (*Chlorophyceae*) mostly inhabit coastal water, where they can absorb large quantities of light, while brown (*Phaeophyceae*) and red (*Rhodophyceae*) algae are dominant in deeper water, where the sunlight is limited (Christaki et al., 2012). Green algae have no other pigments to mask the chlorophyll, while brown algae do contain other pigments that mask their chlorophyll, and one of these dominant pigments is fucoxanthin. Similar to brown algae, red algae also contain other pigments along with chlorophyll, such as phycocyanin and phycoerythrin. These differences correspond to their adaptation to the environment in the algal habitat (Haryatfrehni et al., 2015). The sizes, physical forms and behavior of certain groups of algae are highly diverse, for example, green algae range from microscopic single cells to large tubular, bushy plants; red algae have different physical forms, with both simple and branched filaments; while brown algae are solely multicellular, but they vary in their physical forms (Murugan et al., 2015).

The chemical composition of the mentioned algal groups is still under constant investigation due to the great diversity in terms of the number of different species of macroalgae, as well as the unique composition of their primary and secondary metabolites. They can be a rich source of bioactive compounds, including polyunsaturated fatty acids, polyphenols, polysaccharides and pigments (Michalak and Chojnacka, 2018). Considering that people today are aware of unprocessed healthy foods, without synthetic additives, algae exhibit a great potential for being a source of natural compounds which can be applied in food products and serve as food supplements. Studies showed that all of the mentioned compounds possess bioactivity, such as antioxidant (Matanjun et al., 2008), antimicrobial (Plaza et al., 2010), anti-inflammatory (Khan et al., 2008), antiaging (Fayad et al., 2017), antiproliferative (Gutierrez-Rodriguez et al., 2017) and many other types of bioactivity. Physiologically active compounds isolated from algae are divided into two groups according to their mechanisms: 1) non-absorbable high molecular compounds (dietary fibers) and 2) low molecular compounds, which affect the human homeostasis (Murata and Nakazoe, 2001; Pal et al., 2014). In addition, these valuable compounds can protect the human body and participate in processes against harmful effects within it. For example, algae produce antioxidant compounds used in the fight against free radicals that appear because of the stress conditions in the environment. Free radicals are responsible for many diseases, such as skin diseases, cancer, etc. (Korzeniowska et al., 2018). Antioxidant compounds from natural sources, like algae, can be a great replacement for synthetic antioxidants, being currently used, including butylated hydroxyanisole, butylated hydroxytoluene, *tert*-butylhydroxyquinone and propyl gallate. These synthetic antioxidants are strictly regulated in many countries because they can potentially cause harmful effects on human health (Cornish and Garbary, 2010). Thus, phenolic compounds are the main contributors to the antioxidant activity of algae, which

makes them candidates for implementation in the food, cosmetic and therapeutic industries as natural antioxidants. They are a promising alternative with no harmful effects on the human body, produced with low cost and beneficial effects on our health (Niemczyk et al., 2018).

1.2. The Diversity of Phenolic Compounds Derived from Macroalgae

Polyphenols are the most highly distributed group of phytochemicals in plants. They are secondary metabolites synthesized from two pathways, the shikimate and the acetate (Ross and Kasum, 2002). The simplest polyphenol structure consists of an aromatic hydrocarbon group with a hydroxyl group bonded to it (Holdt and Kraan, 2011). However, these compounds show a wide diversity of structures due to the number of phenol rings that they contain and the types of bonded structural features (Manach et al., 2004). This large group of secondary metabolites is present in both terrestrial and marine environments. Plant polyphenols are derived from the ellagic acid and the gallic acid, while algal polyphenols are derived from polymerized 1,3,5-trihydroxybenzene (phloroglucinol) units (Holdt and Kraan, 2011). Due to their chemical diversity, they can be divided into several classes starting from simple molecules such as phenolic acids, followed by flavonoids, isoflavanoids, stilbenes and lignans, to highly polymerized phenolics such as tannins (Figure 1).

They are present in fruit and beverages (tea, coffee, wine), as well as in vegetables and legumes. Flavonoids account for two thirds of the total intake of polyphenols, while phenolic acids account for the remaining third. However, the bioavailability within polyphenols must also be taken into consideration because it differs significantly, along with their concentration in foods regarding the environmental, genetic and processing factors (Machu et al., 2015).

It is well known that plant polyphenols are essential for plant physiology because they provide resistance against pathogens and predators and they are involved in the process of growth and contribute to the morphology of the plant. These polyphenols are applied for various industrial purposes, such as the production of paper and paints, they serve as additives in the food industry and also as tanning agents in cosmetics. Additionally, they have some applications in the pharmaceutical industry, as antibiotics and anti-inflammatory agents, but they can also be used in the treatment of hypertension, allergies, hypercholesterolemia, etc. (Bravo, 1998). Consequently, their consumption can reduce the risk of different diseases, such as cancer and neurodegenerative and cardiovascular disorders (Gomez-Guzman et al., 2018). They can serve as therapeutic agents and they can be used for the protection of other bioactive compounds from harmful environmental effects (Korzeniowska et al., 2018).

Aside from polyphenolic compounds such as catechins and flavonols, macroalgae are also a rich source of a special type of polyphenols, called phlorotannins, found only in algae. Phlorotannins are a group of complex polymers of phloroglucinol and they represent dominant polyphenolic compounds in brown macroalgae. On the other hand, red and green macroalgae are abundant in bromophenols, phenolic acids and flavonoids (Gomez-Guzman et al., 2015). Generally, red and green algae contain a low content of polyphenols compared to brown algae. The phenol content varies from <1% to 14% of dry seaweed biomass among species, but the content is also dependent on the different parts of thalli and on seasonal variations (Holdt and Kraan, 2011). For example, Flodin et al. (1999) observed that the bromophenol content, in *Ulva lactuca*, changed as the seasons changed and there are high variations between the detected bromophenol compounds.

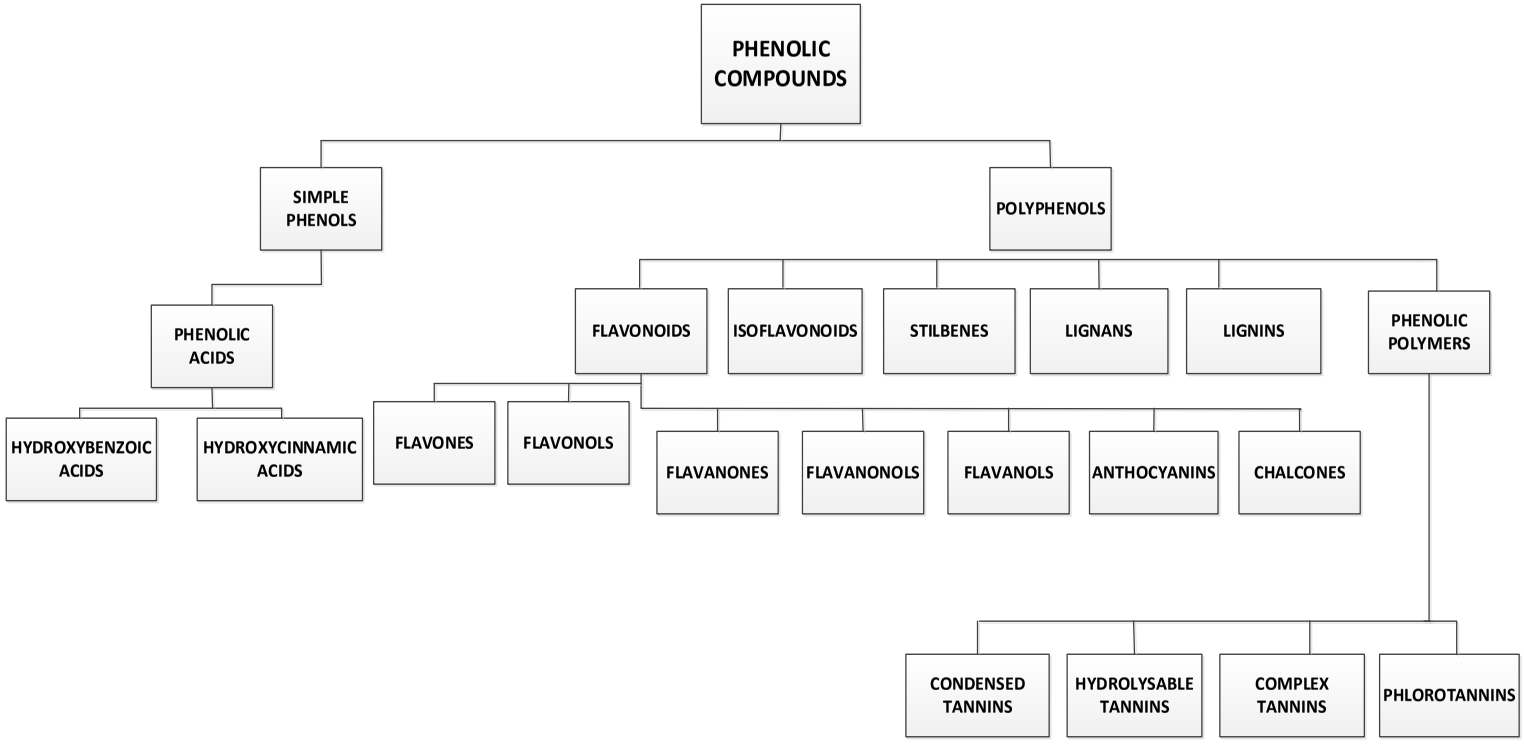
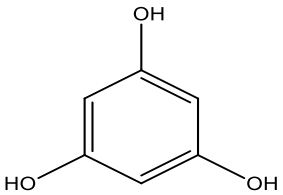


Figure 1. Classification of phenolic compounds (Niemczyk et al., 2018).

Phlorotannins are the most interesting polyphenols found in macroalgae due to their composition and bioactivity, and because they represent integral structural components of the cell wall (Niemczyk et al., 2018). They are composed of polymerized phloroglucinol (1,3,5-trihydroxybenzene) monomer units (Figure 2) biosynthesized through the acetate-malonate pathway or the polyketide pathway. These are highly hydrophilic components and their molecular sizes range from 126 Da to 650 kDa (Murugan et al., 2015). They are synthesized in brown algae for protection in stressful situations, which they achieve by reducing the oxidative damage (Gomez-Guzman et al., 2018). Marine algae, especially brown algae, contain a wide variety of phloroglucinol-based polyphenols, which can be low, intermediate or high molecular weight phlorotannins, with both phenoxy and phenyl units. These units can be bonded with ether, phenyl, both ether and phenyl, and dibenzodioxin linkage. Based on the linkage, phlorotannins are classified into four groups: fuhalols and phlorethols, fucols, fucophlorethols and eckols (Figure 3) (Wijesekara et al., 2010). According to the presence of these unique compounds, it can be said that algae, specifically brown algae, are a valuable source of special ingredients which have a potential use in medicine and in the food industry (Korzeniowska et al., 2018). Their therapeutic properties are being intensively studied and it was shown that they possess antioxidative, anticancer, antibacterial, anti-allergic, antiaging, anti-inflammatory, and anti-HIV properties, which were reviewed by Thomas and Kim (2011). Lower molecular phlorotannins exhibit higher antioxidant activity, which decreases with higher molecular weight. Also, it was shown that they reduce the activity of DPPH and superoxide anion radicals better than antioxidant vitamins such as ascorbic acid and α-tocopherol. It was suggested that phenolic hydroxyl groups attached to the eckol skeleton have the most important role in the radical scavenging activities (Shibata et al., 2007; Gomez-Guzman et al., 2018). Aside from the antioxidant activity, the structure of phlorotannins also has an influence on the bactericidal effects, Nagayama et al. (2002) showed that it increased with the polymerization of phloroglucinol.



Phloroglucinol

Figure 2. The chemical structure of phloroglucinol (Cikoš et al., 2018).

Regarding the available literature, polyphenols are antioxidants with a greatly diverse chemical composition, thereby making the activities of *in vitro* and *in vivo* systems difficult to correlate (Cornish and Garbary, 2010; Holdt and Kraan, 2011). Different algal species provide different total polyphenol content due to the geographical origin of the species, area of cultivation, environmental and seasonal variations. Polyphenols are synthesized when protection against biotic or abiotic factors is needed, and because of that, phenolic compounds are known as stress compounds (Niemczyk et al., 2018). One of these stress situations is oxidative stress, which occurs due to damage caused to important molecules and because of the production of free radicals. The imbalance between the production of free radicals and the

production of antioxidant compounds results in diseases and illnesses. Phenolic compounds stabilize these free radicals by binding with them or by inhibiting certain enzymes, and these are the main reasons why they are known as the most important group with antioxidant properties (Korzeniowska et al., 2018).

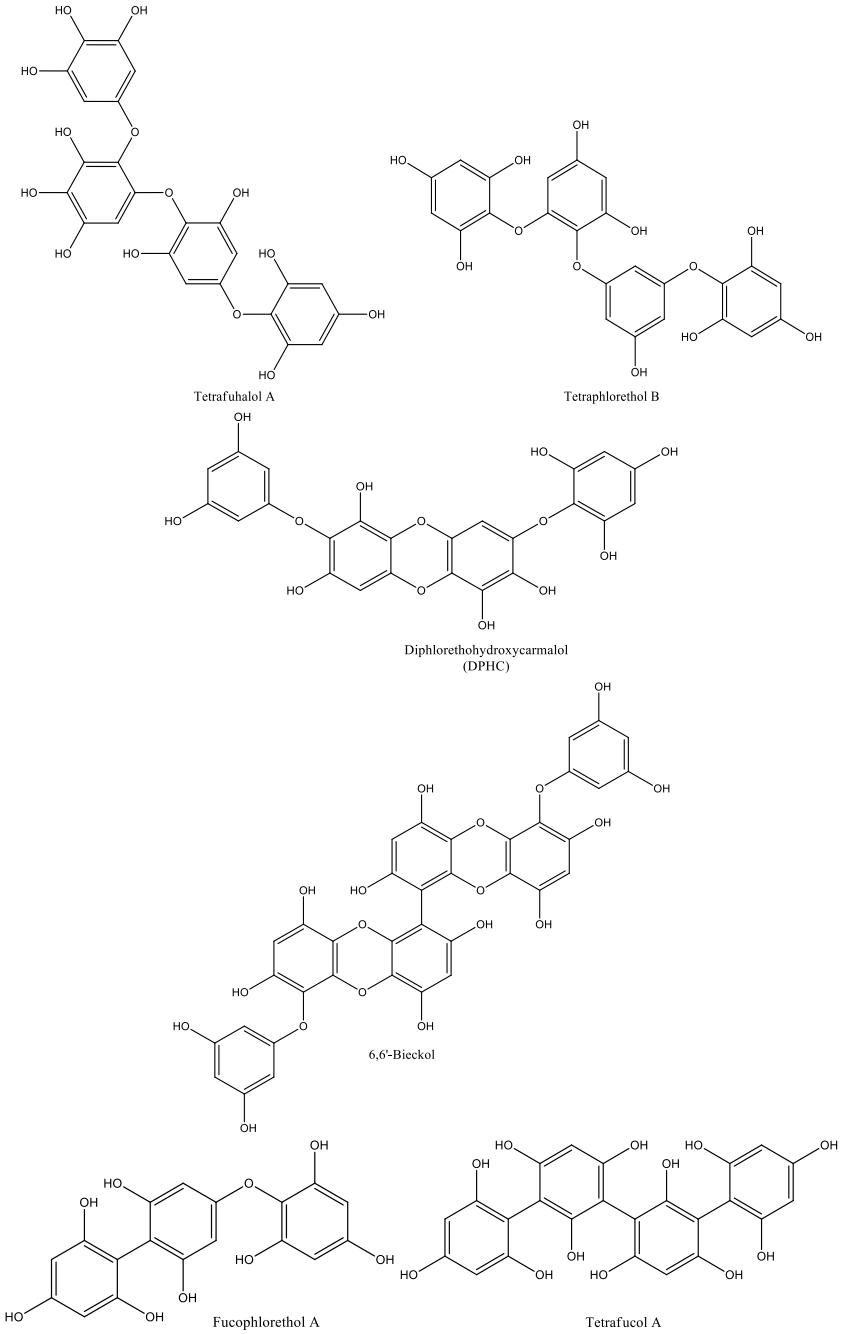


Figure 3. Different types of phlorotannins and their chemical structures: a) Tetrafuhalol A; b) Tetraphlorethol B; c) Diphlorethohydroxycarmalol (DPHC); d) 6,6'-Bieckol; e) Fucophlorethol A; f) Tetrafucol A (Cikoš et al., 2018).

2. THE ANALYSIS OF PHENOLIC COMPOUNDS

The first step in the determination of phenolic compounds is the extraction of these phytochemicals from plant material, which plays a crucial role in the final result, so the extraction technique and the solvent need to be selected properly. The success of the extraction depends on the applied method and the selected process parameters, as well as on the nature of the desired compounds (Azmir et al., 2013). In recent years, there has been a huge interest in algal extracts due to the high demand for natural products, which all contributes to the improvement and the development of novel extraction techniques (Michalak and Chojnacka, 2014). Novel extraction techniques need to be able to provide an efficient isolation of biologically active compounds that can be used in food, cosmetic or pharmaceutical industry. The isolation of compounds depends on the solvent used for the extraction, also on the chemical composition and the type of algae. Solvent polarity is a crucial parameter in phenolic recovery. It is known that by increasing the polarity of the solvent, the amount of the extracted phenolic compounds is higher in the final extract (Azmir et al., 2013; Niemczyk et al., 2018). Aside from solvent polarity, other factors that influence the phenolic chemical profile of the obtained extract are: macroalgae species, season when they were collected and the applied extraction method (Tanniou et al., 2013).

Conventional methods, also known as traditional methods, include Soxhlet extraction, solid-liquid extraction (SLE) and liquid-liquid extraction (LLE). These techniques require the use of large amounts of inorganic and organic solvents, as well as long processing time, there is possible degradation of thermolabile compounds and they are very energy-consuming. Considering that large amounts of waste is released, especially chemicals, these methods represent an environmental issue (Michalak and Chojnacka, 2014; Wang and Weller, 2006). All of these disadvantages and limitations can be overcome and new extraction techniques with shorter time of the process, reduced amounts of organic solvents and performance at lower temperature can be introduced, and the formation of unwanted residue of chemicals can be avoided, which makes them environmentally friendly (Wang and Weller, 2006). The new extraction methods, used most frequently today for the isolation of bioactive compounds from marine macroalgae, include Supercritical Fluid Extraction (SFE), Subcritical Water Extraction (SWE), Ultrasound-Assisted Extraction (UAE) and Microwave-Assisted Extraction (MAE).

2.1. The Extraction and Characterization of Polyphenols in Macroalgae

2.1.1. Conventional Polyphenol Extraction Methods

The extraction of polyphenols from various species of macroalgae using conventional methods, including solid-liquid extraction (SLE) with different solvents, is shown in Table 1. Scientists isolated various types of polyphenols from various macroalgal species, including species from all algal classes: brown, red and green. Alcohols, such as ethanol and methanol and their aqueous solutions are mostly used as extracting solvents, but several other hazardous solvents such as chloroform, petroleum ether and hexane, are also used. The evaporation of solvent is needed for this kind of extraction, and the discharge of these hazardous solvents presents a problem for the environment, which means that this method is

not environmentally friendly. Aside from that, high temperature achieved during the conventional process can cause degradation of thermolabile compounds. Therefore, the novel extraction techniques are preferable.

Table 1. The extraction of polyphenols from marine macroalgae using conventional extraction methods

Macroalgae Species	Extraction Method (Solvent)	Analytical Method	Determined polyphenols	Bioactivity	Reference
Ulva reticulata, Caulerpa occidentalis Cladophorasoicalis, Dictyota ciliolata, Gracilaria dendroides	SLE (ethanol, chloroform, petroleum ether, water)	HPLC	Flavonoids (rutin, quercetin, kaempferol)	Antibac-terial	Al-Saif et al., 2014
Ecklonia cava	SLE (80% aqueous methanol solution)	HPLC, NMR	Phlorotannins (phloroglucinol, eckol, dieckol)	Photo-protective effect, whitening effect	Heo et al., 2009
Porphyra dentata	SLE (methanol)	HPLC-DAD-ESI-MS	Rutin, catechol, hesperidin	Anti-inflamma-tory	Kazlowska et al., 2010
Eisenia bicyclis	SLE (water, ethanol)	HILIC method*	Phlorotannins (eckol, phlorofucofuroeckol-A, dieckol, 6,6'-bieckol, 8,8'-bieckol)	-	Kim et al., 2013
Ecklonia cava	SLE (70% aqueous ethanol solution)	HPLC	Dieckol	-	Kim et al., 2016
Stypocaulonsco-parium	SLE (methanol, ethanol, water, 50% aqueous methanol solution)	RP-HPLC	Gallic acid, protocatechuic acid, catechin, vanillic acid, epicatechin, syringic acid, chlorogenic acid, genitisic acid, caffeic acid, coumaric acid, ferulic acid, rutin, myricetin, quercetin	Antioxidant	Lopez et al., 2011
Fucus guiryi Fucus serratus, Fucs spiralis, Fucus vesiculosus	SLE (methanol:water, 1:1, v/v), SPE	HPLC-DAD-ESI-MS, UPLC-ESI-QTOF/ MS	Phlorotannins (trimers, tetramers, pentamers and hexamers)	-	Lopes et al., 2018
Himanthaliae-longata	SLE (60% aqueous methanol solution), SPE	RP-HPLC-DAD-MS/MS	Phloroglucinol, gallic acid, chlorogenic acid, caffeic acid, ferulic acid, myricetin, quercetin	Antioxidant	Rajauria, 2017
Palmaria spp., *Porphyra* spp., *Himanthaliae-longata, Laminaria ochroleuca, Undaria pinnatifida*	SLE (methanol-water-acetic acid (30:69:1, v/v)	HPLC, LC-MS	Catechin, epicatechin, epicatechingallate, catechingallate, epigallocatechin, epifallocatechingallate	-	Rodriguez-Bernaldo de Quiros et al., 2010

* Hydrophilic interaction chromatography (HILIC) – the main retention mechanism is the liquid-liquid partitioning of the polar analytes through hydrophilic interactions (ion-exchange and hydrogen-bonding between the polar stationary phase and the non-polar mobile phase). In this method, retention depends on the composition of the mobile phase and the functional group of the stationary phase, as well as the structure of the analyte.

However, available literature demonstrates that SLE is an efficient method for the extraction of various types of polyphenols. For instance, Al-Saif et al. (2014) used four different solvents (ethanol, chloroform, petroleum ether and water) for the extraction of flavonoids: rutin, quercetin and kaempferol. Chloroform was demonstrated as the most effective solvent for the extraction of these polyphenols. Among the investigated species, the red algae *Gracillaria dendroides* had the highest amount of all three flavonoids. It is known that red seaweeds are rich in phenolic compounds such as catechol, catechin, epicatechin, hesperidin, rutin and others. Kazlowska et al. (2010) reported that catechol, rutin and hesperidin were present in the red algae *Porphyra dentata* and they suggested that these three phenolic compounds are primary bioactive compounds in the crude extract of this algae. Rodriguez-Bernaldo de Quiros et al. (2010) showed that catechin was present only in red algae, while epicatechin gallate was found only in brown algae. Rajauria (2017) detected a hydroxybenzoic acid derivative, hydroxycinnamic acid derivatives, flavonolos and one special type of polyphenol, phloroglucinol. The results for antioxidant activity showed that phloroglucinol contributes the most to the antioxidant capacity of *Himanthalia elongata*, but other identified phenolic compounds could have synergistic or antagonistic effects on the total activity. Lopez et al. (2011) used various solvents, but those that were less harmful for the environment. The obtained results showed that there is a significant difference in the total polyphenol content and the antioxidant activity according to the applied solvent. The solvent polarity affected the extraction of polyphenols and the antioxidant activity, and water was the best solvent for the extraction, while ethanol provided the lowest phenolic content in the extract. Consequently, HPLC chromatograms showed that the amounts of certain polyphenols changed depending on the used solvent and its polarity. Gallic acid was found to be the dominant phenolic compound in all of the extracts, except in the ethanolic extract, while the amount of protocatechuic acid increased as the polarity of the solvent decreased. The authors concluded that solvent polarity changes the phenolic profile, but it does not change the total amounts of phenolics.

Aside from the isolation of flavonoids and phenolic acids, several scientists reported the extraction of one special group of polyphenols, known as phlorotannins, which are present only in brown macroalgae. Three groups of authors, Heo et al. (2009), Kim et al. (2013) and Kim et al. (2016), based their research on the isolation of major phlorotannins, but they used different solvents for the extraction. Heo et al. (2009) used an aqueous methanol solution, Kim et al. (2013) and Kim et al. (2016) used ethanol and its aqueous solutions. Kim et al. (2013) successfully developed an HPLC method for the determination of dieckol which consists of six phloroglucinol units and functional hydroxyl groups, but it also includes other phlorotannins, such as eckol, phlorofucofuroeckol-A, 6,6'-bieckol and 8,8'-bieckol, which represented the most abundant phlorotannins, while Heo et al. (2009) showed the protective effects of these phlorotannins. It must be taken into consideration that the phlorotannins showed variations in their concentration throughout the year. It was demonstrated that the concentrations are high during the hot temperature season (June-September), while these concentrations started decreasing after September, and the lowest amount of phlorotannins was recorded in February (Kim et al., 2013). The geographical location also affected the degree of polymerization of phlorotannins. The specific feature of the *Fucus* species was that a higher degree of polymerization of phlorotannins was detected in the species located in the environment with higher temperatures and light exposure (Ferreres et al., 2012). Lopes et al. (2018) showed that there is a difference in the chemical composition of brown seaweed

belonging to the *Fucus* genus, but phlorotannins of low molecular weight were characteristic for all the species of *Fucus*.

2.1.2. Novel Extraction Methods for the Isolation of Polyphenols

The most commonly applied novel extraction techniques for the isolation of bioactive compounds from marine macroalgae are Supercritical Fluid Extraction (SFE), Subcritical Water Extraction (SWE), Ultrasound-Assisted Extraction (UAE) and Microwave-Assisted Extraction (MAE). However, some of them are not suitable for the extraction of polyphenols because of the polarity of the solvent used. For example, pure CO_2 used for the extraction in supercritical conditions (SC) is not capable of extracting polyphenols due to its polarity. SC-CO_2 can only extract non-polar constituents or compounds of low polarity because of its non-polarity (CO_2 is a non-polar solvent). However, the extraction of polar compounds can be enhanced by adding a small amount of a polar co-solvent, such as ethanol or methanol, into the process. Therefore, the recommendation is to use other methods which are better for the isolation of phenolic compounds, such as SWE, UAE and MAE. The available data describing the application of the mentioned novel techniques is summarized in Table 2. It clearly shows that there is insufficient data available, about both the application of the novel techniques and about the analysis of the extracts, in order to provide enough information about the extracted polyphenols and about their composition in the extracts, as was previously described for SLE.

Supercritical CO_2 Extraction (SC-CO_2)

Even though polyphenols are polar compounds, some authors used SC-CO_2 for the extraction of polyphenols. CO_2 is usually mixed with a polar co-solvent such as ethanol or water. For instance, Saravana et al. (2017) used sunflower, soybean and canola oil as co-solvents, along with water and ethanol. However, the results showed that these oils were neither suitable for the extraction of polyphenols nor for lipids and other non-polar compounds. Water was the best co-solvent for the extraction of polyphenols due to the highest phlorotannin content found in the extracts. The process parameters also influenced the amount of extracted polyphenols. Roh et al. (2008) noticed that the polyphenol content increased with the increase in pressure, which is due to the higher solvating power of the supercritical fluid. Also, the increased temperature caused the higher polyphenol content, which is explained with the dominance of polyphenol vapor pressure in solvating power. Similar observations were obtained by Saravana et al. (2017), who noticed the increase of phlorotannin content corresponding to the increase in pressure, temperature and the co-solvent flow rate. Two groups of authors, Tanniou et al. (2013) and Sivagnanam et al. (2015), compared the extraction efficiency of polyphenols between the conventional methods (usually SLE) and SFE. The obtained results were contradictory. While Sivagnanam et al. (2015) indicated that the SLE extracts exhibited a higher total polyphenol content (TPC), Tanniou et al. (2013) indicated the opposite, a lower TPC was observed when SC-CO_2 was used with ethanol as the co-solvent. These differences can be explained by using different macroalgal species and by applying different process parameters. The NMR extract profiles analyzed by Tanniou et al. (2013) showed significant differences among the extracts obtained using the other methods. That indicates that the extracted compounds are dependent on the applied extraction method and on the solvent used. SFE is a good method for obtaining extracts with

non-polar compounds from macroalgae, such as lipids and fatty acids, and also pigments like carotenoids (Cikoš et al., 2018).

Ultrasound-Assisted Extraction (UAE)

During UAE, many factors such as ultrasonic power, temperature, time, solid to solvent ratio influence the extraction of polyphenols. Dang et al. (2017) showed that the TPC was most affected by the extraction time, followed by temperature and power. The longer time combined with low ultrasonic power was found to be the most efficient method for the extraction of a higher amount of polyphenols. The temperature had an effect on the diffusivity and the mass transfer. When the temperature was higher, the solvent diffusion rate and the mass transfer increased due to the lower viscosity of the solvent and the surface tension, which caused better dissolution of polyphenols in the extraction medium. On the other hand, Topuz et al. (2016) showed that the solid to solvent ratio had the highest influence on the extraction of polyphenols from red seaweed. In this case, the biological variation of seaweed, as well as the solvent used, must be taken into consideration when explaining the different results. Lee et al. (2013) compared the TPC in the UAE extracts and the SLE extracts. The results showed that the SLE extracts exhibited higher TPC and antioxidant activity. On the other hand, Dang et al. (2017) showed that UAE was better for the extraction of polyphenols and that these extracts had higher antioxidant activity, and they concluded that UAE can serve as a good technique for the isolation and purification of phenolic compounds. However, both authors used different species of macroalgae and different solvents, so that can be the reason for the difference in the obtained results. The Q-TOF-MS analysis of the UAE extracts showed that the ultrasound had no effect on the degradation of phlorotannins in the algae *Ascophyllum nodosum* and that they were extracted in greater amounts compared to the control treatment (conventional extraction) (Kadam et al., 2015). According to the provided information it could be suggested that UAE is a compatible method for the isolation of polyphenols and that further purification can enable the application of phenolic compounds from macroalgae in the food and pharmaceutical industry.

Subcritical Water Extraction (SWE)

SWE operates at high temperatures (50–200°C) and high pressures (50-300 psi), with water in very small amounts as solvent. The high temperature and pressure keep the water below its boiling point, near the critical-region. Water is known as the greenest solvent, so this type of extraction is an environmentally friendly method (Duarte et al., 2014). Several authors tried to apply different enhancers for the extraction in order to obtain higher yield and a larger amount of extracted polyphenols. Sanchez-Camargo et al. (2016) applied enzymes with SWE for determining the possibility of a higher yield, a higher amount of polyphenols and phlorotannin recovery. The enzymes were used to disrupt cell wall polysaccharides which could influence the release of phlorotannins. However, the results showed that the recovery of polyphenols using combined enzyme-subcritical extraction is not better. Vo Dinh et al. (2017) used ionic liquids (IL) as catalysts in SWE+IL experiments. When IL were added, they increased the extraction ability of the subcritical solvent due to the high solvating property of IL in interactions with complex matrices. The antioxidant activity of SWE+IL extracts was higher than in the case of SWE, which is the consequence of the higher TPC in these extracts than in the SWE extracts. The IL exhibited a strong influence on the extraction of bioactive compounds. Also, the SWE+IL provided a higher quality and quantity of extracted phenolics

than in SWE and SLE. The comparison between SLE and SWE was carried out by Heffernan et al. (2014). They compared the conventional extraction (SLE) with SWE and the results showed that the extracts obtained with conventional method using water as solvent produced a higher extraction yield and a higher TPC, which indicates that the majority of soluble compounds in seaweed were of high polarity. Also, the brown algae *Fucus serratus* showed the highest content of polyphenols among all of the investigated species. This can be explained by the fact that phlorotannins are only present in brown algae. The lower concentration of phenolic compounds in SWE extracts can be explained by the loss of thermolabile compounds which occurred during the extraction. On the contrary, Tierney et al. (2012) showed that SWE extracts exhibited a higher yield than SLE extracts. This can be explained by improved diffusivity and enhanced penetration through the particles of the material, which leads to higher extraction capacity. The extraction of phenolics was higher in the extracts obtained using SLE. Even though the extraction yield was higher in the extracts obtained using SWE, the content of phenolics was higher in the extracts obtained using SLE. On the other hand, Vo Dinh et al. (2017) showed that SWE extracts have a higher TPC content than SLE extracts. Although, at a high temperature (250°C) TPC was reduced, which can be explained by the degradation of phenolic compounds. This can be seen on the analyzed chromatograms, where the amount of certain phenolic compounds was reduced at higher temperatures. Aside from that, the solvent was for the extraction also influenced the extraction of polyphenols. Tierney et al. (2012) observed that the use of water as the solvent was the most suitable for the extraction of polyphenols, which reflects the hydrophilic nature of these compounds within macroalgae.

Microwave-Assisted Extraction (MAE)

MAE as one of the novel extraction techniques, also has several advantages, such as shorter extraction time and the reduced amount of solvents. Lou et al. (2010) compared the extraction efficiency of polyphenols by determining the TPC, and it was shown that MAE provided the highest TPC when compared to the other three methods (UAE, Soxhlet Extraction, Heat Reflux Extraction). Shorter time provided the preservation of labile compounds such as phlorotannins, which was shown in the research by Zhang et al. (2018.). They indicated that MAE extracts contained six major phlorotannin chemical groups, while only four groups were detected in SLE extracts. This can be explained by the higher degradation of the cell structure during MAE or the shorter time during the process, which prevented the oxidation of phlorotannins that occurred during SLE (48 hours). The conventional method SLE could not be used to obtain phlorotannins in any significant amount because the cell walls of the macroalgae could not be damaged through stirring. When MAE was applied, the degradation of the cell wall occurred, which enabled the extraction of phlorotannins. The degree of cell wall degradation was investigated using a scanning electron microscope, which showed that the highest distortion of cells walls occurred under MAE conditions. The obtained phlorotannins were analyzed using HPLC and it was shown that the major phlorotannins from *Carpophyllum flexuosum* belong to the fuhalol group. Magnusson et al. (2017) also used *Carpophyllum flexusoum* for the optimization of process parameters used during MAE for phlorotannins, but they also analyzed other macroalgal species commonly used for SLE. They confirmed that the extracts obtained using MAE contain twice the amount of polyphenols compared to the extracts obtained using SLE. Li et al. (2012) have been analyzing the MAE extracts using IR spectral analysis and RP-HPLC and they reported

that the purification of the extracts using ethyl acetate and water enriched the phenolic compounds. Also, they determined the correlation between the TPC and antioxidant activity, and the HPLC analysis demonstrated that phenolic compounds with medium polarity were the major contributors to the antioxidant activity. The examined potential of phlorotannins as natural antioxidants confirms the fact that they are thermally stable and that they have high antioxidant activity. This was confirmed in the research by Zhang et al. (2018), where they showed that algal phlorotannins from *Carpophyllum flexuosum* have great potential for replacing ascorbic acid as natural antioxidants. MAE can be a superior method for the extraction of phenolic compounds from seaweed because the antioxidant activity of MAE extracts was higher compared to the activity of the extracts obtained using conventional methods (Yuan et al., 2018).

2.2. The Analysis of Phenolic Compounds

In the last two decades of the previous century, algae have become the center of attention for many scientists due to the bioactive compounds which can be isolated from them. In the 1950s, algae were used as food and in traditional and folk medicine, especially in China. Nowadays, studies have shown that algae are considered as a valuable source of compounds with many beneficial effects. The most popular algae used for the treatment of diseases such as hyperlipidemia, cancer, atherosclerosis and hypertension are the brown algae *Sargassum* with four main species: *S. fusiforme, S. pallidum, S. horneri* and *S. thunbergii*. Antioxidant activity is one of the most investigated properties of the compounds isolated from marine macroalgae. It is known that these compounds serve as protectors against various diseases, including skin diseases and cancer, by quenching free radicals (Korzeniowska et al., 2018).

As we already mentioned, polyphenols exhibit a broad spectrum of bioactivity. For instance, Holdt and Kraan (2011) reviewed the potential of macroalgal polyphenols for the application in various industries, but a special type of macroalgal-derived polyphenols is attracting special attention from scientists. Phlorotannins, as unique polyphenolic compounds, found only in brown macroalgae, exhibit varied beneficial biological activity. Several reviews that contain information about phlorotannins as agents against breast cancer (Padua et al., 2015), about their antidiabetic functions with no after-effects (Murugan et al., 2015), about the prevention of cardiovascular diseases caused by hypertension, and about the analysis of obesity and diabetes in both *in vivo* and *in vitro* mechanisms are available (Gomez-Guzman et al., 2018). Li et al. (2011) reviewed phlorotannins derived only from brown macroalgae with all the potential health benefits, such as antioxidant activity, enzyme inhibitory effect, bactericidal effect, anti-HIV, anticancer, radioprotective, anti-allergic and other biological activity. Also, they mentioned the bioavailability of marine-algal derived polyphenols, with the conclusion that further investigation with human subjects is needed because bioavailability was studied only on mouse model systems. Similarly, Thomas and Kim (2011) reviewed many potential pharmacological applications of phlorotannins isolated from brown algae. They suggested that it is important to screen phlorotannins from other brown algal species to provide more information on the interactions with human cellular systems.

Phlorotannins and their biological activity are presented in the following section due to the importance of these specific compounds and their potential benefits for human health.

Table 2. The extraction of polyphenols from marine macroalgae using novel extraction methods (supercritical CO2 extraction, subcritical water extraction, ultrasound-assisted extraction and microwave-assisted extraction)

Macroalgae Species	Solvent	Analytical Method	Determined polyphenols	Bioactivity	Reference
Supercritical extraction with CO_2 (SC-CO_2)					
Undaria pinnatifida	SC-CO_2 with ethanol as co-solvent	-	Total polyphenol content	-	Roh et al., 2008
Saccharina japonica	SC-CO_2 with sunflower oil, soybean oil, canola oil, ethanol and water as co-solvent	Colorimetric method	Phlorotannins	-	Saravana et al., 2017
Saccharina japonica, Sargassum horneri	SC-CO_2 with ethanol as co-solvent	-	Total polyphenol content	Antioxidant	Sivagnanam et al., 2015
Sargassum muticum	SC-CO2with ethanol as co-solvent	NMR	Total polyphenol content	-	Tanniou et al., 2013
Ultrasound-assisted extraction (UAE)					
Hormosira banksii	70% aqueous ethanol solution	-	Total polyphenol content	Antioxidant	Dang et al., 2017
Ecklonia cava	Water, 50% aqueous methanol solution, 100% methanol	-	Total polyphenol content	Antioxidant	Lee et al., 2013
Laurencia obtuse	95% ethanol	-	Total polyphenol content	Antioxidant	Topuz et al., 2016
Ascophyllum nodosum	Water, 0.03 M HCl	Q-TOF-MS	Total polyphenol content, phlorotannins	Antioxidant	Kadam et al., 2015
Subcritical water extraction (SWE)					
Fucus serratus, Laminaria digitata, Codium fragile, Gracilaria gracilis	-	-	Total polyphenol content, phlorotannins	Antioxidant	Heffernan et al., 2014
Sargassum muticum	SWE+ enzymes	HPLC-DAD-MS/MS	Phlorotannins	Antioxidant	Sanchez-Camargo et al., 2016
Ascophyllum nodosum, Ulva intestinalis, Pelvetia canaliculata, Fucus spiralis	-	-	Total polyphenol content	Antioxidant	Tierney et al., 2012
Saccharina japonica	SWE+ ionic liquid solution	HPLC	Gallic, chlorogenic, gentisic, procatechuic, *p*-hydroxybenzoic, caffeic, vanillic, syringic acid	Antioxidant	Vo Dinh et al., 2017

Table 2. (Continued)

Macroalgae Species	Solvent	Analytical Method	Determined polyphenols	Bioactivity	Reference
Microwave-assisted extraction (MAE)					
Caulerpa racemosa	20-100% ethanol	IR spectral analysis, RP-HPLC	Total polyphenol content	Antioxidant	Li et al., 2012
Enteromorpha prolifera	10-60% ethanol	-	Total polyphenol content	-	Lou et al., 2010
Carpophyllum flexuosum	H_2O, acetone, ethanol, propan-1-ol, ethyl acetate	-	Total polyphenol content	-	Magnusson et al., 2017
Ascophyllum nodosum, Laminaria japonica, Lessonia trabeculate, Lessonia nigrecens	70% methanol	HPLC-DAD-ESI-MS	Total polyphenol content, hydroxybenzoic acid, p-coumaric acid, dyhydroxybenzoic acid, phlorotannin hexamers and dimers, epigallocatchin	Antioxidant, anti-hyperglycemic, pancreatic lipase and tyrosinase inhibition	Yuan et al., 2018
Carpophyllum flexuosum, Carpophyllum plumosum, Ecklonia radiata	Water	NMR, HPLC-DAD-ESI-MS	Phlorotannins	Antioxidant	Zhang et al., 2018

2.3. Phlorotannins as Biologically Active Agents

Many scientists analyzed phlorotannins from various macroalgal species, but as demonstrated in Table 3, brown algae from the genus *Ecklonia* showed the highest potential for the isolation of biologically active phlorotannins with phloroglucinol, eckol and dieckol, as the most abundant phlorotannins in these macroalgal species. Ahn et al. (2007) purified these three phlorotannins from *Ecklonia cava* and tested them for both scavenging capacities on some radicals such as alkyl, DPPH, hydroxyl and the superoxide anion radical, as well as for their protective effect against H_2O_2-induced DNA damage. Eckol exhibited the highest potential for radical scavenging among the tested phlorotannins, but all three phlorotannins displayed strong antioxidant activity. The DNA damage inhibition activity of eckol was higher than that of phloroglucinol and dieckol, but these two phlorotannins also displayed significant inhibitory activity against H_2O_2-induced DNA damage. This study claims that all of the tested phlorotannins from *Ecklonia cava* have the potential for being used as effective antioxidants. Heo et al. (2009) showed the whitening ability of the three above mentioned phlorotannins through their inhibition of melanin synthesis, where dieckol showed the highest potential, but it was still lower than that of the commercial whitening agent retinol. However, it was higher than that of kojic acid. Also, they investigated the photo-protective effect against cell damage induced by UV-B radiation, where dieckol was the most active among the three investigated phlorotannins. Dieckol is a hexamer of phloroglucinol and due to more functional hydroxyl groups present in its structure, it can be applied in cosmeceutical and pharmaceutical industries as a natural compound for reducing UV-B radiation cell damage and for the inhibition of melanogenesis.

Cho et al. (2012) also studied *Ecklonia cava*, but they investigated the neurological activity of the phlorotannins found in this algae. According to the HPLC analysis, it was shown that the most abundant phlorotannins were phloroglucinol, eckol, eckostonol, triphlorethol-A and dieckol. They investigated the possible use of these phlorotannins for hypnotic effects and for inducing sleep through the binding affinity to the $GABA_A$-BZD receptors *in vivo* using animal models. Dieckol, the main phlorotannin found in *Ecklonia cava*, showed the highest binding affinity, followed by eckol. Their binding activities were similar to those of flavonoids from terrestrial plants. The phlorotannins from *Ecklonia cava* were also studied for potential anti-proliferative activity and anti-allergic activity. Firstly, their anti-proliferative activity was investigated by Kong et al. (2009). They isolated two phloroglucinol derivatives from *Ecklonia cava*; dioxinodehydroeckol and 1-(3',5'-dihydroxyphenoxy)-7-(2'',4'',6- trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin. Several tests were performed to show the inhibition of cell proliferation in human breast cancer cells, as well as the induction of apoptosis, which is known as a promising strategy for cancer prevention. The results showed that dioxinodehydroeckol can potentially have anti-proliferative effects, as well as affect the induction of apoptosis, and it is more effective against MCF-7 human breast cancer cells. This phloroglucinol derivative has great potential for being used as an inhibitor of breast cancer. In addition, it is known that *Ecklonia cava* is used in Korea and Japan as a folk medicine against allergies. Consequently, Li et al. (2008) wanted to show which compounds contribute the most to this activity and they came up with the conclusion that phlorotannins are the main contributors to the anti-allergic effects of this alga. They isolated fucodiphloroethol G, phlorofuco-furoeckol A and eckol, and the first two phlorotannins exhibited strong inhibitory effects. Due to the different structure among

phlorotannins, there were differences in inhibition, because of the linkages between phenol units and the number of hydroxyl groups. They suggested that fucodiphloroethol G and phlorofucofuroeckol A can be implemented in pharmaceuticals but further investigations must be carried out to determine the inhibition of mechanisms.

Table 3. Bioactivity of different phlorotannins

Macroalgae species	Phlorotannins	Bioactivity	Reference
Ecklonia cava	Phloroglucinol, eckol, dieckol	Antioxidant	Ahn et al., 2007
Fucus guiryi, *Fucus serratus,* *Fucus spiralis,* *Fucus vesiculosus*	Total phlorotannin content	Anti-inflammatory	Barbosa et al., 2017
Fucus guiryi, *Fucus serratus,* *Fucus spiralis,* *Fucus vesiculosus*	Total phlorotannin content	Anti-allergic	Barbosa et al., 2018
Ecklonia cava	Phloroglucinol, eckol, eckostonol, triphlorethol-A, dieckol	Neurological	Cho et al., 2012
Cystoseira nodicaulis, *Cystoseira usneoides,* *Cystoseira tamariscifolia,* *Fucus spiralis*	Fucophloroethol, fucodiphloroethol, fucotriphloroethol, 7-phloroeckol, phlorofucofuroeckol, dieckol	Antioxidant, anti-hyaluronidase	Ferreres et al., 2012
Ecklonia cava	Phloroglucinol, eckol, dieckol	Inhibition of melanogenesis, photo-protective effect	Heo et al., 2009
Ecklonia stolonifera	Eckostonol, eckol, phlorofucofuroeckol A, dieckol, 8,8'-bieckol	Antioxidant, α-glucosidase inhibition	Iwai, 2008
Ecklonia stolonifera	Phlorofucofuroeckol A, dieckol, dioxinodehydroeckol	Antioxidant, anti-inflammatory	Kim et al., 2009
Ecklonia cava	dioxinodehydroeckol, 1-(3',5'-dihydroxyphenoxy)-7-(2'',4'',6-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin	Anticancer	Kong et al., 2009
Ecklonia cava	Fucodiphloroethol G, phlorofucofuroeckol A, eckol	Anti-allergic	Li et al., 2008
Ecklonia kurome	Phloroglucinol, eckol, phlorofucofuroeckol A, dieckol, 8,8-bieckol	Bactericidal	Nagayama et al., 2002.

Aside from *Ecklonia cava, Ecklonia stolonifera* was also investigated due to its bioactive compounds. Iwai (2008) investigated the potential of macroalgal polyphenols in *Ecklonia stolonifera* for α-glucosidase inhibitory activity *in vitro*. According to the LC/MS analysis, it was assumed that phlorotannins (eckostonol, eckol, phlorofucofuroeckol A, dieckol and 8,8'-bieckol) were responsible for the antioxidant activity and the α-glucosidase inhibitory

activity. It was concluded that *Ecklonia stolonifera* and its polyphenols can be used as antidiabetic pharmaceuticals. Kim et al. (2009) isolated three phlorotannins from *Ecklonia stolonifera:* phlorofucofuroeckol A, dieckol and dioxinodehydroeckol and they found out that they possess strong antioxidant activity against DPPH radicals, while phlorofucofuroeckol A and dieckol showed inhibitory activity against the production of intracellular ROS. They also tested isolated phlorotannins for anti-inflammatory activity and only phlorofucofuroeckol A exhibited this effect.

Food-borne bacteria and MRSA are still present and they represent a problem in the world due to the infections which they can cause. Plant extracts and tea catechins were used for the prevention of these infections, but Nagayama et al. (2002) showed that macroalgae exhibit great potential for use against these infections. They obtained five purified phlorotannins: phloroglucinol, eckol, phlorofucofuroeckol A, dieckol and 8,8-bieckol, from *Ecklonia kurome* and tested them for bactericidal effects on pathogenic bacteria. The results showed that all phlorotannins, except for phloroglucinol, exhibited antibactericidal activity against all the strains tested. Also, it was shown that these effects increased with the polymerization of phloroglucinol.

Lopes et al. (2018) showed that phlorotannins of the *Fucus* genus are of lower molecular weight, which leads to their possible application as natural antioxidants due to the fact that these phlorotannins exhibit higher antioxidant activity compared to higher molecular weight phlorotannins. Barbosa et al. (2017) determined that phlorotannins of the *Fucus* species have potential to be anti-inflammatory agents due to the effective inhibition of lipoxygenase. Continued investigations on *Fucus* species showed that phlorotannin extracts can reduce the degree of immune cell degranulation and counteract the enyzme systems (hyaluronidase, β-hexosaminidase) which are correlated with allergic diseases (Barbosa et al., 2018). The authors suggested that phlorotannins are multi-target agents in the prevention and reduction of allergic symptoms. Ferreres et al. (2012) determined that *Fucus spiralis* contained a greater amount of higher molecular weight phlorotannins and, consequently, showed the strongest lipid peroxidation inhibitory activity, as well as hyaluronidase inhibition. *Cystoseira nodicaulis* showed the best superoxide scavenging capacity due to the presence of lower molecular weight phlorotannins.

CONCLUSION

This chapter has shown that macroalgae, aside from their use as food, can serve as functional ingredients in food products and that their bioactive compounds can be utilized in cosmeceutical and pharmaceutical products.

The main contributors to the antioxidant activity are phenolic compounds, which are produced in the algae under stress conditions. Nevertheless, the potential of phenolic compounds is beyond their antioxidant activity. Studies have shown that they can be great anti-inflammatory, anti-allergic and antiproliferative agents. These abilities were discovered in phlorotannins, a specific group of macroalgal phenolic compounds found only in brown macroalgae. Since brown algae and phlorotannins have great potential for technological use, scientists have been investigating a wide range of macroalgal species. But, in order to obtain the maximum amount of phlorotannins from macroalgal samples, it is very important to find

the appropriate extraction method. Due to the disadvantages of conventional methods (long time, large amounts of organic solvents, environmental pollution), the novel green extraction technologies are desirable for obtaining bioactive compounds without the traces of the toxic solvent used. Even though SC-CO_2 is mostly used for non-polar compounds, several authors showed that it can also be used for the extraction of phenols, but in a much smaller amount. UAE, SWE and MAE have been the appropriate methods for obtaining polar compounds such as phenols, but the solvents must be chosen wisely. There is little available literature on the topic of extraction of macroalgal polyphenols using modern extraction methods, especially UAE and SWE. MAE showed the most potential for implementation in the industrial isolation of macroalgal phenolic compounds.

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Chapter 106

THE IDENTIFICATION, CHARACTERISTIC FEATURE AND ROLE OF BURROW (NEOICHNOLOGICAL) STRUCTURE IN BIOTURBATION ACTIVITIES OF OCYPODOID AND GRAPSOID CRABS OF PAKISTAN

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ABSTRACT

Burrows constructed by semi-terrestrial crabs affect the abiotic and biotic environmental functioning of the marine ecosystems. These burrows constructed in intertidal areas and protect amphibious crabs from predators, harsh environments and provide molting areas as well as reproductive sites. Understanding about burrow construction and burrowing behavior helps to understand the several aspects of marine ecological system. In turn, the bioturbation activities provide benefits to the surrounding environment by sediment turnover, grain size modification, nutrient recycling and penetration of oxygen into deep down that enhances the microbial activities in the deeper sedimentary layers.

Several environmental factors (temperature, salinity, organic matter, grain size, metal ion) effect density as well as distribution of burrowing crabs e.g., during extreme temperature (low or high) crab digs deep burrows and remain inactive in their burrow to protect them self from desiccation. These studies showed that substrate type, species diversity, stem and root density, predator, seasons, mate display activity affect the zonal and spatial distribution as well as the diameter of a burrow in semi-terrestrial crabs.

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Studies showed that the sediment texture (grain size) is an important factor in the distribution of different crab species. e.g., different species of *Uca* occupy sandy substrate while sentinel crabs (*M. depressus*) occupy muddy and sandy environment. Organic matter content in sediment is also a main contributor of crab distribution. Finer particle size retains more organic matter than coarse sand. Studies showed that within the sandy substrate, burrow density decreases with the increase of particle size as a coarse particle size retain less organic content; therefore the burrow construction and maintenance is also difficult. The study showed that the number of burrow density exceeds than the crab density; that increase the chance to escape from the predator. In addition, the diameter of the crabs burrow also helpful to determine the size of the resident crab as the study based on carapace length (CL) and burrow diameter (BD) showed a significant linear relationship. In general; studies on burrow morphology also showed the variation in shape, size as different species of *Uca* formed vertical burrows with single or branched shaft as well as single or multiple entrance. In sentinel crabs, *M. depressus*, the burrow cast shape were horizontally elongated with a single shaft. It was also observed that the species of *Uca* and *Ocypode* which occupies the high tide zone forms deeper burrows (to avoid extreme environmental condition) as compared to low to mid tide zone species. Ocypodoid crabs also construct a variety of earthen structure (e.g., chimney, barricades, shelter, pyramid, hoods, mud balls and mounds) by using mud or sand. The main purpose of these structures likely to convey the signals to other members of their population, protection from predators, conspecific competitor or to rear offspring as well as decrease the chance of intrusion by wandering crabs.

Keywords:burrow, ocypodoid crabs, substrate, seasonality, niche, earthen structure, sematectonic, anthropogenic effect

1. INTRODUCTION

Burrow considered as the fundamental structure of semi terrestrial crab's life. Digging of burrows begins, when the megalopa start their lives as crab (Hyman, 1920; 1922; Herrnkind, 1968a, b). A variety of burrowing crabs dominated in coastal areas of the world and considered as an important component of the macrobenthic fauna. These crabs distributed in the wide range of substratum along the estuarine and marine environment and actively construct their burrows in the intertidal zone from coarse beach sand to fine clay-rich marshy mud at low tide as well as at high tide area. The principal burrowing behavior of these crabs has been described by various authors (Dembowski, 1926; Altevogt, 1955; Crane, 1975; Katz, 1980; Noman & Pennings, 1998; Saher, 2008) and these burrowing crabs mostly belong to the two super families Ocypodoidea and Grapsoidea.

The burrowing mangrove crabs are important grazers of bacteria and microalgae (Bouillon et al., 2002; Reinsel, 2004); sorted from sediments through specialized structures (maxilliped and setae) present in their mouth cavity and form small irregular balls (feeding pallets ≤3 mm) of retained material (Penha-Lopes et al., 2009). The sub-surface sediment pallets (burrowing pallets) are used to form mounds (diameter ranges from 5 to 7 mm) near the burrow opening of fiddler crabs (Botto & Iribarne, 2000). These crabs stimulate the microbial metabolism, organic matter degradation and nutrient cycling by their bioturbation activities as well as they are also responsible for the floral productivity (Nielsen et al., 2003; McHenga & Tsuchiya, 2008).

The burrowing crabs of sandy coast construct deep and complex burrows may be as deep as 2m (George, 1982) which provide shelter against climatic extremes, predators and also serve as the sanctuary during molting and motherliness (Chan et al., 2006; Lucreziet al., 2009). Burrow construction is primarily an occupation of daylight hours; therefore, it is important to define the micro-environment of burrows as different types of organisms increase the rate of sedimentation. There is a mixture of different features of the environment, such as energy conditions under which the burrowers increase the rate of sedimentation and compactions of sediments that can be determined from a significant analysis of the burrow structural morphology (Richter, 1931; Charkarbarti,1981). Burrower crabs may also determine the concerns, includes the relationship between crab size and opening diameter as well as burrow depth, likewise the relationship between physical environment and crab behavior can be known (Reinech, 1967; Charkarbarti, 1981).

2. BIOTURBATION ROLE AND ECOLOGICAL SIGNIFICANCE OF BURROWS AND BURROWING

In mangrove area, the benthic fauna are typically dominated by burrowing decapods and more than 80% of crabs belong to superfamily Ocypodoidea and Grapsoidea (Rosenberg, 2001; Saher, 2008; Saher et al., 2018). These crabs constitute a dense population on intertidal mudflats and in the mangrove canopy (Skov & Hartnoll 2001; Skov et al., 2002), In addition, they are important grazers of microalgae and bacteria (Reinsel, 2004). Burrowing crabs have been considered as ecosystem engineers due to their conspicuous role in the various processes of the mudflat ecosystem through biotic and abiotic activities (Jones et al., 1994; Kristensen, 2008; Saher & Qureshi, 2012; Saher et al., 2018). The crawling, foraging, burrow construction and maintenance activities of burrows results to increase sediment drainage, soil redox potential, translocation of sediment (organic matter and nutrients), change in sediment erosion threshold and increase the sediment area (Boto & Iribarne, 2000; Kristensen, 2008). In result, they are responsible for the stimulation of microbial metabolism, organic matter degradation, nutrient cycling and floral productivity (Kristensen & Alongi, 2006). These activities have an important effect on the sediment characteristics includes sediment chemistry (Katz, 1980; Montague, 1980; Bertness & Miller,1984; Bertness, 1985; Genoni, 1991; Noman & Pennings, 1998), burrows of crabs facilitate the general aeration of the sediments (Genoni, 1991; Noman & Pennings, 1998). These activities may be affected by anthropogenic eutrophication (McHenga & Tsuchiya, 2008).

The burrows have been attributed for the numerous functions (Figure 1) as are recognized to provide a microhabitat for a community of organisms (Bright & Hogue, 1972; Nielsen et al., 2003; Kristensen & Alongi, 2006) and also known to enhance the movement of tidal waters through sediments thus oxygenating and exchanging nutrients and metabolites that likely enhance the sediment stability (Hoffman et al., 1984; Bertness, 1985; Micheli et al., 1991) as well as increase the rate of the decomposition of plant debris within the sediments (Bell et al., 1978; Valiela et al., 1974; Howarth & Hobbie, 1982; Hoffman et al., 1984; Robertson, 1986; Lee, 1998; McHenga & Tsuchiya, 2008; Saher & Qureshi, 2012; Mokhtari et al., 2016). These burrows also act as an oxygen inlet tube and increase the depth to which oxygen can penetrate or enhance sediment oxygen level (Katz, 1980; Bertness, 1985).

Various authors (Katz, 1980; Montague, 1980; Bertness & Miller, 1984; Bertness, 1985; Genoni, 1991; Noman & Pennings, 1998) have previously studied the general effects of crab burrows on pore water chemistry. The burrows are also speculated to act as an outlet tube for toxins such as sulphide and as well as reducing the accumulation of metabolic products in the sediments (Katz, 1980; Bertness & Miller, 1984). They increase the amount of nutrients and decrease the sulfide concentration in the sediment and contribute to the secondary production like affect the mangrove productivity (Smith et al., 1991; Lee, 1997; Otani et al., 2016). As these factors are very important for the growth of micro- and meio-organisms and likely the overall turnover of organic matter; such faunal mediated disturbances of the physical, chemical and biological structure of sediment systems are known as 'bioturbation'. The bioturbation and fecal pellet production of crabs have been widely known and resulting is an increase in the growth of the cord grass, *Spartina alterniflora* in salt marshes (Montague, 1980; Bertness & Miller, 1984; Bertness, 1985).

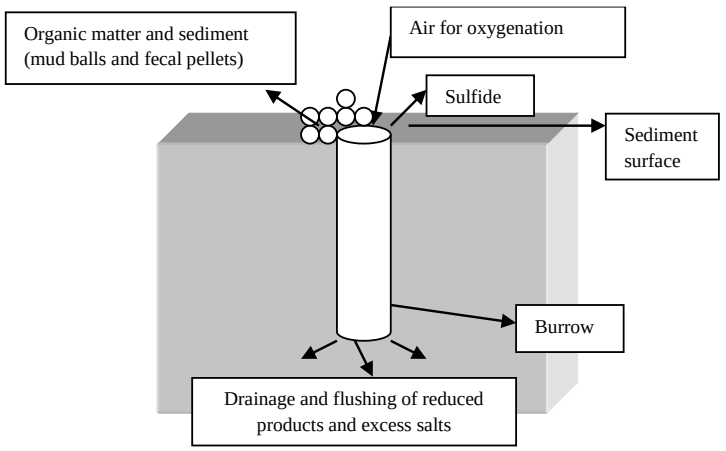

Figure 1. Conceptual diagram of the function attributed to the burrow of crab.

The burrowing activity modifies the grain size in addition to altering the geochemical properties (Warren & Underwood, 1986; Katz, 1980; Wolfrath, 1992; Morrisey et al., 1999; Saher et al., 2016; Mokhtari et al., 2016). These activities enhance the carbon flow in the food chain that may later reach back to the crabs and may also serve other unidentified functions (Genoni, 1991; Otani et al., 2016). Burrows are important in the crab's life as attributes to a number of functions, mainly in order to avoid an excessive wave action, relief from both (hot and cold) ambient temperature and desiccation, provide a refuge from the aerial and terrestrial predators during exposed periods as well as aquatic and terrestrial predators during flooding and ebbing accordingly (Hyman, 1922; Wilkens & Fingerman, 1965; Smith & Miller, 1973; Crane, 1975; Power, 1975; Powers & Cole, 1976; Hyatt & Salmon, 1979; Katz, 1980; Christy, 1982; Thurman, 1984a; Bertness & Miller, 1984; Genoni, 1991; Lim & Diong, 2003; Lim, 2006). These biogenic structures also provide the neoichnological interpretations that support, associate and compare the traces produced by the fossil organisms (paleoichnology) with the current information (neoichnology) for a better understanding of the paleoclimatic conditions, limiting factors of paleoenvironment as well as paleoecology of benthic organisms from the coastal areas.

3. ENVIRONMENTAL FACTORS AFFECTING ON BURROW DENSITY AND DISTRIBUTION

The environmental factors affect the physiological activities of crabs and determine their habitat selection as most of the species live in the diverse habitat of a collective dispersal coastal system that ultimately influenced by environmental inclines (Moore & Chapman, 1986) and ultimately effects on the density and distribution of crab burrows (Mouton & Felder, 1996; Takeda & Kurihara, 1987; Ashton et al., 2003; Saher et al., 2016; Saher et al., 2018). The distribution of Ocypodoid species along the coastal belt is solely dependent on sediment texture (burrow construction), which may be influenced by various physical and chemical factors, including temperature, salinity, vegetation, organics, metal ions and granular size and composition, etc. (Lighter, 1977; Barrass, 1963, Alberto & Fontoura, 1999; Turra et al., 2005; Chan et al., 2006; lucrezi et al., 2009; Saher & Qureshi, 2012). A number of studies have been conducted to determine the inter relationship of burrows, burrowing activity and previously described the physicochemical factors (Table 1).

Table 1. The previous studies conducted on the crab burrows and burrowing activity and their interaction with various physicochemical parameters

Factors affecting crab burrows	Species	References
Temperature, Salinity, organic content	*Paratylo diplax blephariskios*	Owen et al. (2000)
Geochemistry of iron (reduction and oxidation)	*Ucides cordatus* and *Ucamaracoani*	Araújo et al. (2012)
Exchange of organic matter	*Neohelice granulate*	Casariego et al. (2011)
Substrate variations	*Austruca annulipes* and *A. vocans*	Lim et al.(2005)
Different mangrove species	*U. cordatus*	Wunderlich & Pinheiro (2013)
Sediment characteristics (percent organic composition) and vegetation cover	*A. annulipes, A. iranica* and *A. sindensis*)	Qureshi &Saher (2012)
Substratum properties	*A. sindensis*	Saher & Qureshi, 2012
Physico-chemical properties of sediments	*A. rosea, A. triangularis*	Sen et al. (2014)
Environmental heterogeneities	Crab burrows	Wang et al. (2009); Wang et al. (2015)
Substrate penetrability	*Chasmagnathus granulata* and *A. uruguayensis*	Botto & Iribarne (2000)
Acid volatile Sulphide and FeS	*H. crassa*	Morrisey et al. (1999)
Denitrification and organic matter	*C. granulatus*	Fanjul et al. (2007)
Effect of vegetation type and coverage	Different crab species	Smith et al. (2009), Lim &Rosiah (2007), Lim & Heng (2007), Chen et al. 2007, Wang et al. (2014), Li et al., 2015
Substratum characteristics	*A. spinicarpa* and *A. longisignalis*	Mouton & Felder (1996)
Erodibility of sediments	Crab burrows	Needham et al. (2013)
Biotope heterogeneity	*A. tangeri*	Wolfrath (1992)
Sediment characteristics	*Austruca* distribution	Checon & Costa (2017)
Reduced iron (Fe II) and redox potential	*A. triangularis, A. rosea, A. forcipata* and *A. paradussumieri*	Mokhtari et al. (2016)
Nutrients cycling, sediment properties	Crabs	Wang et al. (2010)
Temperature variations	*A. pugilator*	Mat et al. (2017)
Chemical analysis of the sediment and pore water	*C. granulatus*	Fanjul et. al. (2007)

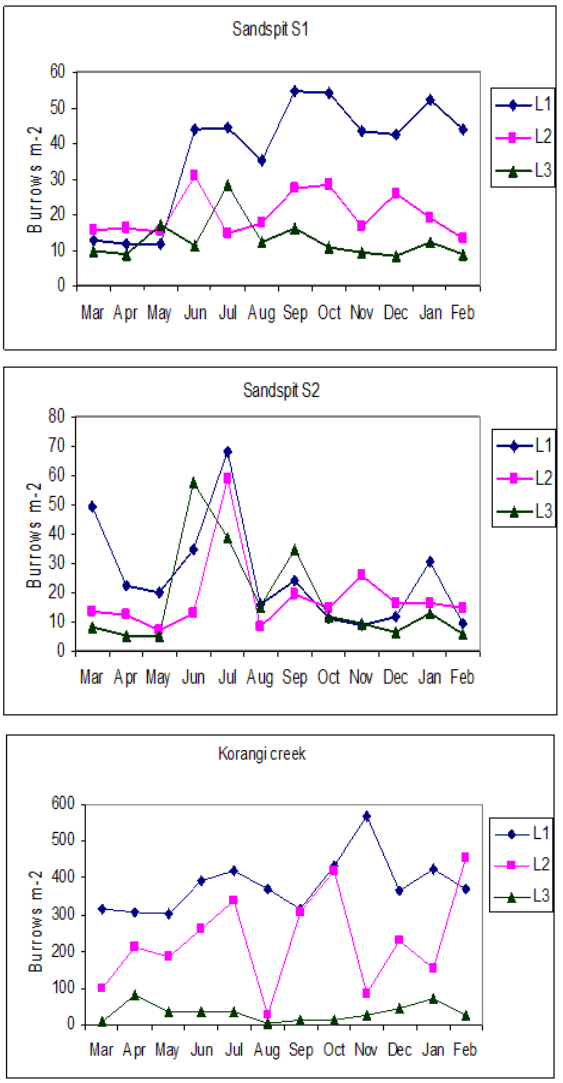

Figure 2a. Monthly distributions of burrow density (m-2) at three levels (L1: low tidal level, L2: mid tidal level, L3: high tidal level) of Sandspit backwater mangrove area (S1 and S2) and Korangi creek.

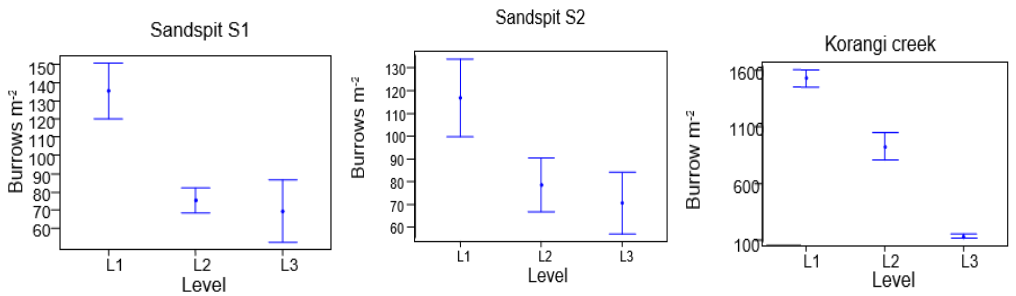

Figure 2b. Distribution of burrows (m^{-2}) at three levels (L1 = low tidal level, L2 = mid tidal level, L3 = high tidal level) at two stations of the Sandspit backwater mangrove area (S1 and S2) and Korangi Creek during March 2001 to February 2002 (Error bars indicate $\pm$ 1 SE).

The burrow openings of the sandy crabs are circular with accumulated sand mounds and are often surrounded by radiating feeding lines left by the crabs (Takahashi, 1932; Chakrabarti, 1981; Chan et al., 2006). There are clearly visible entrances of the sand crab on the surface of sandy beaches that maintained as territory (Vannini, 1976) by counting these holes, that can help to determine the densities of crab (Moss & McPhee, 2006; lucrezi, 2009). The inter-relationship based on the burrow structure, surrounding environment and correlation between them also helps to understand the different aspects of the burrows and physicochemical parameters. The study was conducted throughout the year from three coastal sites (two sites in Sandspit backwaters and one in Korangi creek) and once or twice visited sites (Bhambore, Dhabeji, Ketibunder, Sonmiani, Phitti creek, Manora, Sonari and Bhaira). The monthly burrows data (burrow density and burrow diameter) were collected by counting the total number of burrows present in each 0.25 m^2 quadrat at the three tidal level (from low tide mark to high tide mark in two transect (20 m apart) and the distance of levels (25 meters to 40 meters; depends on the exposed mudflat area during low tide period) at each station of all study sites. The sizes of the burrow opening (burrow diameter N = 10) were measured randomly by Vernier calipers (correction 0.01 mm) within each quadrat.

The monthly variations were observed in the crab burrow density at three tidal levels during each month from the Sandspit (S1 and S2) areas as well as Korangi creek (Figure 2a). The number of the burrows was higher at KC ranged from 16 to 2376 burrows m^{-2} and were in low density at Sandspit ranged from 12 to 376 burrows m^{-2} (Table 2). The density of crabs can be reduced by human disturbance and the burrow density of crabs has been proposed as an indicator of recreational impacts on sandy shores (Barros, 2001).

Table 2. Seasonal and tidal distribution of burrow density(N m^{-2}) from Sandspit backwater mangrove areas (S1 & S2) and Korangi creek mangrove area during study period

Tidal levels	Seasons	N	Sandspit (S1)	Sandspit (S2)	Korangi Creek
Low TL	PRM	6	47.3 ± 20.15	62.0 ± 22.16	1235.3 ± 138.2
	SWM	6	164.7 ± 56.8	83.3 ± 41.5	1578.0 ± 372.0
	POM	6	145.3 ± 82.5	78.7 ± 32.2	1753.0 ± 488.0
	NEM	6	184.7 ± 36.9	77.3 ± 37.2	1547.0 ± 306.0
Mid TL	PRM	6	48.0 ± 30.7	122.7 ± 75.3	658.0 ± 278.0
	SWM	6	140.7 ± 149.9	159.3 ± 104.3	840.0 ± 636.0
	POM	6	49.3 ± 23.96	116.0 ± 83.9	1084.0 ± 741.0
	NEM	6	39.3 ± 12.50	69.3 ± 64.8	1122.0 ± 576.0
High TL	PRM	6	44.0 ± 15.4	25.3 ± 11.2	167.3 ± 163.4
	SWM	6	107.3 ± 104.7	149.3 ± 77.1	102.7 ± 71.0
	POM	6	98.7 ± 27.1	73.3 ± 52.3	79.3 ± 30.2
	NEM	6	63.3 ± 13.3	34.0 ± 17.1	187.3 ± 103.6

The highest number of crab burrows observed at low tidal level (L1) at all stations (Figure 2b) in comparison with mid and high tide level. The temperature patterns explain the several modifications in the activity patterns and temporal niche shifts of crabs (Hut et al., 2012). To regulate their body temperature, the crabs use three principal mechanisms (1) evaporation of water for cooling down the temperature; (2) remaining inactive inside their burrows during the extreme temperature conditions and (3) by an acclimatization process through metabolism either by increasing respiratory enzyme activity or by oxygen

consumption (Vernberg & Vernberg, 1968; Berry 1976; Eshky et al., 1988; Weinstein et al., 1994; Strachan et al., 1999; Lucrezi & Schlacher, 2014). The seasonal variations were also observed in the burrow density as being highest during the Southwest monsoon season in Korangi creek and two stations of Sandspit. The lowest number of burrows were found in pre-monsoon season at S1 of Sandspit and in Northeast monsoon seasons at Korangi creek and S2 of Sandspit (Table 2). The CRD-ANOVA with the nested treatment showed that seasons nested within stations and levels nested between stations and seasons showed the significant differences at Sandspit ($P < 0.00$, $F = 5.87$; $P > 0.00$, $F = 2.85$, respectively). In Korangi creek level nested within seasons showed significant differences ($F = 19.23$). The crabs can tolerate a wide range of temperature outside their burrows ranging from 12 to 50ºC and the crabs become inactive as well as remain inside their burrows, if the temperature rise or decline (seasonal shifting) from the tolerant range (Lucrezi & Schlacher, 2014).

Recruitment (Strachan et al., 1999), winter dispersion (seasonal variation) and growth (Wolcott, 1978) may effect density of crab as well as their burrows. Beach type and exposure appear to impact density (Chan et al., 2006) as well as affect the zonal distribution (Jaramillo et al., 2000). Indeed, during storms the zonal distributions can shift landwards (Alberto & Fontoura, 1999; Hobbs et al., 2008).

There was a marked spatial variation ($F = 5.76$, $p < 0.001$) in density of crabs burrows m^{-2} were recorded from the coastal areas of Pakistan. The highest (722 ± 706) mean burrows density m^{-2} was observed at Sonmiani and the lowest (36.7 ± 14.6) mean burrows m^{-2} were at the Phitti creek (Table 3). Different sediment types and factors other than sediment particle size and organic content may play a significant role to determining the colonization of these burrowing crab species. These results clearly demonstrate the importance and interaction of substratum characteristics in controlling the distribution and abundance of crab and has interesting implication on the interspecific dynamics of the different species population at all sites. These crabs are known to adjust their burrowing activities to a variety of conditions, such as stem density, root mat density, substratum, water, ground temperature, tidal and diurnal rhythms, reproductive activity, the threat of potential predators, seasonal activities and mate display activities (Zucker, 1974; Ringold, 1979; Bertness, 1985; Genoni, 1991).

Table 3. Summary of descriptive statistics of burrows density (m^{-2}) from once visited collection sites during the study period

Sites	N	Mean	SD	Minimum	Maximum
Bhaira	3	91.7	37.5	55.0	130.0
Bhambore	6	674.0	496.0	112.0	1592.0
Dhabeji	3	57.7	24.3	36.0	84.0
KetiBunder	3	267.0	174.0	66.0	376.0
Korangi Creek	3	855.0	646.0	176.0	1462.0
Phitti Creek	5	115.2	148.7	28.0	378.0
Sandspit	6	69.7	36.8	34.0	140.0
Sonari	5	259.2	83.6	190.0	402.0
Sonmiani Bay	6	648.0	673.0	101.0	1966.0

The seasonal variations and zonal distribution were also evident in crabs burrow opening diameters (mm). The burrow diameter (BD) ranged from 4 to 14.6 mm at Korangi creek and 4.7 to 21.1 mm at Sandspit. Spatial variations in the size of the burrow diameter (BD) were

observed with small sized BD at low tide level (L1) and the largest at high tide level (L3) at all stations (Figure 3). The smallest mean BD (4.88 ± 0.59 mm) was observed at Korangi creek during Northeast monsoon season and the largest mean BD (12.6 ± 2.15 mm) was during pre-monsoon season at Sandspit S1. There were also a significant difference in BD between all the once visited sites (Table 4; F = 9.21). CRD-ANOVA with nested treatments showed a significant difference in the crab burrows diameter between stations (F = 25.48), seasons nested in stations (F = 8.94) and levels nested within stations and seasons (F = 2.23) at Sandspit. The mean BD ranged between 7.6 mm at Sonmiani and 14.7 mm at Dhabeji (Table 4).

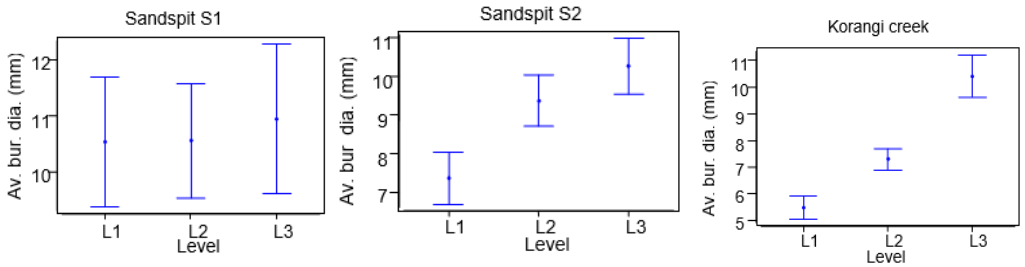

Figure 3. Distribution of average burrow diameter (mm) at Korangi creek and two station of Sandspit backwater mangrove area (Error bars indicate ± 1 SE).

Table 4. Summary of descriptive statistics of burrows diameter (mm) from once visited collection sites during the study period

Sites	N	Mean	SD	Minimum	Maximum
Bhaira	3	10.20	1.567	8.57	11.70
Bhambore	6	8.35	2.72	5.05	11.40
Dhabeji	3	13.96	1.17	13.01	15.26
KetiBunder	3	8.35	1.55	6.67	9.72
Korangi Creek	3	8.07	3.37	4.90	11.60
Phitti Creek	5	12.76	1.88	10.95	15.50
Sandspit	6	8.78	1.29	7.47	11.07
Sonari	5	9.16	0.87	8.00	10.37
Sonmiani Bay	6	8.04	1.80	6.10	10.67

Botto & Iribarne (2000) reported the higher moisture contents in sediments with the crabs as compared to the area devoid of crabs. According to Wang et al. (2009), the sediments of different micro habitat (such as slope, edge and bottom) have the different water holding capacity along the intertidal areas. Therefore, the burrow density of crabs decreases with the increase of water contents in sediments on the slope area. The flooding level also affect the sex ratio of *U. cordatus*, with a predominance of males in less-flooded mangroves, independently of the biological period and a gender balance in the more-flooded mangroves only during the breeding season. The results of previous studies suggested that the tree-species composition and tidal flooding level could have a significant effect on the habitat partitioning of sexes and sizes of the mangrove crab *U. cordatus* (Wunderlich & Pinheiro, 2013).

4. ROLE OF SUBSTRATUM IN MICROHABITAT SPECIFICATION

Spatial variation in sediment properties and heterogeneous characteristics of mangrove sediment create different niches for different species of Ocypodoid and Grapsoid crabs to perform their living activities. Some species prefer fine-grained sediments, while others tend to occupy the coarse sandy substrates. The main example included the fiddler crab species that occupy sandy sediments possess higher numbers of spoon tipped setae on their second maxilliped and these setae are believed to hold sand grains during feeding activity and aid in the removal and sorting organic particles (e.g., bacteria) from the grains of sand (Crane, 1975; Mokhtari et al., 2015). The environmental heterogeneities within the tidal creek microhabitats (such as bottom, slope, edge and flat) play a substantial role in distribution of crab burrows (Wang et al., 2009), Figure 4 illustrated the different intertidal habitat along the coastal belt of Pakistan. Sediment properties varied among the microhabitats, therefore; the crab diversity and distributions also varied among the habitats, but usually crab selected relatively solid sediments to build their burrows.

Figure 4. Habitat diversity and specification of different semi-terrestrial crabs from coastal areas of Pakistan.

The diversity of brachyuran crab considered as a potential indicator of the alterations of mangrove habitats. The age of the mangrove forest is also effected on the brachyuran crab diversity (Sen et al., 2014). The burrowing activities increase the substrate penetrability, for instance, *C. granulata* stabilizes the sediment by placing the fine and cohesive sediment on the surface; *A. uruguayensis* interrupts the surrounding sediments by pelletizing and increase their erodibility. The contrasting activities of these two species may also produce opposing and significant impacts on the structure of the benthic communities due to their impact on

sediment stability (Botto & Iribarne, 2000). As in temperate marshes, fiddler crabs can have significant ecological effects on mangrove communities, serving as ecological engineers by modulating the amount of resources available to marsh plants, and by altering the physical, chemical, and biological state of these soft sediment communities. The crab burrowing significantly increased the mangrove height by 27%, trunk diameter by 25% and leaf production by 15%, compared to mangroves in crab exclusion enclosures. Mangrove height, trunk diameter, and leaf production that variations in crab burrow density were positively associated with the number of crab burrows (Botto & Iribarne, 2000 Bezerra et al., 2006; Mokhtari et al., 2015).

4.1. Organic Matter

The presence of percent organic matter also accountable for the burrow density and crab distribution. The organic matter supply to the benthic environment and *in situ* the primary production is the fundamental determinants of deposit feeder production. The deposit feeder crab species extract their food in the form of organic content from the surface sediment and somewhat depend on the similar nutritional sources for their survival. They are important factor affecting the quantity and quality of the detrital food (Rice & Rhoads, 1989; Saher & Qureshi, 2012; Saher et al., 2018). Various studies showed the influence of organic matter on the presence of crabs along the coastal areas. For instance, organic matter seems higher in the crab dominance area as compared with nearby areas without crabs (Botto & Iribarne, 2000). However in another study, the movement of *Neohelice granulata* enhances the exchange of organic matter among habitats by faecal deposition between marsh and tidal flats with seasonal variations (Casariego et al., 2011).

4.2. Grain Size Composition

The sediment structure and composition determine the crab density. The current study also revealed the inter-relationship between the burrow properties with sedimentological properties (Figure 5a and b). In the current study, the burrow density and the total burrow opening area decreased with the increase in coarse sand (Saher et al., 2018) which suggested that the crabs do not construct burrows more efficiently in coarser sediment likely because this practice is energetically more expensive and time consuming (Grow, 1982). Alternatively, the burrow density increased as the increasing in mean grain size and fine sand (Saher et al., 2018). Finer sediments might provide a more stable substrate for the high burrow density and more complex burrow structure due to the cohesive nature of fine sediments (Takeda & Kurihara, 1987; Rudnick et al., 2005). Takeda & Kurihara (1987) also reported the depth of the water table and silt-clay content in the substratum largely determine the crab burrow distribution. The increase of soil surface area induced the crab burrows and significantly affects the biogeochemical processes by increasing the redox reactions and solute transport (Webb & Eyre, 2004; Wang et al., 2009). As discussed earlier, the burrows on the slope were smaller; however, the density was higher than that at the edge and on the flat. However, no crab burrows were found at the creek bottom. The density of small crab burrows (<10mm) was greater, but that of large burrows (>10mm) was lower in tidal creeks

than in non-creek habitats. Therefore, sediment properties showed a gradual transition from hydrophytic to terrestrial environments on the creek section that caused significant differences in burrow distribution among the microhabitats (Wang et al., 2009).

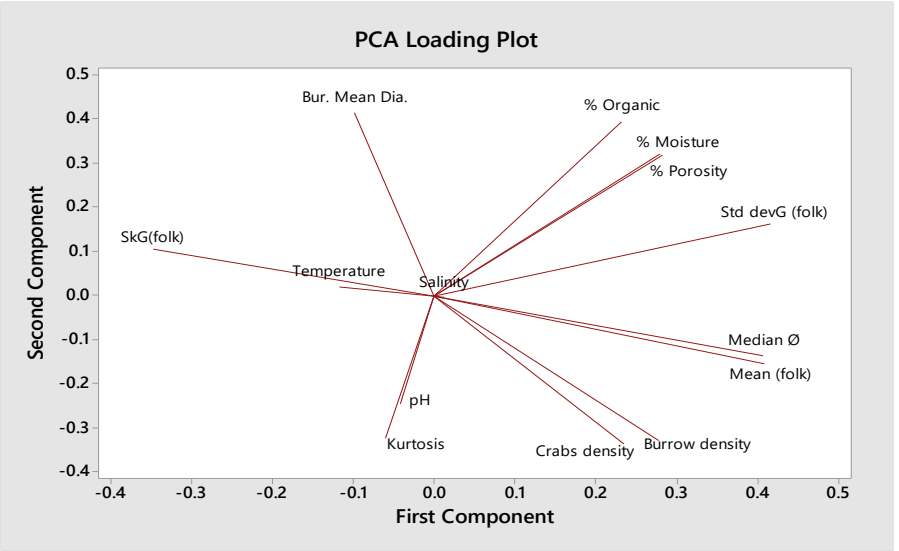

Figure 5a. Relationship of burrow properties with physicochemical properties from coastal environment of Pakistan.

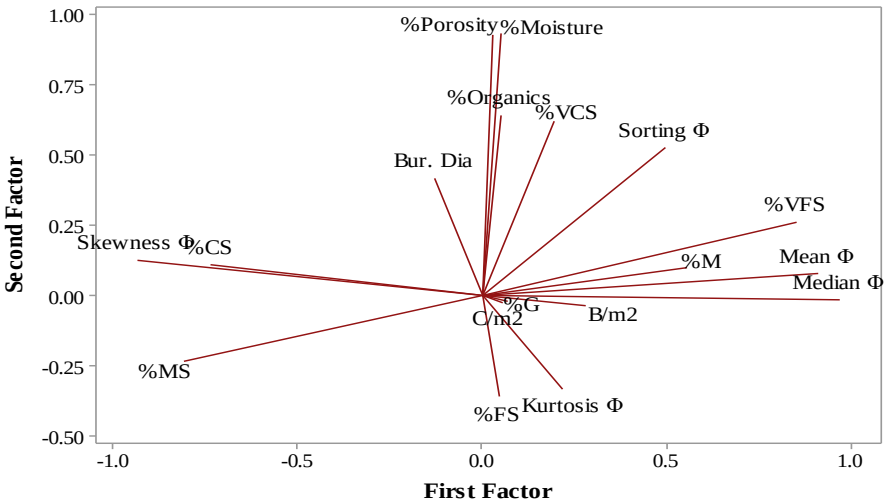

Figure 5b. Factor analysis for Burrow properties (density and diameter) with sedimentological properties from coastal environment of Pakistan.

The factorial analysis showed that the sediment properties i.e., mean, median and sorting coefficient are also important factors that effect the burrow density and distribution. The particle size as well as sorting coefficient were significantly related to abundance of the crab (*Paratylodiplax blephariskios*) as best explained crab distributions from two estuaries (St. Lucia and the Mhlathuze) of southern Africa (Owen et al., 2000). *A. annulipes* was

significantly associated with the sediments containing higher sand contents regardless of shore level, however, *T. vocans* was significantly associated with higher mud content sediments, and therefore, *A. annulipes* are generally found in sandier habitats than *T. vocans* (Lim et al., 2005). The Indus crab (*Austruca sindensis*) distribution primarily depends on sediment size and composition and this species preferred sandier type of sediments (Saher & Qureshi, 2012). The burrows of *H. crassa* were more abundant at muddy areas than the sandy areas (Morrisey et al., 1999). The distribution of fiddler crab (*Uca spinicarpa* and *U. longisignalis*) burrows in coastal marsh habitats described that *U. spinicarpa* preferred to burrow in substrates of higher percent clay (Mouton & Felder, 1996). Sediment characteristics, especially grain size, are usually considered the most important variables affect the crabs distribution (Checon & Costa, 2017) and thus showed during the current study.

The substratum properties were of primary importance in the distribution of brachyuran crabs along the coast of Pakistan. In addition, it seems that the variable structure and composition of sediments facilitate the burrowing ability of different crab species as well as to inhibit and specify their respective habitat accordingly.

5. RELATIONSHIP BETWEEN DENSITY OF CRABS AND CRAB BURROWS

It is notoriously difficult to study the densities of mangrove crabs therefore, the several techniques have been applied and tested for these burrowing mangrove crabs includes; use of pitfall traps, mark and recapture, burrow counts, visual or binocular counts and excavation of fiddler crabs (Nobbs & McGuinness, 1999; Skov & Hartnoll, 2001). Mark and recapture disturbs burrows and creates bias towards size and sex (Skov & Hartnoll, 2001) in the comparison to excavation of crabs is invasive as well as labour intensive and time consuming but gives reliable estimates of crab densities (Firth & Brunenmeister, 1980; Macintosh, 1984). The pitfall traps and binocular counts may underestimate or overestimate as usually depend on the degree of surface activity, but are quicker and non-invasive (Nobbs & McGuinness, 1999).

The number of crabs and the number of crab burrows varied considerably at each level of all three stations in each month (Figure 6). The numbers of burrows have been usually greater than the number of crabs collected from all levels at Korangi creek and both stations of the Sandspit, except a few instances at station 1 and station 2 in the months of March and May, respectively, when the number of crabs exceeded the number of crab burrows (Figure 6). Burrows have been also used to identify and estimate densities of crab population (Aspey, 1978; Krebs & Valiela, 1978; Warren, 1990; Steinke et al., 1993; Mouton & Felder, 1995; Skov & Hartnoll, 2001). At once visited sites, the numbers of burrows were also higher than the numbers of crabs (m^{-2}) at each level, T-test was employed against the null hypothesis that expected burrow frequencies were equal to the number of crabs show the significant difference at Korangi creek, Sandspit S1, Bhaira, Bhambore, Dhabeji, Sonari and Sonmiani. The null hypothesis was rejected and concludes that the expected frequencies of crab burrows were significantly greater than the number of crabs collected at these sites (Table 7).

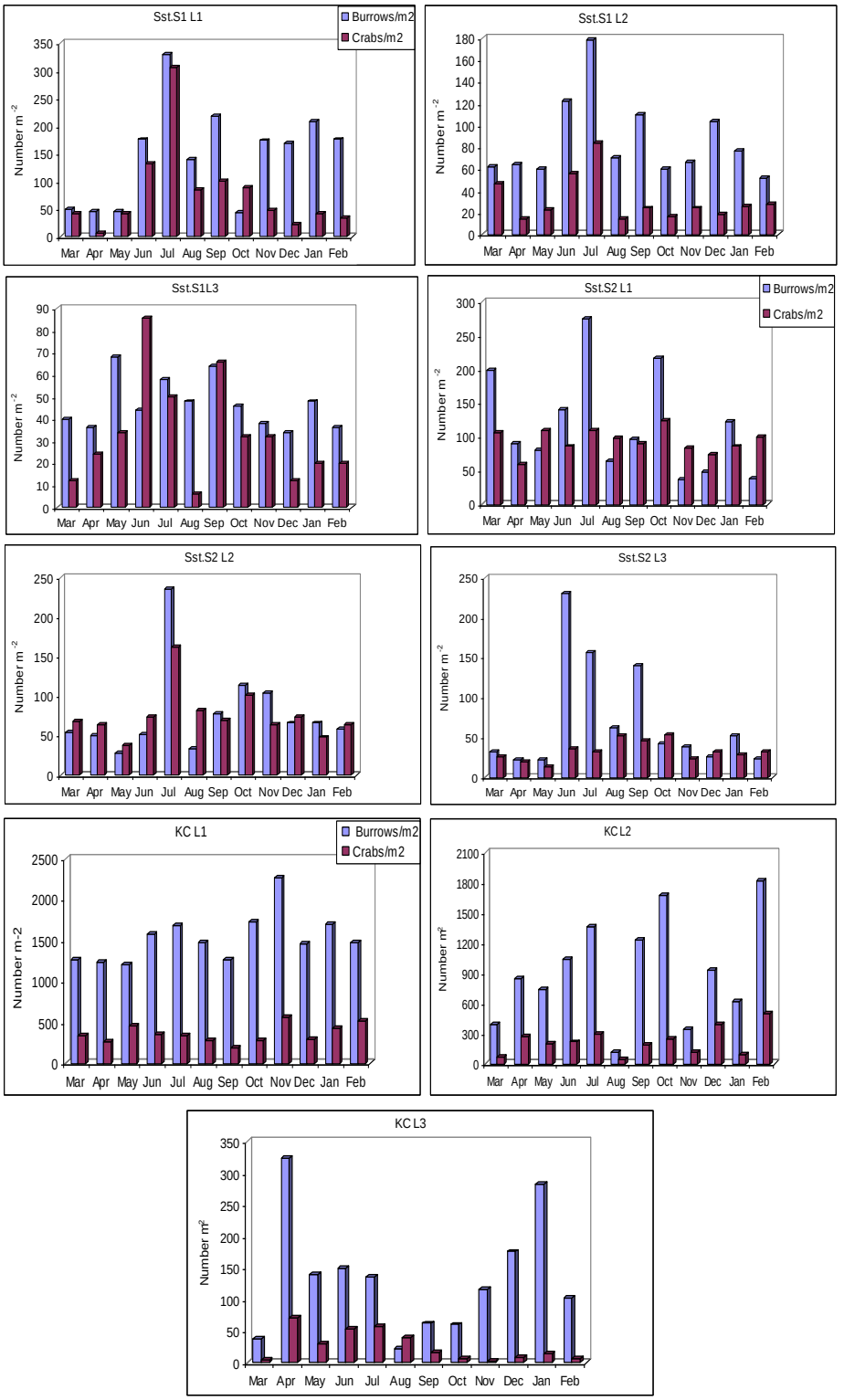

Figure 6. The monthly distribution of number of burrows (m^{-2}) and crabs (m^{-2}) at three levels of S1 and S2 collected from Sandspit back water mangrove area and S3 of Korangi Creek.

Table 5. T-test comparing the numbers of burrows (m^{-2}) and the number of crabs (m^{-2}) at all sites

Site	DF	T-test	P-value
Korangi creek	79	7.81	0.000
Sandspit S1	105	4.78	0.000
Sandspit S2	109	1.44	0.150
Bhaira	8	2.55	0.034
Bhambore	11	3.76	0.003
Dhabeji	11	3.39	0.006
Ketibunder	7	2.28	0.056
Phitti creek	9	1.81	0.100
Sonari	8	4.44	0.002
Sonmiani	15	2.91	0.011

Similarly, burrow counts are related to surface activities related to biotic functions (feeding, availability of food, reproductive activities, agnostic behaviour, predation and recruitment) and abiotic features (substratum preference, harsh conditions, tidal periodicity, etc.) and can result in spatial and temporal variability and over estimates of crab densities (Skov & Hartnoll, 2001). The use of burrow densities as surrogate to crab densities have been established in literature (Aspey, 1978; Macintosh, 1984; Genoni, 1991; Mouton & Felder, 1995; Nobbs & McGuinness, 1999) and burrow densities have been correlated with the crab densities and have been predominantly used (Krebs & Valiela, 1978; Mouton & Felder, 1995). The present results showed that the ratio between number of burrows to number of crabs was greater than one, i.e., more burrow numbers were found compared to the number of crabs. Similar observations have been observed by Genoni (1991) in the salt marsh. The greater number of unoccupied versus occupied burrows in the study area appeared to be the usual characteristic of crab habitats and this has been observed by Crane (1975) and Firth & Brunenmeister (1980). Burrow numbers in excess of crabs (supernumerary burrows) were common and were associated with the topographic heterogeneity of the substratum at higher levels on the shore and the composition of the sediment only accounted for a small fraction of this pattern (Mouton & Felder, 1996). Burrow counts tend to overestimate the population density because a number of burrows are unoccupied or have more than one opening (Frith & Frith, 1977). In many species of crabs, the burrowing depends on the cohesiveness of the substratum, its hardness, the presence of roots, grain-size, and moisture content (Warburg & Shuchman, 1979; Bertness & Miller, 1984; Takeda & Kurihara, 1987; Henmi, 1989; Kelaher et al., 1998; Morrisey et al., 1999).

Mouton & Felder (1995) concluded that the density of burrows despite seasonal variability can invariably be correlated to the crab densities to assess the population densities in intertidal marshes. Genoni (1991) in his study on *U. rapax* observed the high burrow turnover rates by the small sized crabs and concluded that crabs dig additional burrows despite the presence of pre-existing unoccupied burrows and that number of burrows may serve other needs like limited or low food availability to escape from predation and reproductive needs and suggested that early stages were more sensitive to food availability. Previous studies have also shown that megalopae were known to settle on a substratum with high organic content (Crane, 1975). Firth & Firth (1978) and Macintosh (1988) reached a similar conclusion in their studies of fiddler crabs from mangrove shores in Malaysia and Thailand. They also suggested that the higher number of burrows in relation to the real

number of the crabs can be attributed to the crabs' need to improve the opportunity for escape from predators.

6. Relationship of Carapace Length and Burrow Diameter

The size (carapace length) of resident crab species can be determined for each species by using linear equations relationship between carapace length (CL) and burrow diameter (BD). As the crab always enter its burrow sideways (Lim & Diong, 2003) therefore carapace lengths have been used to study the relationship between resident crabs and burrow diameters. A variable number of crabs approximately (n = 40) for each species were randomly collected to analyze the burrow diameter and resident crab-length relationship. The burrow diameters were also measured with the Vernier caliper and the following parameters; sex, carapace width, carapace length (CL) of inhabitant crabs were recorded. The burrow diameter and crab length relationship was evaluated for the selected species of crabs by using the best fit regression line.

Carapace length (CL) = a + b (burrow diameter BD) Equation 1

Where, a, and b are coefficients to be estimated by the model. Data for male and females were pooled, as no difference between the sexes was observed during preliminary regression analysis. A data of male and female crabs were pooled as no significant differences were observed.

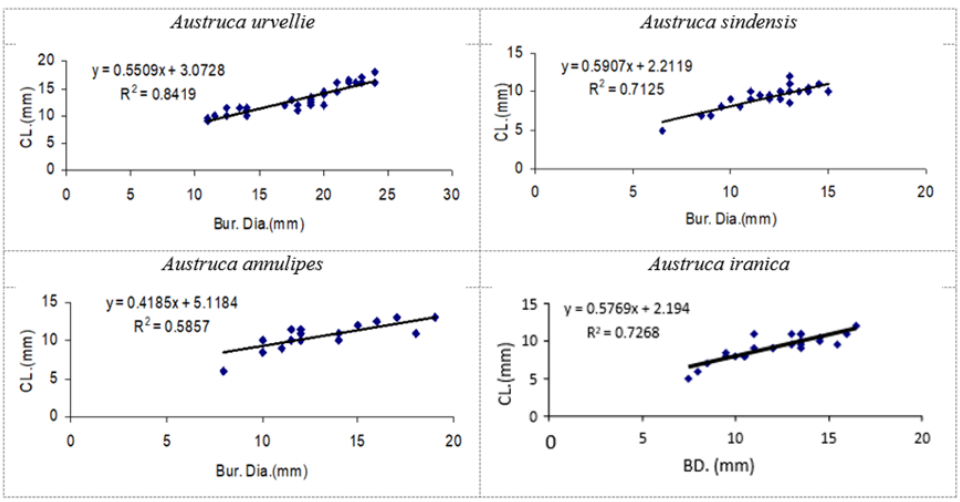

Figure 7. Linear relationships between the carapace length (CL) of crab and the burrow diameter (BD) of *Tubuca urvillei*, *Austruca sindensis*, *A. annulipes* and *A. iranica*.

The current study showed that the size of crabs was positively correlated to the burrow diameter, similar to the finding of Lim & Doing(2003) who found large-sized crabs with large burrow diameters and length. Larger-sized crabs had a greater burrow diameter,

larger burrow volume and bigger chamber diameter than small- and medium-sized crabs. This indicates that the larger-sized crabs generally excavate the larger entrances and reside in more spacious burrows. A good correspondence and significant positive linear relationships were observed in all species of Ocypodoid and Grapsoid crab (Figure 7 and 8).

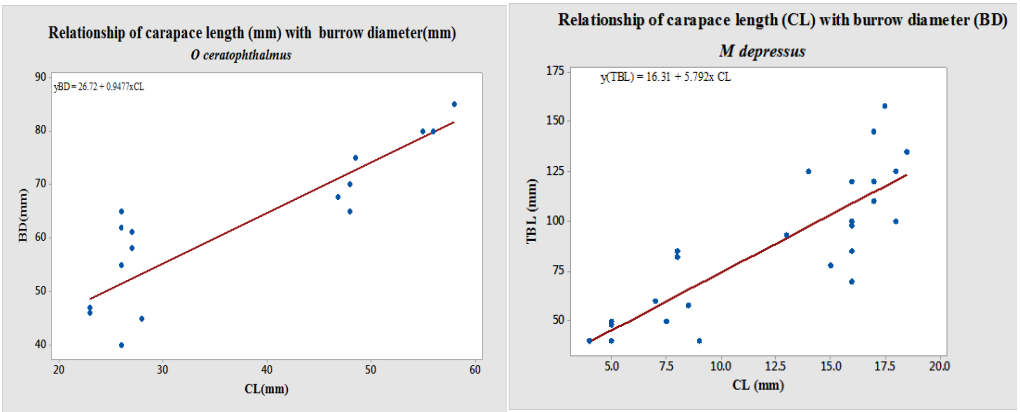

Figure 8. Regression analysis values for the relationship between carapace length and burrow opening diameter in *O. ceratophthalmus* and *M. depressus.*

7. INTER AND INTRA-SPECIFIC VARIATIONS IN BURROW ARCHITECTURE

The burrowing and other bioturbation activities in intertidal coastal areas may strongly affect the substrate topography and granulometry (Kristensen, 2008). The decomposition of the organic matter increased by the macro fauna, with the regular transport of the tidal water increased vertical and horizontal movement of sediment and detritus (Rhoads & Boyer, 1982, Meadows & Meadows, 1991), burrows increased the permeability of the sediment to water and air, stimulates microbial activity (Andersen & Kristensen, 1991) and the access of oxygenated water to areas. These changes in the sediment environment can, in turn influence the distribution and bioavailability of anthropogenic contaminants within the sediment.

The significant amount of information about the burrow structure is available for the larger burrowing species, but smaller burrowing species are highly ignored. Generally, crab species revealed the significant interspecific and intraspecific variations in their burrow morphology according to the biotic and abiotic factors of habitat. The various combinations of sediment, inundation levels as well as vegetation coverage influence the morphological features of crab burrows (Qureshi & Saher, 2012). Shapes of burrow casts generally consisted of at least one long tube varying in length and in the shape of the closed ends (forming L, J, U, and Y shapes) in vertical and complex branching morphologies.

Burrowing behaviour is commonly found in the invertebrate brachyuran crabs inhabiting marine soft sediments. The burrow morphology is species specific, but with the change in sediment characteristics, shore morphology, the species may modify their physical structures (Qureshi & Saher, 2012; Trivedi & Vachhrajani, 2016). Burrow morphology has been usually described by using plaster of Paris, rubber, or epoxy casts and general burrow morphology

ranging from burrow diameter, burrow depth, burrow volume, angle of the burrow with respect to the shore and general burrow design has been studied in several species of crab (Table 6).

Table 6. Literature review for burrow architecture of different crab species

Species	Scientific name	Author
Fiddler crabs		
	A. annulipes, A. iranica, A. sindensis	Qureshi &Saher(2012)
	A. vocans, A. annulipes	Lim (2006)
	A. pugilator	Dembowski (1926); Christy (1982)
	U. rapax	Genoni (1991)
	U. pugnax	Gray (1942); Powers (1975); Katz (1980); Bertness & Miller (1984)
	U. longisignalis, U. spinicarpa, U. vocator, U. subcylindrica	Thurman (1984a)
	U. tangeri	Wolfrath (1992)
Sentinal crab		
	Macrophthalmus japonicus	Otani et al. (2010)
Ghost crab		
	Ocypode ceratophthalma	Chan et al. (2006)
	Ocypode quadrata	Silva &Calado (2014)
Grapsid crabs		
	Eriocheir sinensis	Rudnick et al. (2005)

In the present study, the burrow architecture of some brachyuran crab studied with relation to variation in environmental factors like sediment structure and vegetation cover. Aqueous solution of plaster of Paris was poured in to the crab burrows with the help of a syringe until the burrow was completely filled and was allowed to dry for 30 to 60 minutes (Warburg & Shuchman, 1979), after hardening these casts were carefully excavated by hand. Burrows casts were separately placed in pre-marked poly bags and brought back to the laboratory for further analyses. Different parameters measured for each burrow cast were: (1) burrow diameter (BD), total burrow length (TBL), total burrow depth (TBD), and burrow volume (BV). The volume of the burrow cast were determined by weighing the burrow cast ($\pm$0.1g) and dividing the weight of the burrow cast by the density (2.2 g cm^{-3}) of plaster of Paris (Chan et al., 2006). Data for only complete burrow casts were used for analyses.

For each species, burrows openings with different diameters (N = 30 at least) were randomly selected. Three species of genus *Austruca (Austruca annulipes, A. iranica* and *A. sindensis), two species of genus Ocypode (O. rotundata and O ceratophthalmus)* and single species of sentinel crabs (*Macrophthalmus (Mareotis) depressus*) are common and wider spread along intertidal shores of Pakistan, which were studied in order to investigate intra and interspecific variation of crab burrow architecture according to the habitat and tidal level (Figure 9). The burrow cast experiment for *A. iranica* was performed at three tidal heights as it was possible to identify the patches at three levels.

Fiddler crabs of genus *Austruca* (formerly called *Uca*) are common inhabitants of sandy shores, mudflats, mangroves and estuarine habitats of tropical and subtropical region. A red legged fiddler crab *(A. annulipes*) was mostly associated with fringing mangroves among pneumatophore from mid to low tidal areas. Morphology, size and structure of fiddler crab burrows were determined (Figure 9), however the general morphology of crab burrow is tube

like having straight shaft ended without chamber at the base with a single or double burrow opening (Figure 9). Total 5 different types of burrow shapes were recorded, including straight tube like with single burrow opening, straight tube like with bulb at the end, J shaped with single opening, Y shaped with two burrow opening and V shaped with two openings.

Table 7. Summary of descriptive statistics for difference in burrows morphological characters of three species of genus *Austruca* from Sandspit backwater mangrove area during the study period

Burrow parameters	Variable	N	Mean	Min	Max
Total Burrow length (TBL) mm	*A. annulipes*	40	115.4 ±45.9	45	210
	A. iranica	38	118 ±34.2	62	205
	A. sindensis	40	220.2±71	110	335
Total Burrow Depth (TBD) mm	*A. annulipes*	40	95.7 ±36.5	32	165
	A. iranica	38	92.7 ±29.3	46	145
	A. sindensis	40	150.4 ±62	70	244
Burrow diameter (BD) mm	*A. annulipes*	40	13.70 ±3.0	7	18
	A. iranica	38	10.50 ±3.8	5	26
	A. sindensis	40	12.80 ± 1.9	10	17
Burrow Volume (BV) cm^{-3}	*A. annulipes*	40	8.470 ± 5.8	1.84	23.0
	A. iranica	38	6.69 ± 4.11	1.92	22.4
	A. sindensis	40	34.37 ±16.9	16.6	82.3

Fiddler crab (*A. iranica*) is the most frequently distributed from low to mid tide level of sandy and muddy areas. Indus fiddler crab (*A. sindensis*) inhabitant of high intertidal areas.

(A) Burrows cast of *Austrucairanica*.

(B) Burrows cast of *Austrucasindensis.*

(C) Burrows cast of *Austrucaannulipes.*

Figure 9. Burrows cast in three species of fiddler crabs from Sandspit backwater mangrove areas of Pakistan.

The comparison of the size of burrow cast among three species of *Austruca* showed the mean TBL was 115.4 ± 45.9 mm for *A. annulipes*, 118.0 ± 34.2 mm for *A. iranica* and 220.2 ± 71.0 mm for *A. sindensis* (Table 4.9). The mean BD was 13.7 ± 3.0 mm (*A. annulipes*), 10.5 ± 3.8 mm (*A. iranica*) and 12.8 ± 1.9 mm (*A. sindensis*). The one way ANOVA showed significant differences in TBL, TBD, BD and BV among the three species studied. In *A. iranica*, the spatial variability was observed in morphological characters in burrow casts at three tidal levels. The mean TBL, TBD, BD, and BV at L1 was smaller than the L2 and L3 and one way ANOVA showed significant differences in all burrow cast morphological characters at the three tidal levels. The results also indicated the size specific zonal distribution of *A. iranica* in the intertidal area. The identification of burrow was an important issue to study burrow morphology of *Austruca* species with sympatric distribution and it was quite difficult to determine which burrow belongs to which crab species without identifying a pure distribution patch of each species. The number and size of burrows and size of crabs are related to reproductive cycle, recruitment and growth of these species.

Total burrow length, burrow depth and burrow volume were significantly varied among the different fiddler crab species. It was greater in *A. sindensis as* compared to the other two species (*A. sindensis*<*A. annulipes*≈*A. iranica*). The interspecific differences in burrowing behaviour between three species of *Austruca* demonstrated that these variations depend on variable combination of sediment composition and firmness (Qureshi & Saher, 2012).

The previous studies showed that the burrow depth increased with the distance from the water's edge (approximate mean sea level) increased along the marsh (Mouton & Felder, 1996). Likewise, the distance from the shore appeared to be the main determinant that is influencing the distribution and abundance of crab (Owen et al., 2000). The interspecific differences in burrowing behaviour between three species of *Austruca* (*A. annulipes*, *A. iranica* and *A. sindensis*) showed that these variations largely affected by the tidal height and distance from the shore (Qureshi & Saher, 2012). Mouton & Felder (1996) studied the influence of elevation and landward distance from the shoreline on burrow densities of two fiddler crab (*A. spinicarpa* and *A. longisignalis*).

Table 8. Summary of descriptive statistics for burrow structure (mm) of *Macrophthalmusdepressus* crab from two different sampling areas of Pakistan

Parameters	Sites	Mean	Min	Max
Total Burrow length(TBL) mm	Sandspit	110.7± 23.6	70	158
	Korangi creek	55.3 ± 15.8	40	85
Burrow diameter (BD) mm	Sandspit	32± 6.83	18	48
	Korangi creek	14.3 ± 2.65	10	19
Crab density/m^2	Sandspit	58.0 ± 5.16	52	64
	Korangi creek	168± 43.2	140	232

Sentinel crab (*M. depressus*) usually occupies mid to low tide area in sandy muddy intertidal shores. A total of 40 burrow cast were obtained from two backwaters mean density of crabs was 58/m^2 with CL 16.3 mm (Table 8). At Sandspit backwaters, the mean burrow length (TBL) 110.7± 24.6 was higher than crabs made in Korangi creek (55.3±16.4). The density of *M. depressus* was higher in Korangi creek area (168/m^2) with mean CL = 6.7 mm, whereas in Sandspit BD) was 1.4±0.26 and 3.2 0.68 at Korangi creek and Sandspit

respectively. Crab built horizontal burrow with tube like shaft ended without chamber at the base having single burrow opening (Figure 10).

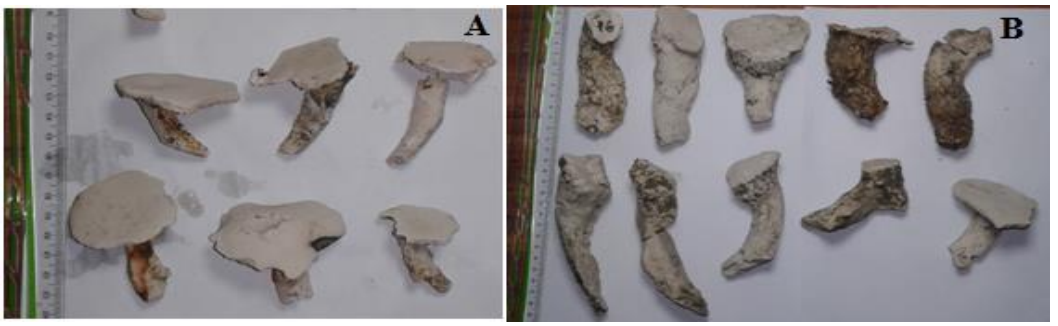

Figure 10. Burrow morphology of *M. depressus* from two different mangrove areas, (A) Korangi creek, and (B) Sandspit from coastal areas of Pakistan.

For three species of fiddler crab, burrows were nearly vertical, straight, and single or branched whereas in sentinel crab it was horizontally elongated with single shaft opening without any branch. Results in the present study showed that the species of crab occupy high zone form longer burrows than species which inhabitant from mid to low tide zones. Deeper burrows maintain lower burrow temperatures in areas that are exposed to sunlight for greater periods of time (Powers & Cole, 1976; Wolfrath, 1992; Lim & Diong, 2003). The ghost crab of genus *Ocypode* Weber, 1795 are nocturnal burrowing species distributed along sandy beaches of subtropical and tropical regions of the world. Two species (i.e., *O. rodondata* and *O. ceratopthalmas*) of ghost crabs frequently distributed along the coast of Pakistan and studied for burrow architecture (Figure 11). A total of 67 burrow casts for *O. rotundata*, with four different types of burrow architecture were obtained (Table 9), including straight tube like (694.1 ± 278.2, 320–1400), J shaped (742.9 ± 53.5, 700–800), L shaped (736.7 ± 241.3, 410–900) and complex branched network (783.3 ± 57.7, 750–850). For 59 burrows of *O. ceratopthalmas*, four different types of burrow cast were obtained i.e., C shaped (493.7 ± 52.4, 405–550), J (465 ± 9.1, 380–550) shaped, L shaped (650 ± 77.5, 600–750) and Y shaped (640 ± 69.6, 550–690) all with single opening (Table 9). The ghost crabs are the carnivore in a food chain of the dune and fore beach ecological environment and have been considered as an indicator of the dune and fore beach environment. Various species of Ocypodoid semi terrestrial group selectively burrow into inter tidal substrata, from coarse beach sand to fine clay-rich marshy mud (Teal, 1958). It appears that the changes in elevation, vegetation and sediment composition determine the dominancy of species in particular environment. As the tidal frequencies and magnitudes, along with temperature, appears to provide an important environmental regulator for feeding, burrowing and courtship (Crane, 1958; Power & Cole, 1976; Montague, 1980). The length of burrows varied depends on tidal levels and size of burrow diameters. The burrows with a greater angle could aid in the survival of the crabs, as more acutely bent burrows provide the better refuge from predators therefore, the open areas have a greater angle (Lim & Heng, 2007).

(A) Casts of burrows of *Ocypoderotundata*

(B) Casts of burrows of *Ocypodeceratophthalmus*

Figure 11. Burrow morphology of two *Ocypode* species from coastal areas of Pakistan.

Table 9. Summary of descriptive statistics for difference in burrows morphology of two species of genus *Ocypode* from Sandspit area

Burrow structure	Species	Mean length ±SD	Min	Max
Straight tube like	*O. rotundata*	694.1 ±278.2	320	1400
C shaped	*O. ceratopthalmas*	493.7 ±52.4	405	550
J shaped	*O. rotundata*	742.9 ±53.5	700	800
	O. ceratopthalmas	465 ±9.1	380	550
L shaped	*O. rotundata*	736±241.3	410	900
	O. ceratopthalmas	650 ±77.5	600	750
Y shaped	*O. ceratopthalmas*	640 ±69.6	550	690
Complex branching structures	*O. rotundata*	783 ± 57.7	750	850

The pattern found for *A. sindensis* and *Ocypode* in the present study is consistent with that found for other crabs with deeper burrows in drier sediments (Takeda & Kurihara, 1987; Wolfrath, 1992; Lim & Diong, 2003). Species and even individuals can produce many different burrow architectures and different species can also produce the same architectures. Burrow morphology is generally influenced by organism morphology, behaviour and their response to variations in environmental stimuli, including biotic and abiotic factors (Bromley, 1996; Bowen & Hembree, 2014).

8. SEMATECTONIC COMMUNICATION THROUGH ALLIED LANDMARK AND STRUCTURES NEAR BURROWS

Most of the crabs are inhabiting in intertidal mud flats and dig burrows to protect them from predation, environmental stresses and also creates unique habitats for infauna but in addition most of them also build earthen structures at their burrow entrance by using moist mud or sand (Crane, 1975; Christy, 1982). One of the basic function of burrow is to use for reproductive activities and through these landmark structures, the male shows up himself to females to attract them for copulation that usually occurs inside the burrows (Christy, 1987;

Netto et al. 2007). The male species of Ocypodoid and Grapsoid crabs construct various structures near their burrow, which are used for sematectonic communication (Wilson, 1975). These structures are named according to their appearance, such as the pillars, (Christy, 1988), chimneys, (Wada & Murata, 2000), mud balls, (Burford et al., 2001), pyramid (sand piles) fence (Crane, 1975; Christy, 1982; Wada et al., 1994), semidome or hoods, (Zucker, 1981; Clayton, 1998), shelters, (Zucker, 1974), and barricades, (Wada, 1994), built by 17 species of genus *Austruca* (formerly called *Uca*), (Burford et al., 2001) which resembles many of *Uca* sand structures, (Christy, 1988a, 1988b, 2001; Muramatsu, 2010; Saher & Qureshi) and the pyramids are used by ghost crabs female to attract them by not displaying themselves (Wilson, 2000). The other role of pyramid construction is to enforce the male territories and maintaining the neighbor distances like other species of family *Ocypodidae* (Oliveira et al, 1998; Mammatus, 2010).

The genus *Uca* is also well known for the behaviour to build the various earthen structures from mud excavated inside from their burrows. Christy (1988) suggested that the pillars of *A. beebei* may function as guide posts to allow the resident male to find his burrow entrance quickly when escaping a predator and/or to attract females to the males' burrows. In the fiddler crab (*A. beebei*) males build a small mud pillar next to their burrow which increases their attractiveness to the females.

Low semi-domes (hoods) *Cleistostoma kuwaitense* construct hood to ensure social spacing (Clayton 1988). *U. terpsichores* also constructed low massive semi-domes (i.e., hoods) on one edge of the burrow entrance as a territorial behaviour (Zucker, 1981). The semi-domes of some species may act as an object to provide wharf to the male, whilst engaged in forceful combat (Christy, 1982). However, in some species hoods play a part to attract females for courtship, for example, in *U. musica*, *U. terpsichores* and *U. beebei* occurrence of hoods reduces the frequency of combats between territorial males and their construction frequency is associated with high densities of male burrows (Zucker, 1981); similar observations were observed in the *A. iranica* along the Pakistan coast (Saher & Qureshi, 2017).

Chimneys are mud mounds walls that encircle the entrance to a crab burrow (Crane, 1975; Thurman, 1984). Different fiddler crab species build the chimneys but the proposed functions vary greatly among the species (Wada & Murata, 2000; Shih et al., 2005; Saher & Qureshi 2017). In some species (*Uca formosensis*) it is built only by males that have recently attracted a female into their burrow to mate while in others, assumed to hide male from rivals while he expands his burrow for the female (Shih et al., 2005; Saher & Qureshi 2017). By contrast, only large reproductive females build chimneys in *Uca urvillei*, *Uca coarctata* and *Uca forcipata* (Crane, 1975; Salmon, 1987). In *U. arcuata*, the chimney decreases the chance of intrusion by wandering crabs (Wada & Murata, 2000) and protect them during incubation. Thurman (1984) proposed that chimneys may help to regulate the environmental stresses (temperature or humidity) in *Uca subcylindrica*.

Sand piles (sand pyramids) made by male ghost crabs (*Ocypode saratan*, *O. ceratophthalmus* and *O. kuhlii*) near the burrow and these structures apparently function in male spacing and to attract female (Linsenmair, 1967). Mounds in *Ilyoplaxpingi* keep the neighbor crabs away from burrow (Wada et al., 1994). The barricade and fence built by *I. pusillus* a mud wall on the near side of a neighboring burrow (barricade) or apart from the burrow to defend the area around their burrows against intrusions by conspecific invasion (Kitaura et al., 1998).

9. ANTHROPOGENIC EFFECTS ON BURROW AND BURROWING

The coastal developmental activities by human beings results in widespread modifications of the beach. Sandy beaches are the most important intentions for outdoor activities of human beings for their leisure time such as by driving off-road vehicles, walking on beaches or dunes, walking over the vegetation, inducing soil erosion and imposing the other drastic changes; however, these regions have various energetic and often sensitive ecosystems. The coastlines of the world are dominated by sandy beach ecosystems (McLachlan & Brown, 2006, Branco et al., 2010). By increasing coastal human population, the natural habitats of sandy beaches are being destroyed in the accelerating rate (Defoe et al., 2009). As the humans are shifting to coastal areas to utilize the habitats as their visiting places, they greatly exploit the natural resources of sandy beaches (Roberts & Hawkins, 1999). These variable types of anthropogenic activities are important and increasing the concern for coastal management (Beatley et al., 2002, Branco et al., 2010), and therefore, ghost crabs can be used for the assessment of human impact on the beach environment (Barros, 2001).

This is necessary to know the ecological responses to urban sewage by the fauna inhabited in the mangrove ecosystem. To understand the effect of sewage on crab directly or indirectly will help further knowledge about crab survival and bioturbation activities to comprehend the effects of organic discharge on the mangrove forests and develop sustainable mangrove wastewater wetlands. The waste water is a potential threat to mangrove forest ecosystem and recently these forests are recently expressed a potential as a natural wastewater treatment in China (Yang et al., 2008). These crabs have the potential to remove the nutrients and organic matter efficiently (Penha-Lopes et al., 2009). The mangrove forest productivity can be enhanced by applying the moderate sewage loadings (Mohamed et al., 2009) also responsible for the enhancement of benthic primary productivity and micro-heterotrophs (Meziane & Tsuchiya, 2002). The increased microalgal and bacterial growth in sewage contaminated mangrove forest ecosystem have no effect on Ocypodoid crabs biodiversity (Yu et al., 1997). Though, under poor hydrodynamic conditions the organic matter may increase above the ecosystem capacity and lead to eutrophication and hypoxic condition (Gray et al., 2002). The crustaceans are considered as most sensitive species to hypoxia; consequently, faunal migration takes place because of lower activity of smaller burrow structures of marine benthic organisms and high mortality may be caused which may further lead to decrease in bioturbation activity (Diaz & Rosenberg, 1995).

On sandy beaches, the crabs construct their burrows generally in discrete zones on beaches. The studies suggest that burrows constructed by the crabs are generally above the high tide level (Grubb, 1971; Jones, 1972; Hartnoll, 1975; Burggren & McMahon, 1988) can be confirmed by the previous studies(Naidu, 1951; Barrass, 1963; Hughes, 1966; Jones, 1972; Vannini, 1980b; Burggren et al., 1988; Burggren & McMahon, 1988; Clayton, 2001; Tureli et al., 2009). Some species have adapted to construct their burrows on the supra littoral Zone (Magnus, 1960; Quijonet al., 2001; Clayton, 2005) whereas even other crabs show the burrow construction on inland of sandy soil (Borradaile, 1903; Pearse et al., 1942; Pearseet al., 1942; Macnae&Kalk, 1962; Jones, 1972; Hill & Hunter, 1973; Horch, 1975; Stoddart, 1984; Strachan et al., 1999; Strachan et al., 1999; Alberto &Fontoura, 1999; Clayton, 2005; Saher, 2008; Lucrezi et al., 2009; Saher & Qureshi, 2013; Lucrezi et al., 2014).

The previous studies recommend human disturbance from pedestrians and vehicles is a major factor affecting density of different sandy crab species (Hughes, 1966; Frey & Mayou, 1971; Chakrabati, 1981; Strachan et al., 1999; Barros, 2001; Blankesteyn, 2006; Maccarone& Matthews, 2007; Moss & Mcphee, 2006; Schlacher et al., 2007; Lucrezi et al., 2008; Hobbs et al., 2008; Brook et al., 2009; Magalhaes et al., 2009; Yong & Lim, 2009; Pombo & Turra, 2013; Hereward & Sluka, 2014; Sankar et al., 2013). Therefore, these previous studies suggest that *Ocypode* species as a good biological indicator, though densities may increase around the food waste, (Hill & Hunter, 1973; Steiner & Leatherman, 1981), while hot weather and short term human trampling can lead them to struggled temporarily and hide under burrows cause reduction in burrow counts (Lucrezi et al., 2009). Tide-dominated beaches also have too many confounding factors impacting density as they are particularly heterogeneous habitats (Turra et al., 2005).

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Chapter 107

ESTIMATING INTRINSIC OPTIMUM TEMPERATURES AND LOWER AND UPPER THERMAL THRESHOLDS FOR THE DEVELOPMENT OF AMERICAN LOBSTER LARVAE USING A THERMODYNAMIC MODEL

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This work is dedicated to Nan Yao, a precious friend and lobster colleague, whom I miss dearly.

ABSTRACT

Temperature strongly affects the development of American lobster (*Homarus americanus* H. Milne Edwards, 1837) larvae. At temperatures below and above the species' lower (T_L) and upper (T_H) developmental thresholds, respectively, successful development should be prevented, while at temperatures between these warmer conditions should result in faster development, except as the upper threshold is approached. Likewise, the species should have an intrinsic optimum temperature for development (T_Φ), at which its larval development rate is high while its other physiological characteristics are in good condition. These thermal parameters and the temperature-dependent development relationships they define mediate the effects of temperature on recruitment to lobster populations and fisheries, so it is important to know them. However, no previous study has defined all three thermal parameters for the development of American lobster larvae. In this chapter, a thermodynamic model, the Sharpe–Schoolfield–Ikemoto (SSI) model, was fit to development data for American lobster larvae through different stages and at different temperatures from eight previously published studies. Bootstrap 95% confidence intervals (C.I.s) were also generated for SSI thermal parameter (T_Φ, T_L, and T_H) estimates to allow them to be compared among larval stages and source studies. The estimates obtained varied widely and significantly among

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studies, with there being no consistent and few significant differences in thermal parameters among stages. Most T_Φ estimates were biologically realistic, but the majority of T_L and T_H estimates were not. When all studies' datasets were combined in the same analyses, the T_Φ, T_L, and T_H for American lobster larval development were estimated to be 16.534-17.326°C, 0.095-7.804°C, and 23.483-25.661°C, respectively. The T_Φ of stage I was significantly lower than those of stages II and III, but all other differences in thermal parameters among stages were non-significant. Further, while all T_Φ and most T_L estimates were considered realistic, all T_H estimates were slightly too low. The SSI model was derived based on enzyme thermodynamics, and thus should provide useful information concerning lobster larval developmental optima and limits. The results found suggest that as ocean temperatures experienced by lobster larvae in nature more often exceed the T_Φ for their development due to climate change, larval development and survival may be impaired, which will have important impacts on lobster recruitment. However, none of the previous studies analyzed were conducted over a wide enough thermal range to allow a conclusive fit of their data to be done with the SSI model. Therefore, a future rearing study conducted over a wide thermal range, followed by analyses with the SSI model, is needed to better define the limiting and optimum temperatures for the development of this species' larvae.

Keywords: American lobster, larva, development, threshold temperature, intrinsic optimum temperature, thermodynamics, Sharpe–Schoolfield–Ikemoto (SSI) model, bootstrap confidence intervals

1. INTRODUCTION

Environmental temperatures have important and powerful impacts on the physiology and ecology of ectothermic animals (Bělehrádek 1935; Anger 2001; Angilletta Jr. 2006; Byrne 2011; Jost et al. 2012; Shi et al. 2011, 2012a, b, 2013, 2017), including lobsters (McLeese 1956; Boudreau et al. 2015; Quinn 2017a). Larval development is a particularly important temperature-sensitive process in the lifecycles of ectotherms, as it must be successfully completed for there to be recruitment to later life stages and for populations to be maintained (Schmalenbach and Franke 2010; Vaughn and Allen 2010; Byrne 2011; Pineda and Reyns 2018). At temperatures that are too low or too high, development cannot be completed; these limiting temperatures are termed the lower (T_L) and upper developmental thresholds (T_H), respectively, and vary among species (Jost et al. 2012; Shi et al. 2017) and perhaps developmental stages of the same species (Campbell et al. 1974; Brière et al. 1999; Quinn 2017a). The rate at which larval development proceeds is also important, as a longer larval developmental period results in greater exposure of larvae to predation risks, environmental stress, possible dispersal away from juvenile habitat, and other sources of mortality, and thus decreases the probability of them surviving the larval period and recruiting to the adult population (Pechenik 1999; Vaughn and Allen 2010; O'Connor et al. 2007; Pineda and Reyns 2018; Gendron et al. in press). Over the majority of temperatures likely to be experienced by a species between its lower and upper thermal thresholds, higher temperatures result in faster development and thus shortened larval durations (Angilletta Jr. 2006; Campbell et al. 1974; Ikemoto and Takai 2000), and the relationship between temperature and development rate is usually linear across much of this range, although near extreme values it becomes nonlinear (Brière et al. 1999; Jost et al. 2012; Shi et al. 2011; 2012a,b; 2013; 2017). However, as

temperatures approach the upper threshold this beneficial effect of temperature breaks down and development speeds up less and less, and then eventually slows rapidly just before the threshold is reached (Schoolfield et al. 1981; Yamamoto et al. 2017). In light of ongoing and future climate change, there is much interest and importance in obtaining a better understanding of the effects of temperature changes on various organisms (Byrne 2011; Caputi et al. 2013; Pinsky et al. 2013). It is particularly important that the thermal thresholds and temperature-dependent development rates of ecologically and/or economically important species are quantified. However, for many species such information has not yet been sufficiently assessed due to the high costs and methodological challenges of needing to rear larvae over a wide range of temperatures to do so (e.g., Quinn 2017b; but see Yamamoto et al. 2017).

The American lobster, *Homarus americanus* H. Milne Edwards, 1837 (Crustacea: Decapoda: Astacidea: Nephropidae), is a large, clawed species of lobster than inhabits the coastal marine benthos of the Atlantic Shelf of eastern North America, from Cape Hatteras, NC, USA to Labrador, NL, Canada (Lawton and Lavalli 1995). This species is presumed to be an important heterotrophic component of many marine communities, and supports the most lucrative and socioeconomically important fishery in the region spanning Cape Cod, MA, USA to Newfoundland, Canada (Wahle et al. 2013). During the spring-summer months (ca. May-October), lobster larvae are released by benthic females, and then spend from 2-8 weeks or more (depending on water temperature) in the water column, living as planktonic and/or pelagic raptorial predators (Phillips and Sastry 1980; Ennis 1995). Lobster larvae develop through three zoea or mysis stages, termed larval stages I, II, and III, which are followed by a single decapodid stage, termed stage IV (and commonly called a 'postlarva') (Hadley 1906; MacKenzie 1988; Ennis 1995); stage IV lobsters are strong swimmers that settle to the benthos when they encounter suitable substrate, after which they moult into the first juvenile instar (stage V) (Lawton and Lavalli 1995). Some strong correlations have been found between the supply of potential settlers (i.e., larvae) to a given locality and its fisheries recruitment (e.g., Miller 1997; Wahle et al. 2004; Quinn et al. 2017; Gendron et al. in press), so it is likely that any factor that impacts larval survival and/or planktonic dispersal patterns (which affect the spatial distribution of settlers) could strongly impact lobster populations and the fisheries they support (Caputi et al. 2013; Boudreau et al. 2015; Jaini et al. 2018). Changing water temperature due to climate change is one such factor, as such change has the potential to expose lobster larvae to temperatures exceeding their developmental thresholds and/or to temperatures that lengthen larval duration (Quinn and Rochette 2015; Quinn 2017a). Therefore, it is clearly important that we know the thermal thresholds and temperature-dependent development rates of American lobster larvae to allow such changes and their potential impacts to be predicted.

A number of studies have investigated the impacts of temperatures on American lobster larval development times (e.g., Hadley 1906; Templeman 1936; Hughes and Matthiessen 1962; Ford et al. 1979; MacKenzie 1988; Hudon and Fradette 1988; Annis et al. 2007; Quinn et al. 2013; Waller et al. 2017; Harrington et al. 2019). Although studies have been done at rearing temperatures within the range of 6.7 to 26.3°C, no single study has been done over this full range, and none have yet investigated the larval development of this species across its full range of potentially biologically relevant temperatures (ca. 0-30°C or higher: Quinn and Rochette 2015). Thus, we do not yet have a detailed picture of its larval development curve, including the point at which rising temperatures cease to increase development rates (Quinn

2017a); although it should be noted that based on results of Templeman (1936) and Ford et al. (1979), development rates may plateau between 19.2 and 23.8°C and begin to decline slightly between 24.2 and 26.3°C. Further, although a handful of studies have attempted to estimate lethal temperature limits of lobster larvae (0°C and 28-35.5°C: Huntsman 1924; Sastry and Vargo 1977; Gruffydd et al. 1979), no comprehensive investigation of the thermal limits of larval development (i.e., using long-term exposures of large numbers of larvae to extreme temperatures) has yet been done (Quinn 2017a).

Until such studies are done, the only means available to assess potential developmental threshold temperatures for lobster larvae is the application of development functions that can estimate such thresholds to data from previous studies (Quinn 2017a, b). For example, Quinn (2017b) applied the linear sum (Winberg 1971), linear rate (Campbell et al. 1974), Bělehrádek (Bělehrádek 1935; McLaren 1963), modified Arrhenius (Guerrero et al. 1994), and Brière-2 (Brière et al. 1999) functions to data from MacKenzie (1988) and Quinn et al. (2013) to estimate T_L and/or T_H thresholds of lobster larvae of 0-8.8°C and 22.5-41.4°C, respectively (Quinn 2017a). However, many of these estimates were not considered biologically realistic and/or were made with limited power (see Quinn 2017b for discussion), and could further not be statistically compared among larval stages or data sources. These functions also did not allow the optimum temperature for larval development to be estimated (see below). There thus remains the potential for better estimates of developmental threshold temperatures to be made for this species.

A prominent and potentially very useful (but also extremely complex) development function that was not examined or applied to lobster data by Quinn (2017b) was the Sharpe–Schoolfield–Ikemoto (SSI) model (Schoolfield et al. 1981; Shi et al. 2011; Ikemoto et al. 2013). This model was derived from studies of enzyme thermodynamics, and equates the limiting temperatures for development (T_L and T_H) to those at which a hypothetical enzyme (presumably one controlling and/or affecting development) has a 50% probability of being in either an active or temperature-inactivated state (Ikemoto et al. 2013). It thus has the potential to produce more realistic and meaningful estimates of thermal limits than many of the other models applied previously to lobsters. This model has been applied extensively in studies of insects and arachnids (e.g., Ikemoto 2005, 2008; Shi et al. 2011, 2012a, b, 2013, 2017; Jafari et al. 2012; Ikemoto and Egami 2013; Padmavathi et al. 2013; Sreedevi et al. 2013; Quinn 2017b), and has recently been applied to a few crustaceans (Yamamoto et al. 2017). The SSI model is represented by the equation:

$$d(T) = \frac{\rho_{\Phi}\frac{T}{T_{\Phi}}exp\left[\frac{\Delta H_A}{R}\left(\frac{1}{T_{\Phi}}-\frac{1}{T}\right)\right]}{1++exp\left[\frac{\Delta H_L}{R}\left(\frac{1}{T_L}-\frac{1}{T}\right)\right]+exp\left[\frac{\Delta H_H}{R}\left(\frac{1}{T_H}-\frac{1}{T}\right)\right]} \quad (1)$$

where $d(T)$ is the development rate (1/development time, in 1/day) at a given absolute temperature, T (in degrees Kelvin (K), such that 0°C = 273.15 K), R is the gas constant (1.987 cal/deg/mol), ΔH_A is the enthalpy (in cal/mol) of a reaction that is catalyzed by a hypothetical enzyme, T_L and T_H are the lower and upper threshold temperatures (in K) at which the enzyme is half active and either half low-temperature inactivated or half high-temperature inactivated, respectively, ΔH_L and ΔH_H are the changes in enthalpy (in cal/mol) associated with the low- or high-temperature inactivation of the enzyme, respectively, T_{Φ} is the intrinsic optimum temperature (in K) of the reaction at which the probability of the enzyme being in an

active state is maximized, and ρ_Φ is the development rate (in 1/day) at the T_Φ assuming no enzyme inactivation (Sharpe and DeMichele 1977; Schoolfield et al. 1981; Shi et al. 2011; Ikemoto et al. 2013).

The intrinsic optimum temperature, T_Φ, is related to the other parameters of the SSI model, and thus may be the most important and informative thermal parameter to estimate (Ikemoto et al. 2013). In previous work, the T_Φ was assumed to be equivalent to 25°C (in the SS model of Sharpe & DeMichele (1977)), but more recent studies using the SSI model have permitted the value of the T_Φ to vary as appropriate for the species and dataset under consideration (Shi et al. 2011, 2012a, 2013, 2019; Ikemoto et al. 2013). Traditionally, the 'optimum' temperature for the larval development of a species was considered to be the temperature at which its development rate was maximized (e.g., Amarasekare and Savage 2012). However, this is not really the case, as an increasing body of work has demonstrated that at temperatures causing the most rapid development possible a number of other physiological processes are impaired, causing reduced growth, survival, etc. (Ikemoto 2008; Martin and Huey 2008; Forster et al. 2011; Corkrey et al. 2012; Shi et al. 2012a, 2013, 2019); indeed, for lobsters this seems to be the case as well (Quinn 2017a; Harrington et al. 2019). Thus, the true intrinsic optimum temperature for the development of a species should be below the one at which development rate is maximized (Martin and Huey 2008; Shi et al. 2019), and algorithms used to estimate SSI model parameters should not constrain the value of T_Φ to be equal to either 25°C or the temperature at which the development rate is maximized.

There is obviously interest in determining the intrinsic optimum temperature for the development of a species under consideration. Many species of insects and crustaceans, for example, are reared in laboratory or hatchery settings for use as food for humans or livestock or to supplement natural stocks of their species (Van Olst et al. 1976; Carlberg and Van Olst 1977; Ford et al. 1979; Quinn 2017b), and to maximize their production and efficiency such efforts should be carried out at temperatures that maximize larval development rates while avoiding negative thermal effects on survivorship and other characteristics. It is also useful to know the optimum temperature for a species when making predictions about its dynamics in nature, as temperatures below and above the optimum can have important negative impacts on larval survival and supplies of recruits (Quinn and Rochette 2015; Quinn 2017a) and threshold temperatures may determine the boundaries of geographic distributions (Boudreau et al. 2015). There is a long history of studies of American lobster, which does suggest that the optimum temperature for larvae of this species is somewhere around 18-20°C (e.g., Van Olst et al. 1976; Carlberg and Van Olst 1977; Ford et al. 1979; Bartley et al. 1980; Quinn 2017a); however, such estimates vary among studies and have not been made while considering the thermodynamics of the enzymes controlling development. Therefore, it may be useful to estimate the intrinsic optimum temperatures for American lobster larval development using the SSI model.

Recently, techniques have also been developed that allow the thermal parameters T_Φ, T_L, and T_H estimated by the SSI model to be compared among datasets based on analyses of bootstrap confidence intervals (Ikemoto et al. 2013). The possibility that such thermal characteristics differ among developmental stages within the same species has previously been raised (Anger 2001; Schmalenbach and Franke 2010; Jafari et al. 2012), and has implications to changes in the timing of events in nature under climate change; for example, if a certain stage is more sensitive to elevated temperatures than others, then it may represent a

bottleneck for recruitment to all later life stages (Gruffydd et al. 1979; Caputi et al. 2013; Quinn 2019). Being able to compare limiting and optimum temperatures for the development of American lobster larvae among stages, and perhaps also among different source populations (e.g., to test for local adaptation; Quinn et al. 2013) would also be highly useful.

Therefore, in this chapter the SSI development model was fit to data for the development of American lobster larvae at different temperatures obtained from the literature using a series of statistical packages and techniques developed by Ikemoto et al. (2013). This was done to generate new and hopefully improved estimates of the lower and upper threshold temperatures for American lobster larval development compared to those produced in previous studies, and to compare these estimates statistically among larval stages and different source studies. During this procedure, estimates of the intrinsic optimum temperature for the larval development of this species were also produced and compared, which has not been done before. The results obtained were discussed in light of previous research on this species, as well as in relation to the species' larval ecology and potential impacts of climate change. The quality of the estimates obtained was also critically evaluated to make recommendations for future studies of this species' temperature-dependent larval development.

2. MATERIALS AND METHODS

2.1. Sources of Development Data

Development times of American lobster larvae at different temperatures were obtained from 8 previously published studies (Table 1). These studies reported development times of American lobster larvae at three or more (up to 25) different temperatures, which were as low as 6.7°C, as high as 26.3°C, and covered a thermal range of 3.2 to 17.1°C (mean: 8.45°C) in total (Table 1). Many additional studies besides these 8 have also examined American lobster larval development times at different temperatures (e.g., Annis et al. 2007; Waller et al. 2017) but only compared two temperatures, meaning their data could not be fit with a complex curve like the SSI model; these other studies' data were thus not used herein.

The studies whose data were used were carried out in either a laboratory or hatchery setting (6/8 studies) or in the field (2 studies), and used larvae that originated from different locations across the species' range, as reviewed by Quinn et al. (2013) (Table 1). Larvae were reared individually (5 studies) or communally (2 studies), or sampled in field plankton tows (1 study) (Table 1). One field study (Hadley 1906) did not rely on plankton tows, but rather held groups of larvae in scrim bags moored in a harbor. Rearing temperatures were controlled and held ~constant in 4 studies, while in 3 other studies temperatures were allowed to undergo natural fluctuations, and 1 study tested both controlled and fluctuating temperatures (Table 1). All 8 studies reported total development times from hatch to the moult to stage IV (i.e., the combined duration of stages I, II, and III), and most (5/8 studies) also reported individual development times through each of stages I, II, and III (Table 1). However, development times for stage IV, and therefore total development times from hatch to the moult to stage V (i.e., the combined duration of stages I, II, III, and IV), were only reported by 3 of these studies (Table 1).

For each study, the mean development time (in days) spent in each stage (I, II, III, and IV) or combination of stages (I-III combined and I-IV combined) at each studied temperature was either taken directly from the published paper (if reported) or calculated from the data presented therein. These mean temperature-dependent development times were then used in all subsequent analyses.

Table 1. Details of American lobster larval rearing studies from which data were obtained

Study	Stages	Temperatures (°C) (range, # tested)	Source population	Rearing conditions	Temperature control
Hadley (1906)	I-III	15.6-22.2 (6.6, 3)	Rhode Island, USA	Communal (field)	Variable
Templeman (1936)	I, II, III, I-III	6.7-23.8 (17.1, 11)	Bay of Fundy and Northumberland Strait, Canada	Individual (lab)	Constant
	IV, I-IV	9.1-19.2 (10.1, 9)			
Hughes and Matthiessen (1962)	I-III	14.0-22.3 (8.3, 25)	Massachusetts, USA	Communal (hatchery)	Variable
Ford et al. (1979)	I-III	16.9-26.3 (9.4, 5)	Hatchery, origin not specified (study done in California, USA)	Individual (lab)	Constant and variable (both tested)
MacKenzie (1988)	I, II, III, I-III, IV, I-IV	9.8-22.0 (12.2, 5)	Southwest Nova Scotia and Northumberland Strait, Canada	Individual (lab)	Constant
Hudon and Fradette (1988)	I	11.3-17.5 (6.2, 12)	Magdalen Islands, QC, Canada	Field sampling	Variable
	II	12.3-17.5 (5.2, 11)			
	III, I-III	14.3-17.5 (3.2, 10)			
	IV, I-IV	14.3-17.5 (3.2, 9)			
Quinn et al. (2013)	I, II, III, I-III	10.3-22.2 (11.9, 4)	Gaspé-Nord, QC, Canada	Individual (lab)	Constant
Harrington et al. (2019)	I, II, III, I-III	14.0-22.0 (8.0, 4)	Maine, USA	Individual (lab)	Constant

2.2. Estimating SSI Model Parameters

The mean development time data for each study and stage or combination of stages at different temperatures were fit with the SSI model using the *OptimSSI* package by Ikemoto et al. (2013) in R v.3.1.1 (R Core Team 2014). This package estimated the parameters T_Φ, T_L, T_H, ρ_Φ, ΔH_A, ΔH_L, and ΔH_H of the SSI model in equation (1) for each dataset, which were output as T-Phi, TL, TH, rho-Phi, HA, HL, and HH, respectively. It also produced χ^2 and R^2 goodness-of-fit values for the fit of the model to each dataset, which were output as Chi-square and R-square, respectively. This package was used because the SSI model is extremely complex relative to other temperature-dependent development functions and is not always

possible to fit to data using all statistical programs' nonlinear regression procedures (Quinn 2017b). Although earlier programs were made to estimate SSI model parameters (Ikemoto 2008; Shi et al. 2011), they tended to be too slow for practical purposes, whereas the package introduced by Ikemoto et al. (2013) is able to estimate parameters very quickly, which is particularly important for its use when calculating bootstrap confidence intervals for these estimates (see section 2.3).

The main inputs to the *OptimSSI* program are temperatures (in °C) and development times (in days); the package then converts the temperatures into degrees Kelvin (K) and the development times into development rates (1/day). The user must also specify what portion of the dataset represents the putative 'linear' portion of the temperature-development curve. Over the specified range, a linear fit is then made of the temperature-development rate relationship (Winberg 1971; Campbell et al. 1974; Ikemoto and Takai 2000), from which initial estimates of the parameters T_L and T_H are made (Ikemoto et al. 2013; Quinn 2017b). These initial estimates are then used as starting values for these parameters in the nonlinear fitting of the data to the SSI model.

For American lobster larvae, very few studies have examined development over a sufficiently broad range of temperatures to provide a good idea of where the 'linear' portion of their temperature-development relationship occurs (Quinn 2017a, b; Table 1). The lower boundary should occur where a decrease in temperature results in a decrease in the development rate that becomes greater in magnitude with each subsequent 1° decrease, while the upper boundary should occur at the point where an increase in temperature ceases to cause an increase in development rate, but rather begins to cause a decrease (Campbell et al. 1974; Jost et al. 2012). Several studies have identified 12°C as a potential lower 'threshold' affecting American lobster larvae, below which survival and settlement decrease markedly and development becomes very slow (MacKenzie 1988; Annis et al. 2013; Quinn 2017a). Meanwhile, the only study that observed slowing development of American lobster larvae with increasing temperature was Ford et al. (1979), who observed slower development at 26.3°C than that at 24.2°C, although Templeman (1936) reported that development time changed very little as the temperature increased from 19.2 to 23.8°C. Therefore, in this chapter the lower boundary of the linear portion of the development curve was set at 12°C and the upper boundary was set at 24.3°C. This was fixed for analyses of all studies and stages due to limited information on whether this should vary among stages or the region of larval origin.

In the calculation of parameter estimates, the *OptimSSI* package initially sets the values of T_L and T_H (in K) to those estimated from the linear fit, T_Φ to 298.15 K (25°C), and ΔHL and ΔHH to −50,000 and 50,000 cal/mol, respectively; for other details of model initialization and parameter estimation algorithms, see Ikemoto et al. (2013). After performing all calculations, the program reports all parameter estimates for a given dataset as its final output. For ease of interpretation, all temperature parameter estimates, although reported in K by the program and used as such within the SSI function, were converted to °C for presentation in all tables and figures herein. Plots of the SSI curves for each stage and study dataset were made in R using the *SSIPlot* function of Ikemoto et al. (2013).

2.3. Calculating Bootstrap Confidence Intervals of Parameter Estimates

Because specialized algorithms are required to estimate parameters of the SSI model, it can be difficult to obtain reasonable estimates of the uncertainty around these parameter estimates, which also means that they cannot always be compared statistically (e.g., among larval stages, studies, populations of origin, etc.). Ikemoto et al. (2013) solved this issue by introducing the use of bootstrapping techniques to estimate 95% confidence intervals (95% C.I.s) of SSI model parameter estimates. Bootstrapping is a family of computerized techniques that perform random resamplings of a dataset or distribution with replacement to derive an estimate of the accuracy or repeatability of some sort of estimate made from a sample (DiCiccio and Efron 1992, 1996; Efron and Tibshirani 1994). Ikemoto et al. (2013) introduced two packages to estimate 95% C.I.s of SSI model parameter estimates using different bootstrap techniques in R. The first of these, *BCaSSI*, provides bootstrapped 95% C.I.s for all parameters estimated by the *OptimSSI* package using both the bootstrap percentile and the bias-corrected and accelerated bootstrap (BC_a) methods (Efron and Tibshirani 1994). The second, *mABCSSI*, provides bootstrapped 95% C.I.s for T_Φ only using the approximate bootstrap confidence intervals (ABC) technique (DiCiccio and Efron 1992, 1996; Efron and Tibshirani 1994), which Ikemoto et al. (2013) identified as the most appropriate approach to use for making comparisons of T_Φ values. Therefore, once *OptimSSI* had generated SSI parameter estimates for a given dataset, these algorithms were used to estimate 95% C.I.s of all parameter estimates in the present chapter's analyses.

2.4. Comparisons of Estimates among Larval Stages and Studies

Comparisons herein were focused on the parameters of the SSI model representing potential thermal thresholds for the development of lobster larvae (T_L and T_H), as well as the estimated intrinsic optimum temperature for larval development (T_Φ). As a first set of preliminary tests, one-way analyses of variance (ANOVAs) were performed in R to coarsely test whether estimated T_L, T_H, and T_Φ values differed among American lobster larval stages, with different studies treated as replicates. Overall mean values of each parameter ± 95% C.I.s were also calculated for each stage or combination of stages across studies and plotted. Whether the results of these tests would lead to different conclusions than those of analyses based on bootstrapped 95% C.I.s (see below) was also considered.

Bootstrapped 95% C.I.s formed the primary basis of comparisons among SSI model parameters among American lobster larval stages and studies. Comparisons among the estimates for a given group (stage or study) were made using analysis of confidence intervals (Cummings et al. 2007). In this approach, if the 95% C.I.s of one group overlap with the mean value of another, then the two groups can be concluded to not be significantly different ($p > 0.05$), whereas if both groups' 95% C.I.s do not overlap with one another's means (i.e., the 95% C.I. of the difference between the two groups does not overlap with zero; Lin et al. 2018; Shi et al. 2017, 2019) then they are significantly different ($p < 0.05$) (Cummings et al. 2007). SSI model parameter estimates were compared in place of mean values in the present chapter, and the 95% C.I.s were those produced by bootstrapping for each parameter.

Two series of comparisons were made. First, each of these estimates was compared among different larval stages (I, II, and III, and IV if data were available) for each study that

provided data on multiple stages (Table 1). Second, each of these estimates was compared among different studies for each stage (I, III, III, and IV) and combination of stages (I-III and I-IV). T_L and T_H estimates were compared using the 95% C.I.s calculated for them based on both the bootstrap percentile and BC_a methods. T_Φ estimates were also compared using 95% C.I.s generated using these two techniques, in addition to using those generated with the ABC method; however, given the conclusions of Ikemoto et al. (2013) more weight was given to the results of comparison of T_Φ estimates based on ABC-derived 95% C.I.s.

Whether overall and per-study estimates of T_L, T_H, and T_Φ values were biologically realistic was also considered. Since T_L and T_H are potential temperatures at which larval development could be impaired and prevented, estimates of these parameters that fell within the range of temperatures over which successful larval development has been observed in previous studies (6.7–26.3°C; Table 1; Quinn 2017a) were considered unrealistic. Estimates of T_L, T_H, or T_Φ beyond the likely range of temperatures that are biologically relevant to American lobster larvae (e.g., those < 0°C and >> 40°C; Quinn and Rochette 2015; Quinn 2017a, b) were also suspected of being unlikely to be true. If $T_L > T_H$, $T_L > T_\Phi$, or $T_\Phi > T_H$, this was also obviously unrealistic. Previous studies have suggested (based on various criteria) that temperatures of ca. 18-20°C are 'optimal' for American lobster larval growth (Van Olst et al. 1976; Carlberg and Van Olst 1977; Ford et al. 1979; Bartley et al. 1980; Quinn 2017a), so T_Φ was expected to be near this range. A T_Φ estimate that was not excessively high, but high enough to be within the range over which higher temperatures begin to slow down development (i.e., beyond the upper bound of the 'linear' portion of the development curve, which was herein considered to be ca. 24.2-26.3°C (Ford et al. 1979)), was also suspected of being unrealistic.

2.5. Combined Analyses Using All Studies' Data

As only a relatively small sample size and limited range of temperatures was captured in most of the individual studies from which data were extracted herein (Table 1), a further set of analyses was done in which all 8 studies' data were combined into a single dataset. All of the procedures described above (sections 2.2-2.4) were then carried out on the combined dataset for each stage and combination of stages. Whether the use of the combined dataset resulted in different conclusions regarding the thermal parameters of lobster larval development than analyses of individual studies' datasets was then examined.

3. RESULTS

3.1. Overall Findings

Estimates of the parameters of the SSI model when fit to development time data of American lobster larvae from different studies and their bootstrapped 95% C.I.s are presented in Tables 2-8. The corresponding temperature-dependent development rate curves based on the SSI model parameters estimated for each of these datasets are plotted in Figures 1-6.

For the majority of study and stage datasets to which the SSI model was fit, a reasonable fit with an $R^2 \geq 0.9$ was achieved (Tables 2-8), and a development curve of the appropriate shape corresponding to the SSI model over the range of biologically relevant temperatures for lobster larvae (ca. 0-40°C) was generated (Figures 1-6). Studies that considered a narrower range of test temperatures produced curves that were more narrow, whereas studies that tested a broader range of temperatures produced wider curves, as would be expected (Table 1; Figures 1-6).

Table 2. SSI parameter estimates for data from Templeman (1936)

	Parameter	Estimate	LCI (BP/ABC)	UCI (BP/ABC)	LCI (BC_a)	UCI (BC_a)
Stage I	T-Phi	17.673	12.484	19.371	12.713	21.378
			16.832	18.049		
	TL	5.378	-138.230	8.744	-132.216	8.898
	TH	24.423	20.528	880.038	-33129.340	29.906
	rho-Phi	0.2399	0.1057	0.3048	0.1256	0.4614
	HA	2.175E+04	1.628E+04	3.335E+04	1.548E+04	3.230E+04
	HL	-6.971E+04	-2.277E+05	-9.702E+03	-5.881E+05	-1.922E+04
	HH	1.562E+05	1.154E+04	3.752E+05	6.515E+04	5.542E+06
	Chi-square	3.694E-02	1.332E-04	7.348E-02	2.936E-03	4.634E-01
	R-square	0.9249				
Stage II	T-Phi	16.786	13.459	19.606	14.004	20.187
			16.619	17.018		
	TL	7.936	6.854	9.254	6.809	8.812
	TH	27.868	19.706	37.513	21.294	134.736
	rho-Phi	0.1828	0.1211	0.2346	0.1298	0.2480
	HA	1.747E+04	1.481E+04	2.428E+04	1.310E+04	2.204E+04
	HL	-8.393E+04	-2.086E+05	-7.447E+04	-1.086E+05	-5.281E+04
	HH	6.863E+04	2.842E+04	2.908E+05	7.548E+03	1.526E+05
	Chi-square	2.417E-03	1.152E-04	3.997E-03	7.981E-04	5.045E-03
	R-square	0.9953				
Stage III	T-Phi	18.240	13.937	20.445	15.161	22.233
			17.882	18.561		
	TL	6.896	4.902	9.508	0.725	9.384
	TH	28.662	18.017	53.484	19.066	70.220
	rho-Phi	0.1387	0.0858	0.1697	0.0978	0.1882
	HA	1.729E+04	1.526E+04	2.035E+04	1.430E+04	1.999E+04
	HL	-5.736E+04	-2.139E+05	-4.339E+04	-1.230E+05	-9.680E+03
	HH	7.083E+04	1.659E+04	6.238E+05	6.549E+03	2.639E+05
	Chi-square	5.241E-03	3.053E-04	6.819E-03	3.509E-03	9.160E-03
	R-square	0.9902				
Stage IV	T-Phi	18.005	12.417	19.649	14.217	24.193
			17.111	18.026		
	TL	-1.252	-129.485	9.854	-144.950	9.479
	TH	48.215	15.865	275.492	18.437	1602.177
	rho-Phi	0.0600	0.0327	0.0711	0.0401	0.0837
	HA	1.736E+04	1.114E+04	2.293E+04	1.256E+04	2.620E+04
	HL	-4.538E+04	-8.102E+05	-9.791E+03	-2.452E+05	-1.366E+03
	HH	3.205E+04	8.354E+03	1.376E+06	1.426E+01	1.313E+05
	Chi-square	2.460E-03	7.735E-06	3.596E-03	1.284E-03	4.378E-02
	R-square	0.9601				

Notes: LCI and UCI = lower and upper 95% C.I.s estimated with the bootstrap percentile (BP), BC_a, or ABC methods; ABC-derived 95% C.I.s were calculated for T-Phi only, and are presented in the same columns as BP-derived 95% C.I.s, but one row beneath them; $aE\pm b = a \times 10^{\pm b}$; N/A = could not be estimated; NaN = non-numeric estimate; T-Phi, TL, and TH estimates are presented in units of °C; for the units and meanings of all SSI model parameters, see equation (1) in the Introduction, and for their abbreviations in this Table and in *OptimSSI* output, see section 2.2.

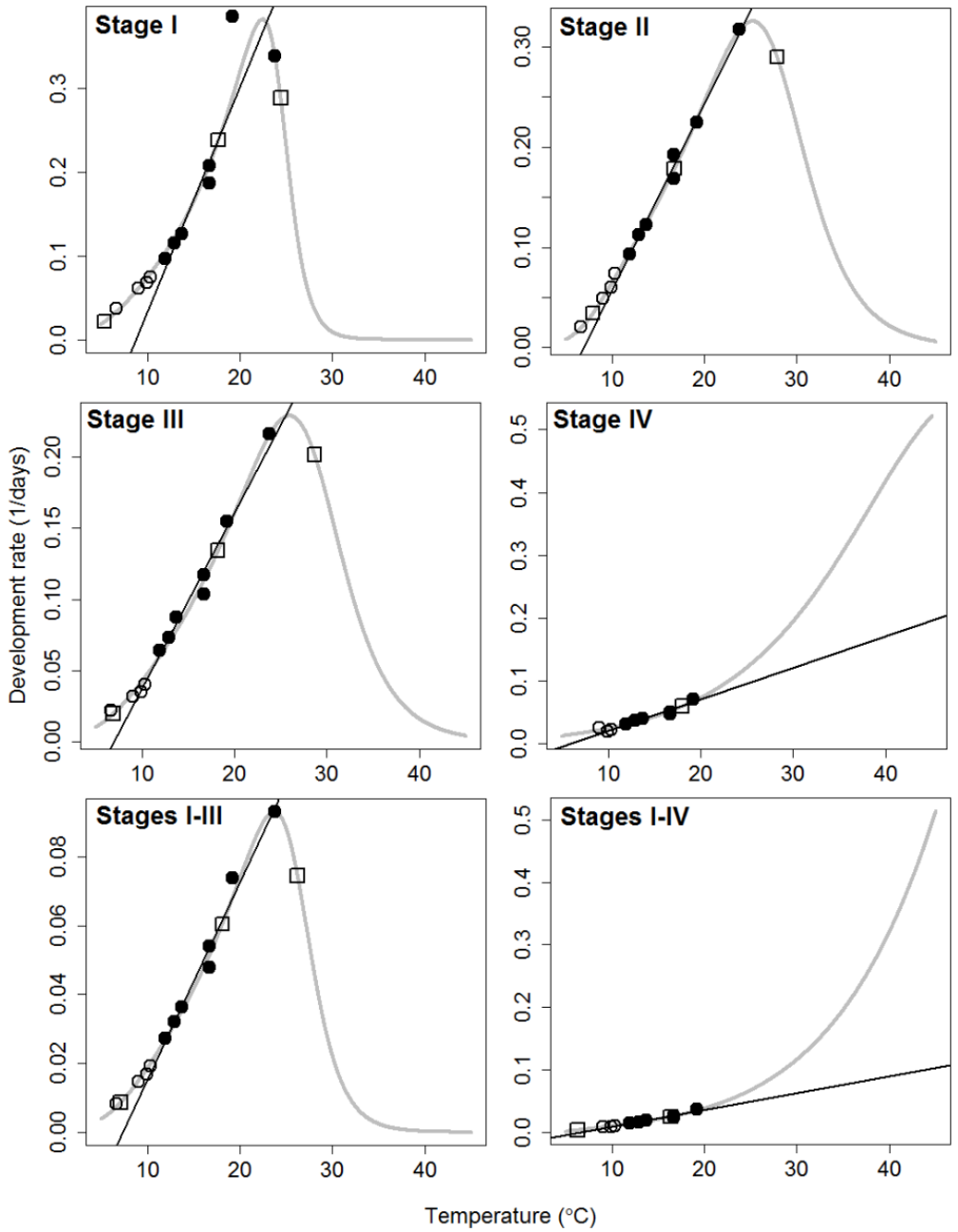

Figure 1. SSI model curves fit to development rates (y-axes, in 1/days) of American lobster larvae through different stages (different panels) at different temperatures (x-axes, in °C), using data obtained from Templeman (1936). Solid circles = observed data within the linear portion of the development curve; open circles = observed data outside the linear range; gray lines = the SSI development curve fit to all observed data; black lines = linear fit of data in the linear portion of the development curve; open squares = estimates of (from left to right) T_L, T_Φ, and T_H based on the plotted data and curves. All plots made in R with the *SSIPlot* function of Ikemoto et al. (2013).

Table 3. SSI parameter estimates for data from MacKenzie (1988)

	Parameter	Estimate	LCI (BP/ABC)	UCI (BP/ABC)	LCI (BC_a)	UCI (BC_a)
Stage I	T-Phi	15.381	12.810	18.215	12.743	18.156
			15.362	15.928		
	TL	7.764	-4.725	9.961	-4.725	9.961
	TH	29.231	17.582	41.979	23.014	43.637
	rho-Phi	0.1947	0.1359	0.2587	0.1352	0.2586
	HA	1.873E+04	1.579E+04	2.040E+04	1.579E+04	2.040E+04
	HL	-9.471E+04	-2.358E+05	-2.325E+04	-2.105E+05	-1.771E+04
	HH	4.741E+04	2.068E+04	2.292E+05	1.932E+04	1.664E+05
	Chi-square	6.853E-05	1.698E-11	6.853E-05	1.966E-05	6.853E-05
	R-square	0.9997				
Stage II	T-Phi	17.590	12.540	19.208	15.508	19.863
			17.357	18.082		
	TL	8.101	-1.960	13.975	-2.538	9.476
	TH	23.374	17.217	49.415	16.545	24.243
	rho-Phi	0.2261	0.1024	0.2740	0.1671	0.2896
	HA	2.305E+04	1.403E+04	2.744E+04	1.403E+04	2.744E+04
	HL	-8.526E+04	-6.527E+05	-4.423E+04	-5.950E+05	-4.423E+04
	HH	1.675E+05	1.688E+04	9.039E+05	3.133E+04	1.217E+06
	Chi-square	3.457E-04	2.724E-11	9.899E-04	1.471E-09	1.040E-03
	R-square	0.9987				
Stage III	T-Phi	15.056	13.031	19.416	12.047	16.884
			15.033	20.345		
	TL	8.991	5.357	10.995	6.548	11.020
	TH	29.734	19.283	59.568	19.959	61.541
	rho-Phi	0.1167	0.0808	0.1916	0.0649	0.1531
	HA	2.305E+04	1.933E+04	3.129E+04	1.933E+04	3.129E+04
	HL	-1.103E+05	-3.791E+05	-5.046E+04	-4.074E+05	-6.158E+04
	HH	3.556E+04	1.908E+04	2.341E+05	1.583E+04	1.122E+05
	Chi-square	2.429E-04	2.288E-11	2.429E-04	8.535E-05	2.589E-04
	R-square	0.9990				
Stage IV	T-Phi	14.363	12.868	23.103	12.536	15.902
			N/A	N/A		
	TL	9.258	-5.514	10.912	0.647	11.147
	TH	42.317	17.054	80.326	23.999	123.036
	rho-Phi	0.0403	0.0343	0.0763	0.0336	0.0448
	HA	1.271E+04	7.508E+03	1.736E+04	7.508E+03	1.736E+04
	HL	-2.072E+05	-6.369E+05	-6.927E+04	-7.715E+05	-7.658E+04
	HH	2.975E+04	1.713E+04	1.540E+06	1.159E+04	9.656E+04
	Chi-square	2.394E-04	3.186E-12	3.422E-04	3.250E-08	3.775E-04
	R-square	0.9927				

Notes: LCI and UCI = lower and upper 95% C.I.s estimated with the bootstrap percentile (BP), BC_a, or ABC methods; ABC-derived 95% C.I.s were calculated for T-Phi only, and are presented in the same columns as BP-derived 95% C.I.s, but one row beneath them; $aE{\pm}b = a \times 10^{\pm b}$; N/A = could not be estimated; NaN = non-numeric estimate; T-Phi, TL, and TH estimates are presented in units of °C; for the units and meanings of all SSI model parameters, see equation (1) in the Introduction, and for their abbreviations in this Table and in *OptimSSI* output, see section 2.2.

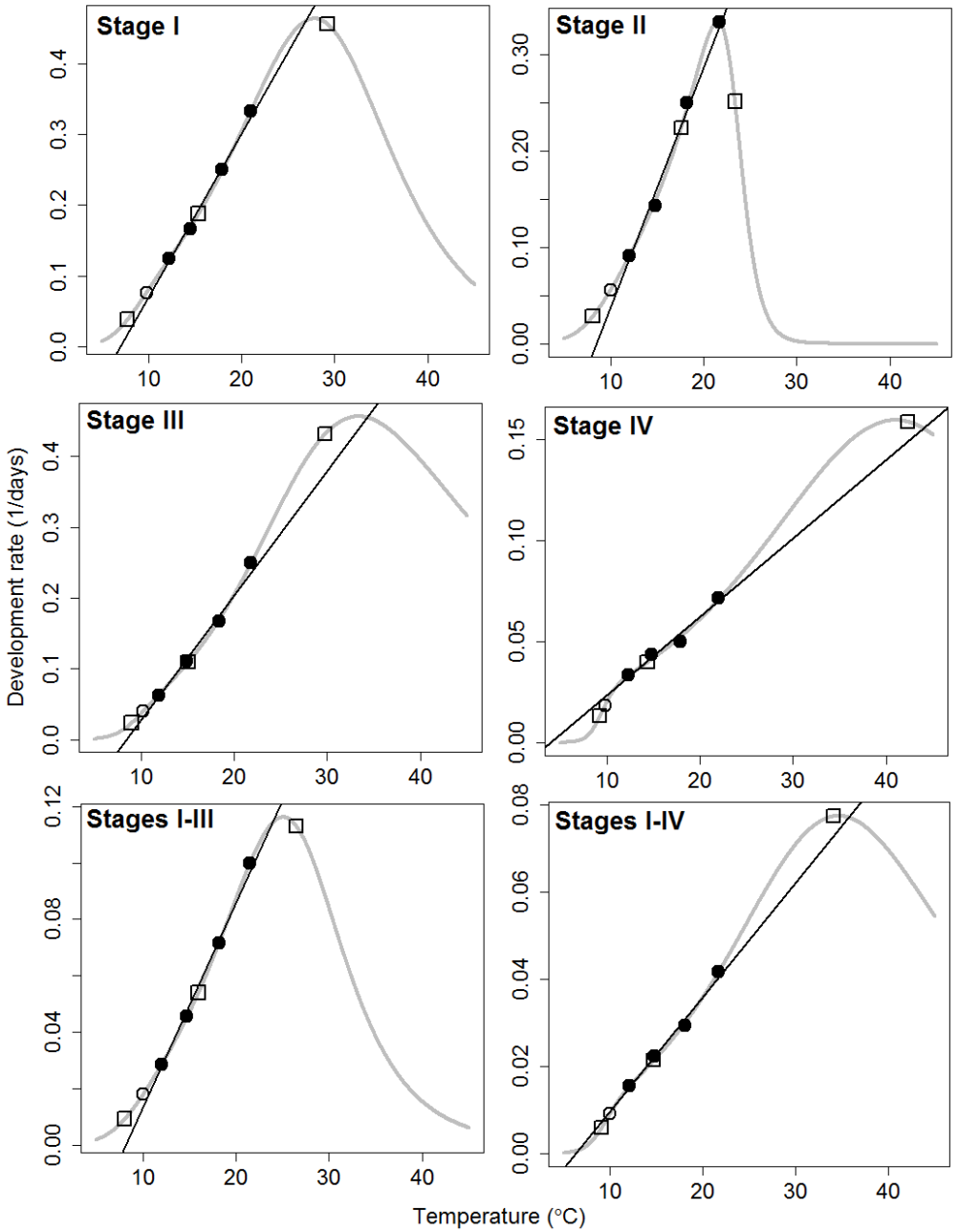

Figure 2. SSI model curves fit to development rates (y-axes, in 1/days) of American lobster larvae through different stages (different panels) at different temperatures (x-axes, in °C), using data obtained from MacKenzie (1988). Solid circles = observed data within the linear portion of the development curve; open circles = observed data outside the linear range; gray lines = the SSI development curve fit to all observed data; black lines = linear fit of data in the linear portion of the development curve; open squares = estimates of (from left to right) T_L, T_Φ, and T_H based on the plotted data and curves. All plots made in R with the *SSIPlot* function of Ikemoto et al. (2013).

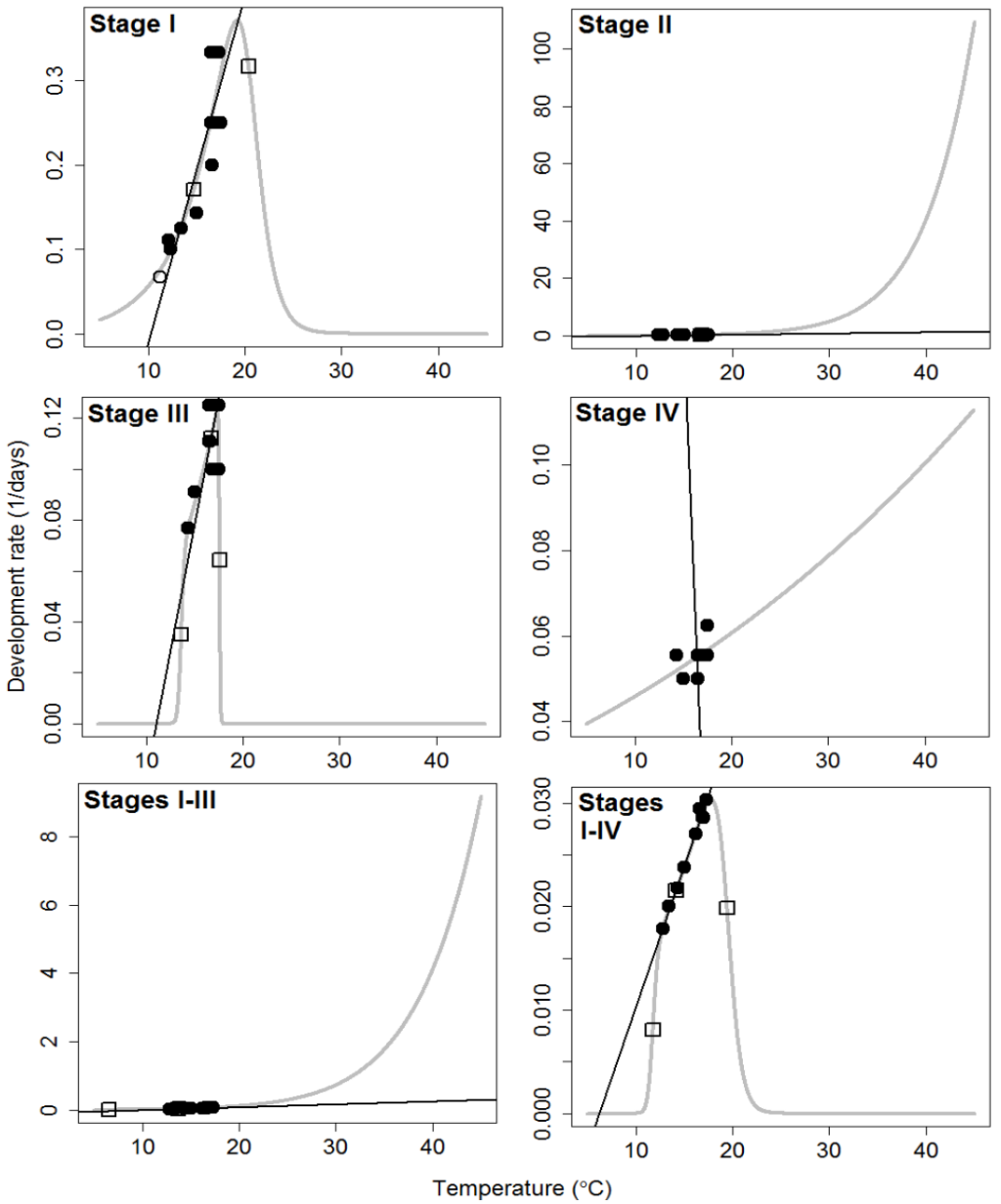

Figure 3. SSI model curves fit to development rates (y-axes, in 1/days) of American lobster larvae through different stages (different panels) at different temperatures (x-axes, in °C), using data obtained from Hudon and Fradette (1988). Solid circles = observed data within the linear portion of the development curve; open circles = observed data outside the linear range; gray lines = the SSI development curve fit to all observed data; black lines = linear fit of data in the linear portion of the development curve; open squares = estimates of (from left to right) T_L, T_Φ, and T_H based on the plotted data and curves. All plots made in R with the *SSIPlot* function of Ikemoto et al. (2013).

Table 4. SSI parameter estimates for Hudon and Fradette (1988)

	Parameter	Estimate	LCI (BP/ABC)	UCI (BP/ABC)	LCI (BC_a)	UCI (BC_a)
Stage I	T-Phi	14.715	12.863	16.957	12.805	16.908
			14.653	17.505		
	TL	-37.113	-117.530	11.704	-253.580	-21.934
	TH	20.394	17.528	439.689	-2705.501	89.515
	rho-Phi	0.1819	0.1132	0.2912	0.1124	0.2888
	HA	3.707E+04	3.005E+04	5.521E+04	2.923E+04	4.857E+04
	HL	-7.219E+03	-3.699E+06	-1.011E+04	-3.024E+03	-8.417E+02
	HH	1.764E+05	1.385E+04	5.843E+06	1.207E+04	5.007E+06
	Chi-square	7.261E-02	1.115E-02	1.241E-01	4.283E-02	7.025E-01
	R-square	0.8251				
Stage II	T-Phi	16.784	N/A	N/A	N/A	N/A
			13.964	16.888		
	TL	-10.535	N/A	N/A	N/A	N/A
	TH	53.795	N/A	N/A	N/A	N/A
	rho-Phi	0.2446	N/A	N/A	N/A	N/A
	HA	3.916E+04	N/A	N/A	N/A	N/A
	HL	-1.052E+05	N/A	N/A	N/A	N/A
	HH	9.623E+04	N/A	N/A	N/A	N/A
	Chi-square	2.653E-02	N/A	N/A	N/A	N/A
	R-square	0.9083				
Stage III	T-Phi	16.698	5.648	37.059	15.406	47.884
			16.200	16.997		
	TL	13.630	-157.033	165.213	5.111	1703.405
	TH	17.599	-108.049	229.838	-340748.550	22.908
	rho-Phi	0.1122	0.0897	4.7534	0.0918	7.7974
	HA	2.487E+04	1.946E+04	4.193E+04	1.707E+04	3.657E+04
	HL	-9.562E+05	-2.785E+06	-2.296E+03	-6.623E+06	-1.758E+05
	HH	3.543E+06	1.300E+04	5.729E+06	1.479E+06	1.913E+07
	Chi-square	7.052E-03	2.744E-04	1.815E-02	1.597E-03	2.141E-02
	R-square	0.6564				
Stage IV	T-Phi	-28.053	N/A	N/A	N/A	N/A
			-49.561	113.625		
	TL	54.007	N/A	N/A	N/A	N/A
	TH	-100.987	N/A	N/A	N/A	N/A
	rho-Phi	2.5219	N/A	N/A	N/A	N/A
	HA	9.969E+03	N/A	N/A	N/A	N/A
	HL	-9.309E+03	N/A	N/A	N/A	N/A
	HH	6.014E+03	N/A	N/A	N/A	N/A
	Chi-square	1.541E-03	N/A	N/A	N/A	N/A
	R-square	0.2141				

Notes: LCI and UCI = lower and upper 95% C.I.s estimated with the bootstrap percentile (BP), BC_a, or ABC methods; ABC-derived 95% C.I.s were calculated for T-Phi only, and are presented in the same columns as BP-derived 95% C.I.s, but one row beneath them; $aE{\pm}b = a \times 10^{\pm b}$; N/A = could not be estimated; NaN = non-numeric estimate; T-Phi, TL, and TH estimates are presented in units of °C; for the units and meanings of all SSI model parameters, see equation (1) in the Introduction, and for their abbreviations in this Table and in *OptimSSI* output, see section 2.2.

However, there were several cases in which the development curve generated was highly abnormal and unlikely to be realistic. In 8 cases, the data to which the model were fitted ended up being treated as located very early in the linear portion of the temperature-development curve, with the temperature at which the maximum development rate occurs and above which the rate declines until the T_H is reached being extrapolated to extremely high temperatures. This problem occurred for data for stages IV and I-IV from Templeman (1936) (Figure 1), stages II and I-III from Hudon and Fradette (1988) (Figure 3), stages II, III, and I-

III from Quinn et al. (2013) (Figure 4), and stage I from Harrington et al. (2019) (Figure 5). Further, in addition to suffering the aforementioned problem data for stage IV from Hudon and Fradette (1988) also produced an inverted linear fit (Figure 3) to produce starting values to estimate T_L and T_H, resulting in extremely high T_L and extremely low T_Φ and T_H estimates for this dataset (Table 4). Therefore, the estimates of T_Φ, T_L, and T_H produced from such datasets are highly suspect and certainly incorrect. The structure of these datasets also in many cases did not permit bootstrap 95% C.I.s to be estimated, or if they were estimated they were often extremely wide and/or highly asymmetrical, limiting the ability for such estimates to be compared across studies or stages (Tables 4 and 5).

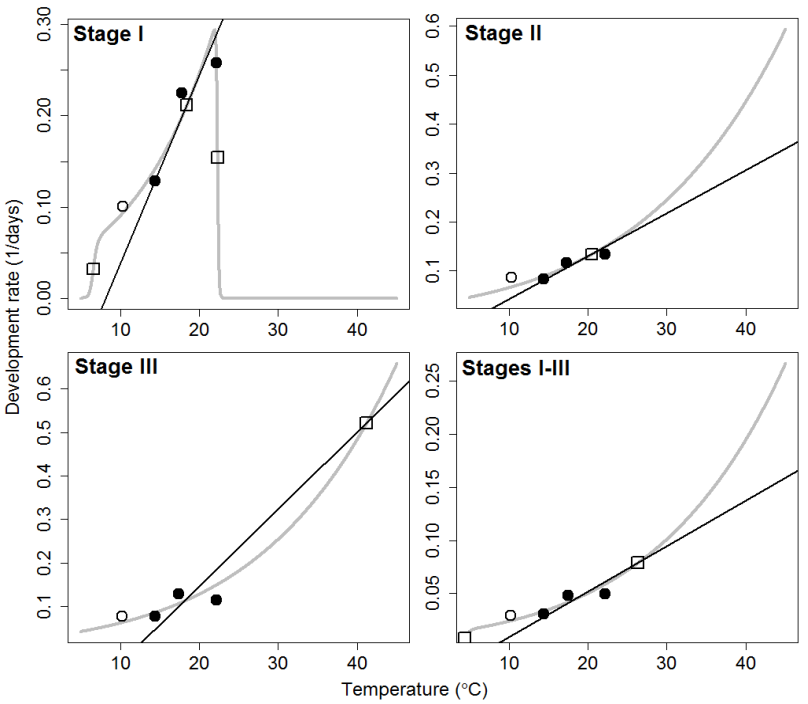

Figure 4. SSI model curves fit to development rates (y-axes, in 1/days) of American lobster larvae through different stages (different panels) at different temperatures (x-axes, in °C), using data obtained from Quinn et al. (2013). Solid circles = observed data within the linear portion of the development curve; open circles = observed data outside the linear range; gray lines = the SSI development curve fit to all observed data; black lines = linear fit of data in the linear portion of the development curve; open squares = estimates of (from left to right) T_L, T_Φ, and T_H based on the plotted data and curves. All plots made in R with the *SSIPlot* function of Ikemoto et al. (2013).

The average estimated value of T_Φ for lobster larvae across all study datasets was 17.44°C (range: 14.72-20.99°C) for stage I, 17.72°C (range: 16.78-20.50°C) for stage II, 21.81°C (range: 16.70-41.17°C) for stage III, 1.44°C (range: -28.05-18.00°C) for stage IV, 18.46°C (range: 13.70-26.33°C) for stages I-III combined, and 15.08°C (range: 14.17-16.373°C) for stages I-IV combined (Figure 8). The extremely low and unrealistic average estimated T_Φ for stage IV resulted from the highly abnormal development curve estimated for this stage from Hudon and Fradette's (1988) data (see above, and Figure 3). Three studies' datasets produced biologically unrealistic estimates of T_Φ: -28.053°C for stage IV (Hudon and

Fradette (1988): Table 4 and Figure 3), 26.333°C for stages I-III combined (Quinn et al. (2013): Table 5 and Figure 4), and 41.172°C for stage III (Quinn et al. (2013): Table 5 and Figure 4). Aside from these extremes, estimates of T_{Φ} for American lobster larvae generally fell within the more biologically realistic range of 13.703-20.503°C (Tables 2-8).

Table 5. SSI parameter estimates for data from Quinn et al. (2013)

	Parameter	Estimate	LCI (BP/ABC)	UCI (BP/ABC)	LCI (BC_a)	UCI (BC_a)
Stage I	T-Phi	18.438	15.155	20.408	15.806	20.408
			15.822	18.442		
	TL	6.621	-141.611	13.554	-141.611	13.554
	TH	22.349	22.349	366.667	NaN	NaN
	rho-Phi	0.2117	0.1500	0.2447	0.1634	0.2447
	HA	1.592E+04	5.419E+03	2.725E+04	5.419E+03	2.725E+04
	HL	-6.125E+05	-6.125E+05	-9.095E+03	NaN	NaN
	HH	2.007E+06	2.300E+04	2.007E+06	4.988E+05	2.007E+06
	Chi-square	5.111E-03	2.394E-12	3.742E-02	3.725E-10	3.742E-02
	R-square	0.9478				
Stage II	T-Phi	20.503	13.854	20.503	18.429	20.503
			20.503	21.411		
	TL	-132.717	-132.717	13.575	NaN	NaN
	TH	71.386	22.400	116.714	24.643	116.714
	rho-Phi	0.1341	0.0863	0.1382	0.0998	0.1382
	HA	1.067E+04	5.068E+03	1.943E+04	5.068E+03	1.943E+04
	HL	-1.239E+04	-5.828E+05	-1.239E+04	-7.244E+04	-1.239E+04
	HH	9.979E+04	5.279E+04	1.577E+06	4.798E+04	1.253E+05
	Chi-square	8.585E-03	1.898E-13	5.392E-02	1.898E-13	5.392E-02
	R-square	0.5845				
Stage III	T-Phi	41.172	11.269	51.897	15.647	51.897
			21.265	41.310		
	TL	-18.797	-141.659	13.710	-145.280	3.724
	TH	71.273	23.108	302.101	23.801	302.101
	rho-Phi	0.5213	0.0823	0.7523	0.0871	0.7523
	HA	1.151E+04	4.400E+03	2.855E+04	4.400E+03	2.855E+04
	HL	-9.766E+04	-6.582E+05	-1.221E+03	-6.582E+05	-4.094E+03
	HH	2.708E+05	3.603E+04	1.672E+06	4.834E+04	1.712E+06
	Chi-square	1.762E-02	1.244E-12	8.547E-02	7.777E-12	8.547E-02
	R-square	-0.0092				

Notes: LCI and UCI = lower and upper 95% C.I.s estimated with the bootstrap percentile (BP), BC_a, or ABC methods; ABC-derived 95% C.I.s were calculated for T-Phi only, and are presented in the same columns as BP-derived 95% C.I.s, but one row beneath them; $aE{\pm}b = a \times 10^{\pm b}$; N/A = could not be estimated; NaN = non-numeric estimate; T-Phi, TL, and TH estimates are presented in units of °C; for the units and meanings of all SSI model parameters, see equation (1) in the Introduction, and for their abbreviations in this Table and in *OptimSSI* output, see section 2.2.

The average estimated value of T_L for lobster larvae across all study datasets was -2.77°C (range: -37.11-7.76°C) for stage I, -22.76°C (range: -132.72-8.10°C) for stage II, 4.57°C (range: -18.80-13.63°C) for stage III, 20.67°C (range: -1.25-54.01°C) for stage IV, 8.90°C (range: 4.44-14.04°C) for stages I-III combined, and 9.05°C (range: 6.30-11.78°C) for stages I-IV combined (Figure 8). Clearly, most of these average estimates are biologically unrealistic, as they are subzero for stages I and II, above temperatures at which successful development has been reported for stages I-III and I-IV combined, and far too high and near the presumed optimum for stage IV. These results likely reflect the high variability in these estimates among study datasets, which was due to many datasets being unable to generate

reasonable estimates of T_L, or indeed to be fit with the SSI model at all (see above). Biologically realistic estimates of T_L (i.e., between 0-6.7°C) were found for only 7 out of 29 (24.1%) cases (Tables 2-8).

The average estimated value of T_H for lobster larvae across all study datasets was 29.99°C (range: 20.39-53.55°C) for stage I, 39.79°C (range: 22.50-71.39°C) for stage II, 34.13°C (range: 17.60-71.27°C) for stage III, -3.48°C (range: -110.99-48.215°C) for stage IV, 41.74°C (range: 23.56-114.76°C) for stages I-III combined, and 42.44°C (range: 19.45-73.78°C) for stages I-IV combined (Figure 8). While most of these averaged estimates are more reasonable, they are quite high, exceeding ca. 40°C for stages II, I-III, and I-IV, and are thus unlikely to be real; further, the average estimate for stage IV is far too low (subzero). As for T_L estimates, high variability and unreliable parameter estimations from many study datasets was likely responsible for these unrealistic estimates. Biologically realistic estimates of T_H (i.e., those > 26.3°C and not much more than 40°C) were only generated in 8 out of 29 (27.6%) cases (Tables 2-8).

Table 6. SSI parameter estimates for data from Harrington et al. (2019)

	Parameter	Estimate	LCI (BP/ABC)	UCI (BP/ABC)	LCI (BC_a)	UCI (BC_a)
Stage I	T-Phi	20.987	14.150	21.486	14.678	21.486
			17.913	21.991		
	TL	3.487	-61.104	12.030	-61.104	7.352
	TH	53.553	18.334	86.202	24.267	86.202
	rho-Phi	0.2415	0.1281	0.2553	0.1322	2.55E-01
	HA	1.619E+04	1.187E+04	1.992E+04	1.187E+04	1.992E+04
	HL	-3.105E+05	-5.638E+05	-5.957E+03	-5.638E+05	-1.832E+05
	HH	1.943E+05	2.004E+04	1.763E+06	3.295E+04	1.763E+06
	Chi-square	1.000E-03	7.961E-12	1.472E-03	6.253E-11	1.472E-03
	R-square	0.9865				
Stage II	T-Phi	16.928	0.389	19.580	-24.925	18.815
			16.740	17.860		
	TL	13.401	-59.558	15.840	-22.294	15.840
	TH	22.504	22.065	435.758	21.487	29.521
	rho-Phi	0.1937	0.1028	0.3237	0.0976	2.64E-01
	HA	2.928E+04	2.122E+03	6.290E+04	2.122E+03	6.290E+04
	HL	-4.108E+05	-1.624E+06	-9.037E+03	-1.624E+06	-8.724E+04
	HH	2.534E+05	1.555E+04	2.483E+06	1.555E+04	2.483E+06
	Chi-square	2.298E-02	1.637E-12	2.874E-02	7.761E-09	2.874E-02
	R-square	0.8642				
Stage III	T-Phi	17.867	14.116	19.098	14.261	19.098
			17.210	19.015		
	TL	12.129	-127.299	15.506	-11.093	15.506
	TH	23.395	22.513	498.116	21.737	31.391
	rho-Phi	0.1548	0.0766	0.1831	0.0776	1.83E-01
	HA	2.447E+04	1.277E+04	4.039E+04	1.277E+04	3.458E+04
	HL	-1.718E+05	-6.985E+05	-9.224E+03	-6.985E+05	-5.333E+04
	HH	1.882E+05	1.214E+04	7.530E+05	2.600E+04	7.530E+05
	Chi-square	2.140E-03	5.282E-12	2.140E-03	1.043E-03	2.140E-03
	R-square	0.9789				

Notes: LCI and UCI = lower and upper 95% C.I.s estimated with the bootstrap percentile (BP), BC_a, or ABC methods; ABC-derived 95% C.I.s were calculated for T-Phi only, and are presented in the same columns as BP-derived 95% C.I.s, but one row beneath them; $aE\pm b = a \times 10^{\pm b}$; N/A = could not be estimated; NaN = non-numeric estimate; T-Phi, TL, and TH estimates are presented in units of °C; for the units and meanings of all SSI model parameters, see equation (1) in the Introduction, and for their abbreviations in this Table and in *OptimSSI* output, see section 2.2.

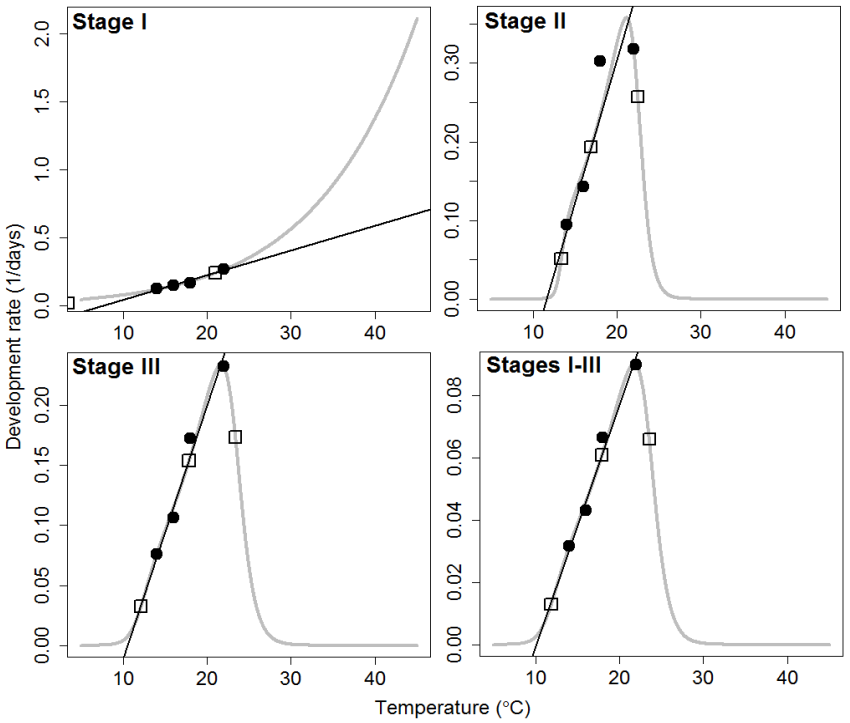

Figure 5. SSI model curves fit to development rates (y-axes, in 1/days) of American lobster larvae through different stages (different panels) at different temperatures (x-axes, in °C), using data obtained from Harrington et al. (2019). Solid circles = observed data within the linear portion of the development curve; open circles = observed data outside the linear range; gray lines = the SSI development curve fit to all observed data; black lines = linear fit of data in the linear portion of the development curve; open squares = estimates of (from left to right) T_L, T_Φ, and T_H based on the plotted data and curves. All plots made in R with the *SSIPlot* function of Ikemoto et al. (2013).

Table 7. SSI parameter estimates for combined stages I-III data from various studies

	Parameter	Estimate	LCI (BP/ABC)	UCI (BP/ABC)	LCI (BC_a)	UCI (BC_a)
Hadley (1906)	T-Phi	18.368	16.014	18.776	16.014	18.776
			17.834	18.420		
	TL	14.040	4.030	14.040	9.383	14.040
	TH	24.216	24.084	33.323	24.084	24.232
	rho-Phi	0.0629	0.0456	0.0660	0.0456	0.0660
	HA	1.940E+04	1.643E+04	2.381E+04	1.643E+04	1.949E+04
	HL	-2.937E+05	-2.937E+05	-6.953E+04	NaN	NaN
	HH	2.160E+05	4.065E+04	2.160E+05	8.899E+04	2.160E+05
	Chi-square	5.543E-07	3.700E-12	5.543E-07	5.284E-12	5.543E-07
	R-square	1.0000				
Templeman (1936)	T-Phi	18.098	14.079	19.584	15.164	22.240
			18.076	18.139		
	TL	7.099	5.501	8.933	5.054	8.688
	TH	26.238	19.498	67.126	13.318	43.816
	rho-Phi	0.0616	0.0385	0.0707	0.0423	0.0833
	HA	1.821E+04	1.570E+04	2.316E+04	1.423E+04	2.265E+04
	HL	-6.595E+04	-1.652E+05	-5.496E+04	-9.950E+04	-1.217E+04
	HH	1.051E+05	1.373E+04	2.277E+05	1.724E+04	3.326E+05
	Chi-square	1.210E-03	7.244E-05	1.733E-03	7.092E-04	2.757E-03
	R-square	0.9916				

	Parameter	Estimate	LCI (BP/ABC)	UCI (BP/ABC)	LCI (BC_a)	UCI (BC_a)
Hughes and Matthiessen (1962)	T-Phi	17.239	16.493	18.357	16.182	17.984
			17.131	17.418		
	TL	6.086	-33.519	14.561	-41.178	14.208
	TH	23.838	21.246	34.841	22.396	777.187
	rho-Phi	0.0486	0.0417	0.0634	0.0397	0.0588
	HA	3.390E+04	2.960E+04	4.201E+04	2.898E+04	4.025E+04
	HL	-7.760E+04	-7.841E+05	-1.270E+04	-3.983E+05	-6.781E+03
	HH	1.579E+05	7.663E+04	8.032E+05	5.296E+04	6.237E+05
	Chi-square	3.694E-02	1.713E-02	5.639E-02	2.331E-02	7.330E-02
	R-square	0.8543				
Ford et al. (1979)	T-Phi	20.012	3.610	23.727	5.482	28.689
			18.551	20.564		
	TL	12.988	-29.860	18.509	-2.142	4043.622
	TH	27.442	3.333	38.263	24.304	90.965
	rho-Phi	0.0678	0.0519	0.9470	0.0518	0.8684
	HA	1.302E+04	6.958E+03	8.297E+04	6.958E+03	1.544E+04
	HL	-1.619E+05	-3.306E+06	-2.826E+04	-3.306E+06	-2.826E+04
	HH	1.606E+05	4.987E+04	4.139E+06	4.067E+04	5.201E+05
	Chi-square	1.069E-03	1.050E-12	1.713E-03	1.020E-08	2.583E-03
	R-square	0.9108				
MacKenzie (1988)	T-Phi	15.972	12.881	18.952	12.871	18.768
			14.615	16.003		
	TL	8.078	4.494	10.910	-0.076	9.309
	TH	26.491	19.839	33.786	21.926	41.598
	rho-Phi	0.0565	0.0339	0.0781	0.0338	0.0766
	HA	2.210E+04	1.723E+04	2.805E+04	1.723E+04	2.805E+04
	HL	-8.073E+04	-1.800E+05	-4.328E+04	-1.195E+05	-3.295E+04
	HH	5.958E+04	3.152E+04	1.412E+05	2.183E+04	1.069E+05
	Chi-square	1.253E-06	1.446E-11	1.253E-06	1.297E-07	2.727E-06
	R-square	1.0000				
Hudon and Fradette (1988)	T-Phi	13.703	13.123	16.897	12.919	16.657
			13.663	17.296		
	TL	6.571	-150.506	11.999	-1.025	14.637
	TH	67.413	17.731	337.263	25.868	1457.406
	rho-Phi	0.0325	0.0279	0.0616	0.0260	0.0567
	HA	3.317E+04	2.894E+04	3.891E+04	2.826E+04	3.757E+04
	HL	-1.449E+05	-5.333E+05	-1.022E+04	-4.664E+06	-7.887E+04
	HH	1.530E+04	1.302E+04	5.122E+05	3.716E+02	4.117E+04
	Chi-square	2.127E-03	4.516E-04	3.135E-03	1.171E-03	4.306E-03
	R-square	0.9408				
Quinn et al. (2013)	T-Phi	26.333	-91.320	35.598	16.478	35.598
			21.957	31.809		
	TL	4.437	-153.003	14.145	-153.003	14.145
	TH	114.756	22.364	755.530	23.493	755.530
	rho-Phi	0.0788	0.0085	0.0788	0.0483	
	HA	1.174E+04	1.268E+03	2.444E+04	1.268E+03	
	HL	-5.451E+05	-2.697E+06	-2.141E+03	-2.697E+06	
	HH	1.858E+05	1.041E+04	3.232E+06	1.031E+04	
	Chi-square	3.501E-03	3.149E-13	1.954E-02	1.756E-12	
	R-square	0.5796				
Harrington et al. (2019)	T-Phi	17.919	14.336	19.171	14.550	19.171
			17.308	17.949		
	TL	11.881	-146.901	15.565	-4.796	15.565
	TH	23.558	21.967	67.928	20.575	34.637
	rho-Phi	0.0612	0.0336	0.0730	0.0337	7.30E-02
	HA	2.284E+04	1.285E+04	3.648E+04	1.285E+04	3.648E+04
	HL	-1.632E+05	-7.560E+05	-1.015E+04	-7.560E+05	-6.119E+04
	HH	1.859E+05	2.957E+04	8.784E+05	3.240E+04	8.784E+05
	Chi-square	6.014E-04	1.691E-12	6.014E-04	1.888E-04	6.014E-04
	R-square	0.9831				

Notes: LCI and UCI = lower and upper 95% C.I.s estimated with the bootstrap percentile (BP), BC_a, or ABC methods; ABC-derived 95% C.I.s were calculated for T-Phi only, and are presented in the same columns as BP-derived 95% C.I.s, but one row beneath them; $aE{\pm}b = a \times 10^{\pm b}$; N/A = could not be estimated; NaN = non-numeric estimate; T-Phi, TL, and TH estimates are presented in units of °C; for the units and meanings of all SSI model parameters, see equation (1) in the Introduction, and for their abbreviations in this Table and in *OptimSSI* output, see section 2.2.

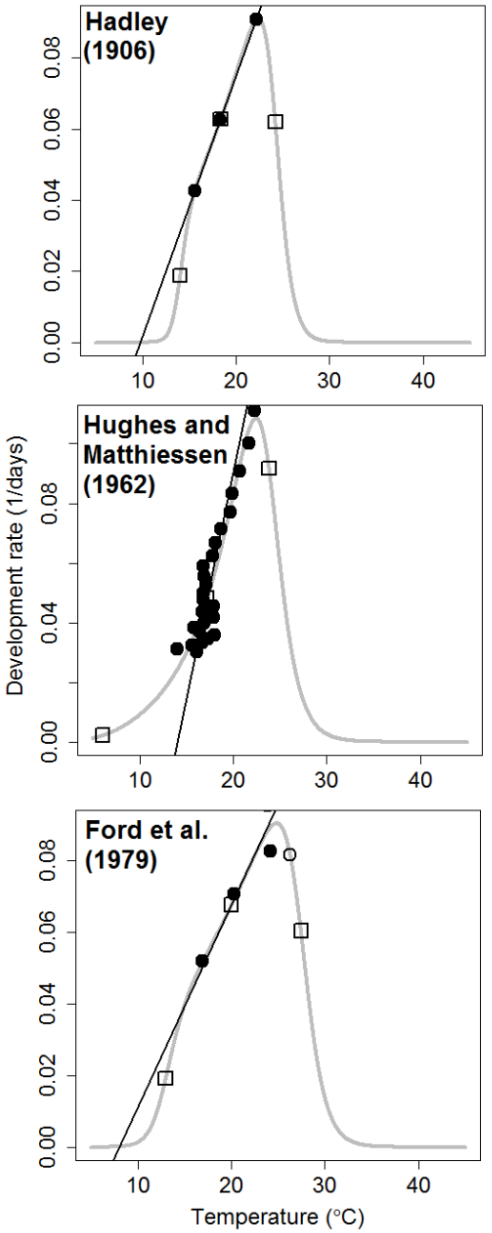

Figure 6. SSI model curves fit to development rates (y-axes, in 1/days) of American lobster larvae through stages I-III combined at different temperatures (x-axes, in °C), using data from three different studies (different panels). Solid circles = observed data within the linear portion of the development curve; open circles = observed data outside the linear range; gray lines = the SSI development curve fit to all observed data; black lines = linear fit of data in the linear portion of the development curve; open squares = estimates of (from left to right) T_L, T_Φ, and T_H based on the plotted data and curves. All plots made in R with the *SSIPlot* function of Ikemoto et al. (2013).

Table 8. SSI parameter estimates for combined stages I-IV data from various studies

	Parameter	Estimate	LCI (BP/ABC)	UCI (BP/ABC)	LCI (BC_a)	UCI (BC_a)
Templeman (1936)	T-Phi	16.373	13.068	18.736	13.492	18.946
			16.125	18.315		
	TL	6.302	-2.575	9.822	-192.688	9.141
	TH	73.779	15.589	238.965	32.371	134721.450
	rho-Phi	0.0259	0.0172	0.0335	0.0180	0.0345
	HA	1.963E+04	1.486E+04	2.433E+04	1.535E+04	2.451E+04
	HL	-8.902E+04	-7.594E+05	-4.606E+04	-3.283E+05	-9.551E+03
	HH	1.259E+04	5.203E+03	7.755E+05	2.691E+01	2.684E+04
	Chi-square	5.639E-04	1.685E-06	7.768E-04	3.282E-04	7.222E-03
	R-square	0.9831				
MacKenzie (1988)	T-Phi	14.700	12.939	19.946	12.510	16.266
			14.526	14.769		
	TL	9.066	3.430	11.199	5.664	11.199
	TH	34.083	17.951	53.108	20.307	78.910
	rho-Phi	0.0220	0.0175	0.0354	0.0164	0.0276
	HA	1.709E+04	1.394E+04	2.264E+04	1.394E+04	2.264E+04
	HL	-1.495E+05	-5.207E+05	-7.393E+04	-4.819E+05	-6.466E+04
	HH	3.383E+04	1.684E+04	4.959E+05	1.558E+04	1.316E+05
	Chi-square	4.240E-05	1.568E-12	5.277E-05	8.424E-06	6.054E-05
	R-square	0.9983				
Hudon and Fradette (1988)	T-Phi	14.169	13.627	16.027	12.904	14.797
			14.059	15.795		
	TL	11.775	-62.283	13.040	8.431	15.072
	TH	19.445	17.154	27.849	17.268	37.392
	rho-Phi	0.0215	0.0201	0.0269	0.0180	0.0232
	HA	1.877E+04	1.698E+04	2.020E+04	1.721E+04	2.046E+04
	HL	-6.019E+05	-2.097E+06	-2.297E+04	-3.390E+06	-1.379E+05
	HH	2.531E+05	6.778E+04	2.920E+06	2.709E+04	1.471E+06
	Chi-square	9.317E-05	3.125E-06	1.461E-04	4.186E-05	2.478E-04
	R-square	0.9841				

Notes: LCI and UCI = lower and upper 95% C.I.s estimated with the bootstrap percentile (BP), BC_a, or ABC methods; ABC-derived 95% C.I.s were calculated for T-Phi only, and are presented in the same columns as BP-derived 95% C.I.s, but one row beneath them; $aE\pm b = a \times 10^{\pm b}$; N/A = could not be estimated; NaN = non-numeric estimate; T-Phi, TL, and TH estimates are presented in units of °C; for the units and meanings of all SSI model parameters, see equation (1) in the Introduction, and for their abbreviations in this Table and in *OptimSSI* output, see section 2.2.

In preliminary analyses, when T_L, T_H, or T_Φ estimates were averaged across studies, there were some apparent differences among stages (Figure 7). Specifically, the T_H and T_Φ estimates were lower and the T_L estimates were higher for stage IV in comparison to those for all other larval stages; all estimates for stage IV also varied much more among studies than did those for other stages (Figure 7). Stage II also appeared to have a lower and more variable T_L than other stages (Figure 7). However, there were no statistically significant differences in these analyses among larval stages in their T_L (ANOVA: $F_{3,14} = 0.943$, $p = 0.446$), T_H (ANOVA: $F_{3,14} = 0.956$, $p = 0.437$), or T_Φ (ANOVA: $F_{3,14} = 2.126$, $p = 0.143$) estimates (Figure 7).

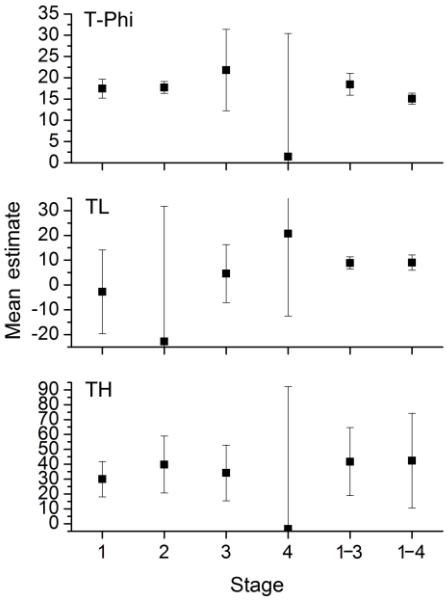

Figure 7. Estimates of the T_Φ (T-Phi), T_L (TL), and T_H (TH) parameters of the SSI model averaged across all study datasets (y-axes, in °C) for each American lobster larval stage or combination of stages (x-axes). Error bars represent ± 95% C.I.s calculated for each mean across study datasets (n = 3-8, depending on stage). Preliminary analyses were done in which each parameter (different panels) was compared across stages with separate one-way ANOVAs, but no significant differences were found (see section 3.1 for details).

3.2. Comparisons among Larval Stages for Each Study

When data from Templeman (1936) were analyzed, the estimated intrinsic optimum temperature for larval development (T_Φ) of stage II was significantly lower than those estimated for all other stages, but the T_Φ of all other stages (I, III, and IV) did not significantly differ from one another (Table 2; Figure 8). The T_Φ was significantly higher for stage II than that for stage I when data from MacKenzie (1988) were assessed and 95% C.I.s were bootstrapped using the ABC method, while that for stage III did not significantly differ from those for these stages (Table 3). The estimated T_Φ for stage IV based on MacKenzie's data was lower than those for stages I-III, but could not be compared to them because its 95% C.I.s could not be bootstrapped using the ABC method (Table 3; Figure 8). The T_Φ values estimated with data from Hudon and Fradette (1988) did not significantly differ among larval stages (Table 4; Figure 8). When data from Quinn et al. (2013) were assessed, T_Φ values for all three larval stages significantly differed, with T_Φ values increasing with stage number (stage III > II > I) (Table 5; Figure 8). The T_Φ value estimated with data from Harrington et al. (2019) for stage I was significantly higher than that estimated for stage II; however, estimates for stages II and III did not significantly differ from one another, nor did those for stages I and III (Table 6; Figure 8).

When 95% C.I.s were bootstrapped using the bootstrap percentile or BC_a methods, the T_Φ values estimated for all datasets did not differ significantly among larval stages (Tables 2-6). Although the lower (T_L) and upper (T_H) threshold temperatures for larval development

estimated for most study datasets both tended to increase with stage number, these did not significantly differ among larval stages based on comparisons using 95% C.I.s bootstrapped using the bootstrap percentile or BC_a methods (Tables 2-6).

3.3. Comparisons among Studies for Each Larval Stage

Comparisons of estimated values of the T_L, T_H, and T_Φ parameters among study datasets for the same larval stage or combinations of stages did not result in consistent overall differences among studies. Often estimates based on data from Hudon and Fradette (1988) (Tables 4, 7, and 8) and Quinn et al. (2013) (Tables 5 and 7) were lower or higher than those based on most other studies' data, but with no consistent directionality or significance (Tables 2, 3, and 6-8). Differences among studies were more often significant for the T_Φ than for the T_L and T_H parameters, especially when T_Φ estimates were compared based on 95% C.I.s bootstrapped using the ABC method (Tables 2-8; Figure 8).

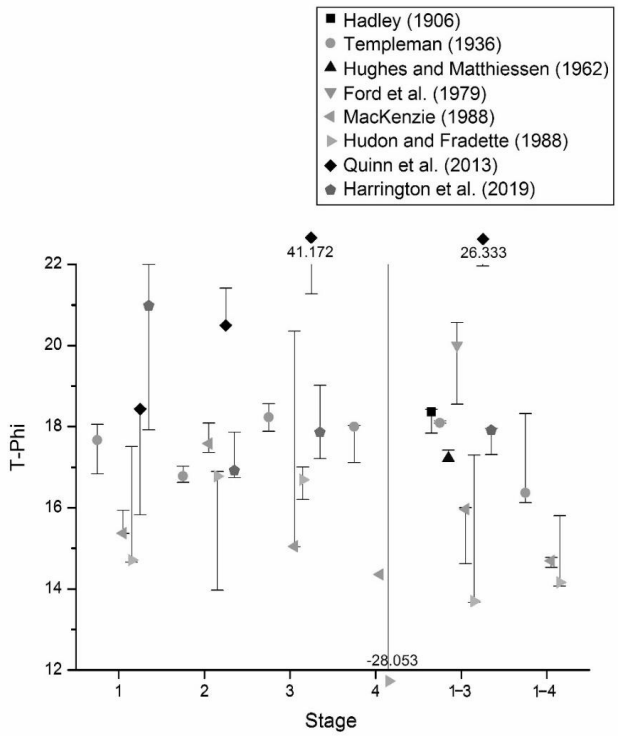

Figure 8. Estimates of the intrinsic optimum temperature (T_Φ or T-Phi) for the development of American lobster larvae estimated with the SSI model for each larval stage or combination of stages (x-axis) using data from different source studies (different symbol types; see Table 1). T-Phi estimates are presented in units of °C (y-axis). Error bars represent the lower and upper bootstrap 95% C.I.s calculated for each parameter estimate using the ABC method in R with the *mABCSSI* package of Ikemoto et al. (2013). For clarity, the y-axis in this figure is constrained to between 12-22°C; 95% C.I.s that extended beyond this range are not shown in their entirety, but the values of the three T-Phi estimates that were outside of this range are presented as numbers at the top or bottom of the graph. T-Phi estimates were compared among stages and studies based on whether their bootstrapped 95% C.I.s and estimates overlapped.

For analyses of T_Φ estimates for data for the total combined development from hatch to stage IV, the estimates based on different studies' data were all significantly different from one another based on their ABC-derived 95% C.I.s (Table 7; Figure 8), with the T_Φ estimated for Quinn et al. (2013) being significantly higher than that estimated for all other studies, and that for Harrington et al. (2019) being significantly higher than those for all other studies except for Quinn et al. (2013) (Table 7; Figure 8). For total development from hatch to stage V, the T_Φ values estimated for data from MacKenzie (1988) and Hudon and Fradette (1988) did not significantly differ from each other according to their ABC-derived 95% C.I.s, but that estimated for data from Templeman (1936) was significantly higher than those for both of these studies (Table 8; Figure 8).

3.4. Results for All Datasets Combined

The SSI model parameter estimates and plots of the SSI development function produced from analyses of all studies' datasets combined are presented in Table 9 and Figure 9, respectively.

If data from all studies were combined, the data for all stages produced reasonable development curves (Figure 9). The T_Φ value estimated for stage I based on data from all studies was significantly lower than those of stages II and III, but all other comparisons of T_Φ among stages were non-significant (Table 9). Comparisons of T_L and T_H estimates based on data from all studies combined among stages based on their bootstrap percentile- and BC_a-derived 95% C.I.s were all non-significant (Table 9), and T_Φ estimates also did not significantly differ among stages when assessed based on these bootstrapped 95% C.I.s (Table 9). Aside from the significant difference between the T_Φ of stage I and those of stages II and III, these comparisons of parameter estimates for all studies' data based on bootstrap 95% C.I.s generally agreed with the conclusions of preliminary analyses using ANOVAs (section 3.1 and Figure 7).

Table 9. SSI parameter estimates for data from all studies combined

	Parameter	Estimate	LCI (BP/ABC)	UCI (BP/ABC)	LCI (BC_a)	UCI (BC_a)
Stage I	T-Phi	16.534	15.087	18.279	13.836	17.334
			16.491	16.595		
	TL	4.936	-28.185	7.997	-22.081	8.384
	TH	23.883	21.581	25.506	22.057	745.066
	rho-Phi	0.212	0.172	0.267	0.136	0.239
	HA	2.254E+04	1.809E+04	2.982E+04	1.786E+04	2.944E+04
	HL	-6.018E+04	-2.715E+05	-2.070E+04	-1.029E+05	-9.947E+03
	HH	1.169E+05	9.241E+04	6.045E+05	1.063E+04	2.216E+05
	Chi-square	3.334E-01	1.309E-01	4.951E-01	2.198E-01	6.263E-01
	R-square	0.759				
Stage II	T-Phi	16.964	15.269	20.865	15.337	21.007
			16.874	17.001		
	TL	5.175	-105.491	8.731	-190.116	7.722
	TH	23.483	21.409	24.980	21.897	139.962
	rho-Phi	0.213	0.156	0.437	0.164	0.490
	HA	2.509E+04	2.053E+04	3.404E+04	2.038E+04	3.335E+04
	HL	-4.970E+04	-7.634E+05	-3.761E+03	-1.096E+05	-1.814E+01

	Parameter	Estimate	LCI (BP/ABC)	UCI (BP/ABC)	LCI (BC_a)	UCI (BC_a)
	HH	1.186E+05	8.783E+04	1.535E+06	2.331E+03	3.260E+05
	Chi-square	3.728E-01	1.232E-01	6.411E-01	2.164E-01	1.215E+00
	R-square	0.688				
Stage III	T-Phi	16.903	15.362	22.498	14.238	18.975
			16.148	20.371		
	TL	5.314	-230.601	9.119	-138.502	9.885
	TH	25.145	22.197	62.618	22.233	1731.213
	rho-Phi	0.121	0.098	0.269	0.084	0.165
	HA	2.041E+04	1.607E+04	2.531E+04	1.498E+04	2.459E+04
	HL	-8.649E+04	-1.769E+06	-8.389E+02	-7.226E+06	-1.302E+04
	HH	1.404E+05	3.162E+04	3.570E+06	1.930E+01	1.074E+06
	Chi-square	1.139E-01	2.593E-02	1.981E-01	5.783E-02	2.753E-01
	R-square	0.821				
Stage IV	T-Phi	16.965	14.792	34.580	14.575	29.177
			16.355	17.618		
	TL	6.949	-3.920	9.560	-51.163	9.344
	TH	24.041	18.225	92.692	18.363	130.129
	rho-Phi	0.0562	0.0444	0.2279	0.0434	0.1760
	HA	1.479E+04	1.161E+04	1.824E+04	1.065E+04	1.788E+04
	HL	-7.394E+04	-2.982E+05	-3.286E+04	-1.809E+05	-5.753E+03
	HH	1.235E+05	1.990E+04	7.927E+05	1.220E+02	3.221E+05
	Chi-square	1.146E-02	2.608E-03	1.842E-02	6.161E-03	2.451E-02
	R-square	0.9043				
Stage I-III	T-Phi	17.326	16.157	18.879	16.326	19.878
			17.085	17.682		
	TL	0.095	-30.387	6.401	-48.507	5.962
	TH	25.661	24.264	26.173	24.590	28.581
	rho-Phi	0.055	0.046	0.068	0.048	0.080
	HA	2.254E+04	2.003E+04	2.620E+04	1.973E+04	2.576E+04
	HL	-3.718E+04	-1.599E+05	-1.393E+04	-9.960E+04	-7.230E+03
	HH	1.057E+05	9.246E+04	2.937E+05	8.296E+04	1.438E+05
	Chi-square	1.089E-01	6.012E-02	1.585E-01	7.240E-02	1.819E-01
	R-square	0.819				
Stage I-IV	T-Phi	16.192	14.331	18.038	14.800	18.742
			15.266	16.735		
	TL	7.804	6.619	9.242	5.928	9.077
	TH	25.526	19.121	31.856	20.035	40.534
	rho-Phi	0.0264	0.0211	0.0317	0.0223	0.0338
	HA	1.793E+04	1.614E+04	2.031E+04	1.578E+04	1.999E+04
	HL	-9.155E+04	-2.010E+05	-7.540E+04	-1.540E+05	-6.630E+04
	HH	8.639E+04	4.612E+04	3.732E+05	2.932E+04	2.541E+05
	Chi-square	1.716E-03	4.193E-04	2.604E-03	1.001E-03	3.787E-03
	R-square	0.9770				

Notes: LCI and UCI = lower and upper 95% C.I.s estimated with the bootstrap percentile (BP), BC_a, or ABC methods; ABC-derived 95% C.I.s were calculated for T-Phi only, and are presented in the same columns as BP-derived 95% C.I.s, but one row beneath them; $aE\pm b = a \times 10^{\pm b}$; N/A = could not be estimated; NaN = non-numeric estimate; T-Phi, TL, and TH estimates are presented in units of °C; for the units and meanings of all SSI model parameters, see equation (1) in the Introduction, and for their abbreviations in this Table and in *OptimSSI* output, see section 2.2.

Based on all studies' data, T_Φ, T_L, and T_H estimates ranged from 16.534 to 17.326°C, 0.095 to 7.804°C, and 23.483 to 25.661°C, respectively (Table 9). All of these estimates of T_Φ, most estimates of T_L (except those for stages IV and I-IV, which were too high, 6.949 and 7.804°C, respectively), and none of the estimates of T_H (all were < 26.3°C) were biologically realistic (Table 9; Figure 9). Compared with estimates based on individual studies' datasets (Tables 2-8), those based on all studies combined were more consistent among stages, were

more reliable in that they had smaller bootstrap 95% C.I.s, and tended to be more realistic (Table 9).

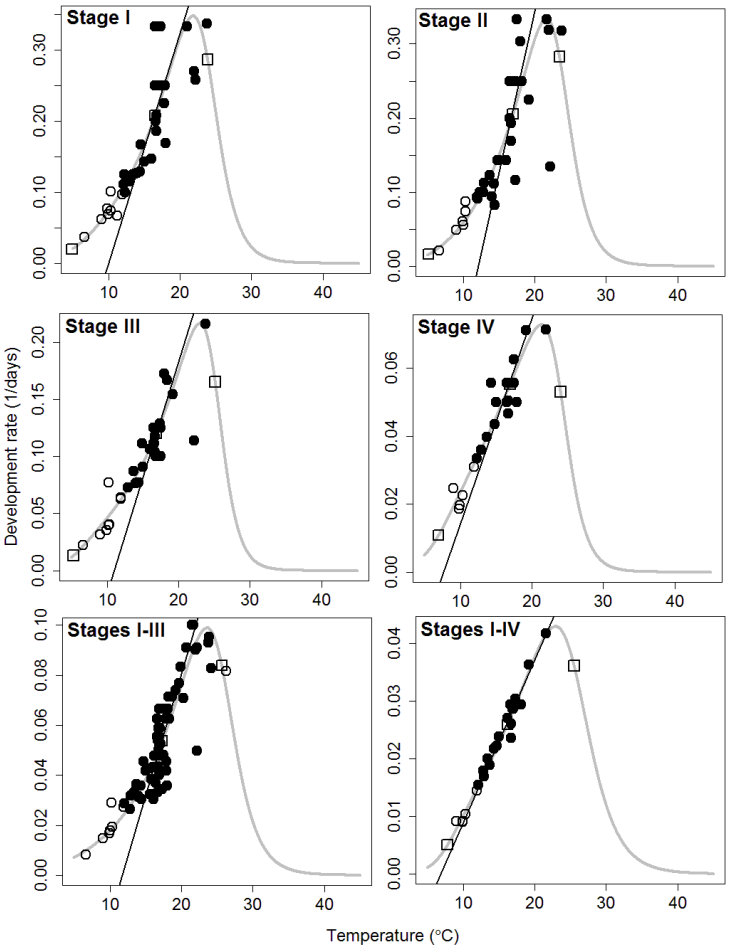

Figure 9. SSI model curves fit to development rates (y-axes, in 1/days) of American lobster larvae through different stages (different panels) at different temperatures (x-axes, in °C), using data from all studies combined (see Table 1). Solid circles = observed data within the linear portion of the development curve; open circles = observed data outside the linear range; gray lines = the SSI development curve fit to all observed data; black lines = linear fit of data in the linear portion of the development curve; open squares = estimates of (from left to right) T_L, T_Φ, and T_H based on the plotted data and curves. All plots made in R with the *SSIPlot* function of Ikemoto et al. (2013).

4. DISCUSSION

4.1. Variability and Issues among Estimates Based on Different Source Studies' Data

This chapter represents the first published application of the SSI development function to American lobster larval development data, as well as the first time that such a thermodynamic

model has been used to estimate the intrinsic optimum temperature for the development of this species' larvae. When the estimated T_{Φ}, T_L, and T_H values for a given larval stage produced for data from different individual studies were compared, however, they were found to vary markedly from one another, and in many cases the biological realism of these estimates and their reliability (inferred based on the width of their bootstrap 95% C.I.s) was very poor. Therefore, the discussion in the present section was restricted to explaining the sources of these variations and issues with individual studies' results, while comparisons of thermal parameter estimates to the published literature and discussion of their implications were saved for the discussion of estimates based on all studies' data combined in the following section (4.2).

The source data came from studies that used different larval source populations, and thus some differences could be expected among studies as a result of geographic differences in temperature-dependent development reflecting local thermal adaptation or acclimation (Quinn et al. 2013; 2017); indeed, the clear differences between the shapes of the development curves (and consequently thermal parameters) derived for data from Quinn et al. (2013) from those for all other studies, which were conducted in more southerly and relatively warm-water regions (Quinn and Rochette 2015), may support the conclusion that that study found evidence of thermal adaptation. However, the magnitudes of the differences found herein in thermal parameters, including among geographically close source locations, were far too great for this be a sufficient explanation by itself.

The data analyzed came from eight different studies spanning more than a century of research on lobster larvae (1906-2019), which obviously varied in a number of additional ways from one another in terms of the temperature range tested, setting (lab vs. field), holding conditions of larvae (individual vs. communal), temperature control (constant vs. variable) (Table 1), and many other ways (e.g., food, photoperiod, sample sizes, etc.; reviewed by MacKenzie (1988) and Quinn et al. (2013)). Such differences among studies were thus more likely reasons for the observed variability, and thus the limiting and optimum temperatures estimated based on any one specific study herein should likely not be considered representative of the entire species' developmental characteristics. The field sampling study of Hudon and Fradette (1988) produced particularly problematic estimates, which was likely due to the limited range of temperatures that can be captured by field sampling (see below), as well as possible errors resulting from not tracking the same larval individuals or cohorts across sampling dates (Annis et al. 2007; Quinn et al. 2013, 2017; Gendron et al. in press). In the future, then, estimation of SSI model parameters based on such indirect field sampling methods should probably be avoided. The small samples sizes included in the data from most of the individual studies examined (Table 1) were also likely too small to permit optimal fitting with the SSI model and the calculation of very precise 95% C.I.s for its parameter estimates (Ikemoto et al. 2013); this justified the analyses performed on combined data (see section 4.2).

The factor that likely had the greatest impact on the differences in the estimated thermal parameters among studies was the difference in the thermal ranges they used (Kontopoulos et al. 2018; Table 1). Although overall a thermal range from 6.7 to 26.3°C was investigated, which should extend into the nonlinear portions of the temperature-development curve and approach the actual T_L and T_H values for lobster larvae, no single study covered this entire range, and some covered very small ranges (e.g., 3.2-6.2°C: Hudon and Fradette 1988) and/or only extended towards very low (Templeman 1936) or high (Ford et al. 1979) temperatures,

but not both. The fitting of a complex nonlinear function like the SSI model to development data can only be accurate if the data cover both the lower and upper nonlinear portions of the development curve in addition to the intermediate linear portion (Schoolfield et al. 1981; Shi et al. 2011, 2017, 2019; Ikemoto et al. 2013). If one or more portions of this range are missing, then estimated thresholds will be placed either just outside of the observed data range (if there is some nonlinearity in the data) or very far beyond it (if the data are highly linear), which in either case results in unrealistic estimates (Jafari et al. 2012; Shi et al. 2012a, b, 2013; Quinn 2017b; Kontopoulos et al. 2018), and will also create quite wide and asymmetrical bootstrap 95% C.I.s. This can also result in a fit to data from one study estimating a threshold within the range of temperatures over which development was observed to be completed in another study; for example, the very high T_L estimate of 12.988°C for data from Ford et al. (1979), who only reared larvae at temperatures higher than 16.9°C, was well within the range of temperatures at which development was completed in most other studies considered (Table 1).

4.2. Interpretation and Implications of Estimates Based on All Studies' Data Combined

To account for the above shortcomings, in this chapter SSI fits were also attempted using all studies' data combined. Although doing this masked much of the variability within the datasets (e.g., the strong differences in the development curves of Quinn et al. (2013) from all others; Figure 9) and was still not able to estimate biologically realistic T_H values > 26.3°C (Table 9), it did allow the widest thermal range possible to be investigated. Further, this approach resulted in much more reasonable and consistent parameter estimates than did analyses with individual study datasets. It also allowed a few patterns to be detected that were missed by preliminary analyses using conventional ANOVAs to make comparisons among stages. Using this approach, the T_Φ, T_L, and T_H of American lobster larvae were estimated to be 16.534-17.326°C, 0.095-7.804°C, and 23.483-25.661°C, respectively (Table 9). Although these estimates did increase, to some extent, with stage number (i.e., thermal parameters of later stages tended to have higher values than those of earlier stages), none of these parameters differed significantly among stages except that the T_Φ of stage I was less than that of stages II and III.

The range of T_L values found was mostly reasonable, although a few were too high, while all T_H estimates were likely too low, but perhaps close to (i.e., within ~2°C of) being reasonable. The range of estimates of T_L and T_H produced agreed in general with previous experimental observations (Huntsman 1924; Sastry and Vargo 1977; Gruffydd et al. 1979; Quinn 2017a) and with those derived using other nonlinear development functions by Quinn (2017b) but with fewer T_L values of 0°C found, perhaps related to the inability for SSI parameter estimates to be constrained in the present analyses (see section 4.3 below). The lack of significant differences in T_L and T_H among stages may disagree with previous studies, in which thermal tolerance was suspected of increasing with stage number (e.g., Huntsman 1924; Gruffydd et al. 1979) or being lower in stage II (Sastry and Vargo 1977), although such results were overall inconclusive (Quinn 2017a). There is evidence from many arthropod taxa that developmental proportions, temperature effects, and thermal thresholds differ among stages (Quinn 2019). However, the rate isomorphy hypothesis developed in other studies of

ectotherms, including arthropods like crustaceans (e.g., Jarošík et al. 2004), alternatively suggests that such thermal developmental threshold should not differ among stages within the same species, and that most apparent differences in these found among stages result from experimental errors. The lack of differences found herein therefore may agree with rate isomorphy, although due to the wide variability in the estimates obtained thus far this is uncertain.

Ultimately, if the thermal optima and limits for development differ among larval stages, this will have important implications to how larvae are affected by temperature changes in nature; for example, if the optima and tolerance limits of earlier stages are lower than those of later ones, then they could be impacted the soonest and most severely by climate warming (Sastry and Vargo 1977; Schmalenbach and Franke 2010; Caputi et al. 2013; Quinn 2019). Although the overall results found herein (as well as some of the results from analyses of individual study datasets) may suggest such stage-specific differences, the magnitudes and significances of the differences among the T_L and T_H estimates were too low and/or variable to conclude that such differences exist based on these data. However, The T_Φ estimates produced through these analyses were much more consistent and realistic, and thus considering them may be more informative. According to Ikemoto et al. (2013), the T_Φ parameter in the SSI model is related to the other thermal parameters (e.g., T_L and T_H) therein as follows:

$$T_\Phi = \frac{\Delta H_L - \Delta H_H}{R \ln\left(-\frac{\Delta H_L}{\Delta H_H}\right) + \left(\frac{\Delta H_L}{T_L}\right) - \left(\frac{\Delta H_H}{T_H}\right)} \quad (2)$$

Therefore, comparisons of more reliable T_Φ estimates may allow inferences to be drawn about the general differences in thermal tolerances, if there are any, among lobster larval stages, including differences in their presumed T_L and T_H parameters.

The overall values and consistency among stages of the T_Φ estimates found herein were interesting. In previous research, the T_Φ of terrestrial insects and arachnids was found to be mainly around 20°C (ca. 15-25°C) (Ikemoto 2005, 2008; Shi et al. 2011, 2012a, 2012b, 2013; Jafari et al. 2012; Ikemoto and Egami 2013; Padmavathi et al. 2013; Sreedevi et al. 2013), while those for decapod crustaceans varied among habitats, being approximately 7-9°C or 19-27°C for species from cold- and warm-water habitats, respectively (Yamamoto et al. 2017) (Shi et al. 2019). The estimates found herein for American lobster are thus reasonable in light of the literature on arthropod developmental optima. Previous studies of American lobsters estimated (based on various criteria) that the optimal temperature for rearing their larvae was ca. 18-20°C (Van Olst et al. 1976; Carlberg and Van Olst 1977; Ford et al. 1979; Bartley et al. 1980; Quinn 2017a), which is close to (but slightly higher than) the intrinsic optimum temperatures estimated herein; these two sets of findings therefore lend some support to one another. The fact that, with one exception, the T_Φ was mostly consistent across stages also agreed with the rate isomorphy hypothesis (Jarošík et al. 2004), but not with other studies that suggested differences in thermal optima among developmental stages (e.g., Sastry and Vargo 1977; Quinn 2019). However, the significantly lower T_Φ of stage I than that of stages II and III does provide some evidence that thermal tolerances increase, if only slightly, among larval stages, which does agree somewhat with past studies (Huntsman 1924; Gruffydd et al. 1979) and make sense in light of the seasonal thermal trajectory from the time of larval release to

settlement across most of the species' range (Ennis 1995; Lawton and Lavalli 1995; Quinn and Rochette 2015).

The implications of lobster larvae developing optimally between 16.534 and 17.326°C are also of interest. Throughout most of this species' range, seawater temperatures ≥ 18°C were historically uncommon during the summer, when larvae are in the water (Quinn and Rochette 2015; Jaini et al. 2018), but recent climate change has resulted in waters being warmer during the summer (Guo et al. 2013; Quinn 2017a). In historically colder parts of the species' range, warming has been associated with increased abundances and fisheries landings of lobsters (Caputi et al. 2013; Jaini et al. 2018), which may be partly associated with lobster larvae more often experiencing temperatures close to or at their intrinsic developmental optimum, in addition to there being beneficial impacts of warming on other life stages (adults, juveniles, and eggs) and processes (e.g., predator-prey interactions: Boudreau et al. 2015). Conversely, in historically warmer parts of the species' range, temperatures exceeding 22°C now occur in the summer, and lobster abundances have declined (Guo et al. 2013; Boudreau et al. 2015); perhaps the fact that lobster larvae are exposed to supra-optimal temperatures in these regions, resulting in stress, physiological impairment, and perhaps some mortality, is related to these recent declines (Quinn 2017a). Throughout the species' range, summer water temperatures experienced by larvae are expected to increase into the future (Guo et al. 2013; Quinn 2017a), so it is possible that more lobster larvae will experience temperatures too far above their optima, perhaps even approaching their T_H, which may lead to further declines. The possibly lower optimum temperature found for stage I than those for later stages could also mean that this stage will be affected first and most strongly by rising water temperatures in the future.

4.3. Findings Relevant to SSI Model Fitting Procedures

In this chapter, comparisons of T_Φ estimates based on their 95% C.I.s bootstrapped using the ABC method detected statistically significant differences among stages and studies far more often than equivalent comparisons based on 95% C.I.s bootstrapped using the bootstrap percentile and BC_a methods. This confirms the conclusion of Ikemoto et al. (2013) that it is most appropriate to bootstrap and compare C.I.s of T_Φ estimates using the ABC method. Congruently, all comparisons of T_L and T_H estimates, which were based on analyses of bootstrap percentile- or BC_a-derived C.I.s only, were non-significant. It seems reasonable to expect, however, that if 95% C.I.s could be calculated for T_L and T_H estimates using the ABC method, then some significant differences might be detected that are lost in comparisons using bootstrap percentile- or BC_a-derived C.I.s because these tend to innately be too wide and variable, particularly for limited datasets such as those used in the present chapter. Although the T_L and T_H of a species are related to its T_Φ (Ikemoto et al. 2013; see equation (2) above), it is still important to know their precise values when making predictions of future changes. Therefore, in the future, a follow-up study to Ikemoto et al. (2013) should be done to develop a program like their *mABCSSI* package that would allow bootstrap C.I.s for T_L and T_H estimates to be calculated using the ABC method.

Many of the thermal parameter estimates derived from the SSI model in the present chapter were also concluded to be biologically unrealistic. This resulted partially because the *OptimSSI* package does not currently contain the means to constrain its parameter estimates.

When performing nonlinear regressions in many statistical packages, for example IBM SPSS Statistics, it is possible to define constraints on development function parameters that can 'force' them to be estimated within bounds defined by the user as 'realistic' (Guerrero et al. 1994); for example, this approach was used by Quinn (2017b) when fitting various complex nonlinear development functions including T_L and/or T_H parameters to arthropod larval development data. Typically this approach achieves a poorer fit than an unconstrained regression would, but avoids estimating unrealistic values for parameters that are defined as having biological meaning (Kontopoulos et al. 2018). Therefore, the addition of a means to constrain thermal parameter estimate values to the *OptimSSI* package (or more accurately a successor to it) should also be pursued.

The evaluation of the biological realism of T_L and T_H may also need to be revisited, or the implications of these parameters as defined in the SSI model to developmental observations considered more carefully. In the SSI model, T_L and T_H are the temperatures at which an enzyme involved in development is half active and half temperature-inactivated (Sharpe and DeMichele 1977; Schoolfield et al. 1981; Shi et al. 2011; Ikemoto et al. 2013), and thus at these temperatures not *all* copies of the enzyme are inactivated (Kontopoulos et al. 2018). Thus, while development would certainly be greatly impaired at or below these temperatures, it would not be entirely impossible. In laboratory studies, larval survival at near-extreme temperatures is often markedly reduced, but some individuals still manage to complete development (e.g., Templeman 1936; MacKenzie 1988). In this case, it is conceivable that they could be experiencing T_L or T_H conditions corresponding to the definitions in the SSI model, but development is still possible (Kontopoulos et al. 2018). This differs from the definition of equivalent lower and upper thermal thresholds in other development functions, at which development is supposed to be impossible (Quinn 2017b). Some of the estimated thermal limits produced in this chapter that were considered biologically unrealistic were only slightly within the range over which previous studies had observed some successful development, although with impaired larval survival (e.g., within 1-2°C of 6.7°C or 26.3°C (Templeman 1936; Ford et al. 1979)). Such estimates may thus in fact be realistic, but would need to be interpreted differently; this possibility should be investigated in a later study.

The definition of which portion of the datasets subjected to analyses with the *OptimSSI* package was within the 'linear' portion of the temperature-development curve also impacted the shapes of the development curves and values of the thermal parameter estimates it produced (*sensu* Campbell et al. 1974; Ikemoto and Takai 2000; Ikemoto et al. 2013). This was because the linear fit produced at this point provided starting values for the values of T_L (and thus indirectly of T_H and T_Φ, since these are interrelated (Ikemoto et al. 2013)) that were then fed into nonlinear regression procedures within this program, and such starting values can impact the final parameter estimates produced through such procedures (Campbell et al. 1974; Quinn 2017b). The data for stage IV analyzed from Hudon and Fradette (1988) herein provide an extreme example of this: due to the very narrow thermal range examined and the wide variability in development rates within it, the linear fit produced ended up having a *negative* slope (Figure 9), implying that development generally *slowed* as temperature increased, and resulting in the T_L produced being *greater* than the T_Φ and T_H (which were < 0°C!) (Table 4, Figure 8). In less extreme cases, the relatively shallow slope of the linear fit produced (e.g., data for Quinn et al. (2013)) and/or the lack or limited amount of data within the nonlinear portion of the development curve led to unnaturally extreme thermal parameter estimates and abnormal development curves (see sections 3.2 and 3.3).

In a series of side analyses (results not shown), for several of the datasets for which biologically unrealistic parameter estimates and/or abnormal development curves were produced using the definition of the linear portion set herein (temperatures > 12°C and < 26.3°C; see section 2.2), changing the bounds of the linear portion produced more reasonable results. However, this procedure of 'optimizing' the linear portion's definition for each dataset was avoided in the main results presented in this chapter because it was considered potentially dubious. As all datasets examined herein were for the same species, it was unclear whether it was justifiable to redefine the shape of the development curve from study to study and/or stage to stage to this extent. It is entirely possible that the shape of the temperature-development curve, including the breath and bounds of its linear portion, could differ among the different populations of the same species examined in different source studies due to geographic variation (e.g., Quinn et al. 2013). However, given that no study has fully spanned the entire range of temperatures over which American lobster larval development is possible (see above and Table 1), we do not currently have sufficient information to conclude exactly where transitions in the development curve occur, much less whether their thermal location differs geographically. The potential sensitivity of the *OptimSSI* package to the definition of the linear portion of the development curve and its implications will bear further investigation, as will the better definition of the full development curve(s) of lobster larvae.

4.4. Need for New Rearing Studies to be Analyzed with the SSI Model

The present chapter produced a large database of SSI model parameters and their bootstrap C.I.s that may be useful for future studies of this species. However, based on the wide variability of the results among stages and source studies, this information cannot, unfortunately, be said to provide conclusive evidence of the exact optimum and limiting temperatures for the development of lobster larvae (although results produced based on all studies' data combined (Table 9) are potentially promising). The chief issue confounding the results is the fact that no one study of American lobster larval development has yet spanned the entire range of temperatures biologically relevant to the larvae of this species, i.e., those extending from its actual T_L to its actual T_H, which must be < 6.7°C (Templeman 1936) and > 26.3°C (Ford et al. 1979), respectively (Quinn 2017a). However, this shortcoming is by no means unique to the American lobster. For the vast majority of marine decapod crustaceans, studies of larval development have historically focused on rearing larvae at a few temperatures within a limited, but presumably most biologically relevant, range to assess their differential survival, growth, development times, etc. at different temperatures (Anger 2001; Quinn 2017b). Studies of crustacean development at thermal extremes and the identification of thermal thresholds have thus been less of interest, and rearing over a wide range of temperatures including extremes is often impractical and expensive for marine larvae, while conversely such studies are commonplace for terrestrial insects and arachnids (Quinn 2017b and references therein; see also: Campbell et al. 1974; Ikemoto 2005, 2008; Shi et al. 2011, 2012a, 2012b, 2013; Jafari et al. 2012; Ikemoto and Egami 2013; Padmavathi et al. 2013; Sreedevi et al. 2013).

However, this is beginning to change (e.g., Yamamoto et al. 2017), and given concerns about the effects of climate change on American lobster fisheries recruitment (Caputi et al. 2013; Boudreau et al. 2015; Jaini et al. 2018; Quinn 2017a) it is time for such an extensive

and thorough study to be done for this species' larvae. For example, a study rearing larvae in the laboratory at as many controlled temperatures as possible (optimally ≥ 10; Ikemoto et al. 2013) between ca. 0-5°C and ca. 27-30°C or higher should help to identify the lower and upper developmental thresholds and better define the shape of the development curve between them.

It should also be noted that most thermal parameter estimates produced for stage IV from a single study's dataset or with all studies' data combined in this chapter tended to be particularly variable and/or unrealistic. This was likely due in part to the low sample sizes available within individual studies for this stage, as well as the low number (3) of studies that provided data for its duration (Table 1). However, because stage IV is the settling stage of the American lobster's lifecycle, its duration can also be affected strongly by other factors besides temperature, especially the availability and type of bottom substrate. For example, in the absence of suitable substrate for settlement (cobble), this stage can be prolonged as a form of settlement delay, whereas if substrate is provided it can be greatly shortened (Phillips and Sastry 1980; Ennis 1995; Lawton and Lavalli 1995). Previous development studies done in the laboratory did not provide stage IV lobsters with substrate (Templeman 1936; MacKenzie 1988), which may have artificially prolonged this stage and also exacerbated developmental and behavioral differences among individuals. Therefore, a future study aiming to assess thermal parameters of stage IV development must also account for the impacts of substrate on its duration, for example by holding stage IV lobsters not only at different temperatures, but also with or without different types of substrate for settlement.

Once such a study has been done, analyzing its data using the SSI model, as done herein, should provide valuable information on the temperature-dependent development of this species' larvae, including their developmental thresholds and optimum temperature(s), which can then be used to predict the effects of future climate change on them. This chapter provides a framework to support the enactment of such a study. Further, the approaches taken herein could and likely should be applied to data for other species of lobsters and other decapod crustaceans, as these also support important fisheries that depend on larval supplies (e.g., Yao and Zhang, 2018) that are potentially impacted by changes in water temperature (Phillips and Sastry 1980; Caputi et al. 2013; see also other chapters in this volume).

CONCLUSION

The estimates of the thermal parameters (T_Φ, T_L, and T_H) of American lobster larval development obtained using the SSI model in this chapter varied widely among studies, but with no consistent and few significant differences in them among stages. Although most T_Φ estimates were biologically realistic, the majority of T_L and T_H estimates were not. When all studies' datasets were combined, the T_Φ, T_L, and T_H for American lobster larval development were estimated to be 16.534-17.326°C, 0.095-7.804°C, and 23.483-25.661°C, respectively. The T_Φ of stage I was significantly lower than those of stages II and III, but all other differences among stages were non-significant. However, none of the previous studies whose data were analyzed herein were conducted over a wide enough thermal range to allow for a conclusive fit of their data with the SSI model to be achieved. Therefore, a future rearing study conducted over a wide thermal range, followed by analyses with the SSI model, is

needed to better define the limiting and optimum temperatures for the development of this species' larvae for use in predicting the potential effects of climate change on its fisheries recruitment.

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Chapter 108

THE CRYPTIC *HOMARUS GAMMARUS* (L., 1758) JUVENILES: A COMPARATIVE APPROACH TO THE MYSTERY OF THEIR WHEREABOUTS

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ABSTRACT

A series of selected biological, ecological, and morphological traits of North Atlantic decapod crustaceans were used to discuss the possible biotope of juvenile European lobsters, *Homarus gammarus* (Linnaeus, 1758), and how to approach filling our knowledge gaps regarding the whereabouts of this life stage in nature. The information examined provided a review of what is known and what is anticipated regarding this species. This comparative and holistic ecological approach will provide useful information for future studies of the 'missing link' in this species' life history – small, juvenile European lobsters. Three hypotheses for the reasons these life stage have not been found are that they are: (1) located too deep to be found in shoreline-based field studies; (2) distributed too scarcely to be found; or (3) living deep inside shelters, which are unapproachable by competitors, predators, and humans. We compared the ecomorphology, life history, habitat, and behavior of *H. gammarus* to those of *Homarus americanus* H. Milne Edwards, 1837, as well as to those of other decapod species living within a similar geographical range to that of adult lobsters. The main differences found among the biotopes of the homarid lobsters were in the diversity of their predators and the number of competitive decapods present, which are both higher in European shallow-water cobble bottom areas. This should have strong impacts on the survival probabilities of settling lobsters. Larvae and adults of *H. gammarus* are found at much lower densities than those of *H. americanus*, although early-stage larvae of both species are regularly caught in light traps and plankton nets. Field and laboratory studies indicate limited dispersal of larvae in *H. gammarus*. In the laboratory, settling juveniles of both species show clear preferences for shelters by burrowing underneath or beside solid objects, which is symptomatic of a cryptic life habit. Juvenile *H. gammarus* show traits that are

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suggestive of adaptations to shallow habitats, even potentially to intertidal life. The higher biodiversity in European shallow waters may cause the young lobsters to stay sheltered deeper within the substrate in shallow places that competitors and predators cannot reach.

Keywords: American lobster, biology, decapod crustaceans, ecology, European lobster, life history, morphology, North Atlantic Ocean

1. INTRODUCTION

Traditionally, the acquisition of information on wild-living species used to be focused on assessing the presence and life habits of mature individuals. However, in the last century the importance of learning about the full life history to understand the ecology of an organism has become apparent, and is now known to be crucial to protecting and managing wild populations sustainably (Sadovy 2001; Denney et al. 2002). Lobsters, particularly the homarid species (*Homarus* spp.), are potentially vulnerable to overexploitation because they are long-lived and of low to medium fecundity (Agnalt et al. 1999; Wahle et al. 2013a). However, the whereabouts of the early life stages of the European lobster, *Homarus gammarus* (Linnaeus, 1758), are still an unsolved mystery (Mercer et al. 2001; van der Meeren 2005).

While little is known about the larger, commercially important decapod crustacean species of the Northern Atlantic Ocean, there are few species for which there are such limited field observations of critical life history stages as for *H. gammarus.* However, it is possible that more could be learned about these concealed life stages by comparing this lobster's living biotope, morphology, and behavior with those of other decapod crustaceans. Comparing behavioral traits is an established method in evolutionary science, which has been used to define phylogenetic relationships and evolutionary connections among taxa (Clutton-Brock and Harvey 1984; Harvey and Nee 1997). Ecomorphological adaptation has also been used as a tool to look at evolutionary habitat shifts in crabs (*Cancer* spp.) by analyzing such traits as size and habitat (Harrison and Crespi 1999). Life history traits include parental investment, which is closely connected to the ecological constraints placed on parents and offspring (Clutton-Brock and Godfray 1991). A comparison of such traits among a selection of North Atlantic decapod crustacean species was presented and discussed in this chapter. The reasoning behind this approach was that evolution and adaptation should have resulted in each species' body shape, pigmentation, environmental requirements, settlement and establishment choices, and behaviors in general being adapted to their optimal living biotope.

This chapter investigated and compared a selection of North Atlantic decapod crustacean species (Figure 1). This included: three nephropid lobsters, the European lobster *H. gammarus*, American lobster *Homarus americanus* (H. Milne Edwards, 1837), and Norway lobster *Nephrops norvegicus* (L., 1758), as well as the limnic noble crayfish *Astacus astacus* (L., 1758); brachyuran crabs, including the brown or edible crab *Cancer pagurus* (L., 1758) and green shore crab *Carcinus maenas* (L., 1758); the European spiny lobster *Palinurus elephas* (Fabricius, 1787); anomurans, such as the spiny squat lobster *Galathea strigosa* (L., 1761) and red king crab *Paralithodes camtschaticus* (Tilesius, 1815); and natantian species,

including the hooded shrimp *Athanas nitescens* (Leach, 1813) and northern deep-water shrimp *Pandalus borealis* Krøyer, 1838.

Figure 1. Selected decapod species with home ranges in the Northern Atlantic region of Europe, from upper-left to lower-right: *Homarus gammarus* (Linnaeus, 1758), *Homarus americanus* H Milne Edwards, 1837, *Nephrops norvegicus* (Linnaeus, 1758), *Pandalus borealis* Krøyer, 1838, *Galathea strigosa*, (Linnaeus, 1761), *Athanas nitescens* (Leach, 1813), *Cancer pagurus* (Linnaeus, 1758), *Paralithodes camtschaticus* (Tilesius, 1815), *Carcinus maenas* (Linnaeus, 1758). Two non-native species, *Homarus americanus* and *Paralithodes camtschaticus*, are present in northeastern Atlantic and Barents Sea waters. 'Lobster-shaped' species in upper section, 'crab-shaped' decapods in the lower section. Photographers: *P. borealis* by Ø. Paulsen/IMR, *A. nitescens* by Nils Aukan, the rest by AK Woll.

The above species are all temperate- to cold-water-adapted and occupy the same trophic level spectrum (2 to 3), but live at different depths, have different habitats and diurnal habits, and face different levels of competition and predation pressure (Williamson 1905; Pearson 1908; Herrick 1911; Crothers 1967, 1968; Christiansen 1969; Ansell and Robb 1977; Cooper and Uzmann 1980; Shumway et al. 1985; Factor 1995; Hunter 1999; Bergström 2000; Ulmestrand and Eggert 2001; Goñi and Latrouite 2005; Poore et al. 2011; Groeneveld et al. 2013; Stevens 2014; Sundet 2014; Füreder 2016). The little information found on *A. nitescens*

was limited to the traits most of the other species have in common (Palomares and Pauly 2018), so this species was not included in the statistical comparisons performed. Even when information had been published on the traits of mature individuals of the rest of these species, information on the juvenile stages is lacking for several of them, not only *H. gammarus*. Some of the information that is available comes from laboratory studies, which may not extrapolate exactly to wild individuals. Nevertheless, our approach is an attempt that may possibly deduce the potential living areas and ecology of the hitherto cryptic life stages of young *H. gammarus*.

2. METHODS

Interspecific similarities and differences in traits among species and their implications were assembled and analyzed for the following categories and types of traits:

- Morphology (Table 1): body shape; shape and relative strength of pereiopods, including chelae; spines in the larval stages; abdominal (tail) flexibility; carapace spines (adults and juveniles); length of second antennae relative to body length; and pigmentation.
- Life history (Table 2): Maximum age (lifespan); age of maturation; maximum size; fecundity; duration of larval period; and size at settling.
- Biotope (Table 3): use of shelter; habitat type; depth; competitors (other decapod species and groups); and predator diversity.
- Behavior (Table 4): Burrowing; tail flip escape response; nocturnal activity; level of aggression; social behavior; and diet.
- Other parameters (Table 5): Temperature range; and trophic level.

The above traits were analyzed and classified using cladistics and principal component analysis (PCA), inspired by the methods used for natural classification by Nelson and Platnick (1981) and Williams and Ebach (2004). Results of statistical analyses were discussed and related to the specific traits of each species, in both its mature and juvenile life stages. The significances of particular traits in relation to life history strategies were also discussed. The traits compared are shown in Tables 1-5.

For analyses, the phenotypic trait categories from Tables 1-5 were represented as numerical variables prior to performing clustering analyses (Tables 6 and 7). Clustering analyses were fitted by the 'Agnes' method (Kaufman and Rousseeuw 1990), implemented in the '*cluster*' package (Maechler et al. 2018) in R v.3.5.1 (R Core Team 2016).

The effects of ecologically important traits (predation pressure and competitive species richness) for the two *Homarus* species were analyzed to look at their possible impacts on survival strategies used in the juvenile phase of these two species.

The PCA conducted reduced N descriptive variables to n independent principal components (PCs). Retaining the PCs that explained the greatest proportions of the variance in the data reduced the dimensionality of the data to allow for greater ease of interpretation of the patterns present.

Table 1. The selected morphological traits (body shape, shape and relative strength of pereiopods, including chelae, spines in the larval stages, abdominal (tail) flexibility, presence of carapace spines (adults and juveniles), length of second antennae relative to body length, and pigmentation) compared among adults and juveniles of the selected decapod species

Species	Body shape	Pereiopods/ claw shape	Chelae shape	Chelae	Larval spines	Flexible tail	Carapace spines	Relative 2nd antennae length	Pigmentation
Adults									
Homarus gammarus (Hg)	Elongate	Strong, pincers	Pointed	Strong	No	Yes	Small	> body	Dark
Homarus americanus (Ha)	Elongate	Strong, pincers	Pointed	Strong	No	Yes	Small	> body	Medium
Paralithodes camtschaticus (Pc)	Wide	Strong, pointed	Pointed	Strong	No	No	None	< carapace	Reddish
Cancer pagurus (Cp)	Wide	Long, strong, pointed	Pointed	Weak	No	No	None	< carapace	Brown
Carcinus maenas (Cm)	Wide	Strong, pointed	Pointed	Strong	No	No	None	< carapace	Greenish
Pandalus borealis (Pb)	Elongate	Long, slender,	Pointed	Weak	Yes	Yes	Yes	> body	Reddish
Palinurus elephas (Pe)	Elongate	Strong	Hooked	None	Yes	Yes	Yes	> body	Contrasting patterns
Nephrops norvegicus (Nn)	Elongate	Slender, pincers	Pointed	Slender	No	Yes	Small	> body	Reddish
Galathea strigosa (Gs)	Elongate	Strong	Hooked	Weak	No	Yes	Small	> carapace	Contrasting patterns
Astacus astacus (Aa)	Elongate	Strong	Pointes	Strong	No	Yes	None	> carapace	Dark
Juveniles, settlers									
Hg juv	Elongate	Thin	Pointed	Slender	No	Yes	Small	> body	Brownish
Ha juv	Elongate	Thin	Pointed	Slender	No	Yes	Small	> body	Dark
Pc juv	Wide	Strong	Pointed	Strong	No	No	Small	< carapace	Reddish
Cp juv	Wide	Long, strong	Pointed	Weak	Yes	No	None	< carapace	Pale
Cm juv	Wide	Strong	Pointed	Weak	No	No	None	< carapace	Patterns
Pb juv	Elongate	Thin	Pointed	Yes	Yes	Yes	Yes	> body	Reddish
Pe juv	Elongate	Thin	Hooked	No	Yes	Yes	Small	> body	Pale
Nn juv	Elongate	Thin,	Pointed	Slender	No	Yes	None	> body	Reddish
Gs juv	Elongate	Thin	Hooked	Weak	Yes	Yes	Yes	> carapace	Pale
Aa juv	Elongate	Strong	Pointed	Strong	No	Yes	None	> carapace	Dark

References: Pearson 1908; Herrick 1911; Crothers 1967; 1968; Christiansen 1969; Ansell and Robb 1977; Cooper and Uzmann 1980; Shumway et al. 1985; Factor 1995; Fogarty 1995; Hunter 1999; Sheehy et al. 1999; Bergström 2000; Ulmestrand and Eggert 2001; Goñi and Latrouite 2005; Agnalt 2008; Poore et al. 2011; Groeneveld et al. 2013; Stevens 2014; Sundet 2014; Füreder 2016; MarLIN 2016; Palomares and Pauly 2018.

Table 2. The selected life history traits (maximum age/lifespan, age of maturation, maximum size, fecundity, duration of larval period, and size at settling) compared among adults and juveniles of the selected decapod species

Species	Lifespan (conservative estimate)	Maturation age, females	Max size (mm CL or CW)	Fecundity	Larval period (northern distribution)	Size at settling (mm CL or CW)
Adults						
Homarus gammarus (Hg)	50	7	160	Medium	NA	NA
Homarus americanus (Ha)	50	5	220	High	NA	NA
Paralithodes camtschaticus (Pc)	15	4	220	High	NA	NA
Cancer pagurus (Cp)	15	3	160	High	NA	NA
Carcinus maenas (Cm)	8	3	70	High	NA	NA
Pandalus borealis (Pb)	6	4	35	Low	NA	NA
Palinurus elephas (Pe)	25	5	150	High	NA	NA
Nephrops norvegicus (Nn)	10	4	50	Medium	NA	NA
Galathea strigosa (Gs)	?	?	53	Medium	NA	NA
Astacus astacus (Aa)	15	3	120	Low	NA	NA
Juveniles, early settlers						
Hg juv	NA	NA	NA	NA	Weeks	10
Ha juv	NA	NA	NA	NA	Weeks	12
Pc juv	NA	NA	NA	NA	Weeks	1
Cp juv	NA	NA	NA	NA	Months	5
Cm juv	NA	NA	NA	NA	Months	1
Pb juv	NA	NA	NA	NA	Weeks	3
Pe juv	NA	NA	NA	NA	Months	25
Nn juv	NA	NA	NA	NA	Months	7
Gs juv	NA	NA	NA	NA	Weeks	1
Aa juv	NA	NA	NA	NA	No free larval period	3

References: See Table 1.

Table 3. The selected biotope traits (use of shelter, habitat type, depth, competitors (other decapod species and groups), and predator diversity) compared among adults and juveniles of the selected decapod species

Species	Shelter	Habitat	Depth (m)	Decapod competitor diversity	Predator diversity
Adults					
Homarus gammarus (Hg)	Burrows under solid objects	Mixed	0 - 50	High	High
Homarus americanus (Ha)	Burrows	Rocky and soft	0 - 100	Low	Medium
Paralithodes camtschaticus (Pc)	In pods (high density spots)	Mixed	0 - 300	Low	Medium
Cancer pagurus (Cp)	In cracks and crevices, in sand	Rocky and soft bottoms	0 - 70	High	High
Carcinus maenas (Cm)	In cracks and crevices, in sand	Rocky	0 - 10	High	High
Pandalus borealis (Pb)	High density	Soft bottoms	9 - 1500	Low	High
Palinurus elephas (Pe)	Under solid objects, in crevices	Rocky bottoms	0 - 200	Medium	High

Species	Shelter	Habitat	Depth (m)	Decapod competitor diversity	Predator diversity
Nephrops norvegicus (Nn)	Deep burrows	Muddy sediments	20 - 800	Low	High
Galathea strigosa (Gs)	In cracks and crevices	Rocky bottoms	0 - 10	Medium	High
Astacus astacus (Aa)	Burrows, under objects	Mixed mud/rocks	0 - 15	Low	Low
Juveniles, early settlers					
Hg juv	Burrows/cracks	Mixed	0 - 10	High	High
Ha juv	In cracks and crevices	Rocky	0 -5	Medium	Medium
Pc juv	Habitat-building organisms	Rocky, kelp forests	0 - 5	Medium	Medium
Cp juv	On sand	Mostly rocky bottoms	0 - 10	High	High
Cm juv	Between sand grains	Sandy	0 - 2	High	High
Pb juv	None	Sandy	> 50 m	Medium	High
Pe juv	Mussels/crevices	Rocky	10 - 90	Medium	High
Nn juv	In burrows	Muddy	20 - 300	Low	High
Gs juv	In cracks and crevices	Rocky	0 - 10	Medium	High
Aa juv	Burrows, under objects	Mixed mud/rocks	0 - 15	Low	Low

References: See Table 1.

Table 4. The selected behavioral traits (burrowing, tail flip escape response, nocturnal activity, level of aggression, social behavior, and diet) compared among adults and juveniles of the selected decapod species

Species	Active burrower	Tail flip escape	Strictly nocturnal	Aggression	Social	Seasonal migration	Diet
Adults							
Homarus gammarus (Hg)	Yes	Yes	Yes	Yes	No	Yes	Omnivore
Homarus americanus (Ha)	Yes	Yes	Yes	Yes	No	Yes	Omnivore
Paralithodes camtschaticus (Pc)	Occasional	No	No	No	Yes	Yes	Omnivore
Cancer pagurus (Cp)	Occasional	No	No	Medium	Medium	Yes	Omnivore
Carcinus maenas (Cm)	Occasional	No	No	Medium	Medium	No	Omnivore
Pandalus borealis (Pb)	No	Yes	No	Low	Yes	No	Planktivore
Palinurus elephas (Pe)	No	Yes	Yes	Low	Yes	Yes	Omnivore
Nephrops norvegicus (Nn)	Yes	Yes	Yes	High	No	No	Omnivore
Galathea strigosa (Gs)	No	Yes	Yes	Low	Medium	No	Omnivore
Astacus astacus (Aa)	No	yes	Yes	Yes	Medium	No	Omnivore

Table 4. (Continued)

Species	Active burrower	Tail flip escape	Strictly nocturnal	Aggression	Social	Seasonal migration	Diet
Juveniles, early settlers							
Hg juv	Yes	Yes	Yes	Yes	No	No	Omnivore
Ha juv	Yes	Yes	Yes	High	No	No	Omnivore
Pc juv	No	No	Yes	Low	Yes	No	Omnivore
Cp juv	No	No	Yes	Medium	No	No	Omnivore
Cm juv	Yes	No	Yes	Low	No	No	Omnivore
Pb juv	No	Yes	No	Low	Yes	No	Omnivore
Pe juv	No	Yes	Yes	Low	No	No	Omnivore
Nn juv	Yes	Yes	Yes	Yes	No	No	Omnivore
Gs juv	No	Yes	Yes	Low	Medium	No	Omnivore
Aa juv	No	Yes	Yes	Medium	Medium	No	Omnivore

References: See Table 1.

Table 5. Other traits (temperature range and trophic level) compared among adults and juveniles of the selected decapod species

Species	Temperature range (°C)	Trophic level
Adults		
Homarus gammarus (Hg)	10 - 22	2
Homarus americanus (Ha)	5 - 25	3
Paralithodes camtschaticus (Pc)	- 1 - 11	2
Cancer pagurus (Cp)	9 - 14	2
Carcinus maenas (Cm)	10 - 25	2
Pandalus borealis (Pb)	0 - 5	2
Palinurus elephas (Pe)	12 - 16	2
Nephrops norvegicus (Nn)	5 - 10	3
Galathea strigosa (Gs)	5 - 20	2
Astacus astacus (Aa)	0 - 18	2
Juveniles, early settlers		
Hg juv	15 - 20	2
Ha juv	12 - 20	2
Pc juv	0 - 3	2
Cp juv	10 - 15	2
Cm juv	10 - 15	2
Pb juv	0 - 5	2
Pe juv	15 - 20	2
Nn juv	8 - 18	2
Gs juv	5 - 20	2
Aa juv	0 - 18	2

References: See Table 1.

In the PCA, the first three and six principal axes explained 59% and 84% of the total variance, respectively. Subsequent analyses were thus based on the first six out of 19 principal component axes.

The traits from all sections were included in the main analyses, but the significance of the different types of traits were discussed in separate sections when comparing species and life stages.

The trait information analyzed was collected from the trait databases at SeaLifeBase.org (Palomares and Pauly 2018) and MarLIN.uk.ac (MarLIN 2016), from scientific papers and books (cited in Tables 1-5), and, for some traits, the authors' personal observations and observations by experienced lobster professionals.

Table 6. The phenotypic trait categories in Tables 1-5 translated into numerical variables prior to fitting in clustering analyses for each and all categories for adults

Trait/code	1	2	3	4
Morphological traits				
Body shape	Elongate	Wide	NA	NA
Pereiopods	Slender	Long	Strong	NA
Pereiopod claw shape	Pointed	Hook	Paddle-like	NA
Chelae	None	Scooped	Pointed	Strong, rounded
Secondary chelae	No	Yes	NA	NA
Rostrum	No	Short	Prolonged	NA
Flexible tail	No	Yes		
Spines on carapace	No	Small	Prominent	NA
Relative 2nd antennae length	> body length	= body	< carapace	NA
Pigmentation	Dark	Medium, brownish	Pale, reddish	NA
Life history traits				
Lifespan (estimated in years)	< 3	3 - 15	> 15	NA
Maturation age, females (years)	< 2	2 - 5	> 5	NA
Max size (mm CL or CW)	< 55	55 - 200	> 200	NA
Fecundity	< 50 000	50 000	> 50 000	NA
Habitat traits				
Shelter	None	Burrow	Cracks	Pods
Habitat	Soft	Algae	Rocky, mixed	Hard
Depth (m)	0 - 10	0 - 50	> 50	NA
Behavioral traits				
Active burrower	No	Occasionally	Yes	NA
Tail flip escape	No	Yes	NA	NA
Strictly nocturnal	No	Yes	NA	NA
Interspecific aggression	No	Medium	High	NA
Social	No	Medium	High	NA
Seasonal migration (females)	No	Yes		
Diet	Planktivore	Omnivorous	Predatory	NA
Local decapod competitor diversity	Low (< 5 species)	Medium (5-10 species)	High (> 10 species)	NA
Predator diversity	Low (< 5 species)	Medium (5-10 species)	High (> 10 species)	NA
Other parameters				
Maximum temperature (°C)	< 5	5 - 15	15 - 25	NA
Trait/code	1	2	3	4
Optimal temperature (°C)	< 5	5 - 10	10 - 20	NA
Trophic level	< 2	2 - 3.9	> 4	NA

References: See Table 1.

Table 7. The phenotypic trait categories in Tables 1-5 translated into numerical variables prior to fitting in clustering analyses for each and all categories for juveniles

Trait/code	1	2	3
Morphological traits			
Body shape	Elongate	Wide	NA
Pereiopods	Strong	Long	Thin
Pereiopod claw shape	Pointed	Hook	NA
Chelae	None	Weak	Strong
Secondary chelae	None	Yes	NA
Rostrum	None	Short	Prolonged
Flexible tail	None	Yes	NA
Spines on carapace	None	Spread	Dense
Relative 2nd antennae length	> body length	= body	< carapace
Pigmenation	Dark	Medium, brownish	Pale, reddish
Trait: Life history			
Lifespan (estimated, in years)	< 2	2-5	> 5
Size at settling (mm CL or CW)	< 1	1-9	> 10
Biotope traits			
Shelter	None	Burrow	Cracks
Habitat	Soft	Hard	Mixed
Depth (m)	< 0.5	0.5-10	> 10
Behavioral traits			
Active burrower	No	Occasionally	Yes
Tail flip escape	No	Yes	NA
Strictly nocturnal	No	Yes	NA
Interspecific aggression	No	Medium	High
Social	No	Medium	High
Seasonal migration (females)	No	Yes	
Diet	Vegetarian	Omnivorous	Predator
Local decapod competitor diversity	Low (< 5 species)	Medium (5-10 species)	High (> 10 species)
Predator diversity	Low (< 5 species)	Medium (5-10 species)	High (> 10 species)
Other parameters			
Maximum temperature (°C)	< 5	5 - 15	15 - 25
Optimal temperature (°C)	< 5	5 - 10	10 - 20
Trait/code	1	2	3
Trophic level	< 2	2 - 3.9	> 4

References: See Table 1.

3. RESULTS

3.1. Overall Comparisons among Decapods

The results of the main clustering analysis are presented in Figure 2. On the dendrogram, the adult and juvenile stages of four species clustered together as each other's nearest neighbors (*A. astacus*, *P. borealis*, *N. norvegicus*, and *P. camtschaticus*). For the remaining species, the nearest neighbor of a group was usually the same life stage of another species. In such cases, the two species formed two clusters, where the first cluster consisted of the two adult stages and the second consisted of the two juvenile life stages; for example, this was found for *H. gammarus* and *H. americanus*. For these species, it can be inferred that the

amount of dissimilarities between species at the same life stage was less than the amount of dissimilarities within the same species between different life stages.

However, for *H. gammarus*, the information on the life habit and biotope of juveniles (< 25 mm carapace length (CL)) included in the above analyses was based on laboratory studies. This was because these juveniles have never been seen in the field, except for a handful of single occasions in different habitats over a 40-year period. Anecdotal observations were made, but not published, of one *H. gammarus* juvenile in an empty mussel shell in shallow water in Sweden (observed by B. Dybern in the 1970s), one in a pearl net for oysters in Denmark (observed by C. Formsgaard Nielsen in 2015), and one in a crack between rocks in Norway (observed by A. K. Woll in 2015, see Figure 4). However, in the laboratory the two species behave very similarly (Wahle et al. 2013a), as is also reflected in the limited juvenile data available in Tables 1-5. The nephropid lobsters clustered together, and separated into one juvenile and one adult cluster (Figure 2), but the juvenile *H. gammarus* was more distinct from the juvenile *H. Americanus* than was expected from the similarities between the adult lobsters. Therefore, although information on wild juveniles is lacking, it seems that based on laboratory observations and inferred ecological traits there are several important differences between juvenile *H. gammarus* and *H. americanus*.

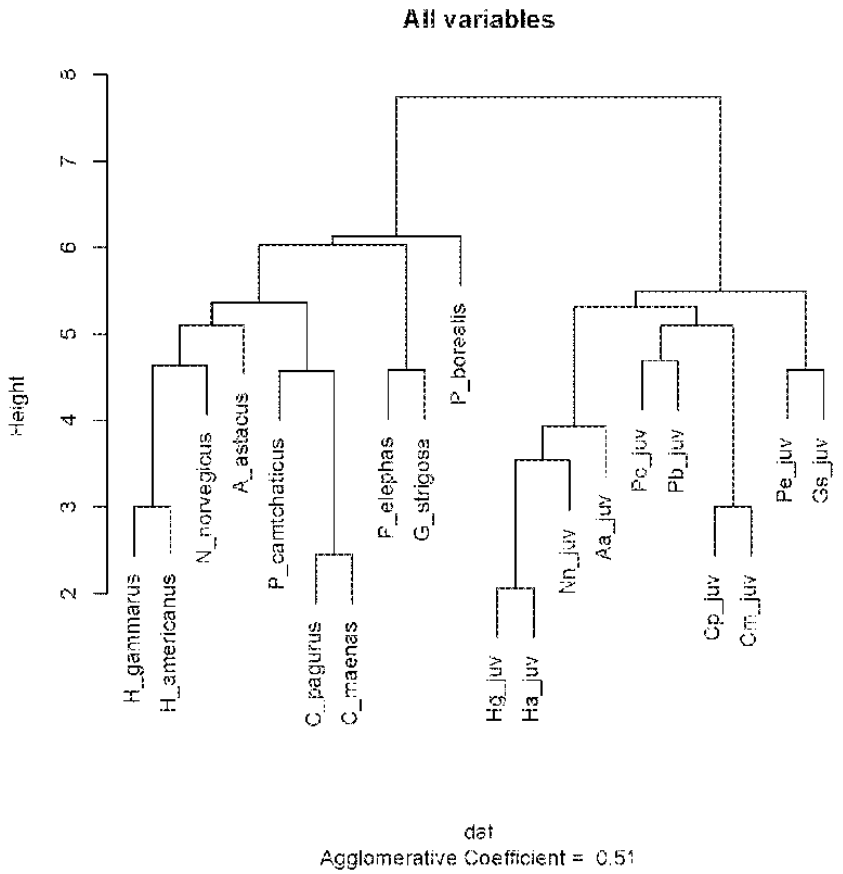

Figure 2. Clustering of the species, as adults and juveniles ('juv'), by the 'Agnes' method, based on data for all the traits listed in Tables 1-5. Agglomerative coefficient: 0.51. Statistics were performed by Francois Besnier, IMR.

3.2. Morphology

If bodily structure and function are closely connected to the ecological niche of a species (Dullemeijr 1974; Alexander 1988; Bock 1994), then it may be feasible to detect common ecological groups in decapods by comparing their morphological traits (e.g., body shape, sense organs, and the function of the tail, chelae, and pereiopods) by looking at their ecomorphology, as well as their functional morphology. The 'Agnes' analyses clustered shrimps, crayfish and lobsters, crabs, including *P camtschaticus* and galatheans, and *P. elephas* into three distinct clusters (agglomerate coefficient = 0.68). For juveniles, the functionality of body shapes and appendages is closely related to their chances of survival. The crab-shaped decapods *C. pagurus*, *C. maenas*, and *P. camtschaticus*, with wide carapaces, also all seem to have relatively short antennae and an abdomen that is no more than a reduced flap tucked underneath the body. The long, slender *P. borealis*, *A. astacus*, *P. elephas*, *H. americanus*, and *H. gammarus* (and, to some extent, the prolonged, although still quite wide, *G. strigosa*) all have long to very long second antennae, and all have strong, flexible abdomens (see Figure 3).

Figure 3. Variation in morphological details between several species, with emphasis on chelae and claws and pereiopods, from upper-left to lower-right: *Homarus gammarus* (L., 1758*)*: chelae, and clawed pereiopods, *Nephrops norvegicus* (L., 1758) chelae and weak pereiopods, *Galathea strigosa* (L., 1761): chelae and hooked claws, *Cancer pagurus* (L., 1758) chelae (left) and straight claws on pereiopods (right), *Carcinus maenas* (L., 1758) chelae and claws, *Paralithodes camtschaticus* (Tilesius, 1815) chelae and claws. Photographer: AK Woll.

From these few morphological traits, it can be deduced that crab-like decapods could have different feeding and survival strategies from those of the more slender (shrimp- or lobster-like) species. The survival strategies of crab-like species appear to involve growing to a larger size (*P. camthschaticus* and *C. pagurus*) and having a more solid carapace with no vulnerable abdomen (*C. pagurus* and *C. maenas*). Conversely, the more slender species are better adapted for speed and making rapid escapes, although the longer, jointed tail they use for these purposes may be more vulnerable to attacks in open spaces.

Crabs and spiny lobsters, which live in the same region and partly in the same biotopes, are usually lighter in color, ranging from brown to spotted, some with starkly contrasting pigmentation providing color-camouflage in the surrounding habitat. They also tend to change pigmentation from lighter and spotted to more homogenous in the first years of life. All the deep-living species examined are reddish in color, from the brightly red *P. borealis* to the pale red *N. norvegicus* and light brownish red king crab. These species do not change their pigmentation as they grow. Red light does not penetrate very far into water, so red colors provide good camouflage in deeper waters. Pigmentation ranges from black through dark blue to dark brown or grayish brown in *H. gammarus* and *A. astacus*. *H. gammarus* tend to be all black in the northern part of their range, usually with some white markings near the mouth, while the coloration of *H. americanus* is more uniform throughout its range, with dark green and reddish brown undertones and less pronounced pale spots in the head region (Figure 3). Both lobster species seem to develop dark pigmentation at settlement, although this probably depends on food quality. Laboratory-reared juvenile lobsters tend to be paler than wild-caught juveniles if they are fed an astaxanthin-deficient diet (D'Agostino 1980; D'Abramo et al. 1983; Lim et al. 1997; Tlusty and Hyland 2005). Juvenile *H. gammarus* also respond with marked changes in pigmentation when their feed is changed by adding astaxanthin or by switching it from *Artemia* to mussels in the laboratory (Wickins and Lee 2002; Drengstig et al. 2003; Kristiansen et al. 2004). The dark pigmentation of lobsters may provide better camouflage in deep cracks and within caverns than the brown and green coloration of crabs. Lobsters are quite conspicuous on open ground, however, so their dark pigmentation can be seen as being linked to a cryptic lifestyle, in which they aim to stay hidden from predators on the sea bottom.

3.3. Life History

The 'Agnes' analyses of life history traits showed that the two *Homarus* species were closely clustered together, and seemed to have traits in common as both adults (agglomerate coefficient = 0.58) and juveniles (agglomerate coefficient = 0.64).

Internal mating, wherein the male deposit a sperm package in the spermathecae of the female, is a common feature of all decapods (Subramoniam 2016). However, besides this, the selection of North Atlantic species examined has a wide range of life history strategies, including very different cycles of reproduction and recruitment. Indeed, fecundity was very dissimilar among these species, ranging from low in *A. astacus* to high in *C. pagurus* (Pearson 1908; Herrick 1911; Cooper and Uzmann 1980; Shumway et al. 1985; Shields 1991; Ingle 1992; Factor 1995; Hunter 1999; Bergström 2000; Poore et al. 2011; Groeneveld et al. 2013; Stevens 2014; Füreder 2016; Bakke et al. 2018) (Figure 4).

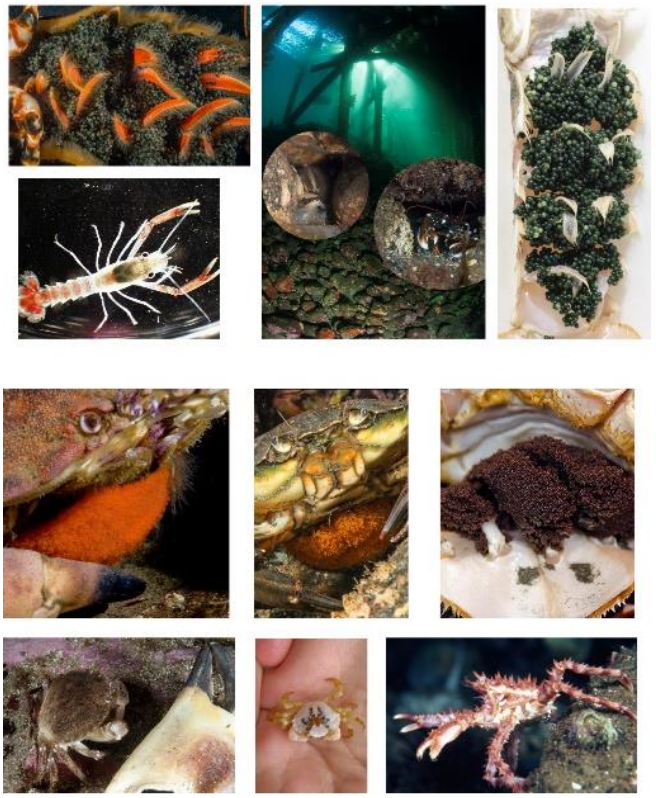

Figure 4. Newly spawned egg clutches and early life stages, listed from upper-left corner to lower-right. Egg clutches: *Homarus gammarus* (L., 1758), *Nephrops norvegicus* (L., 1758), *Cancer pagurus* (L., 1758), *Carcinus maenas* (L., 1758), *Paralithodes camtschaticus* (Tilesius, 1815). Juveniles: *H. gammarus* (stage V, reared) and a small juvenile found in a crevice January, May and August 2016, *C. pagurus*, *C. maenas*, *P. camtschaticus*. Photographers: Egg clutch *H. Gammarus* by Finn Refsnes; Juvenile *H. gammarus* (reared) by GI van der Meeren/IMR and *C. maenas* by IMR, the rest by AK Woll.

Larval development is also widely different among these species, from the larval stages all being completed before hatching and extended parent care being provided to the newly molted juveniles in *A. astacus* to the prolonged larval stages of a year or more in duration in *P. elephas*. All the marine species examined release planktonic larvae at hatch, which then develop through several molts before they metamorphose into the settling stage that then molts into the bottom-dwelling juvenile stage. The fecundity of the different species reflects the results of trade-offs between the costs of parental care versus the survival of the brood (Sastry 1983; Clutton-Brock and Godfray 1991; Denney et al. 2002). The size of the eggs depends on how much energy is stored in each egg, and this ranged from the nearly microscopic eggs of the brachyuran crabs to the slightly larger eggs of *P. camtschaticus*, to the relatively large eggs, which hatch into larger larvae, in *P. borealis*, *G. strigosa*, *P. elephas*, and the three nephropid lobster species (Williamson 1983; Ingle 1992). The number of larval molts and time to maturation also vary. *Palinurus elephas* has an extremely long larval period, but homarid lobsters undergo only four larval molts, and may be ready to settle in less than three weeks if the temperature is optimal. Growth from the juvenile stages to

maturity seems to be related more to the maximum body size of a species than to its clutch and egg sizes (Tables 2 and 3). Size at settling mostly reflects egg size, as for example newly settled *C. pagurus*, which have larger eggs, have carapace widths (CW) of 2-3 mm, while in *C. maenas*, which has smaller eggs, the carapace widths of new settlers are less than 0.5 mm. The first juvenile stage in both homarid species has a CL of almost 10 mm. However, the less fecund *H. gammarus*, with larger eggs, has slightly larger newly hatched larvae.

Age is difficult to determine in decapod crustaceans, as reviewed by Vogt (2012). The maximum age of a species seems to be connected with its general size, with larger species generally living longer, and homarid species continue to grow and produce new batches of recruits for over 50 years. However, *H. gammarus* differs from *H. americanus* in the rates of development during the larval stages. *H. gammarus* larvae are larger at hatch, and they start metamorphosis at stage III, becoming more developmentally advanced sooner than those of *H. americanus* and leading to less change between stages III and IV in *H. gammarus* (Rötzer and Haug 2015). After settling, however, juveniles of *H. americanus* grow faster than *H. gammarus*, and individuals of this species can thus reach larger sizes and weights over their lifetime than *H. gammarus* (Lawton and Lavalli 1995). For the other species examined, the lifespan is limited, and for *C. maenas*, which undergoes a terminal molt at an age of six to eight years, possibilities for further growth are terminated and reproduction is limited to one to two years more after the terminal molt (Crothers 1967, 1968).

The underlying causes for the evolution of dissimilarities in fecundity, larval and settling juvenile sizes, and growth potential between the two homarid species are so far not known, but may be discussed in relation to the different ecosystems wherein these species have evolved (see subsequent sections).

3.4. Biotope

In statistical analyses of the species in relation to their biotopes, a new clustering pattern emerged, which was mainly related to the species' habitat depth, substrate preference, predators, and competition (agglomerated coefficient = 0.59). The habitat preferences of the selection of decapods examined range from deep, soft sea bottoms down to several hundred meters to shallow, intertidal cobble grounds (Williamson 1905; Pearson 1908; Herrick 1911; Crothers 1967; Christiansen 1969; Cooper and Uzmann 1980; Shumway et al. 1985; Factor 1995; Hunter 1999; Poore et al. 2011; Groeneveld et al. 2013; Stevens 2014; Füreder 2016). The habitats preferred by mature lobsters of *Homarus* spp. are rocky to mixed bottoms, in relatively shallow waters, particularly in the summer season, where they excavate shelters underneath protective rocks or other hard objects (Wahle et al. 2013b). Although *G. strigosa*, *P. elephas*, and *C. pagurus* are often found in similar habitats, *P. elephas* is mostly distributed in the southern part of the range of *H. gammarus*, while *C. pagurus* may be found in more exposed waters. *G. strigosa* prefers more sheltered and subtidal sites. *C. maenas* and *P. camtschaticus* can be found on both soft and rocky bottoms, while *P. camtschaticus*, *P. borealis*, and *N. norvegicus* are all found in deeper waters with soft or muddy bottoms, on open ground. High-density patches of *N. norvegicus* are usually found in cohesive mud, where they construct and occupy tunnel systems from which they periodically emerge nocturnally to forage (although females rarely emerge when carrying eggs). This is unlike the

fully epifaunistic *P. borealis* and *P. camtschaticus*, which congregate in large numbers as protection from predator attacks (Shumway et al. 1985; Hunter 1999; Stevens 2014).

Since the 1970s, there has been much speculation about the location of the settling and nursery habitats of commercially important species because of the risk of overexploitation and the need to develop conservation measures. Several laboratory studies have been conducted over the last 50 years examining the survival and growth of newly settled *H. gammarus* and *H. americanus*, but less is known from field studies, especially for *H. gammarus* but also for the deeper-living juveniles of *P. borealis* and *N. norvegicus* and the non-commercial *G. strigosa*.

The early life stages of *H. americanus* were equally unknown until the 1980s, when the newly settled juveniles were found in mixed and rocky cobble nursery grounds in shallow waters, from the lower intertidal zone to a few meters (rarely up to 80 m) down (Cobb 1971; Richards and Cobb 1986; Barshaw and Bryant-Rich 1988; Cobb and Wahle 1994; Lawton and Lavalli 1995; Cowan 1999). Settled juveniles are hardly ever seen until they are several years old, with a CL of more than 35 mm. In Europe, efforts in the early years to find *H. gammarus* juveniles failed (Howard and Bennett 1979; Howard and Nunny 1981), while later attempts to use suction sampling and settlement traps to track wild and released juveniles have not been successful at identifying where this species' juveniles live. During searches for the smallest *H. gammarus* in European cobble grounds by similar methods and in habitats preferred by *H. americanus*, none were found (Linnane et al. 1999, 2001; Mercer et al. 2001; Ringvold et al. 2015). The inability to capture early benthic stage lobsters in cobble was the case even in the vicinity of sites on known adult lobster grounds where large numbers of microtagged hatchery-reared lobsters were released, either directly onto the seabed by divers (Bannister and Addison 1998) or seeded from the surface in shallow waters (van der Meeren and Næss 1993); however, five to eight years later, these juveniles did apparently recruit in significant numbers to commercial lobster catchers at those same sites (Bannister and Addison 1998; Agnalt et al. 1999, 2004). The inference is that the hatchery-reared juveniles adopted the same cryptic behaviors as wild lobsters. During 30 years of lobster research, only three anecdotal observations of possible juvenile lobsters with reasonable certainty are known by the authors, with no patterns (see section 3.1).

The species richness of potential predators of lobsters (Figure 5) in Europe is very high (van der Meeren 2000; Robinson and Tully 2000; Mercer et al. 2001), and includes such highly efficient predatory fishes in the family Labridae as the ballan wrasse *Labrus bergylta* Ascanius, 1767, cuckoo wrasse *Labrus mixtus* L., 1758, goldsinny *Ctenolabrus rupestris* (L., 1758), and several other species of Labridae, as well as gadoid species, particularly the Atlantic cod *Gadus morhua* L., 1758, several species of sculpins, (Cottidae), and flatfishes (Pleuronectiformes) (Barshaw and Bryant-Rich 1988; Wahle et al. 2013b). In addition, octopuses are efficient predators of decapods, like the globally distributed *Octopus vulgaris* (Cuvier, 1797), and also cuttlefish (*Sepia* spp.), although this genus does not occur along the North America Atlantic coast (Young et al. 1998; Jereb et al. 2015). These visual predator species are present, often in high densities, over the entire range of *H. gammarus* (Froese and Pauly 2018), but are not present or are found at lower densities in American waters (Barshaw and Bryant-Rich 1988; Wahle et al. 2013b). Release trials have shown that all of these fishes would predate on newly released or tethered *H. gammarus* up to a size of 35 mm CL within the first day after release (van der Meeren, 2000; Ball et al. 2001; Mercer et al. 2001). MAFF divers saw that during lobster enhancement releases when the water was warm enough for

them to be active, hatchery-reared juveniles could only avoid predators by seeking shelter as quickly as possible (Howard 1983). Released lobsters were not seen in the gut contents of predators after the first 24 h from release, or seen by divers within the first year after release (van der Meeren and Næss 1993). However, recaptures have shown that sufficient numbers of juveniles can survive to boost local stocks if they are released after eight to 10 months in a hatchery and when predation pressure is at the lowest, such as in the early spring (van der Meeren 2001, 2005; Agnalt et al. 2004). Hardly any marine areas in the world have been better investigated than the European coasts, and yet intensive suction sampling and settlement trap deployments in selected, high-density adult lobster grounds with documented recruitment of emergent lobsters have still been unsuccessful in locating small juveniles. However, stage I and II larvae are easily captured, although in low numbers, in light traps and plankton net hauls during a few weeks in the late summer in the coastal waters of Sweden (Öresland and Ulmestrand 2013) and Norway (GI van der Meeren, pers. obs.). Laboratory-reared juveniles show clear selection for specific habitats in release trials, and should thus be even more concentrated after settling than during the planktonic larval stages, yet they have not been found. However, these attempts have revealed that areas with the pebble and cobble substrates in shallow waters that are preferred by the early post-settlement life stages of *H. americanus* are also settling grounds for many decapod species in Europe. This was exemplified by the West Norwegian sampling results from the Lobster Ecology and Recruitment (LEAR) project (Mercer et al. 2001), which is described more below (Figure 6).

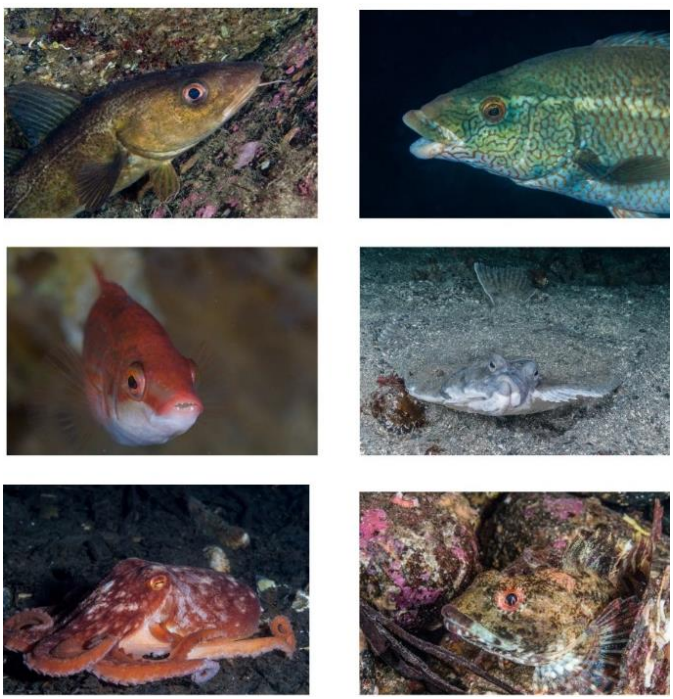

Figure 5. Some of the common and efficient visual predators found in shallow European waters (from upper-left to lower-right): Atlantic cod *Gadus morhua* (L., 1758), ballan wrasse *Labrus bergylta* (Ascanius, 1767), goldsinny *Ctenolabrus rupestris* (L. 1758), plaice *Pleuronectes platessa* (L., 1758), curled octopus *Eledone cirrhosa* (Lamarck, 1798), shorthorn sculpin *Myoxocephalus scorpius* (L., 1758). Photographer: AK Woll.

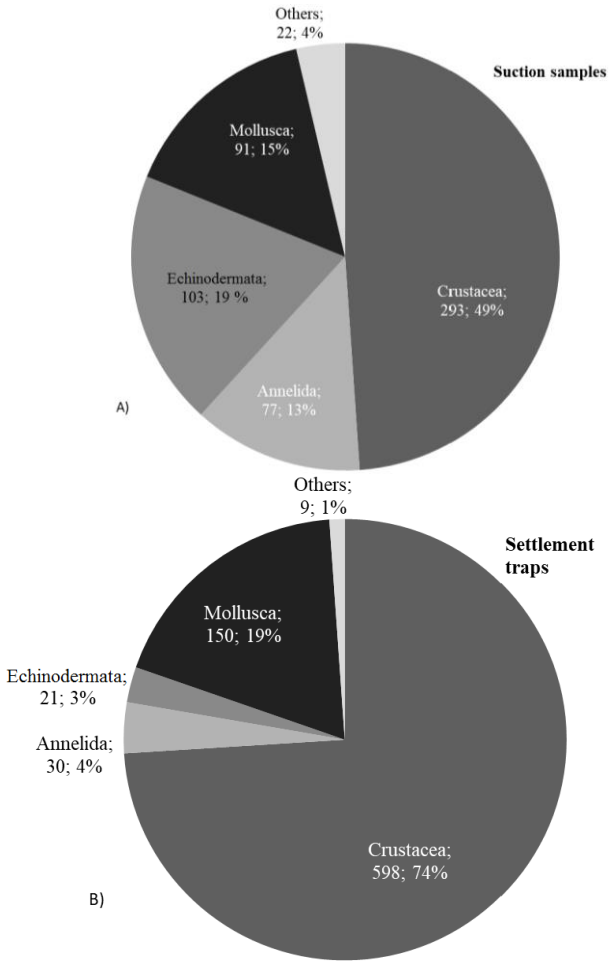

Figure 6. Overall benthic invertebrate diversity found through suction sampling on a mixed cobble- and sand-field (A) and in nine settlement traps with similar substrates (B) at the Vinnes location, Fusa, Hordaland, in western Norway in October 1999. The settlement traps were placed in the field at the beginning of the lobster hatching season in July and recovered after three months. The number of specimens found in each phylum and the percentage of all specimens represented by it is shown. The total sampled area and volume was 1.08 m^2 and 0.012 m^3, respectively. Data are from the LEAR project (Mercer et al. 2001).

The decapods found among cobble during the LEAR project represented not only a high number of species, but also high numbers of individuals, in both suction sampling and settlement traps in natural and lobster fishing areas. Whereas *H. americanus* dominates cobble habitats in North America, a mix of shrimps and several galathean species in all life stages dominated similar habitats in European waters (Figure 7). Small settlement traps provided with cobble and mixed sand and cobble substrates attracted mostly shrimps and juvenile galatheans and brachyurans. Almost 200 small-sized shrimps and nearly 100 galathean juveniles were found per m^2. Although too small to be predators of settling lobsters, both of these group could compete with them for space and food. There are also considerable gaps in our knowledge on the early life stages of galatheans and non-commercial brachyurans. Therefore, unpublished data and results from Norwegian experiments conducted

as parts of the LEAR project were used in the following sections to illuminate some of the similarities and differences in habitat and behavioral traits between laboratory- or hatchery-reared juvenile *H. gammarus* and selected species commonly found in European cobble grounds at 2-10 m depth.

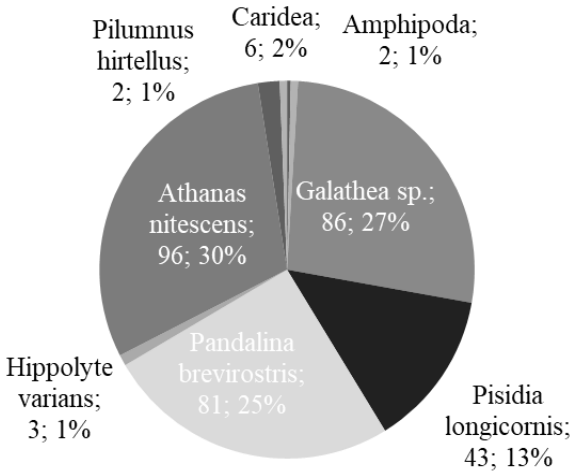

Figure 7. Crustacean diversity in settlement traps recovered in Norway in October 1999. The number of species found in each taxon and the percentage of all specimens represented by it is presented. The total sampled area and volume was 1.08 m^2 and 0.012 m^3, respectively. Data are from the LEAR project (Mercer et al. 2001).

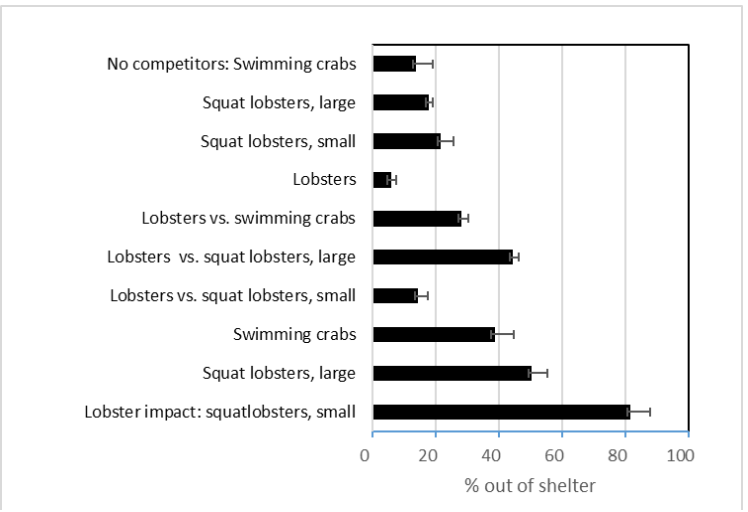

Figure 8. Percentage of time observed out of shelter of newly settled *Homarus gammarus* (L., 1758) with or without different competitors, including *Liocarcinus navigator* (Herbst, 1794) [as *Liocarcinus arcuatus*] (swimming crabs) and/or *Galathea squamifera* Leach, 1814 (squat lobsters), that were either slightly smaller (small) or equal in size to or slightly larger (large) than the lobsters. All specimens used were in the same size range (total length/width: 35 to 50 mm) and were held under a 12/12 h day/night cycle while being observed and counted every 3 h over a 72-h period, in three replicate tanks. The number of lobsters used per tank was 15 individuals, with 15 individuals of each competitor species added per tank as treatments. Data are from the LEAR project (Mercer et al. 2001; Koponen 2003).

A. nitescens, the most common shrimp in the settlement traps and the species most closely resembling stage V of *H. gammarus*, was used to investigate whether and how potential competitors impacted stage V juveniles of *H. gammarus*. One hundred stage V lobsters were paired with 75 *A. nitescen*, sharing space and food in 100 meshed cages (length 135 mm, diameter 70 mm) left on the sea bottom for four months. The 75 lobsters sharing their cages had similar survival (32%) and growth (23.5% in controls and 24.6% overall) to control lobsters, while the weight gain was lower in the shared cages than in the controls (42.2 g in controls vs. 31.3 g overall) (data from the LEAR project: Mercer et al. 2001).

Figure 9. Typical use of biotope (from upper-left to lower-right): *Homarus gammarus* (L., 1758) alert when exposed (left) and in shelter (right), *Nephrops norvegicus* (L., 1758), *Galathea strigosa* (L., 1761), male *Cancer pagurus* (L., 1758) protecting mate, *Carcinus maenas* (L., 1758) partially burrowed in sand. Photographer: AK Woll.

When facing commonly found decapod species in shallow-water cobble grounds in Europe with relatively similar diurnal cycles and shelter preferences to them in the laboratory, naïve newly settled *H. gammarus* were quite effectively able to win and protect covered burrows from potential competitors (Figure 8). The burrowing behavior of *H. gammarus* juveniles was previously described by Berrill (1974). Although lobsters were significantly less affected by their competitors than their competitors were by them (Mann-Whitney U-test: $U = 16.5$, n_1 and $n_2 = 12$, $p < 0.002$), lobsters were out of their shelter significantly more often during the daytime with competitors (e.g., when faced with small squat lobsters: 12.8 ± 3.14% more European lobsters were out of shelter vs. controls; $\chi 2 = 5.99$, $df = 1$, $p = 0.014$). Galatheans and brachyurans can find other shelter options that are unavailable to lobsters. The relatively wide- and flat-bodied crabs can dig into the substrate, while the galathean species can use their hooked claws to cling upside-down to the undersides of rocks and other hard objects (Figure 9). However, the presence of slightly larger competitors still led to more lobsters being out of their shelters, which in the field would expose them more to fish predators (Mercer et al. 2001; Koponen 2003) (Figure 8).

Both homarid species perform very similarly in laboratory settings, but to survive settlement and shelter competition in European waters, *H. gammarus* juveniles in the field may have developed antipredator and shelter selection choices that differ from those of *H. americanus* (Wahle and Steneck 1992; Lawton and Lavalli 1995). The competition for shelter and food in Norway, as in other European cobble-bottomed areas, must be very different from that in American waters (Mercer et al. 2001; Wahle et al. 2013a; Ringvold et al. 2015).

Statistical comparisons of the known and anticipated traits connected to the habitat of homarid species were made using the 'Agnes' clustering analysis and PCA on data for their habitat traits (Figure 10A). *H. gammarus* were placed in a separate branch from adult *H. americanus*, with juvenile *H. gammarus* clustered even more distantly from the juvenile *H. americanus* (Figure 10A). The PCA of descriptive functional traits showed that these species mainly differed along the PC axis that corresponded with the 'Predator diversity', 'Active burrower', 'Pigmentation', and 'Relative 2 antenna length' traits (Figure 10B). The similarities detected in the juvenile and adult *H. gammarus* may have been an artefact caused by the assumed juvenile biotope and optimal habitat being based on observations of juveniles reared in the laboratory and studied in semi-natural habitats (Figure 10C). Since the antennal length, pigmentation, and burrowing habits are known to be similar between homarid lobster species, at least in laboratory studies, the most significant differences in these analyses were thus caused by differences in predator diversity and decapod competitor diversity (Figure 10B) (van der Meeren 2000; Mercer et al. 2000). *H. gammarus* seems to have biotope traits more in line with those of *C. maenas* and *G. strigosa*, which live in shallow waters with high predation pressure and many decapod competitors, as these species were all clustered quite closely in Figure 10C; the adult *H. americanus* was also clustered within this type of habitat.

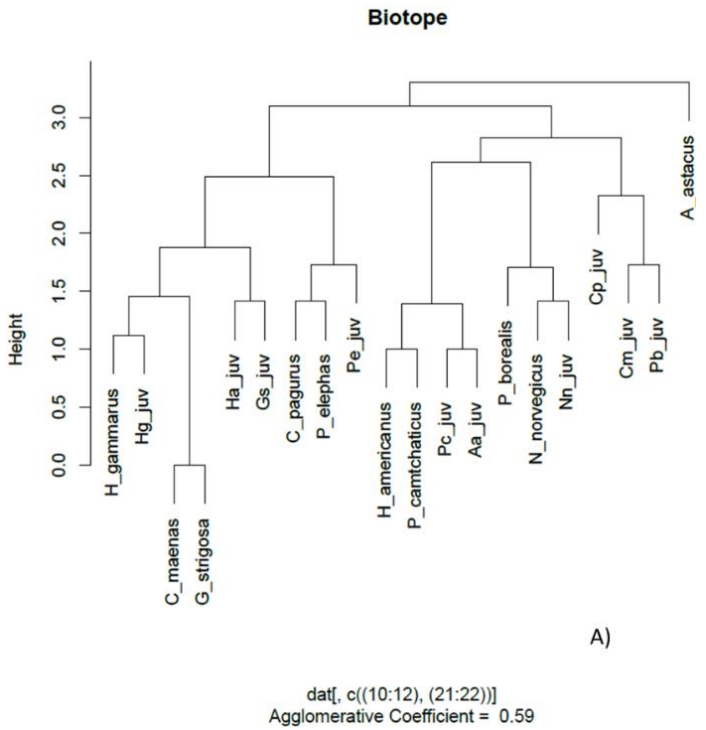

Figure 10. (Continued).

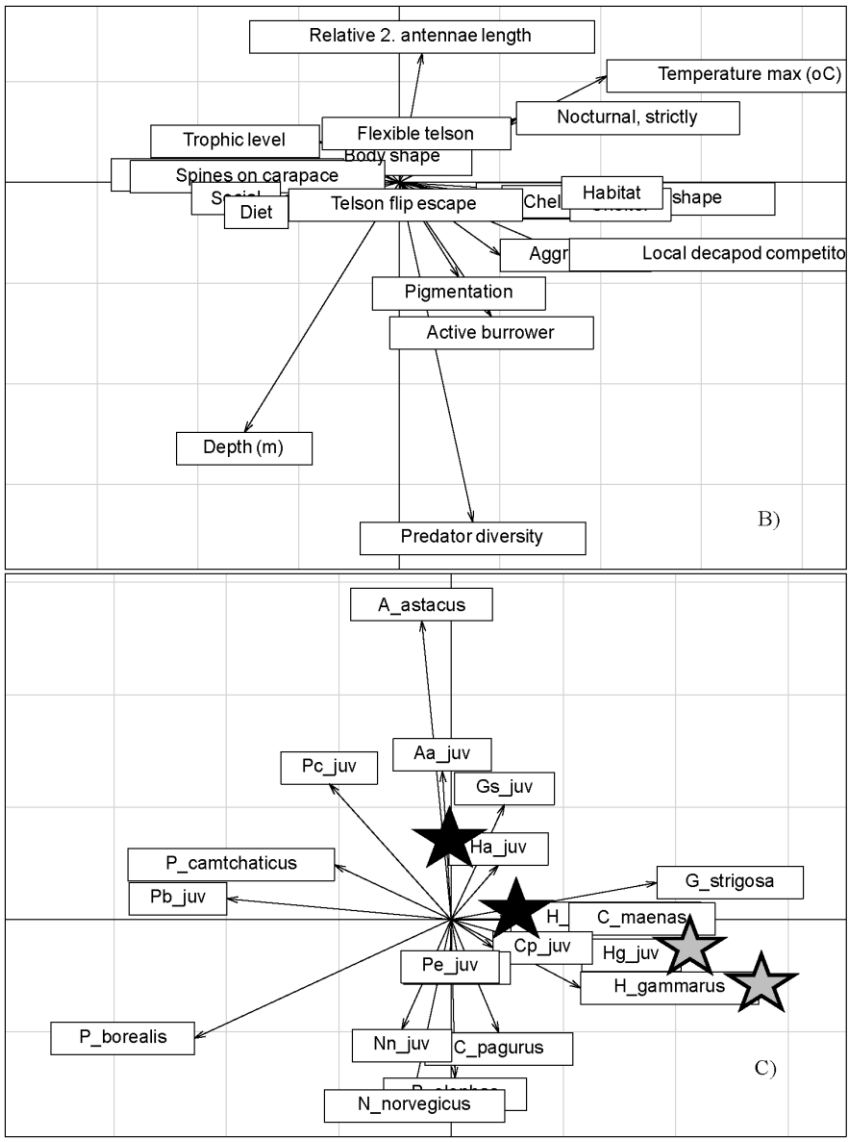

Figure 10. Analyses of similarities and differences among the preferred habitats of all the tested decapods through clustering by the 'Agnes' method (A, previous page) and principal component analyses (PCAs) of the descriptive variables (B) and species (C) along the principal component axes 3 (horizontal) and 5 (vertical). *Homarus gammarus* (L., 1758) are marked with gray stars and *H. americanus* H. Milne Edwards, 1837 are marked with black stars. Statistics were performed by Francois Besnier, IMR.

It is possible to interpret relative abundances of different species in different habitats and how this may impact juvenile lobster settlement and survival in two ways. From one point of view, one might conclude that European lobster juveniles are 'suppressed' by these 'ecological competitors'. On the other hand, the sparse lobster density and cryptic life cycle may be a low-density strategy that has evolved to reduce the prospects of them having to face predation and competition. The outcome is the same, but without careful experiments it is impossible to determine which one is the driver.

3.5. Behavior

The behaviors (Figure 11) of juvenile and adult *H. americanus* have been described in a number of studies (Cobb 1971; Stewart 1972; Barshaw and Bryant-Rich 1988; see also Atema and Voigt 1995, and references therein).

A behavioral trait that is clearly different among the selected species is seasonal migration. Some female *P. elephas* and *C. pagurus*, but not all, migrate as part of their recruitment strategy (Ansell and Robb 1977; Bennet and Brown 1983; Howard 1982; Follesa et al. 2009; Giacalone et al. 2015), and *P. camtshaticus* and some *H. americanus* migrate to deeper waters during the winter season (Stewart 1972; Sundet 2014). However, *H. gammarus*, *G. strigosa*, *N. norvegicus*, *C. maenas*, and *A. astacus* do not migrate seasonally, and are resident in the same areas throughout the year (Edwards, 1958; Klein Breteler 1976; Chapman 1980; Smith et al. 1998; Ulmestrand and Eggert 2003; Moland et al. 2011; Füreder 2016). Still, *H. gammarus* were found emigrating from artificial shelters over time, and the extents of the home ranges of large lobsters may have been previously underestimated (Jensen et al. 1993; Thorbjørnsen et al. 2018).

The homarid species avoid social interactions other than mating. They use body language and olfactory cues to signal dominance and mating and individual status (Atema and Voigt 1995; Skog 2009). Aggression seems to be negatively correlated to the body/chelae size, where female *H. gammarus* are more aggressive than males, and male *H. gammarus* are more aggressive than male *H. americanus* (van der Meeren et al. 2008; Skog 2009). *H. gammarus* does not seem to respond with decreased aggression upon receiving submissive signals from a competitor: After fights between *H. americanus*, the winner will usually ignore a defeated lobster that tucks in its tail and lowers its chelae, but in *H. gammarus* the winner will continue to chase and attack the opponent as long as they are in the same tank (van der Meeren et al. 2008).

Like *H. americanus*, adult *H. gammarus* are nocturnal predators and scavengers (Smith et al. 1998) with very flexible bodies that can easily move both forwards and backwards. As nocturnal foragers, they shelter in caves and self-made burrows during daylight hours. High-density patches of *N. norvegicus* are usually found in cohesive mud, where they construct and occupy tunnel systems from which they periodically emerge nocturnally to forage, although females rarely emerge when carrying eggs. Naïve, hatchery-reared, one-year-old *H. gammarus* show responses to new environmental factors that are similar to those of wild, adult lobsters, which are active burrowers, nocturnal, shelter-seeking, solitary, and aggressive (van der Meeren 1993) (Figure 12). Whether this also applies to wild juveniles is not known. Homarid lobsters, even when reared in hatcheries, are observed to burrow when they have the opportunity, and are well-suited to life in modified interstitial spaces within rocky and complex bottoms, from which they remove sand and build up with rocks to make entrances that are easy to protect. Their antennae provide them with excellent olfactory and tactile senses for navigating their dark world (Cobb 1971; Atema and Voigt 1995).

Although *C. pagurus* and *C. maenas* are sometime seen buried in sand, only the three nephropid lobsters actually dig and shape shelters, such as by making tunnels underneath rocks or in compact sediments. These are all examples of these species' functional morphology and habitat ecology. The crabs are protected by their bulky carapace and may have less need for full body-covering shelters than the less armored homarid species. In laboratory experiments in which sand-covered tank bottoms are offered and used by crabs, *H.*

gammarus juveniles usually prefer to dig underneath artificial shelters put in the tank, while galatheans will use natural crevices in the provided shelters (pers. obs. by the authors).

Figure 11. Interactive behaviors in (from top to bottom): male *Homarus gammarus* (L., 1758) (left) and *Homarus americanus* H. Milne Edwards, 1837 (right), fighting; *Paralithodes camtschaticus* (Tilesius, 1815) podding; *Galathea strigosa* (L., 1761) clinging (mating), *Cancer pagurus* L., 1758 (left) and *Carcinus maenas* (L., 1758) (right) foraging. Photographers: homarid lobsters by GI van der Meeren, the rest by AK Woll.

P. borealis, *P. camtschaticus*, *C. maneas*, and *C. pagurus* do not seek body-covering shelters, but also show other differences from shelter-making lobsters, including camouflage pigmentation and protective behavioral traits. *P. borealis* are good swimmers that live in dense shoals (Shumway et al. 1985). Crabs are often found on softer bottoms, near or in crevices in kelp and alga-covered substrates, environments where they can cover themselves with sand, cling to the surface, or wedge themselves inside crevices. Their pereiopods can fold underneath the body and provide a strong grip on the substrate. *P. camtschaticus* and *P. elephas* are both afforded protection from predators by forming social aggregations and possessing spiny carapaces and long appendages. *G. strigosa* are less social, but still not aggressive like *H. gammarus*. Due to their both flattened and elongated body shape, with a flexible tail tucked underneath the body, and the hooked claws present on all their pereiopods their grip on rocky surfaces and other objects is strong (Figure 9).

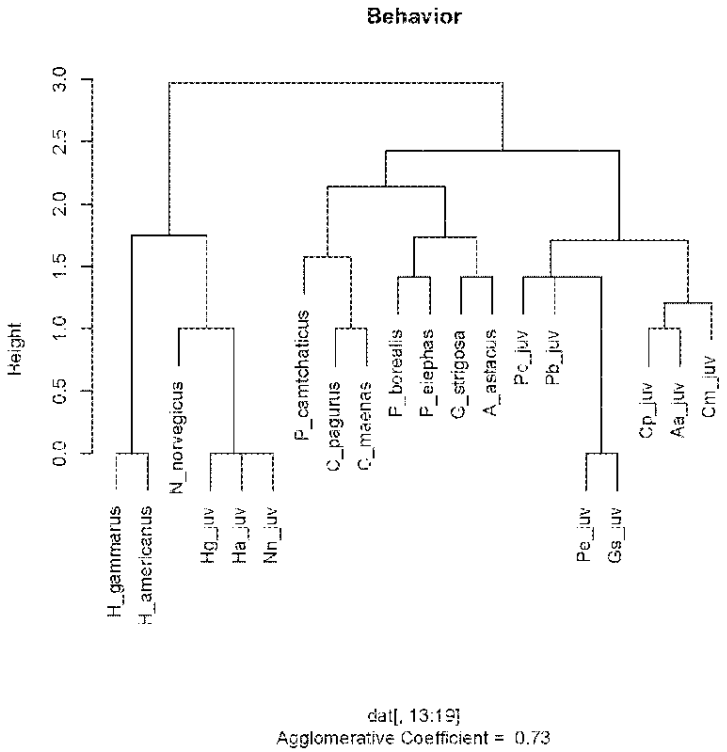

Figure 12. Analyses of similarities and differences between all the selected behavioral traits of the tested decapods ('Agnes' method). Statistics were performed by Francois Besnier, IMR.

Decapod species are found at different depths, from the upper intertidal zone (*C. maenas*), to the upper subtidal zone (*G. strigosa* and *A. astacus*), relatively shallow waters (*H. gammarus*, *C. pagurus*, and *P. elephas*), and deeper waters (*P. borealis* and *N. norvegicus*); some also occupy a wider depth range, from shallow waters down to more than 100 m depth (*P. camtschaticus* and *H. americanus*). Living in the littoral zone requires appropriate responses to the tide. The behavior of *C. maenas* is connected to the tidal cycle, and crabs will move into a shelter when the tide is going out (Crothers 1967, 1968). Typically, they hide under seaweed or rocks, staying humid and sheltered from direct sunlight. In the competition experiments done during the LEAR project, each test was finished by emptying the 0.9 m deep tanks with the experimental animals still in place. In this process, it was observed that the *H. gammarus* juveniles were all hiding underneath the cobble shelters, while the other species sampled from natural cobble grounds at 4 to 10 m depth all reacted by fleeing their shelters, moving out into full exposure on the open sand. The lobsters behaved exactly as the intertidal *C. maenas* would be expected to do (pers. obs. by the authors).

For the early life stages of *H. gammarus*, no previous attempts to document their natural living biotope has yet succeeded, and thus there are no published observations of the behavior of wild juveniles. After more than a century of studies of hatchery-reared lobsters and juvenile releases to the sea, our present knowledge of the juveniles of this species all comes from laboratory studies. Anecdotal information and observations of laboratory-hatched young-of-the-year juveniles in tanks exist, as described earlier in this chapter (section 3.1), which provide varying levels of detail for habitat reconstruction. Searches in nature for this

life stage have been futile thus far. Even after releases of thousands of juveniles from one to 12 months old, very few are seen and none are recaptured in the next three to five years, after which they reappear in traps as adults, in the same location where released (Bannister and Addison 1998; van der Meeren 2003; Agnalt et al. 2004). Notably, a Swedish study suggested that lobster larvae may be retained in the area where they are hatched, which implies that they do seek shelters near to the adults' habitats (Öresland and Ulmestrand 2013). Releases and searches for wild juveniles have been based on knowledge of the habitat of larger lobsters more than 50 mm CL in size and biotopes known to attract *H. americanus* juveniles. However, the lack of actual knowledge of wild *H. gammarus* juveniles' habitat preferences and behavior in the rich biodiversity of European waters make proper monitoring of stock recruitment and conservation measures, including recruitment protection, difficult.

4. DISCUSSION: THE FULL PICTURE

There has been substantial speculation regarding how the juveniles of *H. gammarus* manage to remain elusive. Are the juveniles suppressed by competitors and predators and therefore sparsely distributed, or is the low density of *H. gammarus* versus *H. americanus* a strategy to reduce the prospects of competition and predation? Three main hypothesis have been suggested, that they: (1) stay in deeper waters, (2) settle too sparsely to be detected, or (3) dig deep or hide deep in the substrate, in branched and long burrows or caverns (Mercer et al. 2001).

A bigger-picture perspective is needed to pinpoint how the young of *H. gammarus* differ from those of their closest relative. To evaluate the three hypotheses suggested above, a full ecological picture of this species and others, including their morphology, life history, habitat, and behavior, may be useful. In this chapter, the selection of Northern Atlantic decapod species examined all had several features in common with *H. gammarus*: they need protection when molting, are mainly nocturnal, more or less omnivorous, and live within the same temperature range. However, the species differ from each other in their preferences for habitat depth, bottom quality, and the temperature range they prefer. They also differ in how they avoid predators, the extent of parental care, the duration of the larval period, and in morphology. The most temperate species, *P. elephas*, and the polar *P. camtschaticus* represented the extremes of both temperature preferences and morphology considered, although both share part of their temperature tolerance zone with the homarid lobsters. *P. camtschaticus*, *N. norvegicus*, and *P. borealis* are all commonly found below 100 m depth, well below *H. gammarus*, which is usually found above 70 m depth. These deep-living species have typically reddish pigmentation, less developed chelae, and weaker pereiopods that reflect their life in waters deeper than the sunlit intertidal zone, with reduced diversity of predators and competitors, and shelter from predators in self-made burrows in cohesive mud (*N. norvegicus*) or by gathering in large numbers (*P. camtschaticus* pods, *P. borealis* shoals). The pigmentation, behavior, and habitat of adult *H. gammarus* overlaps with the shallow-living marine species and even the limnic *A. astacus*. Adult lobsters stay in shallow waters even more in *H. americanus*, which are even more tolerant to low temperatures than *H. gammarus* in all life stages; they become inactive in waters colder than 7°C, and temperatures below 5°C inhibit larval molts (Templeman 1936; Nicosia and Lavalli 1999; Wickings and

Lee 2002). Still, hybridization of *H. americanus* females with *H. gammarus* males has been documented in European waters (Jørstad et al. 2011; Öresland et al. 2017). Without doubt, *H. gammarus* belongs to the group of shallow-dwelling species.

The natural living biotope of all animals is functionally reflected in their morphology, life history strategy, habitat selection, and behavior. In *H. americanus*, density-dependent population regulation is likely (Fogarty and Idoine 1986; Ennis and Fogarty 1997; Wahle et al. 2013b), while this is not expected in *H. gammarus* since it is rarely found in densities as high as *H. americanus* (Wahle et al. 2013a). Still, habitat has impacts on the size composition of *H. gammarus* (Howard 1980), as well as conspecific chemical stimuli and interspecific interference competition (Wickins and Lee 2002). Wild-caught post-larval *C. maenas* respond to density-dependent cues that lead to slightly faster growth and the development of stronger chelae, increasing their competitive ability (Duarte et al. 2014).

As nocturnal foragers, adult *H. gammarus* are photophobic and darkly pigmented, fast-moving and highly mobile, and shelter during the daytime in caves and burrows that they usually modify and manipulate to provide better protection. In the northern part of their distribution, *H. gammarus* are black to nearly black in color. Their very dark colors are a good fit for living on rocky and complex bottoms, while their long antennae provide them with excellent senses of smell/taste and touch for navigating in their dark world, including when sitting inside deep caverns and tunnels. In many ways, *H. americanus* is similar in morphology to *H. gammarus*, although with slightly lighter pigmentation, which may indicate that their ecological niches are comparable but not exactly the same. In contrast, *P. elephas* adopts communal living in groups for protection, and is not found inside deep caves (Eggleston and Lipcius 1992; Diaz et al. 2001; Goñi and Latrouite 2005; Buscaino et al. 2011). The crayfish *A. astacus* is as dark as *H. gammarus*, but lives in lakes with no crustacean competitors and only fish and bird predators, where it shelters in natural crevices and protects the brood until the juveniles have completed the second post-hatch molt, making them large enough to be able to flee from visual fish predators (Stein and Magnusson 1976; Söderbäck 1994; Füreder 2016).

The ways in which morphology constrains how decapods can move are also telling. *P camtschaticus* move freely in open habitats, but cannot lift their own weight out of water, tail-flip away from danger, or fold up to effectively cling to the substrate. However, they do not need to use these techniques because they instead obtain protection by moving in pods and outgrowing their predators (Dew 1990). Young ones, which are not protected by size, stay in social groups and are covered in pointed spines all over the carapace. *Pandalus borealis*, which is small in size and unable to grip anything, relies on tail-flip escape behaviors, camouflage pigmentation, and hiding in dense shoals above the seabed for protection. In shallower waters, wide-bodied crabs have relatively hard carapaces, no tail joints and strong chelae and pereiopods, making them increasingly difficult to break and swallow for predators as they grow. Young stages of crabs rely on digging shallow depressions in sandy bottoms, hiding in inaccessible crevices, and a strong grip achieved by folding the chelae and pereiopods around kelp stems or other objects to which they can cling, leading to them having a preference for sheltering in kelp forests or other places with algal cover or complex surfaces (Howard 1982; Fernandez et al. 1993). Both *G. strigosa* and *P. elephas* exhibit typically elongated ‘lobster’ shapes, with quite strong walking pereiopods, and use tail-flips for escape. (Robinson and Tully 2000; Linnane et al. 2001; Ringvold et al 2015). However, galatheans are more dorsoventrally compressed than the cylindrical homarid lobsters and *A. astacus*.

Like brachyurans, galatheans can fold their pereiopods underneath the body and get a strong grip on the surface, as well as cling upside-down by the hooked claws on their strong legs and pointed claws. Lobsters cannot fold their legs underneath the body, but can fold them tightly up on each side of the body. They cannot cling to or grip the substrate or objects, but are shaped well for moving through narrow passages and pushing out sediments from burrows or tunnels. In emerging and adult homarid lobsters, this is the preferred habitat in laboratory studies (Linnane et al. 1999; Jørstad et al. 2001; Wahle et al. 2013a). Even if no *H. gammarus* juveniles have been found in cobble grounds, and extremely few have been found at all, the latter are found in habitats with empty shells or rocky reefs mixed with sandy spots, which can usually be improved by manipulating the entrance and shelter shape. Hatchery-reared *H. gammarus* juveniles released in laboratory tests will shelter faster when the water carries predator odors (Longva Nilsen 2007; Aspaas 2012) and show reduced aggression when a fish is present (van der Meeren 1993), showing how innate responses are still present in intensively cultivated juveniles.

Interestingly, a reaction to water drainage commonly found in intertidal species seems to also be a natural reaction in laboratory-reared *H. gammarus* juveniles. Water drainage induces them to move into drained, but humid and shaded, shelters, whereas galatheans, one of the most common benthic decapod groups in European shallow waters, flee their shelters in the absence of water. Thus, *H. gammarus* acts in the same way as the intertidal *C. maenas*. This could indicate that *H. gammarus* may have evolved to live in the upper littoral zone, perhaps even in the intertidal zone. The nursery grounds of *H. americanus* are shallow as well, but not usually intertidal (Cowan 1999, Wahle et al. 2013b). The innate reaction of *H. gammarus* to shelter in dark and moist places when left out of water indicates that the hypothesis that this species' juveniles have been hard to find because they avoid competition by growing up in deeper waters is not likely to be correct.

Looking at all traits together, the most similar species of those examined are the homarid lobsters (Figure 2 and Tables 1-5), even if they differ in terms of some characteristics of their microhabitats and ecology. In terms of habitat, the rich biodiversity of Europe most probably led to the differences found in juvenile, and to some extent adult, *H. gammarus* and *H. americanus* behavioral, morphological, and life history traits. In Late Medieval Scandinavian literature, before *H. gammarus* was fished commercially, Magnus (1556) and Pedersøn Friis (1599) claimed that foxes, ravens, otters, and men caught small lobsters at low tide by turning stones in the intertidal zone (as cited in Spanier et al. (2015)). No intertidal lobsters have been reported in later centuries. While the fiercest competitor any young lobster will meet is another lobster, the density and biodiversity of other species of competitors and predators is higher in European waters than that found along the North American east coast (and in the biotopes of *H. americanus*). These differences between regions could be the cause for some of the noticeable dissimilarities between *H. gammarus* and *H. americanus*. *H. gammarus* lobsters are more aggressive, showing no tolerance to other lobsters except during mating (van der Meeren et al. 2008; Skog 2009). In *H. gammarus*, each egg is larger and provided with more yolk, causing fewer eggs to be produced per clutch, while the newly hatched larvae are larger and start metamorphosis at larval stage III, leading to them becoming more developmentally advanced stage IV and post-larvae than those of *H. americanus* (Aiken and Waddy 1989; Agnalt 2008; Rötzer and Haug 2015). Larger larvae and juveniles are better prepared to fight for shelter and survival after settlement (Wahle and Steneck 1992).

Although *H. gammarus* is known to live in less dense populations, the catch-per-unit-trap in some locations of *H. gammarus* can be at the same level as that of *H. americanus* (Jørstad et al., unpublished data). The *H. gammarus* stock in the UK and Ireland has been stable and has supported a commercially healthy fishery since 1910 (CEFAS 2014). Stage I and II larvae are easily caught in plankton nets or light traps if these are deployed at hatching time and the following few weeks (Öresland and Ulmestrand 2013). Considering all of the fisheries and field research undertaken along the European coasts, as well as failures to recapture any of the newly released hatchery-reared lobsters that turn up years later in commercial catches (Bannister and Addison 1998; Agnalt et al. 2004), it is not likely that scarcity is the reason that juvenile European lobsters have not yet been found. The UK *H. gammarus* stock is stronger than ever, sustaining a healthy fishery, indicating that nursery ground availability is not limiting the stable recruitment of new lobsters to this stock, which probably has also benefitted from warmer summer ocean temperatures in recent years (CEFAS 2014; Selim et al. 2016). In Scandinavia, it is clear that although the warming seas should be beneficial for the lobsters in this northern portion of their distribution, overexploitation combined with low summer sea temperatures in the 1960s and 1970s resulted in stock collapse (Pettersen et al. 2009). The main cause for the still very low stock in areas outside of lobster marine protected areas, despite benign climate conditions, is still overexploitation (Kleiven et al. 2012; Moland et al. 2013; Thorbjørnsen et al. 2018). The overall picture discussed here indicates that a lack of nursery grounds for juveniles is not plausible, as their innate traits seem to keep them out of the microhabitats of their competitors and common predators.

The full picture points to *H. gammarus* juveniles, much like the adults, living in shallow waters, where they are well-dispersed but not necessarily rare. The cryptic life habit of *H. gammarus* for the first years of its life may have evolved in shallow, highly competitive, and predator-dense biotopes, as a strategy to reduce pressures from competitors and predators by occupying and manipulating their shelters and spaces to keep out predatory or competing species. Thus, the advanced larval metamorphosis, high level of aggression, solitary shelter occupation, and active digging and manipulation of the habitat of *H. gammarus* may all be traits that provide its juveniles access to unique shelters in shallow waters that are so successful they are also unapproachable by humans by all approaches utilized so far.

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Publications from the Last 3 Years:

Prozorkevich D, Johansen GO, van der Meeren GI (2018) Survey report from the joint Norwegian/Russian ecosystem survey in the Barents Sea and adjacent waters, August-October 2017. *IMR/PINRO Joint Report Series*, No. 2/2018: 100 p.

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Publications from the last 3 Years:

Bakke S, Larssen WE, Woll AK, Søvik G, Gundersen AC, Hvingel C, Nilssen EM (2018) Size at maturity and molting probability across latitude in female *Cancer pagurus*. *Fish Res* 205:43-51.

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Chapter 109

BIOLOGIC AND SOCIOECONOMIC HARVESTING STRATEGIES FOR THE CARIBBEAN SPINY LOBSTER FISHERIES

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ABSTRACT

The spiny lobster fisheries exploited by 25 Caribbean countries are subject to heterogeneous harvesting practices, and some of them undergo recurrent socioeconomic crises. Therefore, in this chapter a meta-analysis was conducted with the purpose of providing general management recommendations by evaluating the performance of the five main Caribbean lobster fisheries (those in the Bahamas, Brazil, Cuba, Nicaragua, and the United States of America), as well as the total production of the region. The associated costs, benefits, and social values of a small fishery in southeastern Mexico, in the northwestern Caribbean, were used as reference values. The stocks were assessed by reconstructing the age structure of each population over a 15-year period, and then the catch, profit, direct provisioning of jobs, and profits per fisher were estimated for each fishery in simulations with different ages of first catch (t_c). Based on these, the fisheries mortality (F) values needed to attain the maximum sustainable yield (F_{MSY}) and the maximum economic yield (F_{MEY}) were selected as the optimum harvesting options for different fisheries. The results showed that the yield increased with the t_c, and in three cases the yields at the F_{MSY} were higher than those at the F_{MEY}. The profits were higher at higher t_c values in three fisheries, meaning that they were more profitable if harvested at their F_{MEY} levels. One fishery (that in the Bahamas) was not profitable if harvested at the F_{MSY} level at any age. The social value of most of the fisheries, calculated as the profits/fisher, was the highest at a t_c of 3 years, and again was higher if the F_{MEY} strategy was applied. However, in the Bahamas' fishery the social value at the F_{MSY} was negative at any t_c. Based on the widespread distribution of this species of spiny lobster across the coasts of the Caribbean and the heterogeneous exploitation practices applied to its fisheries, the creation of a multinational organization in charge of regulating and

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managing the fishery in each country is recommended to achieve the sustainable exploitation of this resource within the framework of conservation.

Keywords: Caribbean, optimum yield, *Panulirus argus*, spiny lobster

1. INTRODUCTION

Stock assessment has been a major focus of fisheries scientists' efforts for a long time, and the related exploitation of fished stocks to the level of their maximum sustainable yield has been one of the main goals of fisheries science and management (Beverton and Holt 1957; Cushing 1968; Gulland 1969, 1988; Ricker 1975; Hilborn and Walters 1992; Quinn II and Deriso 1999). This approach has the purpose of acting as a "holding action against the forces of resource depletion" (Walters 1986). In many fisheries, the objective of fisheries management is usually the achievement of the maximum sustainable yield, or *MSY* (Hilborn 2007). However, the maximum economic yield (*MEY*) is a more convenient option, although it is also more difficult to evaluate. Although the basis of studies of the population dynamics of fished species has remained essentially the same, in recent decades fisheries science has made enormous progress relative to traditional views and methods. This is because, since computers became accessible to scientists and fisheries managers for use in their everyday tasks, they have become able to handle enormous amounts of data, which has made it possible to test the outcomes of a wide variety of harvesting scenarios. Unfortunately, many fisheries remain unattended by management efforts or stock assessment studies, meaning that the use of their resources is under free access and they lack harvest controls, and they are thus often depleted (Beddington and Kirkwood 2005). The fisheries of the Caribbean spiny lobster (*Panulirus argus* (Latreille, 1804)) face such challenges, so this chapter focused on analyzing harvest strategies for this species' stocks.

The Caribbean spiny lobster, *Panulirus argus*, belongs to the group of about 20 species of tropical spiny lobsters that are of commercial interest (Holthuis 1991; Phillips et al. 2013). Most details on the life history and fisheries management of this lobster were summarized in the book edited by Phillips (2013). *P. argus* has nocturnal habits, and is associated with coral reef habitats, where it remains hidden in hollows during the day. It is distributed throughout the Caribbean, where it prefers shallow waters but can be found in waters up to 90 m deep or more (Holthuis 1991; Briones-Fourzán and Lozano-Álvarez 2013). It is a gregarious and migratory species. In this region, another species of the same genus (*P. gracilis* (Latreille, 1804)) also occurs, but it is smaller and less abundant than *P. argus* (Holthuis 1991; Briones-Fourzán and Lozano-Álvarez 2013). Lobsters are long-lived species that can live for more than 25 years and grow up to half a meter long, and may reach about 20 kg in weight (Phillips et al. 2013). This species' distribution mainly includes the waters of the Caribbean Sea and the Gulf of Mexico, although some individuals have been found as far north as Massachusetts, northeastern United States of America (USA) (Holthuis 1991; FAO 2019). Towards the south, it can be found until northeastern Brazil (Figure 1), being apparently absent in the waters of the Guianas, where the mouths of several large rivers impose an ecological barrier that may constrain its distribution. It has also been found on the tropical coast of West Africa (Holthuis 1991; FAO 2019).

There is some indirect evidence (Grober-Dunsmore and Keller 2008; Butler IV et al. 2010; Chávez and Chávez-Hidalgo 2012) suggesting that currents in the Caribbean and the long larval periods of spiny lobsters are factors that allow there to be high connectivity among the lobster populations of most islands and coastal zones of tropical America (Chávez and Chávez-Hidalgo 2012; Kough et al. 2013) (Figure 2). In the northern part of this species' range, larvae mainly occur in the water column from June to December (FAO 2019).

Figure 1. Spiny lobster (*Panulirus argus*) distribution; after the FAO (2019) Fisheries GLOBAL Information System (http://www.fao.org/fishery/species/3445/en).

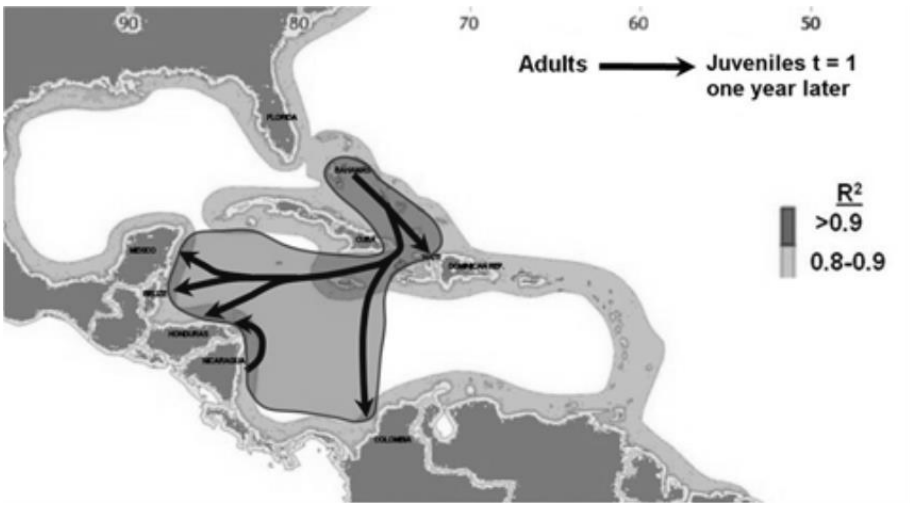

Figure 2. Main pathways of connectivity among spiny lobster populations suggested for the Caribbean waters; after Chávez and Chávez-Hidalgo (2012).

These lobsters have a life cycle that includes larval stages that live in the plankton for about nine months (Kough et al. 2013; Phillips et al. 2013). This characteristic is one of the reasons for this species' wide distribution. At the end of the larval phase, after passing

through a large number of planktonic stages (phyllosomas) and then undergoing metamorphosis, they transform into transparent swimming postlarvae (pueruli) with essentially the same shape as the adult (Briones-Fourzán and Lozano-Álvarez 2013; Phillips et al. 2013). These postlarvae then seek refuge in the reef benthos, where they hide in cavities and grow into juvenile lobsters (Briones-Fourzán and Lozano-Álvarez 2013). In this habitat, they reach sexual maturity after three years, at which point they start breeding (Phillips et al. 2013; FAO 2019).

Spiny lobsters have separate sexes, and although they can reproduce year-round, the main reproductive period takes place from March to September with two main peaks in May and September, corresponding with when water temperatures fall within the range of 23.7 to 28.0°C (Phillips et al. 2013). Females move to deeper water to spawn, and mass migrations occur in the autumn in which groups of animals (up to 50 individuals) move single-file in a certain direction in the daytime, with each animal having body contact with the next in line through the antennae (Phillips et al. 2013; FAO 2019). They lay 800 eggs per g of body weight, with a high fecundity of $> 2 \times 10^6$ eggs per ripe female. Spawning takes place with a one-month lag, when the water temperature is generally above 24°C (Pollock 1997; Phillips et al. 2013). During fertilization, the male deposits a spermatophore on the belly of the female, which by its appearance is known as a 'tarspot', from which the eggs are fertilized after spawning (Phillips et al. 2013). After being extruded from the ovary and fertilized, the eggs are adhered to the filaments of the female's pleopods, where they are carried for about one month (Pollock 1997; FAO 2019). At the end of that process, hatching occurs in protected sites on the reef (Briones-Fourzán and Lozano-Álvarez 2013; FAO 2019). Length at settlement, takes place when postlarvae have a size of 6 mm carapace (Pollock, 1997).

Lobsters are predators that mainly feed upon mollusks and crustaceans (trophic level = 3.39 on a scale from 1 to 5, with 5 being top predators) (Phillips et al. 2013). Importantly, in the year 2000, a pathogenic virus was discovered in juvenile Caribbean spiny lobsters from the Florida Keys, USA. *Panulirus argus* is susceptible to infection by *Panulirus argus* virus 1 (PaV1), the only pathogenic virus known to naturally infect any lobster species, which profoundly affects its ecology and physiology. PaV1 is widespread in the Caribbean, with infections reported in Florida (USA), St. Croix, St. Kitts, and the Yucatan, Mexico (Butler IV et al. 2008; Behringer et al. 2012). The sharing of viral alleles among lobsters from distant locations supports the hypothesis of there being high genetic connectivity among lobsters within the Caribbean, and further supports the hypothesis that postlarvae infected with PaV1 may serve to disperse the virus over long distances (Moss et al. 2013). However, *P. argus* lobsters are able to mitigate PaV1 transmission risk by avoiding infected individuals (Behringer et al. 2012).

The Caribbean spiny lobster is a fisheries resource that is highly prized and exploited in all Caribbean countries and Brazil (Holthuis 1991; Phillips et al. 2013; FAO 2019). The most common commercial sizes harvested have a weight of half a kilogram (0.5 kg) and measure about 30 cm in length (Phillips et al. 2013; FAO 2019). In many Caribbean countries, there is open access to lobster harvesting, although some degree of management is applied in most others. For example, in Belize, the following management regulations are in effect: the minimum legal size is set at a carapace length of 3 inches or 7.6 cm or a minimum tail weight of 4 ounces or 113.4 g; there is a closed season from February 5 to June 14. Possession of diced lobster tail meat is prohibited; no commercial deep-water fishing is allowed, nor is lobster fishing with SCUBA gear; no lobster traps are allowed beyond the front reef, nor

within a distance of 300 m from any coral formation; and it is prohibited to capture 'berried' or egg-bearing lobsters, lobsters with a 'tarspot', or molting lobsters (FAO 2019). Most Caribbean countries apply management practices analogous to those described above for Belize (Phillips et al. 2013; FAO 2019), although with important differences in the specific rules and regulations applied and their degree of enforcement (Hilborn et al. 2005).

The heterogeneity in management practices among shared or connected lobster stocks (Chávez and Chávez-Hidalgo 2012; Kough et al. 2013; Phillips et al. 2013) and the fact that many are open access (Beddington and Kirkwood 2005) has led to uncertainty regarding the sustainability of and appropriate management approaches for Caribbean lobster fisheries (Pauly et al. 2002). For this reason, the present chapter examined the exploited stocks of the Caribbean spiny lobster (*P. argus*) by addressing some guidelines to improve the criteria of assessing the fisheries for this species in the countries within the Caribbean region in which it is exploited, to ensure that this is done sustainably.

2. MATERIALS AND METHODS

2.1. Data, Models, and Analyses

Exploited Caribbean lobster stocks were assessed using catch data for the last fifteen years available at the Food and Agriculture Organization of the United Nations (FAO, 2011) website (http://www.fao.org/fishery/ fishfinder). The values of population parameters came from Ley-Cooper and Chávez (2010). Changes in abundance over time were determined based on changes in the catch data, which were examined in units of metric tons (mt) of fresh weight.

Trends in fishing mortality (F) and estimates of total stock biomass over time were examined. Different fishing scenarios among which the age of first catch (t_c) differed were evaluated based on the F resulting if they were exploited at the level of their maximum sustainable yield (F_{MSY}), as an extreme reference point. The other reference point examined was the F at the maximum economic yield (F_{MEY}). These reference points were determined for each stock at each of the t_c values examined ($1 \geq t_c \leq 6$ years). Tests of t_c values higher than 6 years were deliberately omitted because consumers of this species demand lobsters of around 2 or 3 years old, and the relative value of larger lobsters thus decreases.

The maximum social value of a fishery in a given scenario was determined in two ways. First, the value was assessed in terms of the maximum level of employment or, in other words, the maximum number of fishers, potentially supported by the fishery. The second way in which social value was approached was in terms of the maximum profit per fisher. Economic and social values were assessed in terms of the value per kg landed, the number of fishers per boat, the number of boats, and the number of fishing days, all of which were obtained for the last fishing season in the dataset (FAO, 2011) and then later estimated based on overall trends throughout the 15 years of catch data considered. Costs were estimated by summing the cost/boat/day × the total number of fishing days of the fleet over the fishing season. Profits were subtracted from the total value of the catch minus the total costs. Costs and values were linked to the catch in the semi-automated age-structured FISMO simulation

model (Chávez 2005, 2014), which allowed all of the possible scenarios of exploitation to be tested.

The age of first capture was initialized at 3 years and was maintained at a constant value during the fitting process, but then for the simulations all of the F and t_c values feasible to apply to the data were tested to optimize harvest strategies and design exploitation policies for each stock. The age of first maturity of spiny lobsters occurs at the 3-year mark, after the larval and juvenile stages have been completed (Phillips et al. 2013).

Population parameter values were obtained from published sources (Chávez 2009; Ley-Cooper and Chávez 2010). Estimates of the age composition of the catch were made based on FAO (2011) records (http://www.fao.org/fishery/fishfinder). These estimates were made for each one of the main fisheries in the region (those in the Bahamas, Brazil, Cuba, Nicaragua, and the United States of America; Figure 3) over a time scale of 15 years each, and also from a global assessment of the catch data of all 25 spiny lobster fisheries in the Caribbean pooled together. Catch values per kg without value added were obtained as reference data from a small fishery in southeastern Mexico (Ley-Cooper and Chávez 2010), while assuming that their values were approximately the same as those for all other fisheries in the region.

In each case, total mortality (Z_t) was determined with the exponential decay model as follows:

$$N_{a+1} = N_a \cdot e^{(-z_t)} \tag{1}$$

where N_a is the number of spiny lobsters of age a and N_{a+1} is the number of spiny lobsters of age $a+1$ in reconstructed age-groups. The time units used in this equation are years. The von Bertalanffy (1934) growth equation was used to determine the number of lobsters in each age group, as well as their corresponding sizes in terms of their lengths. These lengths were transformed into their respective weights by using the equation:

$$W = 0.0404 \cdot L^3 \tag{2}$$

where W is total weight (g) and L is total length (cm).

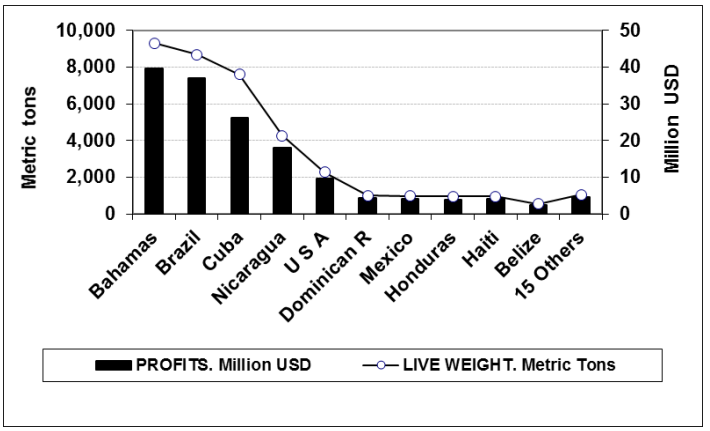

Figure 3. Catch (metric tons) and value (million USD) of the main Caribbean spiny lobster fisheries in the year 2010. Catch data were obtained from FAO (2011) records.

The age structures of each age group (in years) were estimated assuming a constant natural mortality rate. For setting the values of the variables in the initial state, the abundance per age class ($N_{a,y}$) was set using the age-specific abundance, $N_a/\sum N_a$, obtained from equation (1). In subsequent years, the age structure was defined after the number of one-year-old recruits was estimated. These values were used to calculate the catch-at-age as proposed by Sparre and Venema (1992), and were integrated into the FISMO simulation model as follows:

$$Y_{a,y} = N_{a,y} \cdot W_{a,y} \frac{F_t}{(F_t+M)}\left(1 - e^{-(F_t+M)}\right) \tag{3}$$

where $Y_{a,y}$ is the catch-at-age for age a in each year y, $N_{a,y}$ is the number of spiny lobsters at age a in year y, $W_{a,y}$ is the lobster weight equivalent of $N_{a,y}$, F_t is the fishing mortality (as described earlier in this section), and M is the natural mortality coefficient. Given the initial conditions used, the values of $Y_{a,y}$ were adjusted by varying the initial number of recruits, and then linked to equations (1-3) until the following condition was fulfilled:

$$\sum_a^{\lambda} Y_{a,y} = Y_{y(REC)} \tag{4}$$

where $Y_{y(REC)}$ is the yield recorded during the year y, a = 3 years, and $t_\lambda = 3/K$ or the longevity of the species, where K is the growth constant of the von Bertalanffy (1934) growth equation. The value of t_λ was set to 13 years, a value found for this species by assuming that a reasonable life expectancy (L_{max}) is that at which 95% of the population reaches 95% of $L\infty$, the asymptotic length. Thus, the longevity was found by making $L_{max} = 0.95 \times L\infty$ in the von Bertalanffy growth equation and finding the corresponding value of t. The catch equation was used for each year in the time-series analyzed. For the estimation of the natural mortality coefficient (M), the criterion proposed by Jensen (1996, 1997) was adopted, where $M = 1.5K = 0.1793$; the value of K used is further described below. Estimates of the stock biomass and the exploitation rate, $E = [F/(M + F)]$, were made for each age-class in every fishing year analyzed by the model. These values were compared to the E value at the F_{MSY} level. A special case is when E is equal to the F_{MSY} level of fishing mortality, which corresponds to the maximum exploitation rate that a fishery should be able to attain before the stock is overexploited. A diagnosis of the years of the series in which the stock was under- or overexploited was also made, which provided an easy way to recommend either a further increase or decrease in the fishing intensity F.

The annual cohort abundance ($N_{a,y}$) coming from ages older than the age-at-maturity (t_m = 3 years) was used to estimate the annual abundance of adults (S_y) over the years, whereas the abundance of the one-year-old group was used as the number of recruits (R_y). The stock-recruitment relationship was evaluated by using a slightly modified version of the Beverton and Holt (1957) model of the form:

$$R_{y+1} = \frac{a' S_o S_y}{S_y + b' S_o} \tag{5}$$

where R_{y+1} is the number of one-year-old recruits in year $y + 1$, S_y is the number of adults in year y, and S_o is the maximum number of adults in the population. The parameters a' and b' are modified from the original model, such that a' is the maximum number of recruits and b'

is the initial slope of the recruitment line, which was kept constant throughout the simulations done herein. The values of the parameters used as input to simulations are shown in Table 1.

Table 1. Population parameter values used for the evaluation of the main spiny lobster fisheries of the Caribbean

Parameter	Value	Units	Model	Source
K	0.24		von Bertalanffy	Chávez (2001)
L_∞	31	Tail length, cm	von Bertalanffy	González-Cano (1991) (mean)
W_∞	1,619	Live weight, g		González-Cano (1991) (mean)
t_o	-0.17	Years	von Bertalanffy	González-Cano (1991) (mean)
a	0.038		Length-weight	Ley-Cooper & Chávez (2010)
b	3.1		Length-weight	Ley-Cooper & Chávez (2010)
t_c	3	Years (both sexes)		Ley-Cooper & Chávez (2010)
t_m	3	Years		Ley-Cooper & Chávez (2010)
t_λ	13	Years		Ley-Cooper & Chávez (2010)
b'	1.77		Beverton and Holt	Ley-Cooper & Chávez (2010)
M	0.36	Instantaneous rate		Ley-Cooper & Chávez (2010)
E_{max}	0.28		$F_{MSY}/(M + F_{MSY})$	This chapter
Value/kg	33	USD		Ley-Cooper & Chávez (2010)
Cost/day/trip	174	USD		Ley-Cooper & Chávez (2010)

Notes: K, t_o, L_∞, and W_∞ are parameters of the von Bertalanffy (1934) growth model; a and b are obtained from the length-weight allometric equation (3); tc = age of first catch; M = natural mortality; t_m = age of maturity; t_λ = longevity; E_{max} = exploitation rate at the MSY level. To transform carapace length (CL) into total length (L_t), the equation used was $L_t = (CL/0.0275) + 3.2$, which gave $L_\infty = 56$ cm $= L_t$, which was the value used as an input in the model. The resulting W_∞ was obtained by using the length-weight equation (3): $W = 0.0404\ L^3$.

Simulations described the main ecological processes underlying the stock dynamics of the spiny lobster fisheries examined. These allowed different exploitation scenarios, with different combinations of fishing intensities and age-at-first catch, to be simulated to see which scenarios maximized the biomass, profits, and social benefits of each fishery. For this purpose, analytical procedures adopting the principles and views of Chávez (1996, 2005) and Grafton et al. (2007) were used. The model used then estimated the simulated catch based on estimates of the stock biomass and fishing mortality for each year of the series for each of the fisheries examined.

The socioeconomics of a fishery were approached through the explicit consideration of the costs of fishing per boat per fishing day. Herein, these values were assessed based on the number of boats, number of fishers per boat, number of fishing days, and the costs of the 2009 fishing season of a small fishery in the southeastern Mexican part of the northwestern

Caribbean, while assuming that they were representative of all of the Caribbean fisheries examined. The value was set to the selling price at the dock of the spiny lobsters landed in the same fishery; the profit was the difference between the costs and the value. In these simulations, the costs of fishing and catch value per kg were assumed to be constant over time.

The socioeconomic information used allowed the economic trends within each of the fisheries analyzed to be reconstructed over the 15 years analyzed with the aid of the simulation model. This was done by using the estimated fishing mortality for each fishery over time as a reference value to estimate their corresponding value of each of the different economic variables.

Changes in each population's lobster biomass were estimated using the number of survivors in each cohort. The estimation of the potential yield in each case allowed the *MSY* and the *MEY* values for each t_c (where $1 \leq t_c \leq 6$ years) to be found.

2.2. The Simulations

The estimated catch values showed that the level of exploitation at which the rate of change in the catch or profits of a fishery with respect to changes in its *F* was zero corresponded to the point at which the maximum values of these endpoints were attained, which was equivalent to the *MSY* of the catch and the *MEY* of the profits, respectively. A parallel trend in the *F* and t_c values was required for the *MSY* and *MEY* to be found in most cases. The uncertainties of the estimates produced, after Hilborn and Liermann (1998), were expressed as coefficients of variation, estimated for each fishery, but for simplicity their values were deliberately ignored in the presentation of results, and instead the mean tendencies were presented. Parameter values were fixed at some 'best' estimated value herein rather than allowing for uncertainty in their values (Hilborn and Liermann 1998); simulations were done in this way to allow clear trends to be obtained and allow specific harvest recommendations to be addressed.

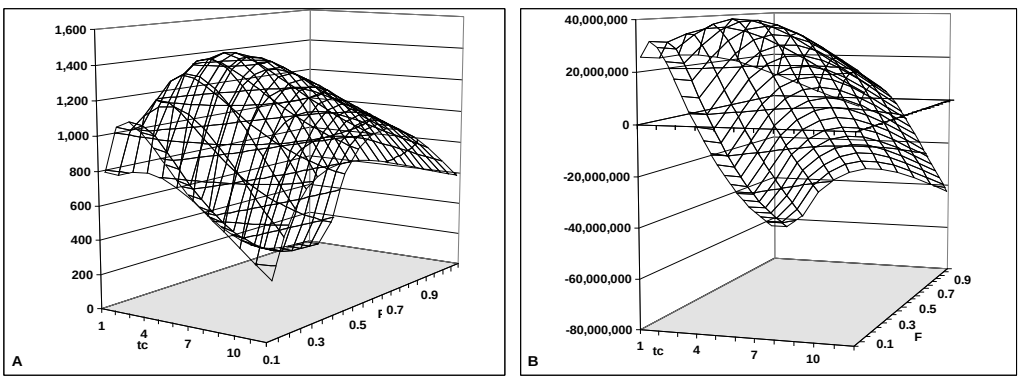

Figure 4. The response of a stock's yield (A) and value (B) to differing values of *F* and t_c. Simulation outputs for the Florida spiny lobster fishery (in 2007) are presented. Potential yield (A) is presented in metric tons, and potential profits (B) are in USD.

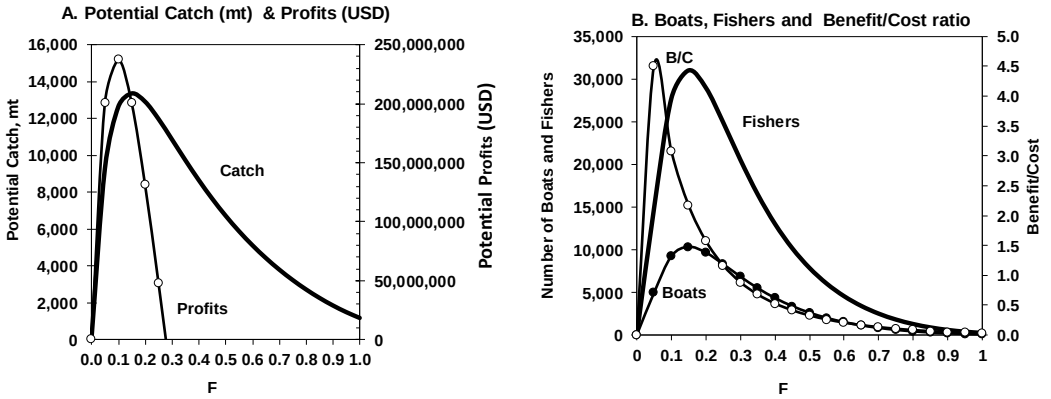

Figure 5. Simulation outputs of potential yield (mt) (A) and potential profits (USD) (B) of the Florida spiny lobster fishery at different levels of F and a constant t_c = 3 years. The *MSY* and *MEY* values are the peaks of the curves for catch and profit, respectively. The *MSY* value corresponds to the catch in the years 1997 and 1999.

2.3. Theoretical Statement

Results of previous stock assessments indicated that yield displays a dome-shaped response surface as a function of F and t_c, as shown in Figure 4A and B. When a single set of outputs as a function of F was examined at a certain t_c, the yield and profits also displayed curves that peaked and attained their maximum values at the corresponding *MSY* and *MEY* (Figure 5A, B). In general, these peak values were found at the same t_c, but the *MSY* was usually attained at a higher F than the *MEY* was. In highly valued fisheries like those of the spiny lobster, the F resulting in the *MEY* generally coincides with that of the *MSY*. Additionally, there should be a certain F value where the maximum yield is attained, declining at higher as well as lower F values, such as in the well-known figure of yield isopleths presented by Beverton and Holt (1957) that displayed the yield per recruit as a function of F and t_c.

3. RESULTS

3.1. Optimum Yields

As result of the examination of the long-term stock responses when attempting to maximize yields, it was found that this variable tended to increase with higher values of t_c. In some cases, the yield at a $t_c \leq 5$ was higher by 20% than that at the current t_c of 3 years, as shown in Figure 6A-C. In three of the fisheries examined (Nicaragua, the Bahamas, and Cuba), the F values required to reach the *MSY* were higher than those needed to reach the *MEY*. In the other fisheries, the F levels at the *MSY* and *MEY* overlapped.

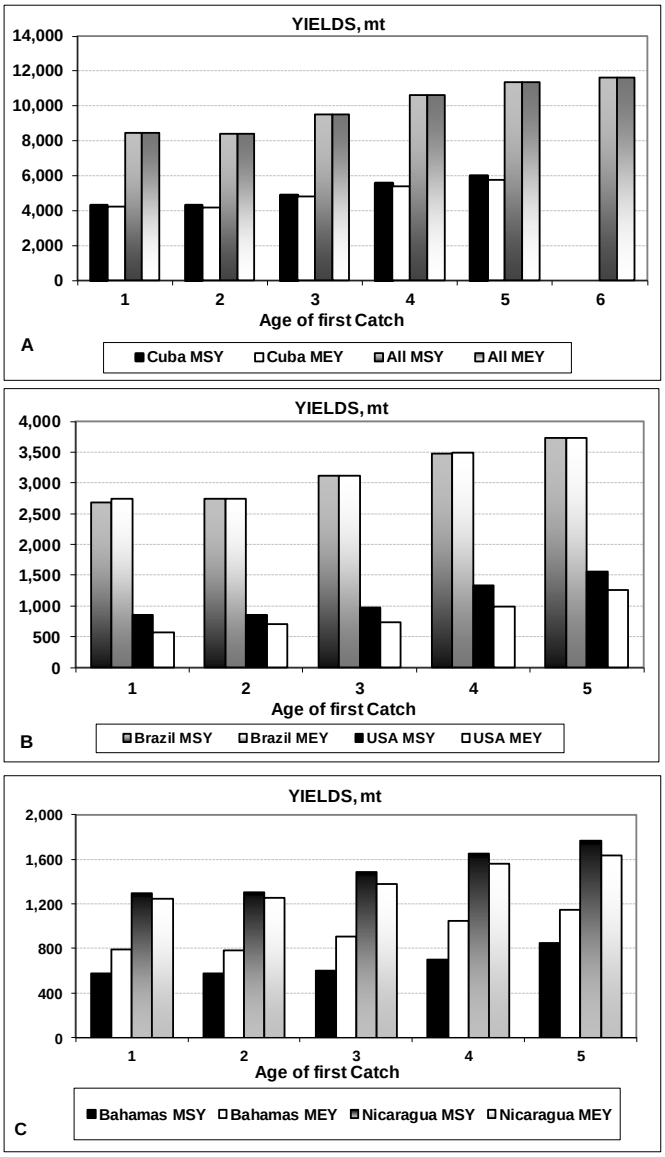

Figure 6. Potential *MSY* and *MEY* of representative Caribbean spiny lobster fisheries as a function of t_c (x-axes) and *F* (not displayed here). Results are presented for all 25 Caribbean fisheries and Cuba (A), for Brazil and the USA (B), and for Nicaragua and the Bahamas (C). In most cases, the yield at the *MSY* was higher than that at the *MEY*, and yields were higher with a higher t_c.

3.2. Optimum Economic Yields

The profits of these fisheries were higher at the *F* values applied to reach the *MEY* than at those required for the *MSY*, as shown in Figure 7A-C. The *MEY* tended to increase as a function of the t_c in the cases of Nicaragua and Brazil. In Cuba and the USA, this increasing trend was not very significant, and higher values were obtained when the *F* of the *MEY* was used rather than that of the *MSY*. These trends depended on the volume of spiny lobsters

landed. The case of the Bahamas was remarkable, because the yields tended to decrease at all t_c values examined, and the profits were negative when the F values required for the *MSY* were applied (Figure 7B). The case of the USA was not so critical because the line of profits was around the economic equilibrium level for this country, with profits being negative at only t_c = 4 years (Figure 7C). Evidently, operating costs are sensitive to the size of the exploited stocks (Hannesson 2007).

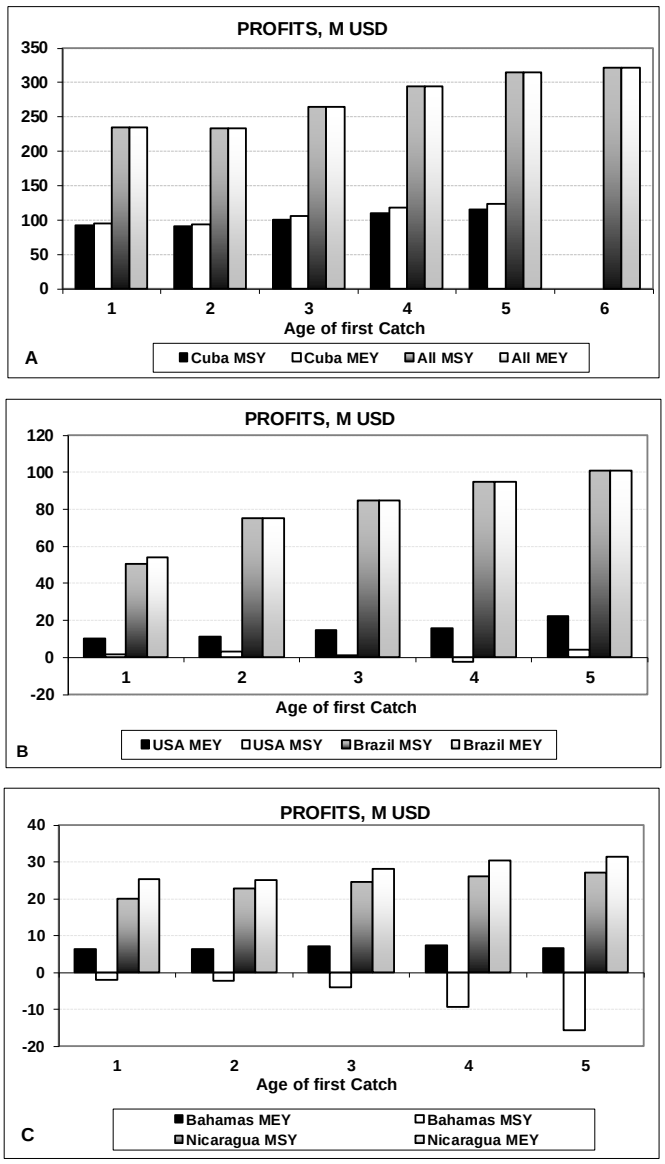

Figure 7. Potential profits (in millions of USD) of representative Caribbean spiny lobster fisheries at the *MSY* and *MEY* as a function of t_c and F. Results are shown for all 25 Caribbean fisheries combined and Cuba (A), for Brazil and the USA (B), and for Nicaragua and the Bahamas (C). In most cases, the yield at the *MEY* was higher than that at the *MSY*. In addition, profits were generally higher with a higher t_c, except in the Bahamas, where the fishery was not profitable in any scenario when the F applied reached the *MSY*.

3.3. Social Benefits

As described earlier (section 2.1), in the simulation model the economic and social values were linked to the costs and value of fishing activities, as well as to the number of fishers involved directly in this activity. Therefore, the model is sensitive to fishing effort and the resulting numbers of fishers, as shown in Figure 8A and B. The same increasing trend was observed for the number of fishers as was previously seen in the case of yield (Figure 7A-C), wherein a higher number of fishermen was required when t_c values were higher as a consequence of the higher catches under these conditions. It was particularly remarkable to observe the case in which all of the fisheries were grouped together (Figure 8B), wherein nearly 30,000 fishers currently participate in all of the Caribbean lobster fisheries with a $t_c = 3$ years, but this could be increased to nearly 50,000 if t_c values were raised to 5 or 6 years. The Brazilian fishery displayed the steepest slope of this growing trend in relation to t_c (Figure 8A). In a similar way as in some of the results already described, when the catch was set at the *MSY*, the number of direct jobs potentially supported by the fishery was the same or higher than that at the *MEY*.

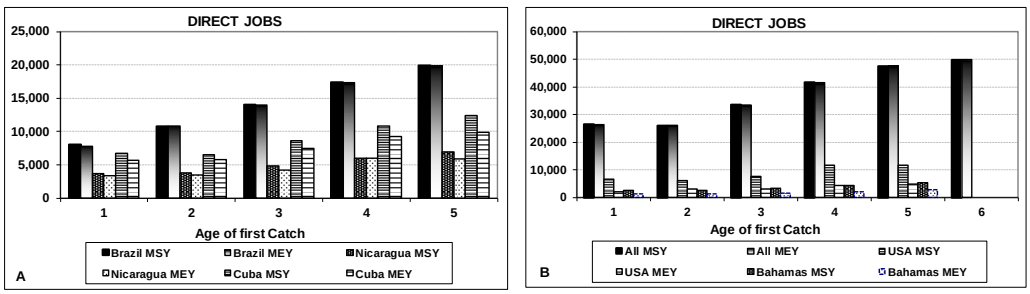

Figure 8. Number of fishers involved in the main Caribbean lobster fisheries and in all of the fisheries combined in relation to t_c. Increases were observed when higher t_c values were used. The catch rate per fisher was maintained at a constant value, representing the mean value under current conditions. Results are shown for Brazil, Cuba, and Nicaragua (A) and for the USA, the Bahamas, and all 25 fisheries combined (B).

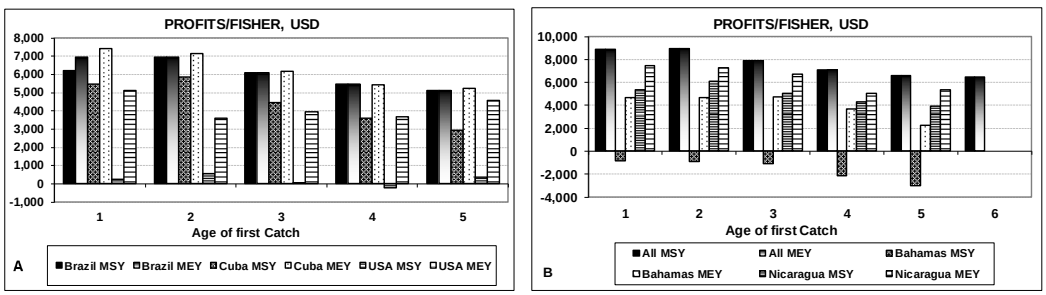

Figure 9. Social benefits, expressed as profits per fisher, of the five main Caribbean spiny lobster fisheries and all of the Caribbean fisheries combined. Results are shown for Brazil, Cuba, and the USA (A) and for the Bahamas, Nicaragua, and all 25 Caribbean fisheries combined (B). The social benefit mainly declined with increasing t_c, and the social benefit was often much higher at the *MEY* than at the *MSY*.

The social benefit of a fishery was expressed herein as the profits obtained per fisher, which was simply calculated by dividing the profits by the number of fishers while assuming that they would have access to the same catch as in current conditions. In all cases except one, the social benefits of the fisheries declined when the stocks were exploited with higher t_c values (Figure 9A, B). The Bahamas fishery was not profitable when the *F* values required for the *MSY* were applied. In the USA, the social benefits were much higher under the *MEY* scenario.

4. DISCUSSION

4.1. The Model Output

To begin, it must be remembered that the figure presented by Beverton and Holt (1957) displaying the relationship of the yield-per-recruit to *F* and t_c did not explicitly take recruitment into account. When a simulation is used as in the present case (Chavez 2005, 2014), a recruitment function must be explicitly defined, as this is a necessary condition to link the simulated cohorts over time. In earlier sections (2.1, 2.2, and 2.3), the simulations were described as being expected to provide the same kind of performance by estimating the stock biomass as the yield-per-recruit curve at high *F* and t_c values. However, this hypothesis has to be rejected. Therefore, it is easy to understand why yields and profits were found to increase when high t_c values were considered because, in this case, the size of the breeding stock would be higher than that under current conditions, where there is a general tendency to catch smaller animals as a consequence of increasing competition among fishers. It must also be kept in mind that there should be a maximum number of adults and recruits that can occur within a stock, which is a limit imposed by the carrying capacity of the ecosystem. For example, in the case of adult biomass, the carrying capacity may be close to the virgin biomass of an unexploited stock, or, in other words, it could be twice the stock biomass at the *MSY* level.

In several instances, the approach presented herein was consistent with the methods used in the management strategy evaluation (MSE) approach, which relies on testing the whole management process through simulations using and specifying different performance measures with which to compare alternative management strategies (Sainsbury et al. 2000). In this chapter, 15-year time series of catch data were chosen to feed the model for two reasons: one was to minimize the effects of climate variability, which may induce significant changes in recruitment rates if longer time series are used; and the other was to be consistent with Jackson et al. (2001), who stated that "Retrospective data not only help to clarify underlying causes and rates of ecological change", but also help to "demonstrate achievable goals for restoration and management."

The estimation of potential yield is one of the goals to which any fishery's stock assessment should aspire, even if it is based on simple relations (Beddington and Kirkwood 2010). In the Introduction (section 1), it was mentioned that the *MEY* is a more convenient option for a target of fisheries assessment than the *MSY* because it is often achieved at lower levels of *F*, thus reducing the risks of overexploiting the stock. This may be a function of the discount rate (Grafton et al. 2007). In addition, in the method applied herein to assess stock

biomass, Hilborn and Walters (1992) can be quoted in saying that "you cannot find the top of a [yield] curve without going beyond the top". The "top" they mention is the *MSY* level. In contrast, when the *MEY* is adopted as a target, when going beyond the top of the *MEY* curve the stock is still underexploited, or at least has not exceeded its reproductive potential. This was the case for about half of the Caribbean spiny lobster fisheries examined in this chapter.

4.2. Management Options

The Caribbean spiny lobster fisheries are a clear example of unsuccessful management systems, wherein access to the resource is open and there is poor ability to monitor its exploitation and implement regulations (Hilborn et al. 2005), and consequently these fisheries have rarely been sustainable (Pauly et al. 2002).

We are living in a world where the political incentive is to maintain the continuous exploitation of fished stocks, which has led to the generally depleted condition of fish stocks worldwide (Rosenberg 2003). Therefore, the purpose of the exercises done in this chapter was to provide some guidelines that could help achieve the sustainable management of Caribbean spiny lobster fisheries (Mora et al. 2009). The fisheries examined herein displayed great heterogeneity. For this reason, it was not possible to make a few general management recommendations that could be feasibly adopted for widespread application. However, based on the results, there are at least three reference points that can potentially be used to develop some planning guidelines, which are the F_{MSY}, F_{MEY}, and t_c. Further, the yield, profits, and social benefit (herein referring to the profits per fisher) are the dependent variables that should be compared when assessing the performance of a fishery under different conditions. The combination of the F and the t_c that produces the highest catch, profits, and social benefit is the feasible targets that should be sought for the management of these fisheries. In this approach, the economic and social aspects of the fisheries were incorporated, which agrees with modern trends in the definition of sustainability (Quinn II and Collie 2005), wherein the costs of management and the windfall profits of fisheries conservation should be shared (Hilborn et al. 2005).

As a final corollary, and after perceiving that the spiny lobster stocks are exploited under heterogeneous conditions and different fishing gears, it is apparent that recurrent socio-economic crises in the exploitation of this resource can and do take place in some countries within the species' distribution. Therefore, it would be useful to create an international organization in charge of regulating and controlling regional access to this resource. This group would be in charge of carrying out stock assessments of the main stocks and assign catch quotas and minimum size limits every year, before the beginning of the next fishing season. In this way, the sustainable exploitation of this common property resource would be ensured. The adoption of these statements as general principles in stock assessment and management is therefore suggested, as well as those more specific principles recommended in a previous paper (Chávez 2007).

CONCLUSION

As result of the analyses carried out in this chapter, the following conclusions were derived:

1. The *MSY* is attained at the same or higher *F* values as those required to reach the *MEY*. The highest values for these variables are obtained when $t_c \geq 5$ years.
2. Likewise, the maximum profits are reached with the *F* values required to achieve the *MEY*. In some cases, when the *F* required for the *MSY* to be reached is applied, the fishery may become non-profitable.
3. The maximum social benefit, meaning the number of jobs directly supported by the fishery, is obtained at the *F* values required to reach the *MSY* and when $t_c \geq 5$ years.
4. The maximum economic benefit per fisher is obtained when the *F* required to attain the *MEY* is applied, and when $t_c \leq 3$ years.

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Chapter 110

MARINE DEBRIS: INTERAGENCY COMMITTEE MEMBERS ARE TAKING ACTION, BUT ADDITIONAL STEPS COULD ENHANCE THE FEDERAL RESPONSE*

United States Government Accountability Office

WHY GAO DID THIS STUDY

Marine debris—waste such as discarded plastic and abandoned fishing gear and vessels in the ocean—is a global problem that poses economic and environmental challenges. The Marine Debris Act, enacted in 2006, requires the committee to coordinate a program of marine debris research and activities among federal agencies. The act also requires the committee to submit biennial reports to Congress that include certain elements such as an analysis of the effectiveness of the committee's recommendations.

GAO was asked to review federal efforts to address marine debris. This report examines (1) how the committee coordinates among federal agencies and the process for determining membership, (2) the extent to which the committee's biennial reports contain required elements, and (3) experts' suggestions on actions the federal government could take to most effectively address marine debris. GAO examined the Marine Debris Act and committee reports, compared committee practices with leading collaboration practices, interviewed federal agency officials, and interviewed a nongeneralizable sample of 14 marine debris experts selected to reflect various sectors and experiences with different types of marine debris.

* This is an edited, reformatted and augmented version of United States Government Accountability Office; Report to Congressional Requesters, Publication No. GAO-19-653, dated September 2019.

WHAT GAO RECOMMENDS

GAO is making four recommendations, including that NOAA establish a time frame for documenting membership and the committee develop processes to analyze the effectiveness of its efforts and identify priority funding. The agency agreed with GAO's recommendations.

WHAT GAO FOUND

The Marine Debris Research, Prevention, and Reduction Act, as amended, (Marine Debris Act) designated six agencies as members of the Interagency Marine Debris Coordinating Committee and specifies that members shall include senior officials from certain other agencies as the Secretary of Commerce determines appropriate. Within Commerce, the National Oceanic and Atmospheric Administration (NOAA) serves as the committee chair. The committee coordinates through sharing information about members' activities to address marine debris, but GAO found that NOAA has not established a process for determining committee membership for agencies not specifically designated in the act. As a result, such agencies may not be included in the biennial reports required by the act which discuss committee members' marine debris activities. NOAA officials said they plan to develop a membership process but have not established a time frame to do so. By establishing a time frame, the committee can more fully benefit from capturing all members' activities.

The committee's biennial reports provide information on members' activities such as education and cleanup, but they do not contain some information required by the Marine Debris Act. Specifically, the reports do not include (1) an analysis of the effectiveness of the committee's recommendations and strategies to address marine debris and (2) recommendations for priority funding needs. Our past work has shown that collaborative entities can better demonstrate progress if they develop a way to monitor and report the results of their collective efforts and identify and leverage resources. By doing so, the committee would be in a better position to know the extent to which it is effectively addressing marine debris and provide Congress with required information about priority funding needs.

Source: National Oceanic and Atmospheric Administration. | GAO-19-653.

Marine debris washed ashore on a beach.

Experts suggested a range of actions—from research to cleanup—the federal government could take to most effectively address marine debris. They stressed that there is not one solution to the growing problem (see figure). Committee officials noted factors to consider, such as cost, when evaluating these actions.

ABBREVIATIONS

EPA	Environmental Protection Agency Interagency
committee	Interagency Marine Debris Coordinating Committee
Marine Debris Act	Marine Debris Research, Prevention, and Reduction Act, as amended
NOAA	National Oceanic and Atmospheric Administration
USAID	U.S. Agency for International Development

September 25, 2019

Congressional Requesters

Marine debris—waste ranging from small, everyday items, such as cigarettes and discarded plastic bottles, to larger objects, such as abandoned fishing gear and vessels found in the ocean or Great Lakes environment—poses economic and environmental challenges and is an issue of growing local, national, and international concern.[1] Marine debris can harm coastal and marine species and habitats, obstruct navigational waterways, cause economic loss to fishing industries and coastal communities, and threaten human health and safety.[2] Debris can enter the aquatic environment directly from domestic or international water- based sources, such as when materials are intentionally dumped in the water or blown off fishing vessels. Debris can also enter the aquatic environment indirectly from land-based sources by washing into waterways that eventually flow to the ocean. Research has shown that a significant amount of marine debris stems from land-based sources, such as improperly managed plastic waste.[3]

[1] For the purposes of this report, we use the term marine debris, but such waste is also referred to as "marine litter" or "marine trash." National Oceanic and Atmospheric Administration and U.S. Coast Guard regulations define marine debris as "any persistent solid material that is manufactured or processed and directly or indirectly, intentionally or unintentionally, disposed of or abandoned into the marine environment or the Great Lakes." 15 C.F.R. § 909.1(a), 33 C.F.R. § 151.3000(a). This definition is also found in the Marine Debris Act Amendments of 2012. Pub. L. No. 112-213, tit. VI, § 608(2), 126 Stat. 1540, 1578 (*codified at* 33 U.S.C. § 1956(3)).

[2] National Oceanic and Atmospheric Administration, *Report to Congress: 2016-2017 Interagency Marine Debris Coordinating Committee Biennial Report* (March 2019).

[3] Jambeck, J.R., et al., "Plastic waste inputs from land into the ocean," *Science*, 347 (2015): pp. 768-771. This study found that five Asian countries contributed the most waste by mass, but many other countries also contributed to the problem including the United States, which ranked 20th on this list. Countries were ranked by mass of mismanaged plastic waste in units of millions of metric tons per year. A metric ton is equal to 1,000 kilograms.

Numerous studies show that plastic is a particularly pervasive and persistent form of marine debris.[4] An estimated 8 million metric tons of mismanaged plastic waste entered the marine environment in 2010 according to one study,[5] and projections show that by 2025 this number could increase to 17.5 million metric tons each year.[6] According to a 2018 United Nations report, studies estimate that the total economic damage to the world's marine ecosystem caused by plastic amounts to at least $13 billion each year.[7] Although chemicals in plastic provide valuable properties such as durability, there is growing concern that these chemicals may be toxic and harmful to marine species.[8] Over time, through exposure to sunlight and wave action, plastic breaks apart into increasingly smaller pieces, eventually becoming tiny particles called microplastics. Marine life may ingest these microplastics, raising concerns about potential health effects for such marine life and any organisms, including humans, which may eat them.[9]

Addressing marine debris is a complex, interdisciplinary issue involving many sectors and levels of government. Multiple federal agencies, often in coordination with state and local governments, Indian tribes, industry, international parties, and nongovernmental agencies, work to prevent, manage, remove, and raise awareness about marine debris. To help address marine debris, the Marine Debris Research, Prevention, and Reduction Act (Marine Debris Act) was enacted in 2006 and amended in 2012 and 2018.[10] The purpose of the Marine Debris Act is to address the adverse impacts of marine debris on the U.S. economy, the marine environment, and navigation safety through the identification, determination of sources, assessment, prevention, reduction, and removal of marine debris.[11]

Among other things, the Marine Debris Act reactivated the Interagency Marine Debris Coordinating Committee (interagency committee) to coordinate a comprehensive program of marine debris research and activities among federal agencies and in cooperation and coordination with nonfederal entities, such as nongovernmental organizations, industry,

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[4] See, for example, the Ocean Conservancy and the McKinsey Center for Business and Environment, *Stemming the Tide: Land-based strategies for a plastic-free ocean* (September 2015); Jambeck, J.R., et al., "Plastic waste inputs," 768-771; National Research Council, *Tackling Marine Debris in the 21st Century* (Washington, D.C.: National Academies Press, 2009); David W. Laist, "Overview of the Biological Effects of Lost and Discarded Plastic Debris in the Marine Environment," *Marine Pollution Bulletin*, vol.18 no 6B.(1987): 319-326.

[5] Written testimony of Jenna R. Jambeck, Ph.D., before the U.S. Senate Committee on Environment and Public Works, May 17, 2016, summarizing the results of Jambeck, et al., "Plastic waste inputs," 768-771. The study estimated a range of 4.8 to 12.7 million metric tons of mismanaged plastic waste, with a mid-scenario estimate of 8 million metric tons.

[6] Jenna R. Jambeck, Ph.D., presentation at American Association for the Advancement of Science panel, San Jose, CA; February 2015.

[7] United Nations Environment Programme, *Single-Use Plastics: A Roadmap for Sustainability* (Nairobi, Kenya: 2018).

[8] Environmental Protection Agency, *State of the Science White Paper: A Summary of Literature on the Chemical Toxicity of Plastics Pollution to Aquatic Life and Aquatic- Dependent Wildlife* (Washington, D.C.: December 2016).

[9] Early research has shown that microplastic-derived toxins may accumulate in organisms and potentially harm other marine life and humans when ingested, but additional research is needed. Environmental Protection Agency, *State of the Science White Paper* (December 2016).

[10] Marine Debris Research, Prevention, and Reduction Act, Pub. L. No. 109-449, 120 Stat. 3333 (2006), *as amended by* Marine Debris Act Amendments of 2012, Pub. L. No. 112- 213, tit. VI, 126 Stat. 1540, 1575-78 (2012); Save Our Seas Act of 2018, Pub. L. No. 115-265, tit. I, 132 Stat. 3742, 3742-3744 (2018) (*codified as amended at* 33 U.S.C. § 1951- 58). The Marine Debris Act Amendments of 2012 changed the name of the act from Marine Debris Research, Prevention, and Reduction Act to Marine Debris Act. Pub. L. No. 112-213, tit. VI, § 602(a), 126 Stat. 1540, 1575 (2012). In this report we use the term "Marine Debris Act" to refer to the act as amended through 2018.

[11] 33 U.S.C. § 1951.

universities and research institutions, states, Indian tribes, and other nations, as appropriate.[12] The act designates a senior official from the National Oceanic and Atmospheric Administration (NOAA), within the Department of Commerce, to serve as the chair of the interagency committee. Other federal agency members designated in the act are the Environmental Protection Agency (EPA), U.S. Coast Guard, U.S. Navy, Department of State, and Department of the Interior.[13] The act also specifies that the committee shall include senior officials from other federal agencies that have an interest in ocean issues or water pollution prevention and control as the Secretary of Commerce determines appropriate.

The Marine Debris Act requires the interagency committee to submit to Congress biennial reports that evaluate progress in meeting the purposes of the act.[14] The biennial reports are to include (1) the status of implementation of any recommendations and strategies of the committee and analysis of their effectiveness, and (2) estimated federal and nonfederal funding provided for marine debris and recommendations for priority funding needs.[15]

You asked us to review federal efforts to address marine debris under the Marine Debris Act. This report examines (1) how the interagency committee coordinates among federal agencies and the process for determining membership and agency representation, (2) the extent to which the interagency committee's biennial reports contain required elements, and (3) experts' suggestions on actions the federal government could take to most effectively address marine debris.

To examine how the interagency committee coordinates among federal agencies and the process for determining membership and agency representation, we reviewed the Marine Debris Act and interagency committee documents, including the committee's charter and the five biennial reports to Congress issued as of March 2019.[16] We also reviewed the most recently available minutes from quarterly committee meetings held from November 2012 through April 2019 to determine the types of topics and activities on which the committee has coordinated and the federal agencies that have participated. We attended five of the interagency committee's quarterly meetings (in May, September, and December of 2018, and April and July of 2019) to directly observe committee coordination among agencies during these meetings. We also reviewed documents from committee member agencies and interviewed and reviewed written responses from those agencies to obtain information on their coordination efforts. Agencies we included were those agencies designated as members in the Marine Debris Act as well as additional agencies identified as members in the

[12] The Coast Guard Authorization Act of 1996 required the Secretary of Commerce to establish a Marine Debris Coordinating Committee. Pub. L. No. 104-324, § 802(b),110 Stat. 3901, 3944-45 (1996). In 2006, the Marine Debris Act reactivated this committee and established it by statute. Officials from the National Oceanic and Atmospheric Administration said they do not have records of committee activities before 2006.

[13] The Coast Guard Authorization Act of 1996 designated NOAA, EPA, the U.S. Coast Guard, and the U.S. Navy as members of the interagency committee. The Save Our Seas Act of 2018 designated the Departments of State and the Interior as members of the committee.

[14] Biennial reports are required to be submitted to the Senate Committee on Commerce, Science, and Transportation, the House Committee on Transportation and Infrastructure, and the House Committee on Natural Resources.

[15] The biennial reports are also to include other required elements listed in 33 U.S.C. § 1954(e). These other elements are about specific agency programs.

[16] The five biennial reports were issued in March 2010, October 2012, September 2014, December 2016, and March 2019. Collectively, the reports include activities the interagency committee members reported conducting between June 2008 and December 2017.

committee's charter.[17] In addition, we compared these agencies' documents and written responses about the interagency committee's coordination with leading practices we identified in our past work on implementing interagency collaborative mechanisms.[18]

To examine the extent to which the interagency committee's biennial reports contain required elements, we compared information contained in the committee's five biennial reports (from 2010 to 2019) to the reporting requirements in the Marine Debris Act. Specifically, two analysts independently reviewed each of the five biennial reports to evaluate information the reports included about (1) the status of implementation of any recommendations and strategies of the committee, (2) analysis of the recommendations and strategies' effectiveness, (3) estimated federal and nonfederal funding provided for marine debris, and (4) recommendations for priority funding needs. The analysts then compared and summarized the results of their analyses. We also interviewed and reviewed written responses from NOAA officials (in the agency's capacity as chair of the interagency committee) and officials from other committee member agencies about steps to develop the biennial reports, including the reports' required elements. In addition, we compared information from the reports and information we obtained from agency officials to leading practices we identified in our past work on implementing interagency collaborative mechanisms.[19]

To obtain suggestions on actions the federal government could take to most effectively address marine debris, we conducted structured interviews with a nongeneralizable sample of 14 experts with expertise in marine debris-related issues. We selected these experts using factors such as the individuals' experience with different types of debris (e.g., abandoned fishing gear or consumer debris) or association with various sectors (e.g., academia or industry). The experts included: (1) academics with expertise in areas such as sources, prevalence, and transport of plastic marine debris; (2) officials representing the plastic manufacturing, food and beverage, and commercial fishing industries; (3) officials from nonprofit organizations with expertise in marine debris removal from coastal areas, litter prevention, and recycling management systems and strategies; and (4) state and local government officials from the District of Columbia, Florida, and Washington with expertise in local litter prevention efforts, derelict vessels, and lost and derelict fishing gear.[20]

We asked the 14 experts to suggest actions the federal government could take to most effectively address different types of marine debris. Specifically, we asked that experts identify up to 5 to 10 actions as well as advantages, disadvantages, and any challenges in potentially implementing these suggested actions. We then categorized the actions based on common themes. To do so, two analysts independently reviewed each expert's description of each action and identified an appropriate category using decision rules the team developed.

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[17] These agencies are: the Department of Commerce's NOAA; the Department of Defense's U.S. Army Corps of Engineers and U.S. Navy; the Department of Homeland Security's U.S. Coast Guard; the Department of the Interior's Bureau of Safety and Environmental Enforcement, National Park Service, and U.S. Fish and Wildlife Service; the Department of Justice; the Department of State; EPA; and the Marine Mammal Commission. In addition, we interviewed officials from the National Science Foundation, the U.S. Agency for International Development, and the Office of the U.S. Trade Representative based on suggestions from interagency committee officials and marine debris experts.

[18] GAO, *Managing for Results: Key Considerations for Implementing Interagency Collaborative Mechanisms*, GAO-12-1022 (Washington, D.C.: Sept. 27, 2012). We used leading practices that are relevant to the requirements for the interagency committee in the Marine Debris Act. These included practices related to participants, resources, outcomes and accountability, and written guidance and agreements.

[19] GAO-12-1022.

[20] We selected these locations using factors such as geographic area and expertise in marine debris issues.

For reporting purposes, we selected several actions within each of the categories to provide illustrative examples of the types of actions experts suggested. Our selection was based on such factors as the number of experts that suggested similar types of actions, the detail provided by the experts, and the availability of supporting information, such as documentation of instances where an action had been taken by state or local governments. Actions suggested by the 14 experts cannot be generalized to actions that might be suggested by other experts, but provide examples of actions federal agencies could take to address marine debris.[21] We also interviewed and received written responses from officials from interagency committee agencies regarding issues that would be important to consider in potentially implementing any of the expert suggested actions. Appendix I presents a more detailed description of our objectives, scope, and methodology.

We conducted this performance audit from October 2017 to September 2019 in accordance with generally accepted government auditing standards. Those standards require that we plan and perform the audit to obtain sufficient, appropriate evidence to provide a reasonable basis for our findings and conclusions based on our audit objectives. We believe that the evidence obtained provides a reasonable basis for our findings and conclusions based on our audit objectives.

BACKGROUND

Marine debris originates from multiple sources and types of materials, entering the marine environment in a variety of ways, as shown in figure 1.

Source: GAO. | GAO-19-653.

Figure 1. Overview of sources and types of marine debris.

[21] We did not limit experts' suggestions to actions that agencies currently have authority to implement. We do not take a position on the merits of, the necessary legal authority for, or the most appropriate entity for the actions suggested by the 14 experts.

Prevalent types of marine debris and their effects include:

- **Plastics and microplastics**. Plastics, including items such as grocery bags, food wrappers, bottles, straws, and cigarette filters, are particularly ubiquitous, having been found in the deepest reaches of the ocean.[22] Microplastics—generally defined as plastic particles less than 5 millimeters in size—are especially pervasive. For instance, one study completed in 2018 found record concentrations of microplastics in Arctic sea ice.[23] Plastic marine debris can damage habitats, entangle wildlife, cause injury via ingestion, impair vessel engines, create navigation hazards, inflict economic loss, and transport non- native species, according to NOAA documents.

Microplastics

Most plastics do not biodegrade, that is, decay naturally and become absorbed by the environment. Instead, plastics slowly break down into smaller and smaller fragments, eventually becoming what are known as microplastics. Microplastics are very small pieces of plastic that are generally less than 5 millimeters in size (about the size of a sesame seed). The formation of microplastics occurs when plastic debris is exposed to sunlight and the plastic begins to weather and fragment.

Microplastics have been found in the stomachs of numerous aquatic organisms including insects, worms, fish, and clams, according to a 2018 study. A study from 2011 showed that once animals ingest microplastics, they can be stored in tissues and cells, providing a possible pathway for the accumulation of contaminants and potentially harming the animals.

Sources: GAO analysis of scientific studies; Sherri Mason/SUNY Fredonia (photo). | GAO-19-653.

- **Derelict fishing gear**. Derelict fishing gear refers to nets, lines, crab pots, and other recreational or commercial fishing equipment that has been lost, neglected, or discarded in the marine environment. According to the Global Ghost Gear Initiative, at least 640,000 tons of derelict fishing gear enters the ocean each year, a weight

[22] According to research published in 2018, there have been multiple sightings of plastic deep in the ocean including a plastic bag found in the Pacific Ocean's Marianas Trench— the deepest part of the ocean and the deepest location on earth. Chiba, et al., "Human footprint in the abyss: 30 year records of deep-sea plastic debris," *Marine Policy* 96(2018) 204-212. Another study estimated that, globally, there are now more pieces of plastic in the ocean than there are stars in the Milky Way. Lavers, et al., "Significant plastic accumulation on the Cocos (Keeling) Islands, Australia." *Nature* 9:7102 (2019): pp. 1-9.

[23] Peeken, et al., "Arctic sea ice is an important temporal sink and means of transport for microplastic," *Nature Communications*, 9:1505 (2018): pp. 1-12.

equivalent to two Empire State Buildings.[24] Derelict fishing gear may entrap sea life, adversely affect marine habitats, present hazards to navigation, and cause other harmful effects (see Figure 2). For example, according to a 2015 NOAA report, derelict fishing gear threatens a variety of fish, turtles, seabirds, whales, and seals, and may be especially problematic for endangered and protected marine species.[25]

Source: National Oceanic and Atmospheric Administration. | GAO-19-653.

Figure 2. Marine life entangled in derelict fishing gear.

- **Abandoned and derelict vessels**. Abandoned and derelict vessels are vessels without identified ownership, in significant disrepair, or both.[26] There are thousands of such vessels in ports, waterways, and estuaries around the United States that have been left to deteriorate by the owner or operator or are the result of a catastrophic weather event, according to NOAA documents. Abandoned and derelict vessels can impede marine transportation by blocking navigable waterways, and, if not visible or well-marked, could pose collision risks to vessel operators. These vessels may also become sources of pollution since they may contain fuel oil or other hazardous materials that can leak into the water as the vessels deteriorate, impacting the local community, marine life, and nearby habitat.[27]

[24] The Global Ghost Gear Initiative is a cross-sectoral alliance committed to finding solutions to the problem of lost, abandoned and otherwise discarded fishing gear (also known as "ghost gear") worldwide, according to its website. Participants in the alliance include the fishing industry, the private sector, academia, governments, and intergovernmental and nongovernmental organizations.

[25] National Oceanic and Atmospheric Administration, *Impact of "Ghost Fishing" via Derelict Fishing Gear* (Charleston, SC, and Silver Spring, MD: March 2015).

[26] For additional information, see GAO, *Maritime Environment: Federal and State Actions, Expenditures, and Challenges to Addressing Abandoned and Derelict Vessels*, GAO-17-202 (Washington, D.C.: March 28, 2017).

[27] According to U.S. Fish and Wildlife Service officials, abandoned and derelict vessels can also leach iron and other contaminants that result in the overgrowth of algae and invasive species that may physically damage coral reefs and other habitats.

Marine debris has garnered increasing interest from the international community. In September 2015, the United Nations General Assembly unanimously adopted an agenda with a set of global sustainable development goals through 2030.[28] One of the goals (goal 14) calls for conservation and sustainable use of the oceans, seas, and marine resources, and includes a target for prevention and significant reduction of marine pollution of all kinds, including marine debris, by 2025. In June 2018, five members of the Group of Seven and the European Union endorsed the Group's Ocean Plastics Charter, which committed them to accelerating implementation of the Group of Seven Leaders' Action Plan to Combat Marine Litter, previously agreed to in 2015.[29] The United States and Japan were the two members of the Group of Seven that did not endorse the charter. Also, in May 2019, the parties to the Basel Convention on the Control of Transboundary Movements of Hazardous Waste and Their Disposal adopted a decision that would, beginning January 1, 2021, require parties to take appropriate measures to ensure that certain plastic waste is reduced to a minimum, taking into account social, technological and economic aspects, among other things.[30]

Marine Debris Act

The Marine Debris Act governs the activities of the interagency committee. For example, it required the interagency committee to issue a report to Congress that included recommendations to reduce marine debris domestically and internationally.[31] In 2008, the committee submitted an interagency recommendation report that contained 25 recommendations intended to guide the federal government's strategies for addressing marine debris (see appendix II for a list of the 25 recommendations). The recommendations were categorized by an overarching topic, such as education and outreach or cleanup. Within each category, the committee then identified specific recommendations. For example, within the education and outreach category, the committee specified three recommendations:

- Demonstrate leadership by distributing educational materials to personnel on the sources and impacts of marine debris as well as methods for prevention with the goal of reducing the federal contribution to marine debris.
- Support public awareness campaigns by providing technical expertise and educational materials and by encouraging private sector participation, when appropriate.

[28] United Nations, *Transforming Our World: The 2030 Agenda for Sustainable Development* (adopted Sept. 25, 2015). The agenda addressed five components of sustainable development (people, planet, prosperity, peace, and partnership), and included 17 goals with 169 associated targets.

[29] The Group of Seven is an informal grouping of seven of the world's advanced economies—Canada, France, Germany, Italy, Japan, the United Kingdom, and the United States—that meets annually to discuss issues such as global economic governance, international security, and energy policy.

[30] The United States is a signatory, but not a party, to the Convention. Parties to an international agreement are those countries that have consented to be bound by the agreement and for which the agreement is in force. Generally, countries express their consent to be bound by an agreement by ratifying, accepting, approving or acceding to it. Countries that have signed the agreement but not consented to be bound to it are obliged to refrain from acts that would defeat the object and purpose of the agreement until the country's intention not to become a party to the agreement is made clear.

[31] Pub. L. No. 109-449, § 5(c)(1), 120 Stat. 3333, 3337 (2006). The report was due on December 22, 2007; the committee submitted the report to the congressional committees in August 2008. This requirement was repealed in 2012. *See* Pub. L. No. 112-213, § 606(a)(1), 126 Stat. 1540, 1577 (2012).

- Engage and partner with state, local, tribal and nongovernmental entities to support coordinated events, such as Earth Day, the International Coastal Cleanup, and other activities that have relevance to marine debris.

The act also requires the interagency committee to submit biennial reports to Congress that evaluate progress in meeting the purposes of the Marine Debris Act. Specifically, these biennial reports are to include:

- the status of implementation of any recommendations and strategies of the committee and analysis of their effectiveness, and
- estimated federal and nonfederal funding provided for marine debris and recommendations for priority funding needs.

Starting in 2010, the interagency committee has issued five biennial reports to Congress, issuing its most recent report in March 2019.[32]

The Marine Debris Act designates six federal agencies as interagency committee members. The six agencies are NOAA, EPA, U.S. Coast Guard, U.S. Navy, Department of State, and Department of the Interior.

The act also specifies that the committee shall include senior officials from other federal agencies that have an interest in ocean issues or water pollution prevention and control as the Secretary of Commerce determines appropriate. The act designates the senior official from NOAA to serve as the chair.

INTERAGENCY COMMITTEE COORDINATES THROUGH MEETINGS, BUT NOAA DOES NOT HAVE A PROCESS FOR DETERMINING COMMITTEE MEMBERSHIP AND AGENCY REPRESENTATION

The interagency committee coordinates primarily through quarterly meetings where agencies share information about their individual activities related to addressing marine debris. Such activities range from education and outreach to research and technology development and are generally driven by the missions and authorities of the agencies. However, we found that NOAA has not established a process to determine the committee's membership. In addition, the Marine Debris Act requires the interagency committee to include a "senior official" from member agencies, but NOAA has not determined the level of official it would consider senior.

[32] In 2019, multiple versions of the Save Our Seas Act 2.0 were introduced in Congress. S. 1982, 116th Cong. (2019); S. 2260, 116th Cong. (2019); H.R. 3969, 116th Cong. (2019); S. 2364, 116th Cong. (2019); S. 2372, 116th Cong. (2019). If enacted into law, most of the bills would require the interagency committee to submit additional reports to Congress. For example, three of the bills would require the interagency committee to submit to Congress a report on innovative uses for plastic waste other than in infrastructure as well as a report on microfiber pollution that includes an assessment of the sources, prevalence, and causes of microfiber pollution.

Interagency Committee Holds Quarterly Meetings to Share Information about Individual Agency Activities Such as Education and Outreach

The interagency committee coordinates primarily through quarterly meetings where federal agencies share information about their individual marine debris-related activities. According to its charter, which was last revised in 2014, the committee is responsible for sharing information, assessing and implementing best management practices, and coordinating interagency responses to marine debris. The charter states that the interagency committee will ensure the coordination of federal agency marine debris activities nationally and internationally as well as recommend research priorities, monitoring techniques, educational programs, and regulatory action. The charter also states that the interagency committee will work to consider the interests of nongovernmental organizations, industry, state governments, Indian tribes, and other nations, as appropriate.

NOAA officials said the main focus of the interagency committee has been to serve as an information-sharing body. The officials said they also seek opportunities to collaborate on individual projects, but the committee does not otherwise collaborate on activities, beyond compiling statutorily required biennial reports.[33] NOAA officials explained that individual agencies each have a unique set of authorities and missions that largely determine their role and involvement in marine debris-related issues. For example, under its Marine Debris Program, NOAA conducts a variety of education, outreach, research, and other activities to identify sources of and address marine debris. In recent years, congressional committee reports accompanying NOAA's annual appropriations have directed the agency to spend a certain amount of its appropriations on its marine debris program.[34] Specifically, these reports directed NOAA to spend $7 million in fiscal year 2018 and $7.5 million in fiscal year 2019 for its Marine Debris Program. The program is also authorized to award grants to, and enter into cooperative agreements and contracts with, eligible entities to identify the sources of, prevent, reduce, and remove marine debris.[35]

In contrast, officials from other agencies on the interagency committee said their agencies have not received such direction or specific appropriations to address marine debris. Rather, the activities these agencies have conducted generally tie to their authority or agency mission.

[33] When the interagency committee was revising its charter, NOAA officials said they discussed expanding the role of the committee beyond information-sharing, but at the time participating agencies collectively agreed that the committee's primary role should be to regularly share information regarding individual agencies' activities and look for opportunities to collaborate, where possible.

[34] 160 Cong. Rec. H475, H508 (daily ed. Jan. 15, 2014) (explanatory statement of the Consolidated Appropriations, 2014); S. Rep. No. 113-181, at 32 (2014) (Senate Report Accompanying the Departments of Commerce and Justice, and Science, and Related Agencies Appropriations Bill, 2015); S. Rep. No. 114-66 at 26 (2015) (Senate Report Accompanying the Departments of Commerce and Justice, and Science, and Related Agencies Appropriations Bill, 2016); 163 Cong. Rec. H3327, H3366 (daily ed. May 3, 2017) (explanatory statement of the Consolidated Appropriations Act, 2017); S. Rep. No. 115-139, at 27 (2017) (Senate Report Accompanying Departments of Commerce and Justice, Science, and Related Agencies Appropriations Bill, 2018); H.R. Rep. No. 116-9, at 614 (2019) (Conference Report Accompanying the Continuing Appropriations Act, 2019).

[35] Grants and cooperative agreements are financial assistance instruments used to transfer a thing of value to a recipient to carry out a public purpose. The difference between the two instruments relates to the amount of involvement between the agency and the recipient during performance: when substantial involvement is not anticipated, agencies use grants; otherwise, they use cooperative agreements. For this report, we use the term "grants" to refer to both grants and cooperative agreements.

Table 1. Activities reported in the Interagency Marine Debris Coordinating Committee's 2016 and 2019 biennial reports

Category	Description of activities	Agencies reporting these types of activities
Education and outreach	Support education and outreach activities related to addressing marine debris, such as developing and distributing educational materials, supporting public awareness campaigns, or partnering with or funding state, local, tribal, or nongovernmental education efforts.	• Bureau of Safety and Environmental Enforcement • Department of State • Environmental Protection Agency • Marine Mammal Commission • National Oceanic and Atmospheric Administration • National Park Service • U.S. Coast Guard • U.S. Fish and Wildlife Service • U.S. Navy
Legislation, regulation, and policy	Identify or help ensure compliance with legislation and regulations and develop or encourage policies and programs to implement practices that address specific types of marine debris.	• Bureau of Safety and Environmental Enforcement • Department of State • Environmental Protection Agency • National Oceanic and Atmospheric Administration • National Park Service • U.S. Coast Guard • U.S. Department of Justice • U.S. Fish and Wildlife Service • U.S. Navy
Cleanup	Support the removal and disposal of marine debris.	• Environmental Protection Agency • Department of State • National Oceanic and Atmospheric Administration • National Park Service • U.S. Army Corps of Engineers • U.S. Coast Guard • U.S. Fish and Wildlife Service • U.S. Navy
Research and technology development	Conduct or sponsor research to monitor, understand the sources of, prevent, mitigate, or reduce the effects of marine debris; support developing new technologies such as ones to produce products using more sustainable or recyclable types of materials.	• Environmental Protection Agency • Marine Mammal Commission • National Oceanic and Atmospheric Administration • National Park Service • U.S. Coast Guard
Coordination	Foster coordination among agencies and with nonfederal partners, such as international, state, and local government agencies.	• Department of State • Environmental Protection Agency • Marine Mammal Commission • National Oceanic and Atmospheric Administration • National Park Service • U.S. Coast Guard • U.S. Fish and Wildlife Service

Source: GAO analysis of Interagency Marine Debris Coordinating Committee reports. | GAO-19-653.

Note: This table reflects those activities reported by the interagency committee in its 2016 and 2019 biennial reports to Congress, which cover activities conducted in 2014-2015, and 2016-2017, respectively.

For example, EPA officials said they have relied on voluntary partnerships with states, industry, and other sources and leveraged existing funds from related programs, such as the agency's stormwater and water quality programs, to support its Trash Free Waters Program. This is a program that encourages collaborative actions by public and private stakeholders to prevent trash from entering water. EPA officials said they also support a number of other activities related to education, outreach, and research, and these activities are a high priority

for the agency, but EPA does not have a line item in its budget dedicated to marine debris activities.[36]

The interagency committee's biennial reports describe general types of activities individual agencies reported conducting—often in coordination with nonfederal partners such as nongovernmental organizations, industry, states, Indian tribes, and other nations—to address marine debris, which include activities in the following categories: (1) education and outreach; (2) legislation, regulation, and policy; (3) cleanup; (4) research and technology development; and (5) coordination (see table 1 for descriptions of types of activities in each category; see app. III for specific examples of activities carried out by agencies).

To help agencies share information, NOAA chairs quarterly meetings where agencies are invited to discuss their individual activities. In reviewing meeting minutes, we found that the meetings were generally well-attended by representatives from multiple agencies. During the meetings, officials discussed marine debris issues and some provided updates on their agencies' activities. For example, at the April 2019 meeting, officials discussed ways in which different agencies may be meeting the sense of Congress on international engagement in the Save our Seas Act of 2018.[37]

At the May 2018 meeting, officials from NOAA and U.S. Coast Guard gave presentations on their agencies' emergency response authorities and efforts. NOAA officials described their actions in response to Hurricanes Harvey, Irma, and Maria in 2017, which included coordinating debris removal activities across federal and state agencies, such as EPA and Florida State's Department of Environmental Protection. U.S. Coast Guard officials also presented information on their marine debris removal activities in response to Hurricanes Irma and Maria. These activities included coordinating with multiple federal, state, and local agencies and contractors to remove or mitigate potential environmental impacts from 2,366 damaged or derelict vessels in Florida and the Florida Keys after Hurricane Irma and 377 vessels in Puerto Rico and the Island of Vieques after Hurricane Maria, according to U.S. Coast Guard officials.

The interagency committee has also used its quarterly meetings to identify opportunities for collaboration among federal agencies and with nonfederal partners, according to NOAA officials. For example, during committee meetings in early 2018, NOAA, the National Park Service, and the Department of State identified an opportunity to collaborate with the German government to bring the Ocean Plastics Lab to the United States. This Lab is an international traveling exhibition that explains the role of science in helping to understand and address plastic pollution in the ocean. NOAA officials said that to collaborate on this effort, officials from three federal agencies served on a steering committee, leveraged volunteers, promoted the Ocean Plastics Lab through outreach efforts to the public and helped staff the exhibits while they were on display in Washington, D.C., during the summer of 2018.

[36] In a March 2019 speech, the EPA Administrator cited marine debris as one of the three top global water priorities for the agency.

[37] Section 102 of the Save Our Seas Act of 2018 stated that it is the sense of Congress that the President should take five actions to respond to marine debris, including to work with representatives of foreign countries that discharge the largest amounts of solid waste from land-based sources into the marine environment, to develop mechanisms to reduce such discharges.

NOAA Has Not Established a Process for Determining Interagency Committee Membership and Agency Representation

We found that NOAA has not established a process to determine interagency committee membership. The Marine Debris Act designates six federal agencies as members of the committee, and also specifies that committee members shall include senior officials from other federal agencies that have interests in ocean issues or water pollution prevention as the Secretary of Commerce determines appropriate.[38] The committee's 2014 charter lists five agencies as members in addition to the six identified in the act, for a total of 11 member agencies.[39] The charter also states that the committee consists of representatives from "any other federal agency that has an interest in ocean issues and water pollution prevention and control," but does not specify the process for documenting membership or how the Secretary of Commerce, or a delegate of the Secretary, will determine that such membership is appropriate, as required by the act.[40]

Various information sources, such as the committee's biennial reports and minutes from quarterly meetings, have provided differing lists of committee member agencies. For example, the committee's March 2019 biennial report and NOAA's website as of July 2019 listed the 11 agencies identified in its charter as members. But, various meeting minutes from meetings held in fiscal year 2019 listed up to 13 members. One agency, the U.S. Agency for International Development (USAID), has regularly attended the committee's quarterly meetings since early 2018 when USAID officials said they were invited to participate on the committee. USAID officials said that their understanding is that USAID is a member of the interagency committee and that this is especially important to recognize given their significant international development assistance related to marine debris over the last few years.[41] However, USAID is not listed as a member on NOAA's website and the agency's marine debris-related activities are not included in the committee's 2019 biennial report. As a result, some agencies may not be included in the required biennial reports on the committee members' marine debris activities.[42]

In April 2019, NOAA officials told us that USAID was a contributing member to the interagency committee.[43] The officials said that "official" member agencies are those six agencies designated by the Marine Debris Act and that they consider other participating

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[38] 33 U.S.C. § 1954(b).

[39] The interagency committee's charter identifies the following members, in addition to those listed in the Marine Debris Act: the Department of Defense's U.S. Army Corps of Engineers, the Department of the Interior's Bureau of Safety and Environmental Enforcement, National Park Service, and U.S. Fish and Wildlife Service, the Department of Justice, and the Marine Mammal Commission. The Marine Debris Act designates the Department of the Interior as a member of the interagency committee, whereas, the interagency committee's charter lists three agencies within Interior as members. For reporting purposes, we counted each of these agencies within Interior as separate member agencies.

[40] The interagency committee first established its charter in 2006 and most recently revised it in 2014. The 2006 and 2014 versions of the charter list the same 11 agencies as members of the committee.

[41] For example, officials from USAID said the agency assists developing countries in preventing and reducing land-based sources of marine debris through a variety of activities. Its activities include working with other countries or international cities to improve working conditions for waste collectors and piloting technology and equipment, such as bamboo trash traps in Vietnam. According to agency officials, USAID's activities in Asia—an area of the world that has been identified as a significant source of land-based marine debris—have resulted in 2.6 million people receiving or engaging in improved solid waste management services.

[42] The biennial reports we reviewed were not consistent in capturing all members' activities.

[43] In April 2019, NOAA officials said USAID and the White House Office of Science and Technology Policy, in addition to those agencies listed in its 2014 charter, were contributing members of the interagency committee.

agencies as "contributing" members.[44] They said it has been the practice of the interagency committee to enable participation and coordination with other agencies, including those who may not be designated as official members.

We found that NOAA does not have a documented process for determining membership on the interagency committee. NOAA officials were unable to locate records from 2006 or earlier documenting the addition of contributing agencies to the committee or the Secretary, or a delegate of the Secretary, making a determination of the appropriateness of such agencies being members. NOAA officials stated the need for the agency to establish a documented process to determine the appropriateness of federal agencies being committee members. The officials said they have started working with NOAA's General Counsel to formalize and document the committee's membership process, and that the process will include a step for the Secretary of Commerce, or a delegate of the Secretary to determine the appropriateness of additional agencies being members. However, NOAA officials did not have an estimated time frame for developing such a process.

Our past work on interagency collaboration has identified the importance of ensuring that relevant participants have been included in the collaborative effort.[45] By establishing a time frame for developing a documented membership process, NOAA and the interagency committee can benefit from capturing all members' activities, and ensuring it provides Congress a complete picture of marine debris efforts across the federal government.

In addition, the Marine Debris Act requires the interagency committee to include a "senior official" from member agencies, but NOAA has not determined the level of official it would consider senior.[46] The interagency committee's charter states that the committee will be composed of "federal agency managers and technical experts," but does not define what is meant by senior official. NOAA officials said that the level of engagement from agency officials has varied over time and often depends on the specific officials participating. The officials said they have had difficulty in the past getting some member agency officials to engage during quarterly meetings and often those that do participate are not decision makers. Specifically, for some agencies, participating officials may not represent the entire agency, but rather a program within the agency, and they may not have decision-making authority, according to NOAA officials. As a result, the officials may not be able to commit agency resources, or they may be uncertain what activities their agency may be able to commit to.

NOAA officials said that it may be helpful to specify the level of official needed to represent the agencies on the interagency committee. The officials said that they have been discussing potential revisions to the interagency committee's charter, and within that broader discussion they are looking into whether the charter should specify what level of official is needed. However, NOAA officials did not have an estimated time frame for revising its charter or determining what those revisions may entail. Our past work on interagency collaboration has identified the importance of ensuring that participants have full knowledge of the relevant resources in the agency, including the ability to commit resources for their agency.[47] By clarifying what is meant by "senior official" such as through revisions to its charter, NOAA would have greater assurance that it has the full engagement of member agency officials who can speak for their agency and commit to activities.

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[44] The interagency committee's charter does not distinguish between official or contributing members.
[45] GAO-12-1022.
[46] 33 U.S.C. § 1954(b).
[47] GAO-12-1022.

INTERAGENCY COMMITTEE'S REPORTS DO NOT CONTAIN SOME REQUIRED ELEMENTS

While the interagency committee's biennial reports provide information on marine debris-related activities of individual agencies, our review found that they do not contain certain required elements. As previously noted, the Marine Debris Act requires the biennial reports to include (1) the status of implementation of any recommendations and strategies of the committee and analysis of their effectiveness, and (2) estimated federal and nonfederal funding provided for marine debris and recommendations for priority funding needs. However, we found that the biennial reports did not include an analysis of the effectiveness of the recommendations implemented or recommendations for priority funding needs.

Implementation of Recommendations and Analysis of Effectiveness

The five biennial reports the interagency committee issued from 2010 to 2019 lay out the committee's 2008 recommendations along with a description of activities taken by individual member agencies related to those recommendations. Specifically, each biennial report references the 25 recommendations the committee first adopted in its 2008 interagency recommendation report, organized into categories (see app. II). The reports then provide a description of activities taken by individual member agencies that fell within the recommendation categories for each preceding 2-year period.[48]

However, we found that the five biennial reports do not include an analysis of the effectiveness of the implementation of the committee's recommendations and strategies as required by the Marine Debris Act. Some of the descriptions of agencies' activities include information on the number of people reached through education or outreach efforts or other quantitative information related to specific activities, but the reports do not include an analysis of the effectiveness of those activities.

NOAA and EPA officials confirmed that the interagency committee did not include an analysis of effectiveness in its biennial reports, stating that undertaking such an effort is beyond the scope of the information-sharing focus of the interagency committee. NOAA officials said that they have attempted to bring member agencies together to discuss how the committee could analyze the effectiveness of its collective efforts, but this has been a challenge because each member has its own priorities and legal authority related to addressing marine debris. Activities to implement the committee's 25 recommendations occur at each individual agency, rather than at the committee level, according to the officials. As such, NOAA officials said each member agency may evaluate the effectiveness of its individual activities and pointed to measures NOAA has in place to evaluate its Marine Debris Program. For example, NOAA estimates the amount of debris removed annually and the number of students it reaches through education and outreach efforts.

EPA officials said that determining a baseline and quantifying the results of specific marine debris efforts to determine effectiveness is challenging, as is the case for other broad, nonpoint sources of pollution. For example, trash enters water bodies through innumerable

[48] The 2014, 2016, and 2019 biennial reports included activities organized by recommendation categories. The 2010 and 2012 biennial reports listed activities by agency, but not by recommendation category.

water and sewer system outfalls, so EPA may focus on strategies to change people's behavior to minimize trash from entering the systems (see Figure 3). But unlike measuring emissions from a smokestack, it is difficult to determine a baseline and then measure and demonstrate progress in terms of trash reduction exiting through the system outfalls. EPA officials said they recognize the need to measure the effectiveness of their efforts related to marine debris—especially as addressing marine debris has become a high priority for the agency—but measuring progress has yet to be determined across all of its various offices and programs that carry out marine debris-related activities. Within the Trash Free Waters program specifically, EPA officials said they take steps to evaluate the effectiveness of the program through a variety of means, such as seeking feedback from stakeholders.

Our past work has shown that collaborative entities—including those addressing complex, cross-cutting issues—can better demonstrate progress and identify areas for improvement if they develop a means to monitor, evaluate, and report the results of their collective efforts.[49] Developing such a means would help the interagency committee ensure that its member agencies are using their authorities and aligning their priorities in the most effective manner possible. Moreover, developing and implementing a process to analyze the effectiveness of the interagency committee's recommendations and strategies, and reporting the results in its biennial reports as required by the Marine Debris Act would better position the committee to determine the extent to which its efforts are making a difference in addressing the complex facets of marine debris.

Source: Laura Stone, Mobile Baykeeper. | GAO-19-653.

Figure 3. Trash capture device to prevent debris from entering waterway.

[49] GAO-12-1022.

Estimated Funding and Recommendations for Priority Funding Needs

The five biennial reports include some estimates of funding for marine debris-related activities, but do not identify recommendations for priority funding needs as required by the Marine Debris Act. Specifically, we found that the reports included estimates for some member agencies' spending related to their marine debris-related activities and estimated nonfederal spending for certain activities.[50] The reports also state that several member agencies conduct activities within multiple programs, offices, and projects indirectly related to marine debris efforts. These agencies do not receive annual appropriations specifically for marine debris activities but instead receive appropriations to fulfill their missions or implement programs, making it difficult to estimate exact spending related to marine debris, according to the reports.

The 2019 biennial report states that the interagency committee's recommendations for priority funding needs are reflected in the President's budget request and operating plan for each member agency in any given fiscal year. NOAA officials said that it would be difficult to identify and communicate priority funding needs outside of these documents, particularly given the complications associated with estimating each agency's individual spending. For example, an EPA official said that EPA's efforts to address marine debris are decentralized and the agency does not receive an appropriation specifically for marine debris-related activities, making it difficult to determine how much the agency spends—or may need to spend—on marine debris. Moreover, NOAA and EPA officials said that because the interagency committee serves primarily as an information-sharing body and each member agency operates independently in identifying resource needs, the interagency committee has not needed to develop a process to identify recommendations for priority funding needs.

However, the Marine Debris Act requires the interagency committee to include recommendations for priority funding needs in its biennial reports, and without a process to identify such recommendations, the interagency committee cannot meet that requirement. Our past work on leading collaborative practices has shown the importance of identifying and leveraging resources, such as funding, in collaborative efforts.[51] By developing a process to identify recommendations for priority funding needs in its biennial reports, the interagency committee could provide Congress with required information about priority funding needs across the federal government to address marine debris.

[50] For example, in the interagency committee's 2019 biennial report, EPA estimated $410,000 in fiscal year 2016 and $320,000 in fiscal year 2017 for its Trash Free Waters Program. The Department of State estimated no funding for marine debris activities in fiscal year 2016 and $1,000,000 in fiscal year 2017 for marine debris-related activities and grants. The interagency committee also stated in this report that it interpreted the reference to nonfederal funding in the act's biennial reporting requirement to mean the required nonfederal match associated with the grants program authorized under section 3 of the Marine Debris Act administered by NOAA's Marine Debris Program. NOAA estimated $2,166,517 in nonfederal match for fiscal year 2016, and $1,944,621 in fiscal year 2017.

[51] GAO-12-1022.

EXPERTS SUGGESTED A RANGE OF ACTIONS THE FEDERAL GOVERNMENT COULD TAKE TO MOST EFFECTIVELY ADDRESS MARINE DEBRIS

The 14 experts we interviewed with expertise in marine debris-related issues suggested a range of actions that the federal government could take to most effectively address various types of marine debris. Their suggestions included increasing or improving actions already being taken by some federal agencies as well as taking new actions. The experts stressed that there is not one solution to the growing, multi-dimensional problem of marine debris. Rather, they said that a multitude of actions involving federal agencies and nonfederal partners—such as international, state and local governments, Indian tribes, industry, and environmental groups—will need to be taken to address the issue.

Experts as well as agency officials we interviewed indicated that there would be a number of factors to consider in evaluating the suggested actions. Some of these factors are overarching, applying to most or all of the actions; others relate to specific actions. For example, several experts and agency officials said that competing priorities and limited resources would be important factors to consider related to all of the suggested actions. Several agency officials also said that their agencies may not have the authority to take some of the actions suggested by the experts, and therefore new legislation would need to be enacted before they could take those actions. Additionally, some actions could result in impacts or costs to particular industries, underserved communities, or consumer groups, and understanding and identifying ways to mitigate such impacts would be important. Moreover, several agency officials said some actions, such as those related to waste management, may be better suited for local or state governments and that those entities would be better-equipped to deal with particular aspects of marine debris.

The following are examples of actions the experts suggested that the federal government could take. We organized the actions into the following five categories, which generally correspond to the categories laid out in the interagency committee's reports: (1) education and outreach, (2) establishment of federal requirements or incentives, (3) cleanup, (4) research and technology development, and (5) coordination.[52]

Education and Outreach

Seven of the 14 experts suggested actions to educate or conduct outreach to the public or specific consumer or industry groups or international governments about ways to prevent, reduce, mitigate, or clean up waste that can become marine debris. A few experts emphasized that education and outreach efforts should be focused on ways to prevent trash from entering the marine environment. Examples of education and outreach actions suggested include:

[52] In our analysis, we categorized actions related to legislation, regulations, policies, or incentives as falling within the category of "establishment of federal requirements or incentives." In analyzing these actions, we did not determine whether laws would need to be enacted or regulations issued, or both, to implement such actions.

- **Domestic education and outreach**. Five experts suggested different types of education or outreach campaigns the federal government could undertake to target certain domestic groups, such as consumers. One expert suggested that the federal government develop a national campaign to educate the public about marine debris. Such a campaign would develop a single message that various entities, including federal agencies and nonfederal stakeholders, could include in advertisements, social media, and other public awareness efforts. The expert pointed to similar state-led campaigns, such as "Nobody Trashes Tennessee," a litter campaign developed by Tennessee's Department of Transportation.[53] This state campaign features celebrities, such as athletes and musicians, in advertisements and involves selling stickers, hats, and other items to help spread the message. However, the expert said that securing collaboration and agreement on a single message across federal agencies and nonfederal stakeholders could pose a challenge and that a national campaign would need a long-term commitment from all parties to be successful.

  NOAA officials said that national campaigns can be expensive and demonstrating results from such efforts can be difficult, especially when they are broad in nature. As a result, these officials said that NOAA's Marine Debris Program targets its education and outreach efforts to a specific audience for a particular type of behavior change or type of debris, such as educating and training high school students to lead "Zero Litter Campaigns" in their schools and communities.
- **International outreach**. Two experts suggested actions the federal government could take to conduct outreach internationally to promote programs, policies, or technologies that can reduce marine debris. For example, one expert suggested the federal government conduct outreach to government officials in countries that have limited waste management infrastructure to demonstrate effective waste management technologies. The expert said that the federal government could partner with private sector companies to demonstrate waste-to-energy technologies, such as gasification and pyrolysis that can convert plastic waste to fuel.[54] According to the expert, demonstrating such technologies would provide information on its benefits, including reducing sources of waste and creating a source of energy to either use or sell.

  Several agency officials we interviewed agreed that international outreach efforts are critical to successfully addressing marine debris and that emphasis should be placed on assisting countries with improving their waste management practices. However, these officials said there are many factors to consider with regard to waste-to-energy technologies. For instance, State Department officials said such technologies may not be supported by civil organizations because of environmental concerns.[55] Waste-to-energy technologies could also entail high upfront capital

[53] To learn more about the Tennessee Department of Transportation campaign, see https://nobodytrashestennessee.com/.

[54] Waste-to-energy is a term used for technologies, such as gasification and pyrolysis that convert waste, including plastics, into fuels or chemicals. Gasification breaks down organic waste (e.g., plastics, wood chips, rice hulls) with heat and controlled amounts of oxygen to produce syngas. The syngas can then be burned as a fuel or can be used as a feedstock to produce other chemicals such as methane and methanol. Pyrolysis heats plastics with no oxygen to produce liquid fuel. According to the Energy Recovery Council's 2018 report, 75 waste-to-energy plants operate in 21 states in the United States.

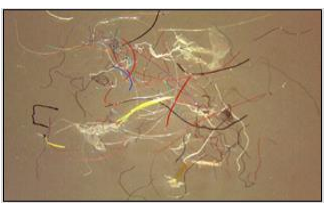
[55] In some cities in the United States, groups have raised concerns with the potential environmental and human health effects of waste-to-energy facilities on surrounding communities. For example, in February 2019, the

investments, and waste-to-energy facilities should adhere to strict environmental standards with monitoring and enforcement to help ensure the technology is not causing negative effects, according to agency officials. As a result, they said it may not be practical for some countries to adopt such technologies. In addition, USAID officials said that promoting waste-to-energy technology presupposes that waste is already being collected in sufficient quantity and quality to serve as a fuel for such technology, but that in some countries waste is openly dumped or burned and therefore sufficient waste may not be available. They cautioned that waste-to-energy technologies can be a part of a response to address marine debris abroad, but would not be sufficient alone.

Establishment of Federal Requirements or Incentives

Eleven experts suggested actions the federal government could take to establish requirements or incentives to address various types of marine debris. Examples included:

Microfibers



Microfibers are a widespread type of microplastic; they have been found on the shorelines of six continents and in oceans, rivers, soils, table salt, and public drinking water, according to scientific studies.

Microfibers enter the marine environment through various pathways. For example, microfibers are shed from synthetic clothing and other materials made of polyester and nylon. These microfibers pass through to waterways because washing machines and wastewater treatment plants typically do not have processes sufficiently refined to remove the fibers. Little is known about other potential sources of microfibers, such as carpet manufacturing; the rate of generation, such as how quickly materials break down and shed microfibers; and any health impacts to humans or wildlife.

Sources: GAO analysis of scientific studies and agency documents; Sherri Mason/State University of New York at Fredonia (photo). | GAO-19-653.

- **Design standards for products**. Five experts suggested establishing federal requirements for manufacturers to design certain products to minimize the chances of material becoming marine debris. For example, two experts suggested the federal government develop design standards for washing machine manufacturers to ensure filters are designed to prevent microfibers from entering wastewater systems and

city council of Baltimore, Maryland, passed an ordinance to place restrictions on two of the city's waste-to-energy facilities because of concerns that the facilities have contributed to asthma and other respiratory illnesses in surrounding communities.

then the marine environment. Three experts suggested the federal government develop design standards to require or incentivize manufacturers to use specific amounts of post-consumer material in developing certain products. For example, one expert recommended requiring the manufacturers of plastic beverage bottles to produce bottles using at least a minimum amount of recycled plastic. According to the expert, this would increase the demand for recycled plastic as a raw material, which in turn would reduce the likelihood that such plastic would end up as waste. The expert said that requiring the use of recycled plastic would likely impose increased costs on manufacturers because virgin plastic—the raw material typically used in producing plastic beverage bottles—is currently less expensive than recycled plastic. Such increases would likely be short term, however, because the increased demand would decrease the price after more of the recycled material is used, according to another expert. Some federal agency officials said that establishing such proposed federal design standards could be difficult due to limited existing statutory authorities.

- **Requirements for fishing gear**. Three experts suggested the federal government establish requirements to mitigate the impact of lost or derelict fishing gear in federal waters.[56] For example, one expert suggested requiring the use of modified fishing gear, such as crab traps with biodegradable escape mechanisms that allow entrapped marine life to escape if the trap is lost or abandoned (see Figure 4). Requiring the use of fishing gear with biodegradable escape mechanisms would likely impose increased costs to the fishing industry, according to the expert, but those costs could be minimized if the federal government offered a subsidy to help purchase required gear. NOAA officials said that it would be challenging to require the use of certain types of fishing gear in part because of the cost to the federal government in ensuring implementation of the requirement. On the other hand, NOAA officials said they promote innovation and voluntary use of certain types of fishing gear through various efforts such as their Fishing for Energy program.[57]
- **Restrictions on single-use plastics**. Four experts suggested that the federal government establish restrictions on the manufacturing or sale of certain single-use plastics. For example, the federal government could establish restrictions on the manufacturing and distribution of plastic bags in the form of thickness or material composition requirements, or production volume limits. Two of these experts also said that the federal government could review existing local, state, and international efforts to restrict single-use plastics to identify best practices so that these types of actions could potentially be scaled appropriately at the federal level. According to the United Nations Environmental Programme, 127 countries and two states have placed various types of restrictions on the retail distribution of plastic bags as of 2018.[58] One expert pointed to research that shows that plastic bags are one of the most abundant

[56] Federal waters typically begin approximately 3 geographical miles from land and extend 200 nautical miles.

[57] Fishing for Energy is a partnership between NOAA, the fishing industry, and other stakeholders to prevent and reduce the impacts of derelict fishing gear in the marine environment by offering no-cost options for disposing of old or unwanted gear and converting gear into energy. For example, metal gear—such as crab pots—is recycled and nonmetal material is brought to a waste-to-energy facility where it is used in the production of electricity for local communities, according to NOAA documentation.

[58] In contrast, several states have enacted legislation prohibiting local governments from regulating the sale of plastic bags.

forms of marine debris and suggested that banning them would therefore significantly reduce the amount of debris entering the marine environment.[59]

Source: Virginia Institute of Marine Science. | GAO-19-653.

Figure 4. Crab trap with biodegradable escape mechanism.

NOAA officials agreed that restricting the sale of single-use plastic bags could help address the marine debris problem, but said that identifying an agency with sufficient legal authority to be responsible for implementing and enforcing any restriction would be important and could be a challenge at the federal level. NOAA and EPA officials said that it would be important to carefully determine and assess trade-offs or other potential impacts before considering these types of restrictions.

Single-Use Plastics

Single-use plastics are any plastic items— such as plastic soda or water bottles—that are intended for use only once before they are thrown away or recycled as defined by the United Nations Environment Programme.

Single-use plastics can have environmental impacts when they are left in the marine environment. For example, single-use plastics may be ingested by hundreds of species of marine wildlife, such as turtles and dolphins, who mistake them for food, potentially blocking their airways and stomachs, according to a 2018 report by the United Nations Environment Programme.

Sources: United Nations Environment Programme; National Oceanic and Atmospheric Administration (photo). | GAO-19-653.

[59] For example, in 2018, the Ocean Conservancy's International Coastal Cleanup found that plastic bags were among the top five most common types of marine debris. *Building a Clean Swell: The International Coastal Cleanup's 2018 Report*, Ocean Conservancy, Washington, D.C.

- **Incentives for waste management**. Four experts suggested actions the federal government could take to provide incentives to local governments to help them improve their waste management and recycling programs. The experts said that waste and water management is typically the responsibility of local governments, but that given the scope and scale of the marine debris problem, the federal government could use its resources to provide incentives to help local governments make improvements. For example, the federal government could provide grants or subsidies to help local governments implement best management practices, such as using trash traps to help remove debris from waterways and prevent it from becoming marine debris. In addition, the experts said that the federal government could provide local governments with resources to help purchase bins with lids to help prevent inadvertent loss of waste or to pay for infrastructure such as trucks and recycling facilities to improve the collection and recycling of waste. According to one expert, transporting materials from consumers to the appropriate waste management or recycling facilities is a significant barrier to achieving better waste management.

  EPA officials agreed with the importance of local waste management efforts. The officials emphasized that it is the agency's mission, in part, to address management of waste to prevent trash, and management of water that carries the trash to the marine environment. The officials said that this is particularly critical for addressing marine debris since an estimated 80 percent of aquatic trash originates from land-based sources. The officials said the agency has provided some funding to local governments to implement mechanisms to capture trash before it enters waterways or to remove trash from water. They added that there is no one size fits all approach, however, to working with local governments. Rather, different localities may have differing needs—such as for funding, information, or technical assistance—and EPA tries to create a climate where localities can identify and best address those needs, according to the officials.

Cleanup

Five of the 14 experts suggested the federal government support marine debris cleanup and removal activities by providing resources to organizations that coordinate cleanup projects (see Figure 5). Several agency officials said that preventing waste from entering the marine environment should be the primary focus of addressing marine debris, but cleaning up existing marine debris continues to be a critical part of the multi-faceted response to the problem, especially after severe weather events such as hurricanes. According to one expert, debris deposited into the marine environment around the Florida Keys after Hurricane Irma in 2017 included construction debris from demolished buildings, household items such as refrigerators and televisions, cars, and boats, among other types of debris. The expert suggested the federal government provide funding and technical assistance to state and local governments to help locate such debris. According to the expert, after a severe weather event, the distribution of debris can vary greatly with ocean and wind currents, and the debris can extend for miles into the ocean. As a result, the expert suggested that the federal government assist with conducting aerial flyovers to locate major concentrations of debris. The flyovers

would employ mapping technology, such as global positioning system equipment and cameras, to locate and map the debris for removal. NOAA officials agreed with the importance of cleanup activities, particularly after severe weather events. In 2018, NOAA provided $18 million to states for the detection, removal, and disposal of debris after the 2017 hurricanes.

Source: National Part Service. | GAO 19-653.

Figure 5. Before and after beach cleanup at a national park.

Research and Technology Development

Ten of the 14 experts suggested actions related to research or technology development. A few experts commended federal research efforts related to marine debris to date but stressed that additional research is needed in multiple areas. Examples of research and technology development actions suggested by experts include:

- **Research on sources, pathways, and location of marine debris**. Five experts suggested the federal government support research on identifying and understanding the various sources, pathways, and location of marine debris. For example, one expert suggested that the federal government conduct a national study to identify where waste is generated, through which types of major pathways it enters the marine environment (such as rivers or stormwater), and where the waste ends up. This study could include a focus on specific pathways, such as where illegal dumping occurs, which has not been researched at the national level, according to the expert. The expert said that federal agencies and others could use the results of such a study to help target education for the public, policy makers, and law enforcement officials on how to prevent and properly dispose of the types of waste that most commonly end up as marine debris. NOAA officials said that illegal dumping tends to be localized, so it may be difficult to carry out research on a national scale, but agreed with the need to better understand sources and types of marine debris since many factors contribute to the problem.
- **Research on effects of marine debris**. Four experts suggested the federal government support research to determine the effects of debris on wildlife and the marine environment as well as on human health. For example, one expert suggested that the federal government conduct or fund research to determine the effects of

microplastics on human health to help the federal government and other stakeholders identify the most appropriate solutions. EPA officials said that this type of research is one among many competing areas related to marine debris research their agency has targeted.

- **Development of technology to address marine debris**. Five experts suggested actions that the federal government could take to develop new technology to help address marine debris. For example, one expert suggested that the federal government fund the development of new technology to recycle hard-to-recycle plastic materials so that these materials are less likely to end up as waste and become marine debris. The expert said that, in particular, plastic materials such as packaging used to preserve food products are not readily recyclable because the technology to recycle these types of plastics is not available or is not economically viable. EPA officials said that even when there is technology to recycle these types of plastics, food contamination is a problem that may prevent them from being recycled. In addition, an increased capacity for recycling may not result in a behavior change on the part of the consumer, which is another factor to consider in evaluating whether to pursue this type of action, according to the officials.

Coordination

Nine experts suggested that the federal government coordinate with local, state, federal, and international governments and other nonfederal partners to address marine debris. Experts emphasized that because marine debris is a complex issue with domestic and international impacts, it requires contributions from and coordination across these many groups. Examples of coordination suggested by experts include:

- **Coordination with stakeholders on management of fishing gear**. Two experts suggested the federal government coordinate to identify ways to prevent fishing gear from becoming a source of marine debris and causing harm to fish and other marine species. One expert suggested the federal government coordinate with stakeholders to identify and implement best management practices for responsible management and use of fishing gear. Specifically, the expert suggested that the federal government coordinate with state agencies, gear designers and manufacturers, fishermen, and other stakeholders to adopt best practices in particular locations such as in the Chesapeake Bay or Puget Sound where there are extensive commercial or recreational fisheries. The expert said it would be important to work with industry stakeholders to avoid the best practices being perceived as unnecessary government intervention. In addition, one of the experts said that adoption of best practices could incur additional costs for activities such as replacing gear, which could be minimized through government subsidies or other incentives. NOAA officials said these types of coordination activities align with current efforts within their Marine Debris Program. For example, in 2016 NOAA partnered with California State University and other stakeholders to encourage the adoption of best practices to prevent the loss of gear used to catch spiny lobster in the Channel Islands in California.

- **Coordination with international governments**. Four experts suggested the federal government increase its coordination internationally such as through developing international agreements and participating in multinational forums. For example, one expert suggested that the United States and other countries enter into an international agreement to prevent further release of plastic into the ocean. Under such an agreement, each country would set a target to reduce the amount of plastic released into the ocean, develop strategies and approaches to meet that target, and measure and report on progress in meeting the target.[60] The expert said that taking actions to meet the target would incur costs and that securing commitments from countries could be difficult. However, the expert said that allowing countries the flexibility to develop their own strategies for meeting their targets could help overcome these difficulties.

  State Department officials said that in addition to coordination with international governments, coordination is needed with other key stakeholders such as waste management and marine debris experts, local leaders, private-sector industry and retail entities, and nongovernmental organizations. This is in part because so much of the international marine debris problem stems from waste management issues at the local level. In some countries, as in the United States, the government may not have the authority to work on waste management at the local level and as a result, understanding this complexity is an important factor to consider in coordinating internationally, according to the officials. USAID officials agreed that coordination with international stakeholders beyond international governments is needed and said that given the local nature of waste management issues that contribute to the international marine debris problem, stakeholders such as local and municipal governments are also important and should be a major focus for coordination and capacity building.[61]

CONCLUSION

Marine debris is a global, multi-faceted problem and multiple federal agencies, along with nonfederal stakeholders such as nongovernmental organizations, industry, states, Indian tribes, and others, have important roles to play in addressing the problem. The interagency committee's sharing of information about its members' activities is a good first step to ensure the agencies are aware of their respective marine debris-related efforts. NOAA, as chair of the committee, has recognized the need to develop a documented membership process, but has not established a time frame for doing so. By establishing a time frame for developing a documented membership process, NOAA and the interagency committee can benefit from capturing all members' activities, and ensuring it provides Congress a complete picture of marine debris efforts across the federal government.

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[60] See *Why We Need An International Agreement On Marine Plastic Pollution*, Stephanie B. Borrelle, Chelsea M. Rochman, Max Liboiron, Alexander L. Bond, Amy Lusher, Hillary Bradshaw, and Jennifer F. Provencher.

[61] USAID officials further said that beyond coordination, many of the experts' suggestions to address marine debris— such as providing incentives for waste management, conducting research on sources, pathways and location of marine debris, and developing new technology to address marine debris—are also needed in developing countries.

NOAA also recognizes that it may be helpful to specify the level of the official needed to represent the agencies through revisions to its charter, but has not determined what those revisions may entail. By clarifying what is meant by "senior official" such as through revisions to its charter, NOAA would have greater assurance that it has the full engagement of member agency officials who can speak for their agency and commit to activities.

The interagency committee's biennial reports provide information on the committee's recommendations and individual agencies' activities to implement those recommendations, but the reports do not include an analysis of the effectiveness of the committee's recommendations and strategies as required by the Marine Debris Act. By developing and implementing a process to analyze the effectiveness of the interagency committee's recommendations and strategies, and reporting the results in its biennial reports as required, the interagency committee would be in a better position to determine the extent to which its efforts are making a difference in addressing the complex facets of marine debris.

Additionally, the interagency committee has not identified required recommendations for priority funding needs. By developing a process to identify recommendations for priority funding needs and including such recommendations in its biennial reports, the interagency committee could provide the Congress with required information about priority funding needs across the federal government to address marine debris.

RECOMMENDATIONS FOR EXECUTIVE ACTION

We are making a total of four recommendations, including two recommendations to the NOAA Administrator and two recommendations to the chair of the interagency committee, specifically:

The NOAA Administrator, in coordination with interagency committee member agencies, should establish a time frame for documenting the committee's membership process. (Recommendation 1)

The NOAA Administrator, in coordination with interagency committee member agencies, should clarify what is meant by "senior official" in the Marine Debris Act, such as through revisions to its charter. (Recommendation 2)

The chair of the interagency committee, in coordination with member agencies, should develop and implement a process to analyze the effectiveness of the interagency committee's recommendations and strategies, and include the results in its biennial reports. (Recommendation 3)

The chair of the interagency committee, in coordination with member agencies, should develop a process to identify recommendations for priority funding needs to address marine debris, and include such recommendations in its biennial reports. (Recommendation 4)

Agency Comments and Our Evaluation

We provided the Departments of Commerce, Defense, Homeland Security, Interior, Justice, and State; EPA; the Marine Mammal Commission; and USAID a draft of this report for their review and comment. The Department of Commerce and USAID provided written

comments, which are reprinted in appendixes IV and V respectively, and discussed below. We also received technical comments from the Departments of Commerce, Homeland Security, the Interior, and State; EPA; the Marine Mammal Commission; and USAID, which we incorporated into the report as appropriate. The Departments of Defense and Justice indicated that they had no comments.

In written comments from the Department of Commerce, Commerce and NOAA agreed with our four recommendations. Regarding our first two recommendations, NOAA stated that its Administrator will establish a time frame for documenting the interagency committee's membership process and, in coordination with the interagency committee, will define the term "senior official" through revisions to its charter so that the term can be consistently applied across all federal agency structures. In forming its definition of "senior official," NOAA indicated that it would consider seniority requirements of similarly situated advisory committees, along with related factors such as the ability to make decisions on behalf of an agency.

Regarding our third recommendation on developing and implementing a process to analyze the effectiveness of the interagency committee's recommendations and strategies, NOAA stated that it agreed with this recommendation to the extent it can be implemented with available budgetary resources. It indicated that the interagency committee lacks the existing resources to require and routinely evaluate the effectiveness of agency activities. Instead, individual agencies are expected to work toward implementing the interagency committee's 2008 recommendations in accordance with each agency's legal and programmatic authorities, mission priorities, and resource limitations. Nevertheless, NOAA stated that to the extent possible it will work with interagency committee members to identify common or easily translatable metrics for evaluating the effectiveness of its 2008 recommendations and include these in the next biennial report to Congress.

Regarding our fourth recommendation, NOAA stated that it agreed with our recommendation, but noted that it does not have the authority to control the implementation of a process for identifying priority funding needs of other member agencies. It stated that the interagency committee's recommendations for priority funding needs are already reflected in the President's annual budget request and operating plan for each member agency. However, NOAA stated that to the extent possible, it will work with interagency committee members to develop a process for identifying priority areas, which can be reflected in each agency's respective budgeting process and shared in the committee's biennial reports. We agree that NOAA does not have the authority to control the implementation of a process for identifying priority funding needs of other member agencies. However, as chair of the committee, NOAA can coordinate with member agencies to develop a process that each individual member agency—under its individual authority and budgetary processes—can use to identify recommendations for priority funding needs to address marine debris. We believe that coordinating such information and providing it in the committee's biennial reports could provide Congress with required information about priority funding needs across the federal government to address marine debris.

In addition, in written comments from USAID, the agency said it is committed to addressing the challenge of marine debris through its programs and in collaboration with interagency committee partners. USAID stated that it has significant opportunities to play an important role in the international response to address marine debris and, as the lead federal agency on foreign assistance, has several programs that target mismanaged municipal waste

in the developing world. For example, USAID stated that the agency's Municipal-Waste Recycling Program has helped reduce land-based sources of ocean plastic waste in four of the top five contributing countries—Indonesia, the Philippines, Sri Lanka, and Vietnam—by providing small grants and technical assistance to a variety of local actors in towns and cities. USAID also stated that it greatly appreciates the work of its interagency committee partners in addressing marine debris and looks forward to continued collaboration with them.

Anne-Marie Fennell
Director, Natural Resources and Environment

List of Requesters

The Honorable Maria Cantwell
Ranking Member
Committee on Commerce, Science, and Transportation
United States Senate

The Honorable Richard Blumenthal
United States Senate

The Honorable Cory A. Booker
United States Senate

The Honorable Christopher A. Coons
United States Senate

The Honorable Mazie K. Hirono
United States Senate

The Honorable Jeffrey A. Merkley
United States Senate

The Honorable Lisa Murkowski
United States Senate

The Honorable Patty Murray
United States Senate

The Honorable Gary C. Peters
United States Senate

The Honorable Brian Schatz
United States Senate

The Honorable Tom Udall
United States Senate

The Honorable Elizabeth Warren
United States Senate

The Honorable Sheldon Whitehouse
United States Senate

APPENDIX I: OBJECTIVES, SCOPE, AND METHODOLOGY

This chapter examines (1) how the interagency committee coordinates among federal agencies and the process for determining membership and agency representation, (2) the extent to which the interagency committee's biennial reports contain required elements, and (3) experts' suggestions on actions the federal government could take to most effectively address marine debris.

To examine how the interagency committee has coordinated among federal agencies and the process for determining membership and agency representation, we reviewed the Marine Debris, Research, Prevention, and Reduction Act, as amended (Marine Debris Act), and interagency committee documents, including the committee's 2008 report with recommendations, charter,[62] and five biennial reports to Congress issued as of March 2019.[63] Specifically, we reviewed meeting minutes from the interagency committee's quarterly meetings from November 2012 through April 2019,[64] to understand the topics and activities the committee has coordinated on and the federal agencies that have participated. We attended five of the interagency committee's quarterly meetings (in May, September, and December of 2018, and April and July of 2019) to directly observe committee coordination among agencies during these meetings. We also reviewed documents from committee member agencies and interviewed and reviewed written responses from those agencies to obtain information on their coordination efforts.

Agencies we included were those agencies designated as members in the Marine Debris Act as well as additional agencies identified as members in the committee's charter (see table 2). In addition, we interviewed officials and reviewed documents from the National Science Foundation, Office of the U.S. Trade Representative, and the U.S. Agency for International Development, based on suggestions from interagency committee officials.[65]

[62] We reviewed the interagency committee's initial charter, developed in 2006, as well as its most recently revised 2014 charter.

[63] The five biennial reports were issued in March 2010, October 2012, September 2014, December 2016, and March 2019. Collectively, the reports include activities the interagency committee member agencies reported conducting between June 2008 and December 2017.

[64] Meeting minutes dating back to November 2012 were those minutes most readily available from the National Oceanic and Atmospheric Administration, the chair of the interagency committee.

[65] We subsequently removed the National Science Foundation and the Office of the U.S. Trade Representative from our review because, unlike the U.S. Agency for International Development, those two agencies have not participated on the interagency committee.

Table 2. Interagency Marine Debris Coordinating Committee member agencies, as identified in its 2014 charter

Department	Agency
Department of Commerce	National Oceanic and Atmospheric Administration
Department of Defense	U.S. Army Corps of Engineers U.S. Navy
Department of Homeland Security	U.S. Coast Guard
Department of the Interior	Bureau of Safety and Environmental Enforcement National Park Service U.S. Fish and Wildlife Service
Department of Justice	Environment and Natural Resources Division
Department of State	
Environmental Protection Agency	
	Marine Mammal Commission

Source: GAO analysis of Interagency Marine Debris Coordinating Committee documents. | GAO-19-653.

From the committee's 2008 report with recommendations, the five biennial reports, and other member agency documents, we summarized activities conducted by member agencies. For reporting purposes, we selected examples from the 2016 and 2019 biennial reports (those most recently available) of activities the agencies have taken to illustrate interagency committee member efforts to address marine debris, to reflect a range of activities across categories of activities and member agencies.[66] In addition, we compared information we received about the interagency committee's coordination to leading practices we identified in our past work on implementing interagency collaborative mechanisms.[67]

To examine the extent to which the interagency committee's biennial reports contain required elements, we compared information contained in the committee's five biennial reports to the statutory reporting requirements in the Marine Debris Act. Specifically, two analysts independently reviewed each of the five biennial reports to evaluate information the reports included about (1) the status of implementation of any recommendations and strategies of the committee, (2) analysis of the recommendations and strategies' effectiveness, (3) estimated federal and nonfederal funding provided for marine debris, and (4) recommendations for priority funding needs. The analysts then compared and summarized the results of their analyses. We also interviewed and reviewed written responses from National Oceanic and Atmospheric Administration (NOAA) officials (in the agency's capacity as chair of the interagency committee) and officials from other members of the committee about steps to develop the biennial reports, including the reports' required elements. In addition, we compared information from the reports and the information we received from the officials to leading practices we identified in our past work on implementing interagency collaborative mechanisms.[68]

[66] The biennial progress reports identified eight categories of activities, but we consolidated activities reported under the "enforcement" and "incentive programs" categories into the "legislation, regulation, and policy" category because of similarities among the activities within these categories. Similarly, for presentation purposes, we consolidated activities reported under the "research" and "technology development" categories into one "research and technology development" category.

[67] GAO, *Managing for Results: Key Considerations for Implementing Interagency Collaborative Mechanisms*, GAO-12-1022 (Washington, D.C.: Sept. 27, 2012). We used leading practices that are relevant to the requirements for the interagency committee in the Marine Debris Act. These included practices related to participants, resources, outcomes and accountability, and written guidance and agreements.

[68] GAO-12-1022.

To obtain suggestions on actions the federal government could take to most effectively address marine debris, we conducted structured interviews with a nongeneralizable sample of 14 experts with expertise in marine debris-related issues. We selected the experts from a list of individuals we identified through interviews with agency officials and through a snowball approach, in which we reviewed relevant literature on marine debris, such as articles the experts authored, to identify other key experts and asked experts to identify other experts for including in this review. We also identified experts through our participation in key marine debris events, such as presenting at the Sixth International Marine Debris Conference.[69] We considered factors such as the individual's experience with different types of debris (e.g., abandoned fishing gear or consumer debris) or association with various sectors (e.g., academia or industry).

Experts selected included: (1) academics with expertise in areas such as sources, prevalence, and transport of plastic marine debris; (2) officials representing the plastic manufacturing, food and beverage, and commercial fishing industries; (3) officials from nonprofit organizations with expertise in marine debris removal from coastal areas, litter prevention, and recycling management systems and strategies; and (4) state and local government officials from the District of Columbia, Florida, and Washington with expertise in local litter prevention efforts, derelict vessels, and lost and derelict fishing gear.[70]

We asked the 14 experts to suggest up to 5 to 10 actions the federal government could take to most effectively address different types of marine debris.[71] We defined the term "actions" to mean any policy, program, effort, or intervention that could be taken by the federal government to prevent, remove, or dispose of marine debris. Actions could include new actions that the federal government may not have implemented or actions the federal government may already have taken. We did not limit experts' suggestions to actions that agencies currently have authority to implement. We do not take a position on the merits of, the necessary legal authority for, or the most appropriate entity for the actions suggested by the 14 experts.

Prior to the interview, we provided experts with background information about our review, the interview methodology, and definitions for key terms to ensure that terminology was used consistently throughout all the interviews. We also reviewed this information with each expert at the start of the interview. For each action, we asked that the expert identify:

- **Name of action;**
- **Type(s) of debris:** (Select any or all of the following types of marine debris that may be affected by the action: consumer-based, abandoned fishing gear, derelict vessels, and/or miscellaneous. If miscellaneous is selected, please explain);
- **Describe this action:** (Briefly describe this action and how it will address (i.e., prevent, remove, or dispose) marine debris and if it is currently being implemented by the federal agencies);

[69] The Sixth International Marine Debris Conference was organized by NOAA and the United Nations Environment Programme. Over 700 participants from 54 countries attended the conference, including international governments and multinational bodies representatives; federal, state, and local government officials; coastal and ocean resource managers; waste management representatives; scientists; academics; and industry representatives.

[70] We selected these locations using factors such as geographic area and expertise in marine debris issues.

[71] One expert suggested 12 actions; we included each of the 12 expert's suggested actions in our analysis.

- **Federal agency(ies)** (Please briefly describe the federal agency(ies) that have implemented or could play a role in implementing the action);
- **Nonfederal partners:** (Please briefly describe the nonfederal partners the federal agencies may need to coordinate with when implementing the action (such as international, state and local governments, nonprofit groups, industry, and/or researchers);
- **Advantages:** (Briefly describe the advantages of the federal agencies implementing the action in terms of the ability of this action to address marine debris, the cost of the action, and the technical and administrative feasibility of implementing the action, or any other advantage that you believe may affect implementation);
- **Disadvantages:** (Briefly describe the disadvantages of the federal agencies implementing the action in terms of the ability of this action to address marine debris, the cost of the action, and the technical and administrative feasibility of implementing the action, or any other disadvantage that you believe may affect implementation);[72]
- **Challenges:** (Describe any factors that may hinder this action from being successfully implemented by the federal agencies and how these factors may be overcome);
- **Examples:** (In instances where the federal agencies have previously implemented the action, please provide examples of how it helped address marine debris. If other entities that are not federal agencies have successfully implemented the action, please provide examples of how the action helped address marine debris);
- **Authorities:** (Briefly describe what legal authorities these actions would be implemented under. If new authorities are needed, please describe them); and
- **Support:** (Provide any studies, reports, or research you are basing your responses on).

We conducted the interviews via teleconference between July 2018 and November 2018. The experts suggested over 70 actions that we organized into five categories based on common themes.[73] Specifically, two analysts independently reviewed each expert's description for individual actions and identified an appropriate category using decision rules the team developed. The analysts then discussed and compared their decisions. For actions the analysts categorized differently, they reviewed the decision rules together and came to agreement on the best category for a particular action. For reporting purposes, we selected several actions within each of the broader categories to provide illustrative examples of the types of actions experts suggested. Our selection of actions was based on a variety of factors, including our analysis of the number experts that suggested similar types of actions, the detail

[72] We defined disadvantages of an action in terms of the ability of the action to address marine debris, the cost of the action, and the technical and administrative feasibility of implementing the action. We defined challenges as the factors that may hinder the action from being successfully implemented by the federal agencies. However, during many of the interviews the experts found it difficult to distinguish between the two terms. As a result, we use the term "challenge" in the report to indicate either a disadvantage or a challenge.

[73] The categories of actions generally correspond to the categories laid out in the interagency committee's biennial reports. However, we merged the categories "legislation, regulation, and policy," "incentive programs," and "enforcement" into the "establish federal requirements or incentives" category. We also merged the categories "technology development" and "research" to make the category "research and technology development."

provided by the experts, and the availability of supporting information, such as instances where an action had been taken by state or local governments. Actions suggested by the 14 experts cannot be generalized to actions that might be suggested by other experts but provide examples of actions federal agencies could take to address marine debris.

We also obtained written and oral responses to questions we asked of agency officials regarding factors their agencies would need to consider in potentially implementing any of the actions identified by the 14 experts. In addition, to corroborate statements from experts and agency officials and provide additional context on marine debris, we reviewed scientific studies and documents from international organizations, such as the United Nations; academic institutions and nonprofit organizations such as the Ocean Conservancy; and federal and state agencies to understand what is known about the types, sources, and effects of marine debris. We identified these studies and documents through various means, such as recommendations from experts and agency officials and authorship by experts. We also interviewed individuals from academia, environmental groups, and industry actively working on marine debris issues and attended the Sixth International Marine Debris Conference held in San Diego, California, in March 2018, to gain an understanding of areas of emphasis in the marine debris community.

We conducted this performance audit from October 2017 to September 2019 in accordance with generally accepted government auditing standards. Those standards require that we plan and perform the audit to obtain sufficient, appropriate evidence to provide a reasonable basis for our findings and conclusions based on our audit objectives. We believe that the evidence obtained provides a reasonable basis for our findings and conclusions based on our audit objectives.

APPENDIX II: RECOMMENDATIONS FROM THE INTERAGENCY MARINE DEBRIS COORDINATING COMMITTEE'S 2008 REPORT

Table 3 lists the 25 recommendations contained in the Interagency Marine Debris Coordinating Committee's 2008 report entitled Interagency Report on Marine Debris Sources, Impacts, Strategies, and Recommendations.[74] According to this report, these recommendations are intended to guide the federal government's strategies with respect to addressing problems of persistent marine debris. Each of the five biennial reports the committee issued subsequent to its initial 2008 report reference the 25 recommendations; the committee has not revisited the recommendations to determine the extent to which any adjustments may be warranted.

[74] The interagency committee submitted this report to relevant congressional committees in response to a requirement in the Marine Debris Research, Prevention, and Reduction Act. Pub. L. No. 109-449, § 5(c)(1), 120 Stat. 3333, 3337 (2006). The report was due by December 22, 2007; the committee submitted the report to the congressional committees in August 2008. This requirement was repealed in 2012. *See* Pub. L. No. 112-213, § 606(a)(1), 126 Stat. 1540, 1577 (2012).

Table 3. Interagency Marine Debris Coordinating Committee 2008 recommendations

Category	Recommendation
Education and outreach	(1) Federal agencies should demonstrate leadership by distributing educational materials to personnel on the sources and impacts of marine debris as well as methods for prevention with the goal of reducing the federal contribution to marine debris. (2) Federal agencies should support public awareness campaigns by providing technical expertise and educational materials and by encouraging private sector participation, when appropriate. These campaigns may target specific threats and audiences to address the diversity of the marine debris issue. (3) Federal agencies should engage and partner with state, local, tribal and nongovernmental entities to support coordinated events, such as Earth Day, the International Coastal Cleanup, and other activities that have relevance to marine debris. These events should include nationwide educational and media outreach efforts to enhance awareness of sources and impacts of marine debris and to provide recommendations regarding specific actions that can be taken to prevent or reduce marine debris.
Legislation, regulation, and policy	(4) The committee should review the findings from the National Academy of Sciences study that will assess the effectiveness of international and national measures to prevent and reduce marine debris and its impacts, and federal agencies should take action, as appropriate.[a] (5) Federal agencies should seek ways to strengthen and enhance their ability to fulfill both regulatory and nonregulatory mandates for marine debris prevention, where appropriate. (6) The committee should coordinate a correspondence group of state, local, and tribal governments to determine the marine debris–related authorities and policies at those levels, including both those that address land-based sources of marine debris and those that address ocean-based sources. The correspondence group will be an important component in the committee's gap analysis of regulatory and nonregulatory authorities that can be used to promote marine debris prevention. (7) Federal agencies, coordinating through the committee, should review existing international policies and strategies regarding marine debris from both land-based and ocean-based sources and develop a white paper outlining possible policies or actions for consideration by the United States. (8) Federal agencies should support voluntary, incentive-based programs that encourage communities to adopt environmentally responsible practices. Examples may include Heal the Bay's "A Day Without a Bag" Program (a southern California nonprofit organization) and the Clean Marina Program, an initiative involving federal agencies and state governments.[b] (9) Federal agencies should work with state, local, tribal, and nongovernmental entities to develop efficient recycling incentive programs for municipalities or appropriate venues.[b] (10) Federal agencies, where appropriate, should evaluate methods by which users of products that contribute significantly to marine debris can be given an incentive to select environmentally friendly alternatives or improve use of recycling infrastructure. Such incentive programs or pilot projects should include regular monitoring and evaluation of their effectiveness.[b] (11) Federal agencies should continue to review enforcement authorities regarding marine debris and items that may become marine debris, enhance the effective use of those authorities as needed and appropriate, and ensure a coordinated approach to enforcement of relevant authorities.[c] (12) In appropriate cases, federal agencies should refer violations of federal law, such as the Act to Prevent Pollution from Ships, Clean Water Act, and Ocean Dumping Act, to the Environment and Natural Resources Division of the U.S. Department of Justice for civil or criminal enforcement action.[c]

Table 3. (Continued)

Category	Recommendation
Cleanup	(13) Federal agencies should work together and contribute to coordinated removal efforts of marine debris and items that can become marine debris in areas under federal jurisdiction, with priority given to heavily impacted areas. (14) Federal agencies should examine how existing programs can be targeted to support difficult marine debris removal efforts. (5) Federal agencies should partner with state, local, tribal, and nongovernmental entities to continue to support and conduct cleanup efforts.
Research and technology development	(16) Federal agencies, coordinating through the committee, should sponsor and conduct research to characterize the nature of marine debris and further investigate reducing, mitigating, preventing, and controlling marine debris and assessing its impacts, with a particular focus on developing cost- benefit analyses for these actions. (17) Federal agencies, cooperating through the committee, should improve efforts to monitor marine debris, including shoreline, floating, and submerged debris, using lessons learned from previous federally funded monitoring efforts. (18) The committee should convene a special session at least once a year to share and discuss the latest research findings on marine debris, with summaries and identified gaps to be passed to the Subcommittee on Integrated Management of Ocean Resources and the Joint Subcommittee on Ocean Science and Technology. (19) Federal agencies, coordinating through the committee, should sponsor and conduct research regarding the attitudes and practices of users of products that contribute to marine debris. In particular, such research should (a) investigate the willingness to alter attitudes and practices in a manner that would reduce marine debris; (b) identify preferences with regard to potential incentive programs and which types of incentives are most likely to produce positive responses; and (c) develop and test incentive programs intended to alter attitudes and/or practices among users of products that contribute to marine debris. (20) Federal agencies should partner with state, local, tribal, and nongovernmental entities to encourage the development of specific technologies that could prevent or reduce the amount of debris entering the marine environment or that could mitigate the impacts of marine debris on navigation, human health and safety, the economy, habitats, and species.[d] (21) Federal agencies should support research, technology development, and use of materials that will not persist in the marine environment.[d]
Coordination	(22) Federal agencies should help sponsor and participate in workshops, conferences, and lectures that address issues related to marine debris and sources of marine debris to encourage the exchange of information that can inform the development of guidelines and implementation of actions to mitigate marine debris impacts. (23) Federal agencies should participate in ongoing international activities to mitigate the impacts and reduce the amount of marine debris. Federal agencies also should support efforts to increase the awareness of such international marine debris efforts and encourage participation of other nations and international organizations in those efforts as well as consider options for new international activities and initiatives to mitigate the impacts and reduce the amount of marine debris. (4) The committee should serve as a central point for coordination of federal efforts to develop new policies, strengthen existing policies, identify new research topics or projects, and address requests from Congress for specific information or actions related to marine debris. (25) Federal agencies should pursue partnerships, as appropriate, with nongovernmental entities to develop, promote, and implement strategies for preventing, reducing, or mitigating the impacts of marine debris.

Source: Interagency Report on Marine Debris Sources, Impacts, Strategies, and Recommendations, 2008. | GAO-19-653.

[a]Tackling Marine Debris in the 21st Century, Committee on the Effectiveness of International and National Measures to Prevent and Reduce Marine Debris and Its Impacts, National Research Council, National Academies Press (Washington, D.C.), 2009.

[b]Categorized as "incentive programs" in the committee's 2008 report. [c]Categorized as "enforcement" in the committee's 2008 report. [d]Categorized as "technology development" in the committee's 2008 report.

APPENDIX III: EXAMPLES OF INTERAGENCY MARINE DEBRIS COORDINATING COMMITTEE MEMBER AGENCIES' ACTIVITIES

The following are examples of activities members of the Interagency Marine Debris Coordinating Committee (interagency committee) reported conducting—often in coordination with nonfederal partners such as nongovernmental organizations, industry, state governments, Indian tribes, and other nations—to address marine debris based on information from the committee's 2016 and 2019 biennial reports and agency documents and interviews.[75] These examples include activities from the categories outlined in the biennial reports: (1) education and outreach; (2) legislation, regulation, and policy; (3) cleanup; (4) research and technology development; and (5) coordination.[76] The examples discussed below do not represent all activities conducted by member agencies, but rather illustrate the nature and type of activities the agencies reported conducting. In addition, the examples include activities from agencies that were identified in the interagency committee's 2014 charter and were included in the committee's most recent biennial reports.[77]

Education and Outreach

Nine of the 11 member agencies reported conducting activities to support education and outreach related to addressing marine debris, such as developing and distributing educational materials, supporting public awareness campaigns, or partnering with or funding state, local, tribal, or nongovernmental education efforts. For example:

- **Online public education**. The Trash Free Waters Program—a program established in the spring of 2013 by the Environmental Protection Agency (EPA) to encourage collaborative actions by public and private stakeholders to prevent trash from entering water— provides information to the public, including online information about actions that can be taken to reduce trash from entering waterways. For example, in 2017, the program produced a series of eight webinars with experts on microplastics with the goal of promoting increased knowledge of the sources, distribution, and impacts of plastics and microplastics in the environment.[78] Additional topics included research on global waste management and

[75] The 2016 and 2019 biennial reports cover activities conducted in 2014-15, and 2016- 2017, respectively. We reviewed each of the five biennial reports issued by the interagency committee between 2010 and 2019, but selected examples of activities from the two most recent biennial reports to include in our report.

[76] The biennial progress reports identified eight categories of activities, but we consolidated activities reported under the "enforcement" and "incentive programs" categories into the "legislation, regulation, and policy" category because of similarities among the activities within these categories. Similarly, for presentation purposes, we consolidated activities reported under the "research" and "technology development" categories into one "research and technology development" category.

[77] These agencies are: the Department of Commerce's NOAA; the Department of Defense's U.S. Army Corps of Engineers and U.S. Navy; the Department of Homeland Security's U.S. Coast Guard; the Department of the Interior's Bureau of Safety and Environmental Enforcement, National Park Service, and U.S. Fish and Wildlife Service; Department of Justice; Department of State; Environmental Protection Agency; and the Marine Mammal Commission.

[78] These webinars are available to the public at no cost, at: https://www.epa.gov/trash-free- waters/trash-free-waters-webinar-series.

mismanagement of plastics, potential replacements for plastic products, and ways to improve the design of materials and products to minimize their environmental impacts.

- **Grants for public awareness projects**. The National Oceanic and Atmospheric Administration's (NOAA) Marine Debris Program awards grants to eligible entities to, among other things, develop projects to educate the public about various aspects of preventing marine debris. For example, in 2014, NOAA awarded one grant to Virginia State's Department of Environmental Quality to develop and implement a social marketing approach to reduce balloon debris. Balloons can end up in streams, rivers, and the oceans where marine animals can ingest the balloons or become entangled by their attachments, causing injury or death. This project aimed to help educate the public about the importance of refraining from releasing balloons in parks or outside schools, churches, wedding venues, or other events where balloons may be common.
- **Sea Partners Program**. Through its Sea Partners Program established in 1994, the U.S. Coast Guard Auxiliary conducts education and outreach to waterway users such as boaters, fishermen, marina operators, marine industry, and the general public with information on protecting the marine environment.[79] For example, its Sayreville, New Jersey unit reaches an annual average audience of about 10,000 people, according to a program document, including youth groups, primary and secondary education science classes, senior citizen groups, and others. Topics presented include an introduction to marine pollution and oil spills and environmental pollution and recreational boating.

Legislation, Regulation, and Policy

Nine member agencies reported conducting activities to identify noncompliance or help ensure compliance with laws and regulations and develop or encourage policies and programs to implement practices that address specific types of marine debris. For example:

- **Notice for offshore oil and gas operators**. In November 2018, the Bureau of Safety and Environmental Enforcement renewed a notice for offshore oil and gas lessees and operators in the Gulf of Mexico that clarifies and provides more detail about marine trash and debris awareness training. Specifically, the notice stated that all offshore employees and contractors active in offshore operations are to complete marine debris awareness training annually. The notice further specifies that lessees and operators are to provide the bureau with an annual report that describes their training process and certifies that the training process was followed.

[79] The U.S. Coast Guard Auxiliary was established pursuant to statute. Its mission is to promote and improve recreational boating safety; provide trained crews and facilities to augment the U.S. Coast Guard and enhance safety and security of our ports, waterways, and coastal regions; and support U.S. Coast Guard operational, administrative, and logistical requirements.

- **Criminal enforcement of environmental laws**. The Department of Justice prosecuted two shipping companies in 2017 for, among other things, falsifying records regarding disposal of garbage from a ship, in violation of the Act to Prevent Pollution from Ships.[80] Specifically, the ship's crew was instructed to throw plastic garbage bags filled with metal and incinerator ash overboard without recording the incidents in the ship's record book. The companies pled guilty and were, among other things, sentenced to pay a $1.5 million fine and make a $400,000 community service payment.[81]
- **Policies for financing waste management infrastructure in Asia**. The Department of State helped convene a meeting in Japan in 2016, under the Asia-Pacific Economic Cooperation framework, to discuss policy changes needed to overcome barriers to financing waste management infrastructure in the Asia-Pacific region to prevent and reduce debris from entering the marine environment.[82] The meeting brought together government officials from the economic cooperation, representatives from industry, international financial institutions, and experts. Ministers of the economic cooperation endorsed nine recommendations developed at the meeting.[83] State Department officials said they have continued to work with Asian governments, industry, and nongovernmental organizations to encourage policy changes and spur financial support for increasing waste management infrastructure and addressing land-based sources of plastic and in Asian countries. For example, at a 2017 meeting on waste management, State Department officials informed Asia-Pacific Economic Cooperation officials of the social and economic impacts of marine debris resulting from mismanaged waste in the region. Officials also said they used the meeting to connect economic cooperation officials with private sector stakeholders to encourage policy changes intended to enable private investment in waste management.

Cleanup

Eight of the 11 member agencies reported conducting a variety of activities to support the removal and disposal of marine debris, often in partnership with others, such as state governments. For example:

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[80] *United States v. Thome Ship Management Pte, Ltd and Egyptian Tanker Company*, No. 17-cr-00075 (E.D. Tex.).

[81] The community service payment was made to the National Fish and Wildlife Foundation, a nonprofit conservation organization that awards grants for the protection and restoration of the nation's fish, wildlife, plants and habitats from monies arising from legal and regulatory actions involving natural resources and the environment.

[82] The Asia-Pacific Economic Cooperation is a regional economic forum established in 1989 to leverage the growing interdependence of the Asia-Pacific region. Consisting of 21 member countries, its aim is to create greater prosperity for the people of the region by promoting balanced, inclusive, sustainable, innovative and secure growth and accelerate regional economic integration.

[83] Meeting participants developed nine recommendations, including setting waste management targets at economy-wide and municipal levels as well as building waste management performance indicators and a methodology to track progress against economy-wide and municipal waste targets, maintain an economy-wide waste database, and encourage and acknowledge frontrunner cities for their overall waste and sanitation achievement through competitive award and certification.

- **Debris removal grants**. In 2016 and 2017, NOAA's Marine Debris Program awarded $2.4 million in grants to 25 entities such as state and tribal governments in 17 coastal states and U.S. territories for projects including community cleanups, crab trap recovery, and derelict vessel removal. For example, in September 2017, the program awarded a grant to the Makah Indian Tribe to remove three sunken vessels from the Makah Marina within the Makah Tribe Indian Reservation on Washington's Olympic Peninsula.
- **National Park cleanup**. National Park Service staff conducted coastal cleanups across the various regions of the National Park System during 2016 and 2017. For example, in fiscal year 2017, park officials from Biscayne National Park, located off the coast of Southern Florida and comprised mostly of water, partnered with the Coastal Cleanup Corporation, a nonprofit organization, to organize 252 volunteers in removing 14,000 pounds of debris from the park.
- **Maintaining navigation channels**. The U.S. Army Corps of Engineers has authority to remove accumulated snags, obstructions, and other debris located in or adjacent to federally-maintained navigation channels. The Corps' operations and maintenance appropriation is available to pay for the removal of obstructions to navigation, and the Corps is sometimes directed to use this appropriation for drift removal. For instance, in fiscal year 2018, the explanatory statement accompanying the Corps' annual appropriation directed the Corps to use about $9.9 million of its appropriation for drift removal in New York Harbor. Debris the Corps removes typically consists of lumber, trees and branches, large waste items like tires, and large plastic items, according to Corps' officials.

Research and Technology Development

Five of the 11 member agencies reported coordinating activities to conduct or sponsor research to monitor, understand the sources of, prevent, mitigate, or reduce the effects of marine debris or to support developing new technologies such as using more sustainable or recyclable types of materials. For example:

- **Research grants**. Since 2006, NOAA's Marine Debris Program has supported at least two marine debris research projects that address questions such as monitoring marine debris, identifying fishing gear improvements and alternatives, or better understanding the environmental or economic impacts of marine debris. For example, in 2016, NOAA awarded a contract to a private research and consulting firm to conduct an economic study on how marine debris affects the economies of tourism-dependent coastal communities around the United States. The purpose of the project was to evaluate changes in tourism spending based on changes in the amount of marine debris to help prioritize areas of the United States where future prevention and removal efforts may be needed. NOAA officials said they expect the final report to be issued by the end of 2019.

- **Microplastics workshop**. In June 2017, EPA hosted a Microplastics Experts Workshop that convened experts from academia and other federal agencies, including NOAA, the U.S. Geological Survey, and the Food and Drug Administration, to identify microplastics research needs. The effort resulted in a 2018 report that identified four main areas where additional research is needed: (1) standardization of research methods, (2) debris sources and fate, (3) ecological risk assessment, and (4) human health risk assessment. EPA is using the report to consider how the agency can best address these high- priority microplastics research needs as it develops the agency's larger environmental research agenda, according to EPA officials.
- **Development of new fishing gear**. In 2016, the Marine Mammal Commission awarded a grant to the New England Aquarium to test a ropeless fishing gear prototype intended to prevent whale entanglements in fishing gear. According to a document from the Commission, entanglement in fishing gear is the number one direct cause of marine mammal injury and death, including the endangered Northern Atlantic right whale.[84] The Commission has used the results of this effort to emphasize the potential for ropeless gear to reduce and prevent entanglement in meetings with lobster and crab fishermen on the east and west coasts.

Coordination

Seven of the 11 member agencies reported conducting a variety of activities to foster coordination among member agencies and with nonfederal partners, such as international, state, and local government agencies. For example:

- **Global Partnership on Marine Litter**. In 2012, the United Nations launched the Global Partnership on Marine Litter, a voluntary network of international governments, nongovernmental organizations, academia, private sector companies, and others with the goal of protecting human health and the global environment primarily by reducing and managing marine debris.[85] Interagency committee members, including NOAA and EPA, are partners to the global partnership. For example, from 2012 through 2017, the NOAA Marine Debris Program Director served as the Steering Committee chair of the global partnership. EPA has coordinated with the global partnership in Latin American and Caribbean countries to help develop a regional strategy for addressing marine debris in those regions and

[84] Fishing gear entanglements cause the majority of right whale deaths and also contribute to declining calving rates through the prolonged health effects of nonlethal entanglements, according to a report from the Marine Mammal Commission.

[85] Specifically, following recommendations made in the Manila Declaration on Furthering the Implementation of the Global Programme of Action for the Protection of the Marine Environment from Land-Based Activities issued in January 2012, the Global Partnership on Marine Litter was launched in June 2012 at the United Nations Conference on Sustainable Development (Rio + 20) in Brazil. Among others, objectives of the partnership include reducing the impacts of marine litter worldwide on economies, ecosystems, animal welfare and human health, and enhancing international cooperation and coordination through the promotion and implementation of the Honolulu Strategy, a global framework for the prevention and management of marine debris that was developed after the Fifth International Marine Debris Conference in 2011.

through in-person meetings and with other global partnership staff and NOAA colleagues through the steering committee.

- **Sister Cities initiative**. In 2015, the State Department announced the creation of a "Sister Cities" initiative with China to share best practices related to waste management and preventing marine debris. As part of the initiative, in November 2016, a Chinese delegation, comprised of central government officials and officials from Weihai and Xiamen, visited Chicago, New York City, and San Francisco to study U.S. practices in addressing marine debris. In November–December 2017, a U.S. delegation comprised of U.S. government officials and a New York City official, visited Xiamen, Weihai, and Beijing to learn about Chinese waste management practices. The partner city relationships were formalized with a memorandum of understanding between San Francisco and Xiamen in July 2016, and New York and Weihai in December 2017 to work together to address marine debris.
- **State emergency response guides and regional action plans**. NOAA's Marine Debris Program has coordinated with coastal managers, nongovernmental organizations, industry, academia, and other groups to develop state marine debris emergency response guides. For example, in 2016 and 2017, NOAA coordinated with Florida, Georgia, Mississippi, North Carolina, and South Carolina to develop individual guides for those states. According to NOAA officials, federal, state, and local officials used the Florida response guide during the 2017 and 2018 hurricane seasons to inform responding agencies which agency has jurisdiction and to better coordinate marine debris removal efforts after an event. In addition, NOAA coordinated efforts to develop, enhance, and implement regional action plans for the Great Lakes, the Gulf of Maine, the Gulf of Mexico, the Mid-Atlantic, the Southeast, California, Florida, Hawaii, Oregon, and Washington regions. The purpose of the action plans is to bring stakeholders together to prevent and reduce marine debris throughout the United States, according to NOAA documents. For example, NOAA officials said that under the Hawaii action plan, several federal agencies and nongovernmental organizations worked together to purchase and maintain bins to collect used fishing line for recycling.

APPENDIX IV: COMMENTS FROM THE DEPARTMENT OF COMMERCE

UNITED STATES DEPARTMENT OF COMMERCE
The Secretary of Commerce
Washington, D.C. 20230

September 18, 2019

Ms. Anne-Marie Fennell
Director
Natural Resources and Environment
U.S. Government Accountability Office
441 G Street, NW
Washington, DC 20548

Dear Ms. Fennell:

Thank you for the opportunity to review and comment on the Government Accountability Office's (GAO) draft report entitled *Marine Debris: Interagency Committee Members Are Taking Action, But Additional Steps Could Enhance the Federal Response* (GAO-19-653).

The Department of Commerce agrees with GAO's four recommendations regarding the National Oceanic and Atmospheric Administration and the Chair of the Interagency Marine Debris Coordinating Committee. Enclosed is our response and recommended technical changes to the draft report.

Should you have any further questions, please contact MaryAnn Mausser, GAO Liaison, at (202) 482-8120 or MMausser@doc.gov.

Sincerely,

Wilbur Ross

Wilbur Ross

Enclosure

Department of Commerce
National Oceanic and Atmospheric Administration
Response to the GAO Draft Report Entitled
Marine Debris: Interagency Committee Members Are Taking Action, But Additional Steps Could Enhance the Federal Response
(GAO-19-653, September 2019)

General Comments

The Department of Commerce's National Oceanic and Atmospheric Administration (NOAA) appreciates the opportunity to review the Government Accountability Office's (GAO) report on the Interagency Marine Debris Coordinating Committee (IMDCC) and Federal efforts to address marine debris. The report's analysis of IMDCC's current operations and the obligations of IMDCC and member agencies under the Marine Debris Act (33 U.S.C. § 1954) presents and analyzes the Federal Government's actions to fulfill this Congressional mandate.

NOAA Response to GAO Recommendations

The draft GAO report made four recommendations:

Recommendation 1: "The NOAA Administrator, in coordination with interagency committee member agencies, should establish a time frame for documenting the committee's membership process."

NOAA Response: NOAA agrees with this recommendation. The NOAA Administrator will establish a time frame for documenting the IMDCC's membership process that provides opportunities for both IMDCC and NOAA-based input. The Marine Debris Act, as amended by the Save Our Seas Act of 2018, requires six named agencies to participate in IMDCC. In addition to these agencies, IMDCC shall include such other Federal agency members that have an "…interest in ocean issues or water pollution prevention and control…" as the Secretary of Commerce determines appropriate. NOAA, in consultation with IMDCC, will determine objective criteria for evaluating Federal agency interest and identify other factors to consider as part of the membership process. NOAA is continuing to formalize and document the committee's membership process.

Recommendation 2: "The NOAA Administrator, in coordination with interagency committee member agencies, should clarify what is meant by "senior official" in the Marine Debris Act, such as through revisions to its charter."

NOAA Response: NOAA agrees with this recommendation. NOAA will work with IMDCC to revise the charter, which was last updated in 2014. As part of this process, NOAA, in coordination with IMDCC members, will define the term "senior official" so that the definition can be consistently applied across all Federal agency structures. In forming its definition, NOAA will consider seniority requirements of similarly situated advisory committees, along with related factors, such as the ability to make decisions on behalf of an agency and general awareness of an agency's available resources.

Recommendation 3: "The Chair of the interagency committee, in coordination with member agencies, should develop and implement a process to analyze the effectiveness of the interagency committee's recommendations and strategies, and include the results in its biennial reports."

NOAA Response: NOAA agrees with this recommendation to the extent it can be implemented using available budgetary resources. IMDCC is primarily a coordinating body focused on information sharing, and its biennial report highlights agency activities implementing the 2008 report recommendations (including 25 recommendations organized into eight overarching topics). However, IMDCC itself lacks the existing resources to require and routinely evaluate the effectiveness of these activities. Instead, individual agencies are expected to work toward implementing the 2008 report recommendations in accordance with each agency's legal and programmatic authorities, mission priorities, and resource limitations. To the extent possible, NOAA will work with IMDCC members to identify common or easily translatable metrics for evaluating the effectiveness of the 2008 recommendations and implement these in the next biennial report to Congress.

Recommendation 4: "The Chair of the interagency committee, in coordination with member agencies, should develop a process to identify recommendations for priority funding needs to address marine debris, and include such recommendations in its biennial reports."

NOAA Response: NOAA agrees with this recommendation, but notes that NOAA does not have the authority to control the implementation of a process for identifying priority funding needs of other member agencies. NOAA believes that IMDCC's recommendations for priority funding needs are already reflected in the President's annual budget request and operating plan for each member agency. To the extent possible, NOAA will work with IMDCC members to develop a process for identifying priority areas, which can be reflected in each agency's respective budgeting process and shared in IMDCC's biennial reports.

APPENDIX V: COMMENTS FROM THE U.S. AGENCY FOR INTERNATIONAL DEVELOPMENT

Anne-Marie Fennell
Director
U.S. Government Accountability Office
441 G Street, N.W.
Washington, D.C. 20226

Re: Marine Debris: Interagency Committee Members Are Taking Action, but Additional Steps Could Enhance the Federal Response (GAO-19-653)

Dear Ms. Fennell:

I am pleased to provide the formal response of the U.S. Agency for International Development (USAID) to the draft report produced by the U.S. Government Accountability Office (GAO) titled, *Marine Debris: Interagency Committee Members Are Taking Action, but Additional Steps Could Enhance the Federal Response* (GAO-19-653).

USAID remains committed to addressing the challenge of marine debris through our programs, in collaboration with interagency partners. Given the global nature of the problem, USAID has significant opportunities to play an important role in the international response, and is already doing so by providing technical assistance on the international titles of the draft "Save Our Seas 2.0" legislation. Through programs such as the Municipal-Waste Recycling Program, a partnership with Circulate Capital, and the new Clean Cities Blue Ocean initiative, USAID continues help developing countries manage these challenges.

I am transmitting this letter and the enclosed comments from USAID for inclusion in the GAO's final report. Thank you for the opportunity to respond to the draft report, and for the courtesies extended by your staff while conducting this engagement. We appreciate the opportunity to participate in the complete and thorough evaluation of our efforts to combat marine debris.

Sincerely,

Frederick Nutt
Assistant Administrator
Bureau for Management

Enclosure: a/s

COMMENTS BY THE U.S. AGENCY FOR INTERNATIONAL DEVELOPMENT ON THE DRAFT REPORT PRODUCED BY THE U.S. GOVERNMENT ACCOUNTABILITY OFFICE (GAO) TITLED, MARINE DEBRIS: Interagency Committee Members Are Taking Action, but Additional Steps Could Enhance the Federal Response (GAO-19-653)

The U.S. Agency for International Development (USAID) would like to thank the U.S. Government Accountability Office (GAO) for the opportunity to respond to this draft report. We appreciate the extensive work of the GAO's engagement team.

USAID appreciates the GAO's recognition of the challenge posed by marine debris and the incorporation of Agency's input on this important topic. In particular, USAID appreciates that the GAO's final report acknowledges the importance of municipal waste as a significant source of marine plastic pollution (p. 1), as well as recognizes USAID's participation in the Interagency Marine Debris Coordinating Committee.

As noted in the *Science* article cited in Footnote 3 on page one, the majority of plastic ocean debris comes from mismanaged municipal waste in the developing world, where waste-management systems, infrastructure, and governments struggle to keep pace with increasing amounts of trash from growing urban populations in riverine and coastal areas. As the lead Federal Agency on foreign assistance, USAID has several programs that target this aspect of the problem:

- Municipal-Waste Recycling Program (2016–2021): Reduces land-based sources of ocean plastic waste in four of the top five contributing countries—Indonesia, The Philippines, Sri Lanka, and Vietnam—by providing small grants and technical assistance to a variety of local actors in towns and cities.
- Partnership Circulate Capital Partnership: In June 2019, USAID's leadership launched a partnership, managed by Circulate Capital and funded by multinational companies, that leverages more than $100 million to incentivize private investment in the recycling value-chain in South and Southeast Asia.
- Clean Cities, Blue Ocean: USAID recently awarded a new $50 million contract to TetraTech to implement a program aimed at preventing land-based sources of ocean plastic pollution. The program will build capacity and commitment for the "3Rs"—reducing, reusing, recycling—and solid-waste management in urban and peri-urban settings, particularly in riverine and coastal areas.

USAID greatly appreciates the work of its interagency partners that are engaged in addressing marine debris, and looks forward to continued collaboration with them.

In: Encyclopedia of Marine Biology (12 Volume Set) ISBN: 978-1-53619-529-3
Editors: Steffen Fischer and Jonas Abend © 2021 Nova Science Publishers, Inc.

Chapter 111

THE EFFECTS OF MARINE DEBRIS ON BEACH RECREATION AND REGIONAL ECONOMIES IN FOUR COASTAL COMMUNITIES: A REGIONAL PILOT STUDY*

National Oceanic and Atmospheric Administration
Marine Debris Division

EXECUTIVE SUMMARY

Marine debris is a persistent problem in many coastal areas of the United States. There are a variety of potential economic losses associated with marine debris, including effects on commercial fisheries, effects on waterfront property values, costs incurred by local governments and volunteer organizations to remove and dispose of marine debris, and more general "existence" values reflecting the public's preference for a clean environment. This study evaluates two types of economic loss that result from the effects of marine debris on beach recreation: the loss of recreational value to beach visitors, and the regional economic impact from reduced spending on beach visits in a particular region.

The goal of this study was to better understand the economic effects of changes in the amount of debris on beaches. The results may help federal, state, and local agencies structure future debris abatement and mitigation projects to maximize social benefits provided by coastal resources. To address these goals, we collected data from four coastal areas in the United States: Gulf Coast beaches in Alabama, Atlantic Ocean beaches in Delaware and Maryland, Lake Erie beaches in Ohio, and Pacific Ocean beaches in Orange County, California.

* This is an edited, reformatted and augmented version of National Oceanic and Atmospheric Administration Marine Debris Division, Abt Associates, 14553, dated July 2019.

Recreational Value

Recreational value is a monetary measure of the enjoyment people get from participating in beach recreation, or their "willingness to pay" for recreation and clean beaches.

We estimated the effect of marine debris using two different economic concepts: *the value of recreation and the economic impacts of recreation*. The value of recreation is a monetary measure of the enjoyment people get from participating in beach recreation. It can also be described as people's willingness to pay for recreational access to beaches, or for policies that improve beach recreation. The economic impact of recreation is a measure of the effect of beach recreation on spending by consumers and businesses in the region. It includes both direct spending on recreational activities and the effects of direct spending in stimulating the local economy. Because spending in some regions may increase as a result of a decrease in spending in other regions, amounts calculated for different regions should not be added together. Because they are interpreted in fundamentally different ways, economic impacts should also not be added together with estimates of recreation value.

Economic Impacts of Recreation

Economic impacts of recreation measure the effect of beach recreation on spending by consumers and businesses in the local economy.

We measured recreational value and economic impacts for two hypothetical scenarios involving marine debris on beaches: a *reduction in debris to almost none* (defined in the study as one piece of debris per 500 square feet of beach), and a *doubling of debris*.

Study Design

This study evaluated the relationship between marine debris and recreational beach use by recruiting participants at beaches in the four selected coastal areas. Those willing to participate in the study were sent a mail-in survey with questions about their beach recreation, their opinions about marine debris, and how their recreation would change if there were different amounts of debris on beaches.

The data from the surveys were used to estimate the total effect of changes in marine debris on the number of beach visits in each area. These estimates in turn were used in a nationwide recreation model originally developed during the natural resource damage assessment for the *Deepwater Horizon* oil spill (English et al., 2018) to estimate the change in recreational value from the hypothetical changes in debris. In addition, the estimates of changes in visits were incorporated into a regional input/output model to determine the regional economic impacts of marine debris (Figure ES-1).

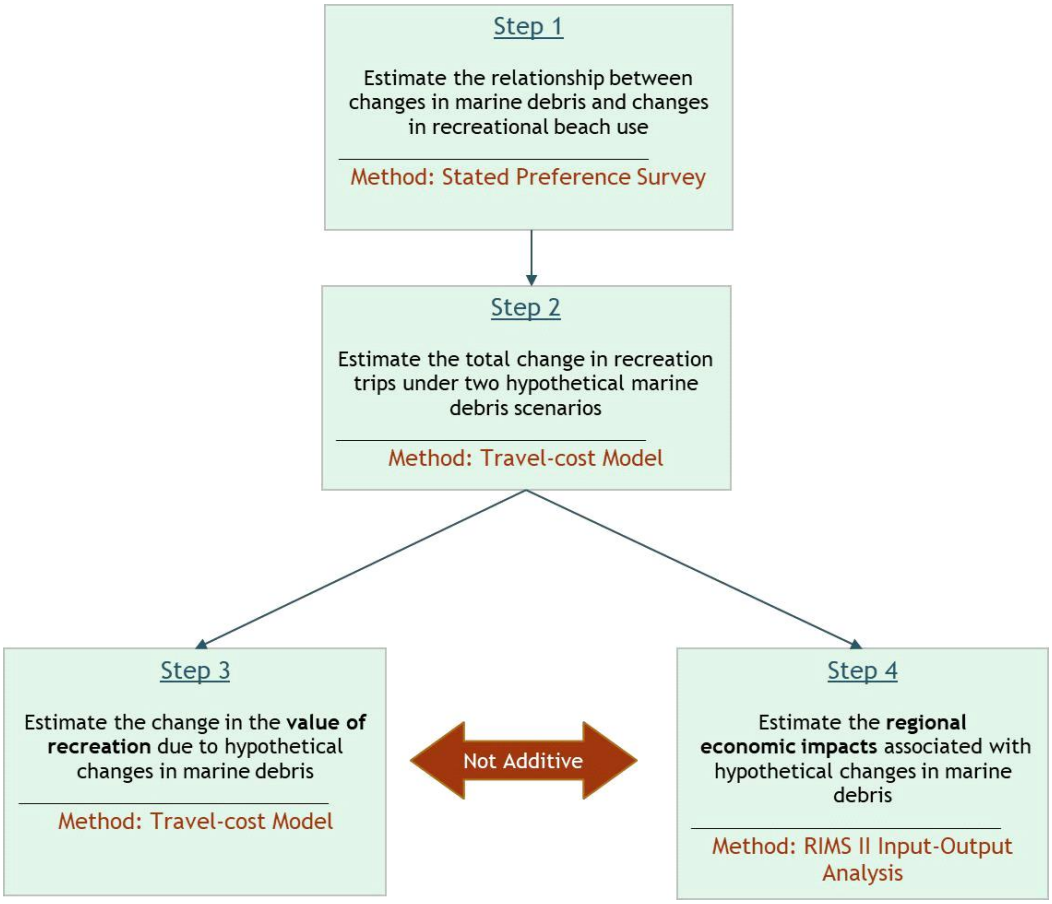
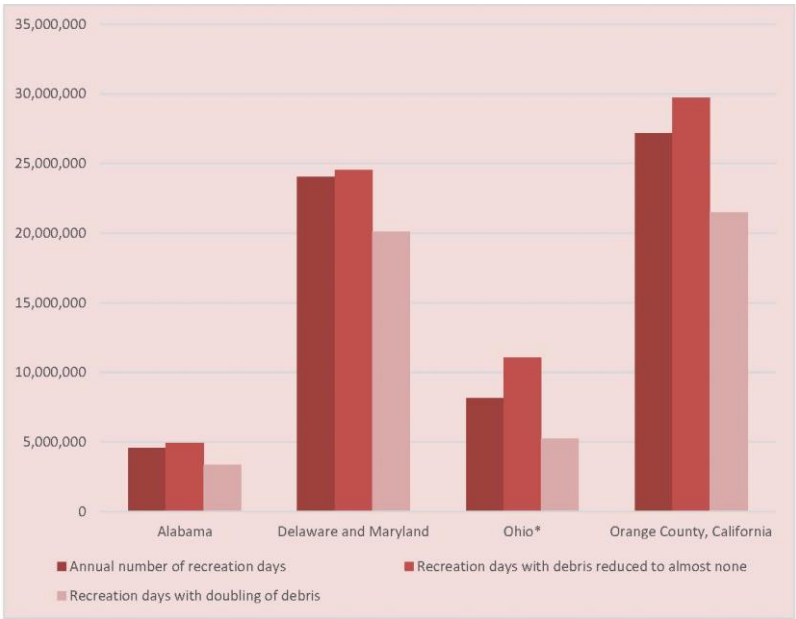


Figure ES-1. Steps for estimating changes in recreational value and regional economic impacts associated with changes in marine debris on beaches.

Survey Results

The results of the mail survey indicate a potentially strong relationship between marine debris and beach recreation. The estimated effect of a reduction in debris to almost none is an increase in recreation days of between 2.2% and 9.5% for the three ocean coasts, and in increase of 35.4% in Ohio.[1] The increase in the number of beach visits ranged from 369,000 visitor days per year in Alabama to 2.9 million visitor days per year in Ohio (Figure ES-2). A doubling of debris would result in an estimated decrease in recreation days of between 16.3% and 26.5% for the three ocean coasts, and a decrease of 35.6% in Ohio. The decrease in the number of beach visits ranged from 1.2 million visitor days per year in Alabama to 5.7 million visitor days per year in Orange County.

[1] Numbers in the text and tables of this report have been rounded for presentation. Calculations performed on these rounded numbers may not reproduce final results.



* Ohio estimates account for multiple-day trips only.

Figure ES-2. Changes in the annual number of recreation days if the amount of marine debris is reduced to almost none, and if the amount of marine debris doubles.

Recreational Value

The change in recreational value is calculated based on the change in the number of recreation days. For example, the nationwide recreation model from the *Deepwater Horizon* research estimates the annual number of recreation days at Alabama beaches is 4.55 million. Based on the results of our survey, if marine debris at those beaches were reduced to almost none, recreation days in Alabama would increase by 8.1%, for an estimated increase of approximately 369,000 recreation days. Based on the nationwide recreation model, recreators value each day of recreation at Alabama beaches at $27.27. Thus if the a reduction in debris to almost none results in an additional 369,000 days of recreation, the resulting change in the value of recreation is estimated to be $10.1 million ($27.27 per day x 369,000 days). Estimates of recreation value for all four study areas are summarized in Table ES-1. For Alabama, Delaware/Maryland, and Orange County, California, the results account for trips of all lengths, including single-day and multiple-day trips. For Ohio, the national recreation model was able to provide results for multiple-day trips only.

Our results indicate that changes to recreation associated with marine debris provide substantial value to recreators. If marine debris were reduced to almost none, the estimated annual increase in recreation value is $10.1 million in Alabama, $19.8 million in Delaware/Maryland, $88.0 million in Ohio (multiple-day trips only), and $129.7 million in Orange County, California (Table ES-1). If the amount of marine debris on beaches were to double, the estimated annual decrease in recreational value is $32.3 million in Alabama, $140.9 million in Delaware/ Maryland, $83.9 million in Ohio (multiple-day trips only), and $275.1 million in Orange County, California.

Table ES-1. Recreation value from changes in debris on beaches (2018 dollars)

Scenario	Alabama	Delaware and Maryland	Ohio[a]	Orange County, California
Annual number of recreation days	4,552,112	24,014,592	8,155,158	27,143,415
Debris reduced to "almost none"				
Percent change in recreation days	8.1%	2.2%	35.4%	9.5%
Change in days	368,525	536,341	2,889,191	2,571,725
Value per day	$27.27	$36.81	$30.46	$50.43
Change in recreation value	$10,051,517	$19,741,209	$88,006,606	$129,689,616
Doubling of marine debris				
Percent change in recreation days	-26.5%	-16.3%	-35.6%	-20.9%
Change in days	-1,206,006	-3,915,792	-2,907,188	-5,682,362
Value per day	$26.82	$35.99	$28.87	$48.41
Change in recreation value	-$32,347,029	-$140,914,688	-$83,935,614	-$275,077,340

a. For Ohio, estimates account only for multiple-day trips and exclude the value and quantity of single-day trips.

Regional Economic Impacts

As noted previously, changes in recreational beach visits because of changes in marine debris quantities would have cascading economic impacts on regional economics. We expressed the regional economic impacts using two key metrics:

- *Value added*: The value of gross output less intermediate inputs. The value of this metric is equal to the sum of compensation of employees, taxes on production and imports less subsidies, and gross operating surplus.
- *Employment*: Number of full- and part-time jobs (including proprietors' jobs).

We calculate that a reduction of debris to almost none would contribute an additional $29 million in economic activity (measured as value added) in Alabama, $27.8 million in Delaware and Maryland; $206.0 million in Ohio, and $137.8 million in Orange County, California (Table ES-2). This economic activity is estimated to provide between 464 and 3,703 jobs. Conversely, a doubling of debris is estimated to cost the local economies $96.3 million in Alabama, $203.2 million in Delaware and Maryland, $207.3 million in Ohio, and $304.5 million in Orange County, California; resulting in a loss of between 2,198 and 4,254 jobs (Table ES-2).

In summary, the results of this study indicate that the amount of marine debris on beaches have a substantial effect on recreational value and regional economies, outside of the costs municipalities incur to remove debris. Beachgoers surveyed indicated that they would increase their beach visits somewhat if marine debris were eliminated, and they would decrease beach visits substantially if the amount of marine debris on the beach were to double. These results can be used by policy and program evaluators to help understand how programs aimed at reducing marine debris levels can provide both significant value to recreators and contributions to the regional economy.

Table ES-2. Regional economic impacts of changes to debris levels at the four study areas (2018 dollars)

	Alabama	Delaware and Maryland	Lake Erie beaches in Ohio	Orange County, California
Average visitor spending per day	$112.40	$72.74	$76.72	$89.49
Reduce debris to almost none				
Change in visitor days	308,365	478,410	2,823,268	2,092,920
Change in visitor spending	$34,660,000	$34,802,000	$216,593,554	$187,294,000
Change in jobs	672	464	3,703	1,925
Change in economic activity (value added)	$29,423,000	$27,834,000	$205,976,434	$137,830,000
Doubling of debris				
Change in visitor days	-1,009,130	-3,492,845	-2,840,854	-4,624,417
Change in visitor spending	-$113,427,000	-$254,086,000	-$217,943,258	-$413,837,000
Change in jobs	-2,198	-3,386	-3,726	-4,254
Change in economic activity (value added)	-$96,288,000	-$203,211,000	-$207,259,895	-$304,542,000

1. INTRODUCTION

Marine debris is a persistent problem in many coastal areas of the United States. There are a variety of potential economic losses associated with marine debris, including effects on commercial fisheries, effects on waterfront property values, costs incurred by local governments and volunteer organizations to remove and dispose of marine debris, and more general "existence" values reflecting the public's preference for a clean environment. This study evaluates two types of economic loss that result from the effects of marine debris on beach recreation: the loss of recreational value to beach visitors, and the regional economic impact from reduced spending on beach visits in a particular region.

Research suggests that litter on beaches detracts from visitors' enjoyment and reduces the amount and value of recreation on coastal beaches (Ofiara and Brown, 1999; Brouwer et al., 2017; Krelling et al., 2017; Leggett et al., 2018). Marine debris may reduce the likelihood that people return to the same location, particularly among first-time visitors (Ballance et al., 2000; Schuhmann, 2012). Effects on beach recreation have implications for regional economies because tourism and spending by beach visitors is significant in many coastal communities (Kosaka and Steinback, 2018; Office for Coastal Management, 2019).

Visitors may perceive a decline in the natural beauty of an area if marine debris is present. Visitors may also perceive potential physical harm due to cuts or bacterial infections, which would have economic costs in terms of medical expenses and lost wages if such harm were to occur (Campbell et al., 2016). In contrast to debris or litter along the roadside or in parks, there is a high potential for dermal contact with marine debris on beaches as visitors frequently go barefoot, lie directly on the sand, and dig in the sand. The existence of numerous volunteer efforts to remove debris from beaches (Zielinski et al., 2019) and the fact

that many municipalities regularly rake beaches to remove debris are also indications that beach visitors prefer cleaner beaches.

Recreational Value

Recreational value is a monetary measure of the enjoyment people get from participating in beach recreation, or their "willingness to pay" for recreation and clean beaches.

This study was designed to determine how the quantity of marine debris on beaches affects the number of days that recreators will visit the beach, and how those changes in behavior translate to lost recreational value and regional economic impact. The goal of this study was to better understand the economic effects of changes in the amount of debris on beaches. The results may help federal, state, and local agencies structure future debris abatement and mitigation projects to maximize social benefits provided by coastal resources. To address these goals, we collected data from four coastal areas in the United States: Gulf Coast beaches in Alabama; Atlantic Ocean beaches in Delaware and Maryland; Lake Erie beaches in Ohio; and Pacific Ocean beaches in Orange County, California (Figure 1).

Economic Impacts of Recreation

Economic impacts of recreation measure the effect of beach recreation on spending by consumers and businesses in the local economy.

We estimated the effect of marine debris using two different economic concepts: the *value of recreation and the economic impacts of recreation*. The value of recreation is a monetary measure of the enjoyment people get from participating in beach recreation. It can also be described as people's willingness to pay for recreational access to beaches, or for policies that improve beach recreation. The economic impact of recreation is a measure of the effect of beach recreation on spending by consumers and businesses in the region. It includes both direct spending on recreational activities and the effects of direct spending in stimulating the local economy. Because spending in some regions may increase as a result of a decrease in spending in other regions, amounts calculated for different regions should not be added together. Because they are interpreted in fundamentally different ways, economic impacts should also not be added together with estimates of recreation value.[22] We measured these effects for two hypothetical scenarios of marine debris levels: a *reduction of debris to almost none* (defined in the study as one piece of debris per 500 square feet of beach), and a *doubling of debris*.

The first step in this study was an evaluation of the relationship between marine debris and recreational beach use using a survey. The data from the survey were used to estimate how changes in marine debris would influence the number of beach trips that recreators would take. These data in turn were used in a nationwide recreation model developed during the *Deepwater Horizon* oil spill (English et al., 2018) to estimate the value of recreation. In

[2] For additional information, Stynes (2005) provides a review of concepts and methods for estimating the economic significance of recreational uses of public lands and provides a distinction between valuation and impact studies, aimed at non-economists, and Rosenberger et al. (2017) provides an overview of recreation valuation.

addition, the data on lost trips were incorporated into a regional input/output model to determine the regional economic impact of marine debris (Figure 2).

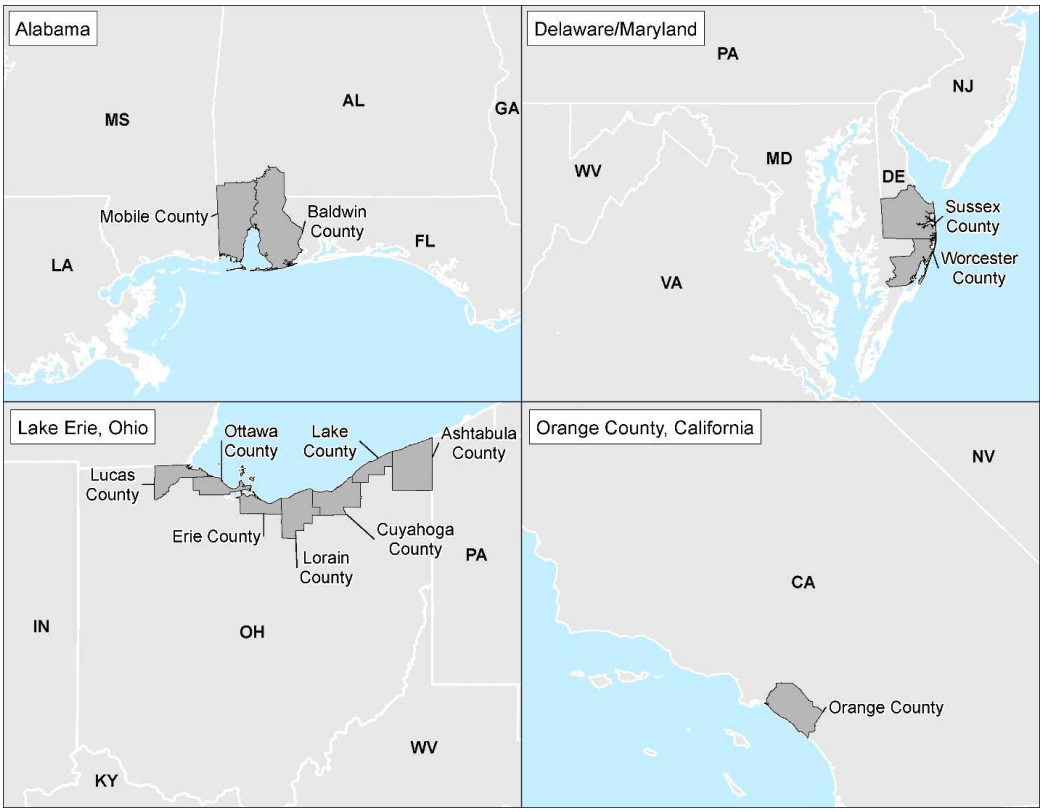


Figure 1. Study areas that define where beachgoers were interviewed and where regional economic impacts were evaluated.

As will be explained in subsequent sections, the scope of the recreational value study includes almost all beach recreation by residents from throughout the United States, occurring at beaches in each of the four study areas. For the assessment of regional economic impacts, the scope of the study includes the regional economies of the coastal counties where the beaches in each study area are located. Each study area was delineated to match a group of beaches that was aggregated into a single regional destination in data developed for the *Deepwater Horizon* oil spill assessment (English et al., 2018). The *Deepwater Horizon* dataset, collected from June 2012 to May 2013, is the basis for the nationwide recreation model.

The survey utilized stated preference techniques, an economic valuation method in which survey respondents are presented with hypothetical choice scenarios and asked what they would do in each scenario. When the choices involve changes in recreation behavior, the particular stated-preference technique is called "contingent behavior." In our study, respondents were asked how many more or fewer recreation trips they would take to beaches in a given study area if levels of marine debris decreased or increased. While stated-preference surveys have a long history of use in economics, it is worth noting the potential uncertainty in stated-preference methods because what people say they would do may not

always reflect what they would actually do. Studies examining the accuracy of contingent behavior methods include Haener et al. (2001), Grijalva et al. (2002), and Morgan and Huth (2011).

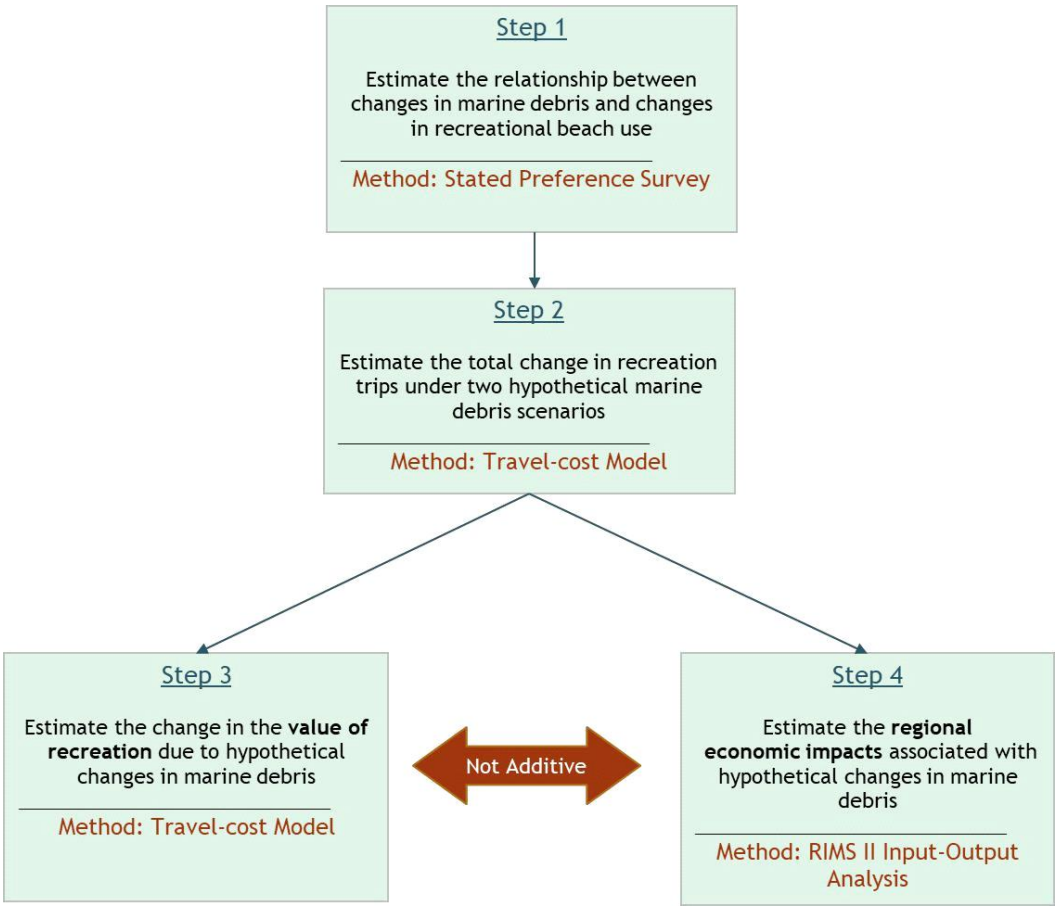


Figure 2. Steps for estimating changes in recreational value and regional economic impacts associated with changes in marine debris on beaches.

For this study, estimating recreational value and regional economic impacts required benefit function transfer, a way of taking economic data and analysis developed for one purpose and revising it to be applied to a new research problem. We adapted recreation data and analyses developed for the *Deepwater Horizon* oil spill assessment (English et al., 2018) to evaluate recreation changes in our four study areas, including estimating the public's value for changes in marine debris in monetary terms. Finally, we analyzed regional economic impacts using the Regional Input-Output Modeling System (RIMS II) developed and maintained by the U.S. Bureau of Economic Analysis (U.S. BEA, 2018, 2019).

Below we describe the key steps of the study, grouped into the three major components: the marine debris survey, the model of recreation value, and the economic impact analysis.

1.1. Marine Debris Survey

The marine debris survey (Section 2) was a mail survey where previously identified beachgoers were asked a series of questions about how marine debris affects their behavior. Specifically, the surveys involved the following steps:

- Interviews conducted onsite at beaches in each study area to collect information about the recreation trip the respondent was taking at the time of the interview, the respondent's demographic characteristics, and the respondent's address for use in a follow-up mail survey
- Implementation of a mail survey that asked respondents about their recreation activities at beaches in the study area during the previous year, the amount of debris they have seen on those beaches (ranking debris levels on a 1-to-5 scale, where 1 is almost no debris and 5 is a "high amount" of debris), and their response to hypothetical changes in the amount of debris on those beaches
- Development of onsite and mail-survey sampling weights that accounted for each respondent's likelihood of being selected into the sample, to help ensure that the opinions of sampled respondents accurately represent all beachgoers
- Analyses comparing respondents' demographic characteristics with their answers to the hypothetical debris scenarios to identify key characteristics that most influence preferences for marine debris
- Adjustment of mail-survey sampling weights such that mail survey respondents match the much larger sample of onsite respondents with respect to the key demographic characteristics, further improving the representativeness of mail survey respondents
- Calculation of the impact of potential changes in marine debris, including a reduction to almost no debris and a doubling of debris, on the number of recreations trips in each study area.

1.2. Nationwide Recreation Model

The key result from the marine debris survey is the estimated percentage change in the number of trips to each study area resulting from the two debris scenarios (reduction to almost zero debris and doubling of debris). The percentage changes were incorporated into a nationwide model of recreation to estimate the resulting changes in total recreation trips and value. The primary steps in implementing the recreation model were:

- Adapting the nationwide model of recreation trips using data available from the *Deepwater Horizon* oil spill assessment (English et al., 2018) to apply to the four study areas using benefit function transfer
- Assessing the consistency of survey and model results with previous research, including a previous study in Orange County, California (Leggett et al., 2018) that used data on actual recreation choices (revealed preference) rather than hypothetical choices (stated preference) to value the effects of marine debris on recreation

- Adjusting model parameters to reproduce the percentage changes in trips from the marine debris survey, leading to final estimates of the total change in recreation trips and the total change in recreation value from the two study scenarios in the four study areas.

1.3. Regional Economic Impact Analysis

The change in the number of recreation trips estimated using the national recreation model was also used to estimate regional economic impacts for the marine debris scenarios. A change in recreation trips results in a change in visitor spending, which we use to estimate the economic impacts of the two marine debris scenarios using input-output models. The primary steps in the economic impact analysis were as follows:

- Calculation of the proportion of trips in each study area coming from outside the local region, and the average number of recreation days per nonlocal trip, leading to an estimate of the increase or decrease in the number of nonlocal recreation days for each marine debris scenario
- Estimation of the average expenditures per recreation day in each study area using the National Economic Ocean Expenditure Survey (NORES) data on recreation expenditures (NOAA, 2012; Kosaka and Steinback, 2018)[3]
- Estimation of the regional economic impact of increased spending by visitor day in each study area by first mapping the NORES expenditure category to the appropriate RIMS II industry and then applying industry-specific RIMS II multipliers
- Calculation of estimates of the regional economic impacts from changes in beach recreation for each of the two marine debris scenarios (reduce debris to near zero and double the debris) in each of the four study areas.

The details of the study and the results are presented in subsequent sections. Section 2 presents information on the marine debris survey. Section 3 presents the model of recreational value, and Section 4 presents the regional economic impacts model. This is followed by the literature cited and appendices.

2. THE MARINE DEBRIS SURVEY

The marine debris survey consisted of an onsite survey conducted at beaches in each study area, and a follow-up survey mailed to people who had been interviewed onsite and agreed to participate in the follow-up survey. The purpose of the onsite survey was to recruit people for the mail survey from the target population of beachgoers in each area, and to briefly collect minimal data on the respondents' trips, opinions, and characteristics. The purpose of the mail survey was to ask recreators about the effect of marine debris on their

[3] Kosaka and Steinback (2018) recently published the NOAA (2012) NORES data. We were originally provided the data in 2017. For this report, we converted all dollar values to 2018 USD.

recreation choices, and to collect more extensive data on respondents' opinions and characteristics.

The main focus of the mail survey was to ask respondents the number of trips they took to the beach during the previous twelve months and how many more or fewer trips they would have taken under two contingent behavior scenarios: (1) if there had been almost no debris on beaches in the study area, and (2) if there had been twice as much debris on beaches in the study area. The term contingent behavior refers to survey questions that ask respondents how their recreation choices would change in response to changes in the quality of recreation sites. The mail survey also asked respondents to estimate the amount of debris on beaches in their area, and included other questions about their opinions and knowledge of debris on beaches.

Data collection occurred in 2018, with onsite interviews conducted from July 24 to September 3 and mail surveys sent out and received between October 5 and December 6. Response rates varied across the study areas and between the two stages of the study (onsite vs. mail). The overall response rate for the onsite survey was 76.7%. Accounting for recruitment into the mail survey and the mail-stage response rates, the final overall response rate for the mail survey was 19.0%. The number of responses provided to specific questions, as well as the number of missing responses to certain questions, was calculated and evaluated.

We weighted the sampling to help ensure that data from the survey was representative of the target population of people who used beaches in each study area. Sampling weights are critical in analyzing survey data whenever the sample design deviates from simple random sampling (Schaeffer et al., 2012). The weights include a base weight to control for differences among respondents in the probability of being selected into the sample, which was influenced by factors such as the amount of time a respondent spent at the beach. To further improve representativeness, the weights also include adjustments to make mail-survey respondents more similar to the much larger onsite sample with respect to key demographic characteristics, such as the proportion of people with a college degree.

Results of the marine debris survey included average ratings of the amount of debris on beaches and the total percentage change in the number of trips to beaches in each study area under the scenarios of a decrease or an increase in debris.

2.1. Survey Design

Below we first describe a pretest for the marine debris survey that was conducted in Orange County, California. A pretest is a common way to evaluate and refine a survey before final implementation. We then describe the final onsite and mail surveys and explain the reasons for each of the questions that were included. The surveys are provided in Appendix A.

2.1.1. Onsite and Mail Survey Pretest

During the survey pretest, we conducted onsite interviews at eight beaches in Orange County on Wednesday, September 27, 2017 and again on the following Saturday, September 30. The onsite interviewers asked several brief questions about each respondent's recreation and also elicited the respondent's address for completion of a follow-up mail survey. We

intercepted 777 recreators and obtained onsite interviews with 504 of them. We obtained addresses for the follow-up mail survey from 345 onsite respondents.

On December 6, 2017, we mailed surveys to the 345 addresses and sent reminder postcards one week later. For any valid addresses from which we had not obtained a completed survey, we sent a follow-up survey on December 20 and a second reminder post card on January 18, 2018. By the end of January, we had received 49 completed surveys. The U.S. Postal Service returned a total of 64 surveys as undeliverable due to invalid addresses.

One issue identified in the pretest was a low response rate of only 17.4% in the mail stage of the study. We believed this was due to scheduling the first mailing before the December holidays and the second mailing after the holidays, an interruption necessitated by the timeline for completing the pretest. In fact, the final mail response rate in Orange County was 30.8% in the final study. Response rates for the full study are discussed in more detail below.

In the pretest, we also found that the item nonresponse rate was high for the contingent behavior questions. "Item nonresponse" refers to missing responses to particular questions in completed surveys, as opposed to nonresponse from those who did not return the survey. In the question about a reduction in debris, 3 out of 49 respondents (6.1%) did not provide a usable answer of either no change or a specified increase in trips. In the pretest question about a doubling of debris, 9 out of 49 respondents (18.3%) did not provide a usable answer of either no change or a specified decrease in trips. The formatting of the contingent behavior questions in the pretest required people to fill out a small table with the abbreviated question "How many more trips if there had been almost no garbage or manmade debris?" in the row heading and "day trips" and "overnight trips" as column headings.

To address this item nonresponse rate, we revised the original question to be two separate and complete questions about day trips and then overnight trips, with the belief that this might improve the clarity of the questions for some respondents. However, the rate of item nonresponse did not improve and as reported below it in fact increased to 7 out of 52 (13.4%) for a reduction in debris and 13 out of 52 (25.0%) for a doubling of debris in the final Orange County results. We do not believe the changes in formatting or wording had an effect on nonresponse or that other refinements could have induced more people to answer the question. High item nonresponse rates are typical for contingent behavior questions (Eisworth et al., 2000; Whitehead et al., 2010), possibly because such questions require an open-ended "fill in the blank" response. Some respondents may be unsure how their recreation would be affected in hypothetical situations and may find it difficult to make a quantitative estimate of the change in their annual total number of trips.

The pretest also included "probing" questions that asked respondents about the design of the survey, particularly the graphical representations. There is a page in the survey that shows a picture of a beach with an area outlined in red and defined as 500 square feet or "an area approximately equal to three parking spaces" (see Appendix A). Pictures are also included that show different amounts of debris that could potentially be found in a 500 square-foot area of a beach. In the pretest, probing questions asked about the pictures and descriptions. Regarding the area of beach defined as 500 square feet, 89.8% of respondents said the description was clear or somewhat clear. Regarding the pictures of debris, 83.7% of respondents said they were able to find a picture that accurately reflected the amount of debris on beaches in their area. These results were considered satisfactory and no changes were made to the description or pictures of marine debris.

One additional concern addressed in the pretest was the possibility that people associated debris on beaches with other types of pollution. A probing question in the pretest asked "When you answered the questions about your trips, were you thinking primarily about garbage or manmade debris, or were you also thinking about other types of pollution, such as runoff from factories or farms?" Of the 49 respondents in the pretest, 71.4% said they were thinking primarily about debris on beaches. This was viewed as acceptable and did not lead to any changes the survey.

2.1.2. Onsite Survey – Regional Pilot

Using an onsite survey to reach people on the beach and recruit respondents for the mail survey ensured that the sample included only respondents who visited beaches in each study location. Reaching an equivalent number of beachgoers using a random sample of addresses would have required considerably more effort and expense, especially given that some beachgoers may live hundreds of miles from the coast in areas where the participation rate for beach recreation is quite low.

The onsite sampling involved intercepting people at beaches in each study location. The beaches were selected to represent the various types of beach experiences available, including more- and less-developed beaches. Interviews were conducted on both weekdays and weekend days to reach people taking anything from short visits to longer vacations. Although we had obtained statistically robust estimates from just 49 observations in the pretest, the target number for completed mail surveys was 100 in each study area to allow for potential shortfalls in recruitment or response rates.

The onsite interviewers collected data to assist in developing sampling weights, including the size of the party from which each respondent was selected (we interviewed only one person in a given party); the number of hours they would spend at the beach on the day of the interview; and for those engaged in a multiple-day trip, the number of days during the trip when they would spend time at the beach. The onsite survey also included several demographic questions, such as age and education, and several attitudinal questions about beach characteristics such as the presence of marine debris. Finally, we asked the respondent's name and mailing address. Those who did not agree to participate in the mail survey were asked their zip code so that data collected onsite could be used in calculating the proportion of trips from outside the local area.

2.1.3. Mail Survey – Regional Pilot

Four versions of the mail survey were developed for the four study areas. Differences across versions included different maps showing the beaches specific to each study area. The wording used to refer to beaches also differed across survey versions, such as "ocean beaches in Delaware and Maryland" or "Lake Erie beaches in Ohio." All other aspects of the mail survey were identical for each of the study areas.

The first question in the mail survey asked respondents to review the map of beaches and indicate the beaches familiar to them. This served to familiarize respondents with the set of beaches they would be asked about in subsequent questions.

The next two questions asked respondents about the total number of their single-day and multiple-day trips during the previous year to all beaches in the study area. Respondents' total number of trips throughout the year were used as a baseline to which changes in trips, estimated in later questions, could be compared.

Question 4 of the mail survey asked about the importance to respondents of 13 beach attributes when they chose which beaches to visit. The attributes included things like water quality, the presence of natural debris on the beach, scenic beauty, how crowded a beach is, and presence of manmade debris. These questions allowed us to evaluate the importance of marine debris in a qualitative way. They may also have encouraged respondents to think carefully about how they respond to marine debris relative to other beach characteristics when answering the contingent behavior questions.

Question 5 asked respondents to report which beaches they visited over the last year and to rate the level of marine debris they encountered at each beach on a scale of 1-to-5 using the photographs of debris amounts provided. The debris ratings allowed us to characterize current debris levels, at least in relative terms. Current debris levels are the starting point for the contingent behavior questions, which ask about changes in debris that are proportionate to current levels.

To specify the size of the beach area respondents were asked to evaluate, the survey included a photograph that showed an area of beach outlined in red. The area was described as 500 square feet, or approximately the area of three parking spaces. Below the beach photo were pictures showing different amounts of debris that could be found in an area of the size shown. The debris photos included cigarette butts, plastic straws, and other common items found on beaches in the United States (NOAA, 2018).

Questions 6 and 7 asked whether respondents would have changed the number of trips they took over the past year to the beaches in a given study area under two hypothetical scenarios: (1) “If there had been almost no garbage or manmade debris at beaches,” and (2) ”If there had been twice as much garbage or manmade debris at beaches.” These questions were to be used for estimating the total percentage change in trips in each scenario, the key input to the recreation model.

Questions 8 through 12 asked whether respondents were concerned about the presence of various types of garbage or manmade debris while visiting a beach, the types of debris they have actually seen on beaches, their understanding of the sources of debris found on beaches, and whether they had participated in beach cleanup efforts. These questions seek to provide helpful context for evaluating marine debris policies. This series of question also asked respondents whether they think marine debris is a problem on beaches in the study area. This question was used to adjust mail survey sampling weights to be consistent with onsite respondents, as described in Section 2.3.

Questions 13 through 19 asked respondents to report the number of adults and children in their household as well as their gender, age, ethnicity, race, education level, and income. These questions were used to investigate the relationship between the response to changes in marine debris levels and demographic characteristics and some of them were used to adjust the sampling weights in Section 2.3.

2.2. Survey Implementation

The survey schedule, the response rates for each stage of the survey, and the total number of completed surveys are reported below for each study area. The sampling procedures are also described, including the approach to selecting respondents during the onsite survey.

Sample statistics showing the final number of responses and missing responses for key questions in the final mail survey data are presented and discussed.

2.2.1. Survey Schedule and Response Rates

We conducted the survey in 2018, beginning with an initial onsite interview with respondents during the late summer, followed by a mail survey in the fall. Those who participated in the onsite interview were asked if they were willing to take part in the mail survey and if so, to provide their address. Although the mail survey asks about a full year of beach activity, we wished to reach visitors in the fall so that their activities during the peak summer beach season would be fresh in their minds. We scheduled our initial onsite interviews in the late summer to avoid an extended period between the initial onsite interviews and the final mail survey.

Table 1 shows the schedule of data collection in each study area. The number and timing of days spent interviewing onsite varied based on weather and the availability of interviewers. A minimum of eight person-days were spent interviewing onsite in all study areas, distributed approximately evenly between weekdays and weekend days. We avoided bad weather days to maximize the number of interviews. While such a practice underrepresents low-activity days and would be problematic in a survey designed to estimate total activity, in our study totals come from the nationwide recreation model. Even if visitors on bad weather days differ systematically with respect to their preferences about marine debris, the small number of people using the beach on bad weather days would mitigate the effects of any such difference when calculating the response to marine debris in the total population of beachgoers.

The field period for the mail survey was approximately two months. The initial mailing took place on October 5, 2018. Reminder postcards were sent October 15, though in Alabama the reminder was delayed one week to avoid contacting people during the immediate aftermath of Hurricane Michael. The second mailing of the mail survey took place November 5. In the three areas where the target of 100 completed surveys had not been met, an additional reminder postcard was sent on November 21.

In total there were 1,327 completed onsite surveys, with a response rate of 76.7% (Table 1). Of the 802 addresses provided for a follow-up survey, 61 were invalid. This left 741 valid addresses for the mail survey, a recruitment rate of 55.8%. The overall mail response rate was 44.4%, with a total of 329 completed mail surveys. The cumulative response rate was 19.0%. This is a typical response rate in survey research, and low levels of response are not necessarily indicative of bias (Groves and Peytcheva, 2008; Pew Research Center, 2012). As described in Section 2.3, the sampling weights for mail respondents in each study area were adjusted so that mail respondents were similar to onsite respondents with respect to key characteristics. This technique can improve representativeness by taking advantage of higher response rates in earlier stages of the study.

The number of onsite interviews, response rates and recruitment rates varied considerably among the four study areas. In Ohio, a low number of onsite interviews was partly offset by a high mail response rate. Onsite response, recruitment, and mail response were all high in Delaware/Maryland, leading to more than the targeted number of completes. Low onsite and mail response rates in Orange County were ultimately responsible for the low number of completed mail surveys in that area.

Table 1. Survey implementation

Implementation stage	Alabama	Delaware and Maryland	Ohio	Orange County, California	Total
Onsite survey					
Field period	August 11–September 3, 2018	August 10–30, 2018	July 27–August 29, 2018	July 24–29, 2018	July 24–September 3, 2018
Completed surveys	533	353	195	246	1,327
Response rate	88.7%	91.2%	76.9%	50.3%	76.7%
Recruitment for mail survey	249	219	104	169	741
Recruitment rate	46.7%	62.0%	53.3%	68.7%	55.8%
Mail survey					
Field period	October 5–December 6, 2018	October 5–December 6, 2018	October 5, 2018–December 6, 2020	October 5, 2018–December 6, 2021	October 5, 2018–December 6, 2022
Completed surveys	99	116	62	52	329
Response rate	39.8%	53.0%	59.6%	30.8%	44.4%
Cumulative response rate[a]	16.5%	30.0%	24.4%	10.6%	19.0%

a. The cumulative response rate accounts for the onsite response rate, the recruitment rate, and the mail response rate. Mail survey responses were reweighted to be representative of respondents to the onsite survey in each study area.

2.2.2. Onsite Sampling Procedures

Onsite sampling procedures are important in ensuring representativeness and developing sampling weights. On the beach, interviewers approached a party of people and randomly selected one adult over 18 for an interview. Random selection ensures that interviewers do not oversample any particular type of person, which could lead to results that are not representative of all beachgoers. Interviewing only those over 18 is standard survey practice to avoid issues of parental consent. For random selection, interviewers picked the person farthest to the right-hand side of the party from wherever the interviewer was standing. Interviewers generally made a judgement about who was over 18 but if unsure, the interviewer would politely explain the situation and ask whether someone was over 18. When asking an address, the full name of the respondent was also elicited because questions in both the onsite and mail survey, including demographic characteristics and preference-based responses, were specific to an individual.

Interviewers walked from party to party along a beach. Interviewers proceeded in one direction from the main access point of a beach, and then returned to the main access point and proceeded in the other direction. The extent of the total sampling area was defined as the area easily accessible on foot from the main access point, determined by the interviewer's discretion. When interviewers had spent at least 1.5 hours at a beach, or had approached every party, they would proceed to the next beach in a prescribed order. The order was agreed upon by the research team in advance, based on the proximity of beaches and logistical efficiency in getting from one beach to the next. In some cases the list was broken into two groups of beaches that were geographically close to one another, with sequential sampling of beaches proceeding separately for each group.

When starting a new day of sampling, the interviewers began at the next beach on the list after the beach where they had last conducted interviews. The list included at least eight beaches located throughout each study area, which helped to ensure reasonable representation of the different types of beaches in the area. Including significantly more than eight beaches would have entailed excessive time requirements for interviewers to become familiar with the location and layout of beaches, find where public parking is available, check whether permission is required to sample at a beach, and other issues.

Maps of the beaches included in each study area are provided as part of the mail surveys provided in Appendix A. Below is a list of beaches where onsite surveys were administered:

Alabama

- Cotton Bayou Beach
- Orange Beach
- Gulf State Park Pavilion
- Gulf Shores Public Beach
- Alabama Point/Florida Point
- Dauphin Island West End Beach
- Dauphin Island Public Beach
- Dauphin Island East End Beach.

Delaware/Maryland

- Rehoboth Beach
- Dewey Beach
- Conquest Road Beach
- Bethany Beach
- Cape Henlopen Beach
- Assateague Island National Seashore
- Ocean City (Boardwalk)
- Assateague State Park.

Ohio

- Euclid Beach Park
- Headlands Beach State Park
- Fairport Harbor Lakefront Park
- East Harbor State Park
- Cedar Point Beach
- Nickel Plate Beach
- Headlands Beach State Park
- Camp Perry Beach
- Edgewater Park Beach.

Orange County, California

- Balboa Beach
- Doheny State Beach
- Bolsa Chica
- Huntington City Beach
- Huntington State Beach
- Newport Beach
- Crystal Cove State Park Beach
- Laguna Beach.

Because of the high prevalence of single-day respondents on Ohio beaches, samplers skipped every other single-day respondent in an attempt to reach more multiple-day respondents. This feature of sampling was addressed in the sampling weights, described in Section 2.3.

2.2.3. Sample Statistics

We compiled descriptive statistics of the unweighted onsite and mail survey sample data prior to describing the development of sampling weights (Table 2). Section 2.3 discusses how we used these data to create sampling weights.

The number of single-day and multiple-day trips from the onsite sample is important for several reasons. Statistics on the origin of trips, as well as the number of recreation days in a multiple-day trip, were taken from the onsite data. These statistics determine the proportion of recreation days coming from outside the local area. A small sample of multiple-day trips could make these statistics, and the regional economic analysis in which they are used, less reliable. The proportion of trips that are single-day trips was also taken from the onsite data and this statistic was used to make final adjustments to the recreation model as part of the benefit function transfer, described in Section 3. Finally, the value of recreation is often expressed in terms of a value per recreation day, an approach we also take in our findings. This calculation again uses the number of recreation days per multiple-day trip from the onsite data.

The sample statistics also show that all beaches in each study area, as enumerated in the mail surveys, had been visited by least one respondent to the mail survey. Almost all beaches received a debris rating from at least one respondent. The total number of debris ratings provided for all beaches ranged from a low of 182 ratings for beaches in Ohio to a high of 281 ratings for Delaware/Maryland beaches (Table 2).

We aimed to have good representation of both single-day and multiple-day trips in the mail survey. It could be important in determining the total response to the debris scenarios if people view the importance of marine debris differently when planning these different types of trips. The number of people taking at least one multiple-day trip was lower in Ohio and Orange County, California. Multiple-day trips in both areas appear to be better represented in terms of the absolute number of trips.

Table 2. Selected descriptive statistics from samples collected via both the onsite and mail surveys

	Alabama	Delaware and Maryland	Ohio	Orange County, California
Onsite survey				
Single-day trips	212	153	222	175
Multiple-day trips	357	200	47	92
Mail survey				
Beaches visited by at least one respondent	10	20	22	22
Beaches rated for debris by at least one respondent	10	17	21	20
Beach ratings (person-beach pairs)	261	281	182	197
Respondents who took single-day trips	59	62	60	48
Respondents who took multiple-day trips	70	83	12	16
Single-day trips	1,108	733	630	1,937
Multiple-day trips	549	478	32	57
Respondents reporting an increase in trips, "almost no" debris	7	3	12	7
Respondents reporting no change in trips, "almost no" debris	83	106	43	38
Missing responses, "almost no" debris	9	7	7	7
Respondents reporting a decrease in trips, doubling of debris	40	27	35	22
Respondents reporting no change in trips, doubling of debris	25	61	11	17
Missing responses, doubling of debris	34	28	16	13

Data from the contingent behavior questions are divided into three categories: those reporting a change in trips, those reporting no change in trips, and missing responses (Table 2). The number of people reporting a change in trips is somewhat low for the scenarios where debris is reduced, but this is not likely to present a problem for the final results. For example, only three people in Delaware/Maryland report an increase in trips in response to a reduction in debris. This fits with the evidence that debris levels are already perceived to be quite low and suggests that most people would not be significantly affected by a further reduction. Indeed, confidence intervals calculated in Section 2.4 indicate that results for all debris scenarios are statistically quite precise.

The number of missing responses is high and suggests that many people had trouble answering the contingent behavior questions. Overall, 9.1% of respondents did not provide an answer when asked about a reduction in debris and 27.7% did not provide an answer when asked about a doubling of debris. We do not believe this is due to the formatting or wording of the questions, which are relatively straightforward. It may be due in part to the fact that the scenarios involve a large area that includes many beaches, some of which may be unfamiliar to the respondent. Questions focusing on a single beach familiar to the respondent may have been preferred, but would not have been feasible given the need to match scenarios to the aggregate sites defined in the *Deepwater Horizon* data. A high rate of missing responses, often 25% or more, appears to be typical for contingent behavior questions (Eisworth et al., 2000; Whitehead et al., 2010). When performing analysis and computing results, respondents

who did not provide an answer to the questions about changing their trips in response to changes in debris were assumed to make no change in their trips. This approach is considered conservative and valid for stated-preference analysis (Carson et al., 2003).

There were a small number of missing responses for other mail-survey variables, such as age, education, and gender. The percentage of missing responses for all variables used in the analysis is shown as part of the summary statistics presented in Appendix B. The greatest number of missing responses for any variable was 6.7% for the question asking about household income, but this variable was not used in the analysis. Of the variables used in the analysis, the highest rate of missing responses was 4.0% for the question about level of education. In preparing the data for analysis, missing responses for all demographic variables were filled in using a random draw from all non-missing responses for the same variable (Andridge and Little, 2010).

2.3. Development of Sampling Weights

Sampling weights ensure that survey data is as representative as possible of the population of interest. A thorough description of sampling and weighting procedures can be found in Schaeffer et al. (2012). In this study, the population includes all people recreating at beaches in each study area. The first step in developing the sampling weights was calculating the base weights, which ensure that differences in the probability of being selected into the sample do not lead to over- or under-representation in the sample. For example, people who go to the beach frequently are more likely to be intercepted in the onsite surveys, so these respondents are given lower weights to ensure they are not overrepresented in the data. Even when accurately represented, the most active recreators are still likely to be more influential than other respondents in estimates of total recreation trips and value.

The second step in developing the sampling weights was adjusting the base weights so that mail-survey respondents represent as accurately as possible the much larger group of onsite-survey respondents. Reweighting to a larger sample with a higher response rate can make the data more representative of the target population. This adjustment to the weights involved first estimating a model that showed how certain key respondent characteristics and opinions were positively or negatively associated with respondents' response to the marine debris scenarios. We then adjusted the sampling weights so that the proportion of reweighted mail-survey respondents with each key characteristic matched the proportion for the analogous group of respondents in the onsite survey. While adjustments for some of the variables led to significant changes in the weights, we found that results were robust to the reweighting and there were only modest changes in the estimated total effect of the debris scenarios.

2.3.1. Base Weights

Base weights account for sample selection probabilities, which are determined by the sample design and by behavioral variables elicited in the onsite and mail surveys. Because data from the surveys are used to calculate proportions only and statistics are not expanded to the full population, selection probabilities and sampling weights can be expressed in relative rather than absolute terms (Piazzi, 2010).

There are four components used in calculating the base weights, reflecting four variables that determine the relative probability of selecting each respondent into the sample. The first variable is party size, or the number of people in the group from which an individual was selected during onsite sampling. The probability of selection for an individual is inversely related to party size, so the first factor for calculating relative selection probabilities is f_1 = 1/party size. The second variable is the number of hours the respondent spent at the beach during the day of the onsite interview. The probability of selection is directly proportional to time spent at the beach, so the second factor is f_2 = number of hours at the beach. The third variable is the number of days the respondent went to the beach during the trip when he or she was interviewed. The probability of selection is directly proportional to the number of days in the trip, so f_3 = number of days in the trip. The fourth variable is the number of trips the respondent took during the year. This is again directly proportional to the selection probability, so f_4 = the number of trips the respondent took during the year.

The final base weights are inversely proportional to selection probabilities, so weights are calculated using the inverse of each of the above factors. Also, relative weights are scaled so that the sum of the weights equals the sample size, which is equal to the number of completed surveys shown in Table 1. Using *i* to represent individual respondents in the sample, *fi*1 through *fi*4 to represent the above factors for individual *i*, and *Nia* to represent the mail-survey sample size for area *a* in which individual *i* was interviewed, the base weighs are calculated as

$$w_{ib} = N_{ia}\frac{\left(1/f_{i1}\right)\left(1/f_{i2}\right)\left(1/f_{i3}\right)\left(1/f_{i4}\right)}{\Sigma_i\left(1/f_{i1}\right)\left(1/f_{i2}\right)\left(1/f_{i3}\right)\left(1/f_{i4}\right)} \quad (1)$$

Although mail surveys were addressed to the specific individual who was interviewed onsite, in some instances the gender or age reported in a mail survey was different from what was recorded onsite, suggesting that someone else filled out the mail survey. We retained these surveys in the data and assumed that onsite variables needed for weighting procedures, such as party size and number of hours on the beach, could be applied to the mail respondent. In the 16 cases where mail survey respondents reported taking no trips during the previous year, we assumed they took a single trip. This assumption is required to calculate a sampling weight for these individuals. The same assumption was also used when calculating total baseline trips, which was the starting point for computing a percentage change in trips in the marine debris scenarios.

In certain instances, noted below, we used weights derived solely from the onsite survey. Weights for the onsite survey are calculated as

$$w_{io} = N_{iao}\frac{\left(1/f_{i1}\right)\left(1/f_{i2}\right)\left(1/f_{i3}\right)}{\Sigma_i\left(1/f_{i1}\right)\left(1/f_{i2}\right)\left(1/f_{i3}\right)} \quad (2)$$

N_{iao} is the sample size for the onsite survey in area *a* where *i* was interviewed, equal to the number of completed onsite survey shown in Table 1. The final factor f_4 is omitted

because the number of trips respondents take during the year is obtained only in the mail survey.

2.3.2. Reweighting Mail Respondents

Respondents' characteristics and opinions are often related to their preferences. Using the mail-survey data, we developed a model to find which respondent characteristics and opinions, if any, helped predict how people would answer the contingent behavior questions about marine debris. The model estimated the relationship between respondents' stated change in trips for each scenario and eight explanatory variables, including the respondent's age, education, and gender; whether there were children in the respondent's household; whether the respondent felt that debris was a problem on local beaches; and the importance to the respondent of free or inexpensive parking, no crowds, and no debris. The model specification was a logit choice model, which is widely used is recreation applications (Train, 2003). Details of the model are described in Appendix C. In the model, three key variables were significant predictors of a response to debris: age, education, and whether the respondent felt marine debris was a problem on area beaches.

We then compared mail respondents to onsite respondents with respect to the three key variables. For any variable where mail respondents were significantly over- or under-represented relative to onsite respondents, we reweighted the responses. For example, in the mail survey, 23% of respondents in Alabama were 45 years old or younger and 77% were older than 45. In the onsite survey, the percent frequencies were 52% for those 45 or younger and 48% for those older than 45. Therefore, we reduced the representation of older respondents in the mail survey could improve representativeness. A table showing detailed percent frequencies for all three key variables in the mail and onsite surveys is given in Appendix C.

Based on a review of the percent frequencies, we chose to reweight respondents in all four regions by all three variables, with one exception: for Ohio, the percentage of people who viewed marine debris as a problem was the same in the onsite survey and mail survey. Since it remained nearly the same after reweighting by age and education, reweighting by the two variables age and education was determined to be sufficient.

Table 3 shows the amount that representation of the key demographic groups changed before and after reweighting. The reweighing procedure changed the representation of all three key variables, in some cases by a factor as high as 2 or as low as 0.5. However, the results concerning the impact of marine debris changed only modestly, as shown in the last two rows of Table 3 for each study area. The largest absolute change was the estimated response to a decrease in debris in Alabama, which rose from 5.4% to 8.1%.

2.4. Results of the Marine Debris Survey

Below we describe the main results of the marine debris survey. For the complete set of weighted mail-survey statistics, see Appendix B. The main results include the proportion of trips that are single-day trips and the number of recreation days in a multiple-day trip. Both come from the onsite data and are used later in the modeling and analysis. We also report three of the importance ratings from Question 4 of the mail survey (q4 variables in Appendix B) that are helpful in providing context for people's response to marine debris. Finally, we

report the percent change in trips for the two scenarios in each of the four study areas, the key inputs to the nationwide recreation model that calculates the number and value of trips.

Table 3. Representation of demographic categories and estimated response to debris scenarios before and after reweighting mail respondents

Region/statistic	Before reweighting	After reweighting	Ratio: after/before
Alabama			
Age ≤ 45 (proportion)	0.23	0.52	2.3
Age > 45 (proportion)	0.77	0.48	0.6
Education ≤ bachelors (proportion)	0.38	0.57	1.5
Education > bachelors (proportion)	0.62	0.43	0.7
Problem = yes (proportion)	0.36	0.45	1.3
Problem = no (proportion)	0.64	0.55	0.9
Change in number of trips when debris is reduced to almost none	5.4%	8.1%	1.5
Change in number of trips when debris doubles	-27.0%	-26.5%	1.0
Delaware and Maryland			
Age ≤ 55 (proportion)	0.54	0.79	1.5
Age > 55 (proportion)	0.46	0.21	0.5
Education ≤ bachelors (proportion)	0.47	0.50	1.1
Education > bachelors (proportion)	0.53	0.50	0.9
Problem = yes (proportion)	0.33	0.18	0.5
Problem = no (proportion)	0.67	0.82	1.2
Change in number of trips when debris is reduced to almost none	3.5%	2.2%	0.6
Change in number of trips when debris doubles	-14.8%	-16.3%	1.1
Ohio[a]			
Age ≤ 55 (proportion)	0.48	0.66	1.4
Age > 55 (proportion)	0.52	0.34	0.7
Education = high school (proportion)	0.41	0.25	0.6
Education ≠ high school (proportion)	0.59	0.75	1.3
Problem = yes (proportion)[a]	0.64	0.63	1.0
Problem = no (proportion)	0.36	0.37	1.0
Change in number of trips when debris is reduced to almost none	34.9%	35.4%	1.0
Change in number of trips when debris doubles	-34.7%	-35.6%	1.0
Orange County, California			
Age ≤ 45 (proportion)	0.51	0.75	1.5
Age > 45 (proportion)	0.49	0.25	0.5
Education ≤ graduate degree (proportion)	0.44	0.24	0.5
Education > graduate degree (proportion)	0.56	0.76	1.4
Problem = yes (proportion)	0.55	0.49	0.9
Problem = no (proportion)	0.45	0.51	1.1
Change in number of trips when debris is reduced to almost none	8.8%	9.5%	1.1
Change in number of trips when debris doubles	-20.4%	-20.9%	1.0

a. In the mail weights for Ohio, observations were not adjusted to match the onsite data with respect to the proportion of people saying marine debris was a problem because the proportions were both 64% before adjustments. After adjusting for age and education the mail proportion saying debris was a problem dropped to 63%, but this small difference was viewed as acceptable.

2.4.1. Population Statistics

We calculated statistics from the onsite survey using the onsite weights described in Section 2.3.1 and statistics from the mail survey were calculated using the final mail sampling weights, including the demographic adjustments described in Section 2.3.3 (Table 4). To calculate standard deviations, we used the formula for weighted standard deviations. To calculate standard errors, we used a jackknife variance procedure (Tukey, 1958) in which any given statistic was calculated separately *Na* times, once with each of the *Na* observations removed, where *Na* refers to the number of respondents in a given study area.

Table 4. Selected population statistics from the onsite and mail surveys by region

Statistic	Alabama		Delaware and Maryland		Ohio		Orange County, California	
	Value	St. Dev. or St. Err.	Value	St. Dev. or St. Err.	Value	St. Dev. or St. Err.	Value	St. Dev. or St. Err.
Onsite survey statistics (with standard errors)								
Proportion of trips that are single-day trips	70.6%	2.5%	72.3%	2.4%	97.7%	0.7%	88.2%	2.2%
Average number of recreation days in a multiple-day trip	3.10	0.10	3.21	0.11	1.98	0.14	2.42	0.13
Proportion of recreation days from outside the local area	83.7%	1.7%	89.2%	1.8%	97.7%	1.2%	81.4%	3.9%
Mail survey statistics (with standard deviations)[a]								
Average importance rating: manmade debris on the beach	4.75	0.55	4.42	1.20	4.77	0.48	4.40	0.77
Average importance rating: good water quality	4.76	0.59	4.58	0.86	4.76	0.55	4.30	0.71
Average importance rating: no natural debris on the beach	2.67	1.36	2.43	1.30	3.00	1.28	2.18	1.27
Mail survey statistics (with standard errors)								
Average debris rating	1.65	0.06	1.60	0.07	2.27	0.10	2.09	0.12
Change in the number of trips – "almost no" debris	8.1%	0.40%	2.2%	0.02%	35.4%	1.56%	9.5%	0.60%
Change in the number trips – doubling of debris	-26.5%	0.58%	-16.3%	0.37%	-35.6%	0.84%	-20.9%	0.77%

a. Population statistics for demographic variables appear in Appendix B.

The proportion of trips that are single-day trips ranged from 70.6% in Alabama to 97.7% in Ohio. This reflects a variety of factors, including the appeal of beaches for those who wish to spend a few hours at the beach relative to those who wish to spend several days at the beach. It also reflects the number of people who live within a distance close enough to make single-day trips feasible, versus the number people who live at distances better suited to multiple-day trips. The average number of days in a multiple-day trip is determined by similar factors, and in our data it is generally inversely related to the proportion of single-day trips.

We also obtained estimates of the proportion of single-day trips from the mail survey data. Those numbers are 63.7% in Alabama, 61.0% in Delaware/Maryland, 94.5 % in Ohio, and 93.2% Orange County. We chose to use the onsite data in our calculations due to the larger number of observations available in the onsite data and the higher accuracy that is likely to result when people are reporting about the trip they are currently on rather than recalling trips over the course of the previous year.

Three importance ratings from Question 4 of the mail survey are useful in providing context for people's preferences about marine debris. The importance ratings were presented in the survey as a scale from 1 to 5, with 1 representing "not important," 3 representing "somewhat important," and 5 representing "very important." The first rating in Table 4 is for the importance of manmade debris. The average ratings were quite high, with a majority of respondents in all study areas giving this factor a rating of 5, or "very important." However, to the extent that there is variation, it is consistent with other results of the survey. For example, the average debris rating at beaches in Alabama is slightly higher but quite close to that of Delaware/Maryland. The difference in the estimated response to changes in debris may therefore be due to differences in preference more than differences in current conditions (recall that the scenarios describe changes in debris that are proportionate to current levels). Indeed the average importance rating for debris is higher in Alabama, supporting the idea that preferences are a factor in explaining the divergent results. Perceptions of current debris levels in Orange County are somewhat higher than those in Alabama, but the reported response to changes in debris are similar in the two areas. Orange County has the lowest importance rating for debris, which again suggests that differences in preference may be offsetting the difference in debris levels in determining the response to changes in debris levels.

Other important ratings shown in Table 4 include water quality and the presence of natural debris such as kelp or seaweed. Water quality is related to manmade debris in that both factors involve a disruption to the natural environment. The presence of natural debris is related to manmade debris in that both factors involve debris on the beach. All three factors show a similar pattern when comparing across regions, with the highest levels of importance in Ohio and the lowest levels of importance in Orange County. Since the importance ratings are not used in calculating final results, the precision of the average ratings is not important. Instead of standard errors, standard deviations are reported for these statistics to assist in understanding the variation in ratings across respondents.

The average debris ratings indicate that Ohio beaches have the highest levels of debris, while Alabama and Delaware/Maryland have comparatively low levels of debris. The average debris ratings were calculated as the weighted average of all ratings provided by respondents for all beaches in a given study area. The average ratings retained the same 1-to-5 scale used in data collection.

There are at least two alternative ways to aggregate the debris ratings. One alternative approach would involve averaging the ratings for each beach and then taking an average of the beach-specific ratings. Relative to the first method, this would place a greater emphasis on ratings for any beaches rated by only small number of people, since all beaches would get an equal weight in the average. Both measures lead to similar ratings for all study areas except Orange County, California, where the average rating increased to 2.43 using this alternative method. We think a single average grouping all ratings together is preferred because it

represents beaches in proportion to their familiarity to people who use the area, which is likely to better represent people's overall impression of debris in each study area.

A second method for calculating an average of debris ratings would involve first converting the ratings to actual estimated debris amount using information provided to survey respondents. For example, the rating of "1" would correspond to 1 piece of debris per 500 square feet, as shown in pictures provided in the mail survey. Likewise a rating of "5" would correspond to 16 pieces of debris per 500 square feet. We found that this alternative method generated ratings that varied dramatically across beaches. Importantly, there was a slight negative correlation between the debris averages for specific beaches calculated using this method and the onsite measurements of debris for those beaches. As described in Section 3.2, when the 1-to-5 debris ratings were averaged for the same beaches, the correlation with the onsite measurements was quite high. Although they can be interpreted in relative terms only, we retain the original 1-to-5 ratings when calculating summary measures of debris in the four study areas.

2.4.2. Effects of Marine Debris on Recreation Trips

The final estimates of the change in demand for trips that results from the two debris scenarios are shown in the last section of Table 4. For a reduction in debris to almost none, the percentage change in the number of trips is 8.1% in Alabama, 2.2% in Delaware/Maryland, 35.4% in Ohio, and 9.5% in Orange County, California. For a doubling of debris, the percentage change in the number of trips is -26.5% in Alabama, -16.3% in Delaware/Maryland, -35.6% in Ohio, and - 20.9% in Orange County, California. Standard errors are reported, and all estimates have high statistical precision. To evaluate the implications of these results for total trips, recreation value, and regional economic impacts, the percentage changes are used as an input to the nationwide recreation model, described below.

3. RECREATIONAL VALUE MODEL

The key result from the marine debris survey was the percentage change in recreation trips from two potential debris scenarios in each study area: a decrease in debris to almost none and a doubling of current debris levels. In this section, we estimate the implication of these percentage changes on the total number and value of recreation trips using a nationwide model of people's recreation choices.

The basis for this analysis is the nationwide recreation model that was developed using data collected in 2012 and 2013 by experts working on behalf of state and federal agencies to assess recreation impacts from the *Deepwater Horizon* oil spill (English et al., 2018). The *Deepwater Horizon* data includes complete information on recreation trips for the Southeast United States, but only includes information about trips lasting two nights or more in other areas. We adapted these data to examine the recreational value in this study, a method known as benefit function transfer. Specifically, we used data from onsite surveys to adjust the model so that it would correctly estimate all trips in the three study areas outside the Southeast. However, we found that Lake Erie beaches in Ohio were too different from other areas to

effectively complete the benefit transfer, and only impacts to multiple-day trips were estimated for that study area.

We evaluated the reliability of our methods by comparing key results to external sources. We found that the estimated effects from changes in debris in our study were either comparable to or somewhat higher than the effects estimated in a previous study in Orange County (Leggett et al,, 2018), depending on the scenario examined. The results are not directly comparable because our model accounts for all trips and the previous model included single-day trips only. We found that respondents' ratings of the relative amounts of marine debris on beaches were highly correlated with previous onsite debris measurements, but that converting the ratings to absolute amounts of debris did not give reliable estimates. We also found some evidence that people using beaches at different times of the year may have a different response to marine debris, suggesting that additional efforts to contact recreators at beaches throughout the year could yield somewhat different results. The final estimates of changes in recreation trips and value for the two scenarios and the four regions are presented in Section 3.4.

3.1. Nationwide Recreation Model

The nationwide recreation model developed for the marine debris study is a type of travel-cost model. It involves a system of demand functions where the price is the cost of traveling to recreation sites and the quantity is the number of trips people take to the sites. The model is based on data collected for the assessment of beach recreation losses following the *Deepwater Horizon* oil spill. An overview of data collection methods, procedures for cleaning the data, and calculation of model inputs are provided in a series of memoranda that are available in the *Deepwater Horizon* administrative record.[4] Specific memoranda are referenced below for details not provided in this report. An overview of the *Deepwater Horizon* beach recreation assessment can be found in English et al. (2018).

3.1.1. Nationwide Coastal Recreation Data

The *Deepwater Horizon* data were collected in a telephone survey of 41,708 respondents conducted over a 12-month period beginning June 2012. This was after the effects of the *Deepwater Horizon* spill had ended, according to onsite studies of recreation activity (Tourangeau et al., 2017). The sample for the survey was drawn from the full population of the contiguous 48 states. The data include information about all recreation trips to beaches in the Southeast United States. For other coastal areas the data include information only about trips lasting at least two nights away from home and originating from outside the Southeast. Specifically, respondents in eastern Texas, Louisiana, Mississippi, Alabama, Florida and southern Georgia were asked about all their beach trips to coastal areas from Texas to Georgia. These respondents were not asked about their trips to other areas of the country. Respondents from the remainder of the contiguous United States were asked about all their coastal trips to anywhere in the contiguous United States lasting at least two nights. Respondents from outside the Southeast were not asked about shorter trips because all of their

[4] The technical memoranda in the *Deepwater Horizon* administrative record are available at https://www.diver.orr.noaa.gov/deepwater-horizon-nrda-data#.

trips to the Gulf Coast from Louisiana to Florida, the focus of the *Deepwater Horizon* assessment, were at least two nights. On the other hand, these respondents were not asked to limit their answers to Gulf Coast destinations, because the additional information about overnight trips elsewhere in the country was viewed as potentially valuable in understanding the substitution of longer trips to areas outside the Gulf after the spill.

For this marine debris analysis, we simplified the *Deepwater Horizon* dataset in two ways. First, we eliminated trips with travel distances greater than 750 miles. This improved consistency of the data across regions. For example, while the full dataset includes trips from California to Alabama but omits trips from Alabama to California, we limited the geographic focus of the model in all regions. The 750-mile cutoff still retains 95.6% of all trips in the data to areas outside the Gulf, and a higher percentage of trips to areas within the Gulf where single-day and one-night trips are a large portion of total trips. Second, when reporting our results we avoid making a distinction between trips of two nights or more and multiple-day trips, a term which also includes trips with an overnight stay of just one night. Instead, we refer only to multiple-day trips as distinct from single-day trips.

For all estimates based on the *Deepwater Horizon* data, we made adjustments to convert trips of two nights or more into an estimate of all multiple-day trips, using the ratio of total multiple-day trips (including one-night stays) to trips of two nights or more. This ratio was estimated for each study area using data from the onsite marine debris survey, which asked respondents about the length of their trips and their overnight lodging.

In addition to information about recreation trips, the *Deepwater Horizon* data include information about respondents' demographic characteristics, such as age, income, education level, and gender. Sampling weights were developed for each respondent using sample selection probabilities as well as adjustments that matched key demographic groups in the sample to population statistics from the U.S. Census (English, 2015a, 2015b). All respondents were included in the recreation model regardless of whether they took coastal recreation trips or not.

The *Deepwater Horizon* data groups coastal beaches into 76 aggregate sites covering all coastal areas of the continental United States, including the Great Lakes. Travel costs from respondent origins to the 76 destinations were constructed using a weighted average of driving and flying costs (Leggett, 2015). The weights for a given respondent and site were equal to the proportion of people driving or flying for trips reported by respondents with similar incomes who traveled a similar distance. Driving costs were based on driving distances and travel times as calculated using PC*Miler software. The cost per mile of driving, including gasoline and per-mile automobile depreciation, was estimated to be 25 cents per mile based on information from the American Automobile Association (2012). The cost per hour of travel time was set equal to one-third of the respondent's household income divided by 2,080. This procedure for estimating an individual's value of travel time is consistent with evidence in the literature (English et al., 2015). Flying costs were computed using the 30th percentile of fares for round-trip tickets as reported in the Airline Origin and Destination Survey, a 10% sample of all airline tickets collected each year by the U.S. Bureau of Transportation Statistics. Using the 30th percentile was intended to eliminate the effects of high fares related to business travel (Leggett, 2015).

3.1.2. Model Structure

The nationwide recreation model developed for the marine debris study uses a common mathematical structure called nested logit (Train, 2003). A nested logit model consists of a set of demand functions, one for each of the recreation sites in the model. It also allows sites that are similar to one another to be grouped together so that the greater amount of substitution between similar sites can be reflected in the model's behavior predictions.

The nesting structure in the nationwide recreation model first groups the 76 sites into distinct regions where sites may be more similar to each other than they are to sites outside each region. This allows the model to account for the possibility that if one site is affected by a decline in quality people are more likely to switch to other sites within a given region of the country rather than, say, switching between Lake Erie and the New Jersey shore. The regions were defined based on an exploration of the best model fit and were selected to be the Gulf Coast, the Atlantic Coast from Florida to Virginia, the Mid-Atlantic region from Maryland to New York, the Northeast from Connecticut to Maine, the Great Lakes, southern California from San Diego to Santa Barbara, and the remainder of the West Coast from San Luis Obispo, California to Washington State.

The nested-logit structure also includes a "participation" component, which is a separate nest describing substitution into and out of beach recreation. This allows for changes in the total number of trips to all sites in response to changes in beach amenities in addition to switching of trips between sites. Each site in the model is represented by an estimated constant that reflects the total effect of all site attributes, such as the size of each site and quality characteristics.

As noted earlier, single-day trips and one-night trips are not included in the data for sites outside of the Southeast United States. This would cause problems in model estimation because demand functions must be fit to data that shows an increase in trips as price decreases. In other words, the model expects complete data that would show people close to the beach taking more trips than people far from the beach. Since most trips from close to the beach are single-day trips, the exclusion of single-day and one-night trips means that low prices correspond to fewer trips rather than more trips in the *Deepwater Horizon* data.

To overcome this problem, we do not attempt to fit the model to trips originating less than 125 miles from a given site. Instead, we fit the model's prediction of total demand for all observations within 125 miles of a site to a constructed total number of trips that accounts for trips of all lengths. We chose the 125-mile threshold because in the data for Southeast sites, 88.3% of all one-night trips originated from within 125 miles of the trip destination. Adding in single-day trips, the 125-mile area accounted for 99.2% of all trips. This total was constructed using the average relationship between the number of longer and the number of shorter trips at sites in the Southeast, where complete data on trips is available. Specifically, we found that the total number of single-day and one-night trips to Southeast sites was six times greater than the total number of trips lasting two nights or longer. For a given site, we therefore multiplied the number of trips lasting two nights or more, as reported in the data, by a factor of six to estimate the number of single-day and one-night trips to the site. Finally, we added together the constructed total number of single-day and one-night trips to the total number of trips lasting two nights or more that also originated from within 125 miles of the site. In model estimation, this constructed total was matched to the sum of model predictions for all respondents living within 125 miles of the site.

We made one final adjustment to ensure accurate trip predictions for study area sites. The factor of six times as many short trips as long trips used above is an average. Therefore it is only an approximation for any given site. For the study area sites, we adjusted this factor until model predictions matched information from the onsite marine debris survey. For example, onsite data for Orange County, California indicates that 88.2% of trips are single-day trips. The number of single-day trips was calculated as the model's estimate of total trips minus the number of multiple-day trips reported in the data. Using the average factor of six times as many short trips as long trips, our initial model estimated that 79.1% of trips in Orange County were single-day trips. We adjusted the constructed total number of trips within the 125-mile threshold until the model predictions matched the onsite estimate of 88.2%. A similar adjustment was performed for the Delaware/Maryland site. No adjustment was needed for Alabama, since complete trip information was available for Gulf Coast sites.

For the Ohio study area we did not attempt to make an adjustment because the original model predicted almost no single-day trips. By contrast, the onsite data indicated that 98% of trips were single-day trips. We also found that the average distance people traveled to Ohio beaches for trips lasting at least two nights was considerably lower than the average distance in other regions of the country. We concluded that our model was not able to accurately estimate total trip demand for the Ohio study area. We therefore limited our results to multiple-day trips estimated directly from the *Deepwater Horizon* data and the onsite marine debris survey. The failure of the model to predict total trips for Ohio also indicates there is some uncertainty about using the model's estimate of value per day for multiple-day trips. However, with this caveat in mind we exclude only single-day trips and report both trip demand and value for multiple-day trips in the Ohio estimates.

3.1.3. Estimated Model Parameters

The estimated coefficients and standard errors for the nationwide recreation model are shown in Table 5. The sample size 41,708, and the model was estimated using the maximum likelihood procedure in Aptech Gauss 12 software. The travel cost coefficient is negative and highly statistically significant (Table 5), indicating that people have a strong preference for sites that are closer and less expensive to access. The nesting structure of the model involves estimating scale parameters that characterize the effect of the nested groupings. The scale parameter for grouping the sites into the seven regions defined above is highly significant (Table 5), indicating that sites within regions are good substitutes for one another and that the selected nesting structure is preferred to a model without the regional nests. The scale parameter for participation is also highly significant (Table 5), indicating that this component of the nesting structure is also important to the model.

Most demographic variables are statistically significant determinants of the demand for recreation trips, and the coefficient on income is highly significant. The constants representing the attractiveness of each of the 76 sites to some extent represent variation in the quality but to a large extent represent variation in the size of the defined sites. Site constants cannot be interpreted in relation to zero but only in relation to each other, so the *p* values are omitted and the lowest constant for any site was normalized to zero. Most site constants are omitted from Table 5, but we report the estimated constants for the four study areas.

The last eight parameters in Table 5 show the adjustments needed to simulate the two debris scenarios in each of the four areas. For example, to simulate the 20.9% decrease in trips (see Table 5) from a doubling of debris in Orange County, California, a value of 0.338

must be subtracted from the site constant for Orange County. This causes the model's prediction of trips in Orange County to decline from 27.1 million to 21.5 million, a decline of 20.9%. No standard errors are reported for the debris-scenario parameters because they are not estimated as part of the model but are found by searching for the value that matches model predictions to the percentage changes from the marine debris survey.

Table 5. Parameter estimates from the recreation demand model using *Deepwater Horizon* nationwide recreation data

Variable	Coefficient	Standard error	*p* value
Travel cost	-0.027	0.002	0.000
Scale parameter, regions	2.373	0.148	0.000
Scale parameter, participation	1.270	0.084	0.000
25k ≤ income < 50k	-0.786	0.145	0.000
50k ≤ income < 75k	-1.113	0.171	0.000
75k ≤ income < 100k	-1.409	0.190	0.000
100k ≤ income < 150k	-1.578	0.190	0.000
150k ≤ income	-2.859	0.375	0.000
% urban	-0.626	0.103	0.000
Age/100	-3.778	1.577	0.017
(Age/100)2	5.313	1.482	0.000
High school diploma	0.518	0.159	0.001
College degree	-0.210	0.115	0.068
Full time	-0.444	0.189	0.019
Part time	-0.586	0.204	0.004
Retired	-0.615	0.223	0.006
White	-0.646	0.196	0.001
Male	-0.006	0.137	0.967
HH members ≥ 18	-0.010	0.160	0.949
HH members < 18	0.162	0.078	0.037
Site constants for the four study areas			
Alabama	7.763	0.852	
Delaware/Maryland	6.814	1.500	
Ohio	3.118	1.400	
Orange County, California	5.846	0.835	
Parameter for simulating debris scenarios			
Alabama, "almost no debris"	0.088		
Delaware/Maryland, "almost no debris"	0.033		
Ohio, "almost no debris"	0.465		
Orange County, California, "almost no debris"	0.136		
Alabama, doubling of debris	-0.340		
Delaware/Maryland, doubling of debris	-0.263		
Ohio, doubling of debris	-0.640		
Orange County, California, doubling of debris	-0.338		

3.2. Comparisons to External Sources

In this section we evaluate key results of the marine debris study using comparisons to external sources. To the extent that results from different sources are similar, the comparison

provides some assurance that our estimates are accurate and robust. To the extent that results from different sources diverge, it is worth assessing the potential reasons and possible implications. The discussion in this section refers to results presented in Table 6.

Table 6. Consistency with external sources and benefit-transfer calibration. See text for discussion of these values

Statistic	Value
Effects of debris on recreation: Comparison of study results to previous revealed-preference estimates in Orange County	
Scenario: Eliminating marine debris (or reducing to almost none)	
Change in value per baseline trip, marine debris study	$5.58
Percent change in the number of recreation trips, marine debris study	9.5%
Change in value per baseline trip, 2014 revealed-preference study[a]	$6.05
Percent change in the number of recreation trips, 2014 revealed-preference study[a]	16.0%
Scenario: Increasing marine debris by 50%	
Change in value per baseline trip, marine debris study	-$6.26
Percent change in the number of recreation trips, marine debris study	-10.9%
Change in value per baseline trip, 2014 revealed-preference study[a]	-$2.31
Percent change in the number of recreation trips, 2014 revealed-preference study[a]	-6.1%
Seasonal consistency: Comparison of survey respondents intercepted on the beach in summer versus fall in Orange County	
Change in trips from reducing debris to "almost none," fall 2017 pretest[b]	4.9%
Change in trips from doubling of debris, fall 2017 pretest[b]	-16.3%
Change in trips from reducing debris to "almost none," summer 2018 respondents[c]	8.8%
Change in trips from doubling of debris, summer 2018 respondents[c]	-20.4%
Debris ratings: Comparison of debris ratings to onsite debris measurements	
Correlation between debris ratings and onsite measurements	0.87
Benefit function transfer: Comparison and calibration of single-day trip predictions[d]	
Delaware/Maryland	
Percent of all trips that are single-day trips, marine debris survey (onsite survey)	72.3%
Percent of all trips that are single-day trips, nationwide recreation model unadjusted	54.4%
Percent of all trips that are single-day trips, nationwide recreation model adjusted	72.3%
Orange County, California	
Percent of all trips that are single-day trips, marine debris survey (onsite survey)	88.2%
Percent of all trips that are single-day trips, nationwide recreation model unadjusted	79.1%
Percent of all trips that are single-day trips, nationwide recreation model adjusted	88.2%

a. These values are from a revealed-preference pilot study of marine debris impacts in Orange County, California, that was conducted by NOAA from July 2013 through January 2014 (IEc, 2014, Exhibit 29). Dollar values are in 2018 dollars, adjusted for inflation using the CPI (Federal Reserve Bank of St. Louis, 2019).

b. A pretest of the marine debris survey was conducted in Orange County, California, using methods that were similar to those of the final study. There were 49 completed mail interviews and 343 completed onsite interviews in the pretest.

c. Pretest results could not be reweighted to match onsite data because attitudinal questions such as "Do you think marine debris is a problem" were not included in the onsite survey for the pretest. We therefore compare pretest results to full-study results that have not been adjusted to match the onsite sample. As shown in Table 4, the differences between adjusted and unadjusted full-study results are modest.

d. The parameters for the Delaware/Maryland site and the Orange County, California, site were adjusted so that model predictions of single-day trips matched information obtained in the onsite surveys. No adjustment was made in Alabama because the *Deepwater Horizon* data includes complete information on trips of all lengths throughout the Southeastern United States. No adjustment was made in Ohio because the site was determined to be sufficiently different from other coastal sites that model predictions of single-day trips would not be reliable.

3.2.1. Effects of Debris on Recreation

Economic methods for valuing environmental quality fall broadly into two categories: revealed-preference methods and stated-preference methods. Revealed-preference involves inferring the value of environmental amenities based on choices people make about how to spend their time and money. For example, if people avoid nearby beaches with high levels of debris and instead travel further to get to clean beaches, we can infer they have a value for clean beaches. Stated-preference methods involve inferring value based on how people say they would spend their time and money in hypothetical circumstances. For example, people could be asked in a survey whether they would travel further to get to clean beaches. A comparison between revealed-preference and stated-preference results is one of the most common ways of evaluating the performance of studies using either method.

The first results in Table 6 compare our stated-preference estimates of the effect of debris on beach recreation to revealed-preference estimates from a previous study in Orange County, California (IEc, 2014; Leggett et al., 2018). The IEc (2014) study involved a survey of Orange County residents about their single-day trips to 31 local beaches in the summer of 2013. The study used a model of recreation choice to estimate the importance of marine debris to beach recreation based on which beaches people went to and the attributes of those beaches, including the amount of marine debris. Exhibit 29 of the IEc (2014) report shows model results for several debris scenarios. For comparison to our study, we selected two scenarios and two measures of the effects of marine debris for each scenario.

The first scenario we selected involves the elimination of debris at all 31 beaches in the IEc (2014) study. We compared this to our scenario of a reduction to almost no debris for beaches in Orange County. The scenarios are not directly comparable, because 13 of the 31 beaches in IEc (2014) were outside of Orange County, and the elimination of debris involves a greater change than a reduction in debris to almost none. The second scenario involves an increase in marine debris of 50% at all 31 beaches in IEc (2014). While still different from the scenario in our study with respect to the number of beaches, we were able to simulate the 50% increase by assuming a linear relationship between debris levels and the importance of debris to recreators. Specifically, we took the parameter of -0.338 for a doubling of debris and reduced it by half, to -0.169. This revised parameter was then entered into our recreation model to simulate the effects of a 50% increase in debris. A linear relationship between measured site attributes and their importance in a recreation model is an assumption used in many studies, including IEc (2014).

The first measure of effects we selected was the "benefit per baseline trip to impacted sites," as presented in Exhibit 29 of IEc (2014). This is the total change in recreation value resulting from the change in debris divided by the baseline number of recreation trips before the change. This measure is not directly comparable between the two studies, because in our study recreation trips includes both multiple-day trips and single-day trips, while IEc (2014) examined single-day trips only. However, it is preferable to normalize values to a per-trip amount since we expect that a total measure of value would be much higher in our study, given that our study includes trips by residents from throughout country rather than just residents of Orange County. The second measure of effects we selected was the percentage change in recreation trips resulting from a change in debris. There is no definitive reason why this percentage change should be higher or lower in either study, given a comparable scenario. However, the fact that the scope of the studies differ in both the types of trips

analyzed and extent of the population could also lead to a divergence in this measure of results.

For the scenario of a reduction in debris, our model estimated a value of $5.58 per baseline trip for Orange County. This is quite close to the value of $6.05 in IEc (2014). We adjusted all dollar values from IEc (2014) upward by a factor of 1.08 to account for inflation between 2013 and 2018 using the CPI (Federal Reserve Bank of St. Louis, 2019). The percentage increase in trips was 9.5% in our study, compared to 16.0% in IEc (2014). Due to the difference in scenarios, with a reduction in debris to almost none in our study rather than the complete elimination of debris, a smaller percentage change in our study is expected. The comparison of per-trip values is more ambiguous. A lower value per trip from a smaller change in debris in our study would be at least partially offset by the fact that the per-trip value for multiple-day trips (and therefore for all trips combined) is likely to be greater than the per-trip value for single-day trips only. Overall, we view the comparison for this scenario to be consistent with expectations. The lower effects in our study make sense given the smaller change in debris, but the difference in less pronounced with respect to value because of the inclusion of higher-value multiple-day trips.

For the scenario of a 50% increase in debris, our model estimated a value of -$6.26 per baseline trip. This compares to a value of -$2.31 in IEc (2014). The percent change in trips was -10.9% in our model, compared to -6.1% in IEc (2014). In this instance both measures of the change are definitively higher in our study. However, the divergence is sufficiently modest that it could be explained by differences in the scope of the two studies with respect to trips and population. One methodological difference that could also play a role is the assumption in IEc (2014) that the importance of debris to beachgoers is linear with respect to debris amounts over all ranges. In our study, we allow the effects of debris increases to be independent of the effect of debris reductions. If the response to a given change in debris is higher at higher levels of debris, the greater flexibility in debris response permitted in our model could help explain the greater divergence of results in the second scenario.

3.2.2. Seasonal Consistency

Respondents to the marine debris mail survey reported their beach recreation trips, and the effects of changes in debris on their trips, for the period of an entire year. However, respondents were first contacted over a period of just several weeks in July, August, and September 2018. It is possible that preferences differ for those using beaches during late summer versus the full population of beachgoers that could be contacted with more extensive onsite sampling. The differences may be greater in areas where beach use continues throughout most or all of the year, such as Alabama and California.

To investigate this potential source of bias we compared results from the final survey in Orange County, California with earlier results obtained from the pretest phase of our study, also conducted in Orange County. Onsite recruitment for the full study in Orange County occurred in July 2018 and onsite recruitment for the pretest occurred in late September 2017. The pretest results for a reduction in debris showed a 4.9% increase in trips and the pretest results for a doubling of debris showed a 16.3% decrease in trips. In the pretest, our methods for reweighting mail survey respondents to match onsite respondents could not be applied because the pretest onsite survey did not ask whether debris was a problem at area beaches, one of the variables used in the reweighting. For a more consistent comparison, Table 6 therefore reports results from the final survey that are also calculated using the onsite base

weights only. These results show an 8.8% increase in trips and a 20.4% decrease in trips for the two scenarios.

The results from the pretest suggest a potentially lower response to debris by people contacted in fall rather than late summer. This comparison indicates that some bias may result from sampling people onsite over a period of a just a few weeks, and that a more extensive study that involves sampling onsite over the full year or full beach season could lead to different estimates.

3.2.3. Debris Ratings

Respondents were asked to estimate the amount of marine debris on beaches in a given study area. It is important to understand the accuracy of respondents' estimates for at least two reasons. First, the debris estimates were used to characterize current conditions in the four study areas and provide context for other study results. For example, if it is true that current debris levels are higher on Lake Erie beaches relative to other study areas, then the high estimated response to the debris scenarios, which involve changes proportionate to current levels, would be expected. Second, if the debris ratings are consistent with external evidence then it suggests that respondents pay attention to debris on beaches and are aware of the relative amounts of debris on beaches. This indicates that respondents have some context for providing accurate responses to the hypothetical debris scenarios.

We compared the ratings of debris levels from survey respondents with estimates of debris from onsite measurements conducted by NOAA in the summer of 2016. The comparison accounts for all 13 beaches that were included in both studies: Ocean City, Maryland (Boardwalk area); Lewes Beach, Delaware; South Bethany Beach, Delaware; Fenwick Isle, Delaware; Bolsa Chica, California; Cedar Point Beach, Ohio; East Harbor State Park, Ohio; Nickel Plate Beach, Ohio; Port Clinton City Beach, Ohio; South Bass Island, Ohio; Fort Morgan, Alabama; Gulf State Park, Alabama; and Orange Beach, Alabama.

The correlation between respondent ratings and onsite measurements was 0.87. Consistent with the average rating calculated for a study area, the average rating for a given beach was calculated as the weighted average of all ratings provided for the beach. The high correlation with onsite measurements suggests that people have a high awareness of the relative levels of debris at beaches. However, as noted earlier, the consistency between respondent perceptions and onsite measurements applies only to the 1-to-5 debris ratings. If these ratings are converted into absolute measures of debris based on the debris levels associated with each numeric rating (1 item per 500 square feet for a rating of 1, up to 16 items per 500 square feet for a rating of 5), the resulting measure is not correlated with the onsite debris measurements. For this reason we used only the 1-to-5 numeric ratings in this study.

3.2.4. Benefit Function Transfer

The *Deepwater Horizon* data has complete information about the number of trips to sites in the Southeast United States. In other coastal areas, the data only include longer trips with at least two overnight stays. As described earlier, for all model sites outside the Southeast United States we accounted for shorter trips using a constructed total number of trips for an area within 125 miles of each site. The constructed total was based on the ratio of longer trips to shorter trips for sites in the Southeast United States, and the result is what we call the "unadjusted" model in the last section of Table 6. In the final "adjusted" model, the

constructed totals in Delaware/Maryland and Orange County, California were further increased to ensure that the proportion of single-day trips predicted by the model exactly matched the proportion of single-day trips estimated from the onsite surveys. This type of adjustment falls under the classification of "benefit function transfer" in the economics literature (e.g., Wilson and Hoehn, 2006; Navrud and Ready, 2007).

The last section of Table 6 shows the original estimates from the unadjusted model, as well as the effect of the final adjustments. We include these results in Table 6 because they play a role in evaluating the reliability of the nationwide recreation model and the use of benefit function transfer in two ways. First, the unadjusted estimates of the percentage of single-day trips is lower in Delaware/Maryland (54.4%) than in Orange County, California (79.1%). A similar difference across regions is apparent in the adjusted percentages of 72.3% for Delaware/Maryland and 88.2% for Orange County, California. This suggests that the model is accurately accounting for important factors that differ across regions. For example, a large population density close to the coast in California would lead to predictions of a large number of single-day trips.

Second, the final adjustments illustrate how we are able to combine information from the *Deepwater Horizon* data with information from the marine debris survey to estimate the total number of trips as accurately as possible. The unadjusted model, while developed from reasonable assumptions, would likely be less reliable than the final adjusted model.

3.3. Caveats and Uncertainties

There are a variety of caveats and uncertainties associated with the methods described above. We summarize them here and discuss their implications.

Stated preference methods have the advantage that they can directly examine a specific issue. If researchers wish to know the effect of a particular beach attribute on beach recreation, a hypothetical scenario can be developed in which only the attribute of interest changes. This differs from revealed-preference methods, in which two beaches that have different levels of one attribute will almost certainly differ with respect to other attributes as well. This makes stated-preference methods applicable to a wider variety of research problems than revealed-preference methods, and less likely to be biased due to the influence of confounding factors. The drawback of stated-preference data is that responses to hypothetical scenarios, and the resulting estimates of value, may differ from the values implied by people's actual choices.

The comparison of our results in Orange County, California to results from an earlier revealed-preference study in the same location provides some assurance that both studies are reliable. For a reduction in debris, the scenarios in the two studies were not exactly comparable and the more modest effects measured in our study could be attributable to the assumption of a smaller change in debris. For an increase in debris, our study measured a greater response than the previous study. The difference was within a modest range that could be explained by the more extensive scope of our study. While the previous study involved single-day trips by the local population, our study encompassed virtually all trips by people from throughout the county.

Another source of uncertainty was the high item nonresponse rate for the contingent behavior questions. Overall, 9.1% of respondents did not provide an answer when asked

about a reduction in debris and 27.7% did not provide an answer when asked about a doubling of debris. It is not possible to know whether or how these respondents may differ from those who answered the contingent behavior questions. In our calculations we assumed these respondents made no change in their trips in response to changes in debris. This could be viewed as conservative. Other options would include filling in the missing responses using the average of the available responses, or analyzing the demographic characteristics of respondents who did not provide an answer and filling in missing responses using other similar respondents. These alternative options would have led to an increase in the size of the estimated change in trips and value from the debris scenarios.

We used onsite sampling to efficiently reach a target population of beachgoers. Some aspects of our onsite sampling procedures were randomized. For example, we selected randomly from each party of people on the beach and used party size to control for selection probabilities. Other aspects of our sampling procedures were nonrandom. For example, we divided beaches into more developed and less developed categories and ensured that both types of beach were included in our onsite surveys. This procedure was similar to stratified sampling. However, a fully random stratified sample would have involved drawing a random selection of days and times for conducting interviews at each type of beach, and then incorporating the sampling rates for each beach stratum into the sampling weights. Without these procedures, we cannot guarantee that our sample accurately represents beachgoers who use different types of beaches in the correct proportion. Likewise, the representation in our sample of times of day and weekdays versus weekend days is unlikely to match the correct proportions of a fully randomized sample. As discussed earlier, our sample does not represent the various seasons throughout the year.

Our comparison of results for different seasons, discussed above, suggested that preferences about marine debris may vary somewhat for people using the beach at different times of the year. It is also possible that a randomized selection of days, times, and beaches, resulting in a sample that better represents all beachgoers, would have led to changes in the estimated effect of debris on recreation. There is no reason to suspect a specific type of sample-selection bias, such as sampling onsite at beaches with high debris levels and therefore biasing the sample toward those who are tolerant of debris. However, there is no basis at this time for estimating the direction or magnitude of any effect of the nonrandom aspects of the onsite sampling.

Onsite sampling also omits from the study population those people who do not currently go to the beach in a given study area but would go if beaches had less debris. This means that some people who would benefit from a reduction in debris are excluded from the study and that the effects of a reduction in debris could be underestimated. This type of bias could be significant if current debris levels are high enough to significantly change not just the number of trips people take, but the number of people who take any trips in a given study area.

Our estimates of the total change in recreation trips and total change in recreation value for the two debris scenarios was based on a benefit-function transfer using the *Deepwater Horizon* data set. Benefit transfer is widely used in economic analysis because it allows researchers to evaluate policy or environmental changes without the potentially prohibitive expense of a full original study. The use of benefit transfer is supported by numerous precedents in the literature and guidance from government agencies (Wilson and Hoehn, 2006; Navrud and Ready, 2007; U.S. EPA, 2014). Benefit function transfer, which involves adapting an economic model to a new research problem, is considered preferable to simpler

forms of benefit transfer, such as applying values calculated in a previous study without the ability to revise the calculations.

The most significant assumption in the nationwide recreation model is that longer trips lasting at least two nights away from home provide sufficient information about the quality of beaches and the preferences of recreators to reliably estimate a recreation demand model. There are situations where this would not be the case. For example, two beaches may be equally attractive to area residents taking day trips, while only one of the beaches may be developed with hotels and beachfront resorts. The number of overnight trips to the less developed beach would not be a good predictor of the number of day trips. However, this type of issue may diminish as sites with divergent characteristics are aggregated together. Continuing the example of overnight accommodations, most of the 76 aggregate sites in our model are likely to be large enough that a lack of suitable hotels or campgrounds would not be a significant factor limiting overnight recreation trips.

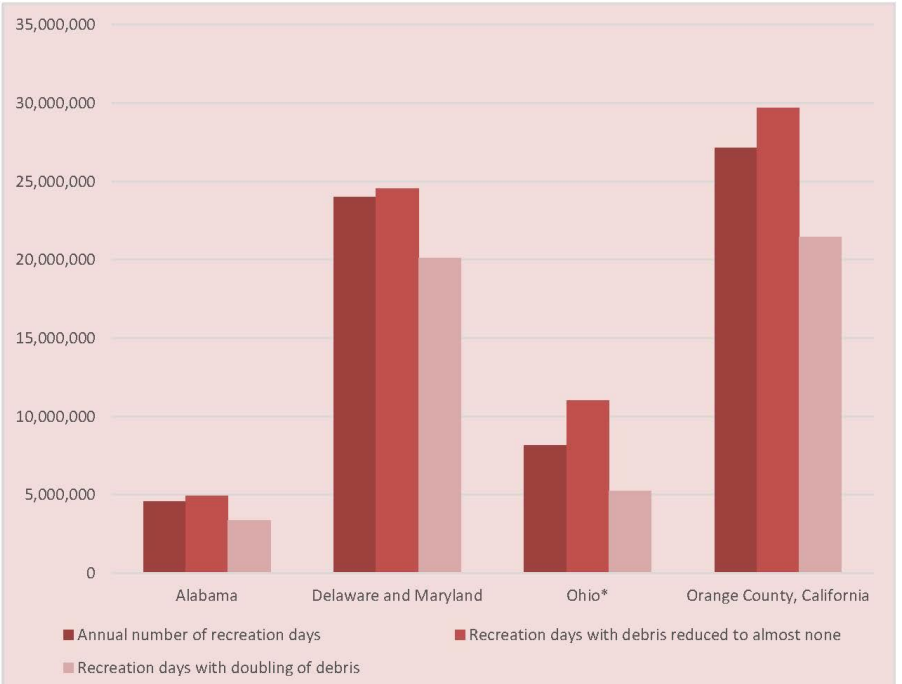
The aggregation of beaches into larger areas would not diminish the effect of differences in beach attributes if beaches in one area are consistently different from beaches in another area. As noted earlier, this appears to be the case for Ohio beaches. For Delaware/Maryland and Orange County, California, where trips data from the nationwide model were incomplete, we believe the benefit function transfer is reliable. This is reflected in the fact that model parameters demonstrated consistency with a standard model specification, including a nesting structure that grouped sites into regions and that included a component for recreation participation, as shown in Table 5. Reliability of the benefit function transfer is also reflected in the fact that only modest adjustments were required to match model predictions in Maryland/Delaware and Orange County, California to information from the onsite data, as shown in Table 6.

3.4. Effects of Marine Debris on Recreation Value

The recreational value model shows noteworthy effects of marine debris on beach recreation for the two scenarios and four study areas examined in the marine debris survey (Figure 3, Table 7). All amounts reflect annual total trips and value by residents living within 750 miles of the coast in the contiguous 48 states. As described previously, the two scenarios are a reduction in debris on beaches to almost none and a doubling of debris, and the four study areas are Gulf Coast beaches in Alabama, Atlantic beaches in Delaware and Maryland, Lake Erie beaches in Ohio, and Pacific beaches in Orange County, California. While confidence intervals were provided for earlier results derived directly from the marine debris survey, results in Table 7 rely on benefit function transfer and therefore do not include confidence intervals. The combination of multiple data sources and researcher judgments make confidence intervals difficult to estimate or justify in most benefit transfer contexts (McConnell, 1992).

The annual number of recreation days under "baseline conditions" ranges from 4.5 million in Alabama to over 27.1 million in Orange County (Table 7). Baseline conditions describes debris levels prior to the changes introduced in the hypothetical scenarios. The baseline number of recreation days was estimated using the nationwide recreation model, developed from data collected in 2012 and 2013 for the *Deepwater Horizon* oil spill assessment. Where possible the model was adjusted to match information collected as part of

the marine debris survey. We assume that beach attributes and recreation activity at beaches in the four study areas have not changed significantly over the past several years and that the two sources combined in this study accurately summarize the starting point for the debris scenarios.



* Ohio estimates account for multiple-day trips only.

Figure 3. Changes in annual number of recreation days if the amount of marine debris doubles, and if the amount of marine debris is reduced to almost none.

Table 7. Recreation value from changes in debris on beaches (2018 dollars)

Scenario	Alabama	Delaware and Maryland	Ohio[a]	Orange County, California
Annual number of recreation days	4,552,112	24,014,592	8,155,158	27,143,415
Debris reduced to "almost none"				
Change in days	368,525	536,341	2,889,191	2,571,725
Value per day	$27.27	$36.81	$30.46	$50.43
Change in recreation value	$10,051,517	$19,741,209	$88,006,606	$129,689,616
Doubling of marine debris				
Change in days	-1,206,006	-3,915,792	-2,907,188	-5,682,362
Value per day	$26.82	$35.99	$28.87	$48.41
Change in recreation value	-$32,347,029	-$140,914,688	-$83,935,614	-$275,077,340

a. For Ohio, estimates account only for multiple-day trips and exclude the value and quantity of single-day trips.

The estimated change in days is the percentage change in trips for each scenario, calculated from the marine debris survey and presented in Table 5, multiplied by the baseline number of days. The change in recreational value is derived using the nationwide recreation model. The value per day is the change in value divided by the change in days. The value per day is usually reported in economic studies of recreation as a common point of comparison across studies. Differences in the value per day across study areas reflect modeling assumptions, differences in recreation preferences by beachgoers in each study area, and differences in income, which is an input to travel cost for beachgoers in each area. The decision to limit recreation trips to a threshold of 750 miles, necessitated by limitations in the *Deepwater Horizon* data, is likely to reduce per-day values in areas with high demand from greater distances.

For a reduction to almost no debris, the estimated annual increase in recreation value is $10.1 million in Alabama, $19.8 million in Delaware/Maryland, $88.0 in Ohio (multiple-day trips only), and $129.7 million in Orange County, California. For a doubling of debris, the estimated annual decrease in value is $32.3 in Alabama, $140.9 in Delaware/Maryland, $83.9 million in Ohio (multiple-day trips only), and $275.1 million in Orange County, California.

4. REGIONAL ECONOMIC IMPACTS MODEL

Coastal recreation is a significant component of the economies of coastal communities. A recent NOAA National Marine Fisheries Service study found that coastal recreation in the United States accounts for $225 billion in gross domestic product (GDP; Kosaka and Steinback, 2018). The four study areas included in our analysis were selected because coastal recreation and tourism is an important part of the local economy in each area. Table 8 shows the total economic contribution of coastal recreation and tourism for the four study areas (Office for Coastal Management, 2019).

Table 8. Economic contribution of coastal recreation and tourism by study area (2018 dollars)

	Alabama	Delaware/Maryland	Ohio	Orange County, California
Employment[a]	17,252	16,509	38,224	44,240
Wages ($million)	$313.9	$368.7	$682.5	$1,145.6
GDP ($million)	$642.4	$849.6	$1,514.1	$2,337.3

Source: Office of Coastal Management, 2019.
a. Employment numbers are for 2015.

The total economic impact of recreation visits includes direct expenditures and subsequent flow-on impacts, which includes both indirect and induced expenditures. Direct expenditures include money that non-local residents spend while visiting and participating in recreation activities in coastal communities (e.g., park entrance fees, gas, equipment, retail purchases, lodging). Local businesses that benefit from direct spending then spend additional money on goods and services that they need to operate their businesses. These are termed indirect expenditures. Direct and indirect spending generates employment in the local region,

creating additional income for households, which generates further spending known as induced expenditures.

To estimate the economic impact associated with marine debris, we multiplied the total change in recreation days for residents who live outside each study area by estimates of spending per recreation day. We include spending from only non-local residents because economic impact analysis assumes that residents who live in a given region and choose not to spend money on a particular good will spend the money on something else in the local economy. As noted earlier, for the economic impact assessment we define the scope of our analysis to be the coastal counties where the beaches in each study area are located.

Our analysis of economic impacts from marine debris can be summarized in the following three steps.

1. We began with the change in the number of nonlocal recreation days estimated in the previous steps for each of the four study areas and for each of the two scenarios in the marine debris survey.
2. We used the NORES dataset to estimate expenditures per recreation day in each of several categories and multiplied the expenditures per day by the change in recreation days. The result is an estimate of the change in direct expenditures in each study area.
3. We then multiplied direct expenditures in each category by the appropriate RIMS II multipliers (U.S. BEA, 2018, 2019). For each expenditure category there are several multipliers for several different types of indirect and induced economic impacts.

In the remainder of Section 4, we describe details of the estimated expenditures per day and the RIMS economic multipliers. In Section 4.1 we present the expenditures per recreation day associated with recreation activities impacted by marine debris. In Section 4.2, we estimate the economic impacts of a coastal-recreation day using the RIMS multipliers; and in Section 4.3, we present the economic impacts of our two marine debris scenarios – a reduction of debris to almost none and a doubling of debris – in the four tourism-dependent communities.

4.1. Trip Expenditures

As our first step in conducting the economic impact analysis, we estimated the direct effects by determining the average spending per day by non-local visitors to the study areas. We included all expenditures that visitors make during their visits, not just those associated with recreational activities. This is based on the fact that the primary purpose of recreation trips in our data was to visit the beach, so a canceled trip to the beach is likely to be a canceled trip to the study area. The NORES data (NOAA, 2012) provide comprehensive expenditures for recreation trips, including spending on hotels, restaurants, transportation, entrance fees, etc.

The NORES data included estimates of trip expenditures for the Pacific, Northeast, Mid-Atlantic, South Atlantic, and Gulf of Mexico regions. There are also estimates of overall average expenditures for all regions. For each study area, we used the region that included that study area. Because the NORES study did not include estimates for the Great Lakes, we

applied the U.S. average expenditures to estimate the trip expenditures for the Lake Erie, Ohio region.[5]

Although the NORES data included separate estimates for local and nonlocal recreation trips, we did not use this breakout. Their "local" regions are much larger than our coastal county areas. For example, the NORES Pacific region considers all recreators from California, Oregon, and Washington to be local, whereas our study defines recreators from outside of Orange County as "nonlocal." Thus, we used the average trip expenditures across all respondents to be more representative of our nonlocal respondents.

The NORES data provide average daily spending on trip-related items for trips involving a variety of recreational activities. Each trip is identified with a single activity based on the respondent's "most preferred" activity on the trip. NORES included eight types of coastal recreation activities. We used the four activities likely to involve trips to the beach:

- Viewing or photographing the ocean or coast
- Beachcombing, tidepooling, or collecting items
- Water contact sports
- Outdoor activities not involving water contact that occur near the ocean or coast because of the view or access to the water (e.g., sunbathing, walking, camping).

Table 9. Average visitor spending per day in the four regions examined in this study (2018 dollars)

	Alabama	Delaware and Maryland	Ohio	Orange County, California
Average visitor spending per day	$138.55	$92.06	$96.61	$89.49

Spending by category varies by the type of activity.[6] We calculated the average visitor spending per day (Table 9) using the weighted average expenses and participation in the various marine debris-related beach activities.

To apply the RIMS II multipliers, we kept the average expenditures separate by category. For example, visiting recreators spend money on lodging, food, and transportation. These expenditure types have different impacts on the local economy and thus different multipliers. This is explained in more detail below. The detailed list of the average expenditures by category is included in Appendix D.

We next apply the changes in the number of recreation days estimated in Section 3 to the average expenditures per day for each of our four study areas and two debris scenarios. The results are described in the following section.[7]

[5] We checked the appropriateness of applying the U.S. average expenditures to the Ohio region using the Consumer Expenditure Survey (U.S. BLS, 2017). In the year the expenditure data were collected, 2012, average annual expenditures in the Midwest and Cleveland metropolitan area were 97% of the U.S. average.

[6] For example, the average auto fuel expense in the Pacific region is $17.48 for "viewing or photographing the ocean" and $22.10 for beachcombing participants.

[7] The aggregated changes in visitor spending are not used in the analysis. As described in the next section, in order to apply the RIMS II multipliers, we must keep visitor spending disaggregated by expenditure category. The expenditure categories are then mapped to the RIMS II industries, and the multipliers applied. These totals are presented here to illustrate the scale of money flowing into the economy.

4.2. Economic Impacts of Coastal Recreation

The next step in estimating the potential impacts of a change in marine debris levels on beaches in our four study areas is to estimate the economic contribution of visitor expenditures on the local economy. Coastal recreation contributes to the local economy by bringing outside money into the economy in the form of visitor spending. Visiting recreators spend money on a number of goods and services, including hotel rooms, food, and retail, which affect several local industries, including restaurants, hotels, retail shops, and other tourist-related enterprises. These industries directly affect the economy by purchasing intermediate goods, such as restaurant supplies and wholesale goods, and by providing jobs. The industries that provide intermediate goods and services to the recreation and tourism industry purchase their own intermediate goods and services form other local industries, and the pattern repeats itself. Thus, the original money from visitor spending creates a multiplier effect on the local economy. At every stage, some portion of expenditures goes toward goods or services generated outside the local area. This is known as "leakage" and is incorporated in the calculations of multiplier effects (Bess and Ambargis, 2011).

Economists use input-output (I/O) analysis to estimate multiplier effects. I/O analysis entails calculating the extent to which direct activities – in our case, increased spending from tourists – stimulate further economic effects, spreading employment and income, thus accounting for linkages among industries (University of South Carolina, 2009). That is, I/O analysis accounts for the production linkages between different industries of the local economy, and in turn, calculates economic impacts using a multiplier effect. We used RIMS II multipliers developed by the Bureau of Economic Analysis (U.S. BEA, 2018, 2019).

We quantified the economic impacts of a visitor coastal recreation day using four metrics: output (sales), value added (GDP), earnings, and employment (full- and part-time jobs). These metrics are defined formally as follows (Bess and Ambargis, 2011):

- *Gross output*. Sum of the intermediate inputs and value added, where intermediate inputs are defined as goods and services that are used in the production process of other goods and services and are not sold in final-demand markets; also measured as the sum of the intermediate inputs and final use. Multipliers measure the total industry output per $1 change in final demand.
- *Value added*. The value of gross output less intermediate inputs. The value of this metric is equal to the sum of compensation of employees, taxes on production and imports less subsidies, and gross operating surplus.
- *Earnings*. Sum of wages and salaries, proprietors' income, and employer contributions for health insurance excluding contributions for social insurance. Multipliers measure the total household earnings per $1 change in final demand.
- *Employment*. Number of full- and part-time jobs (including proprietors' jobs). Multipliers measure the total number of jobs per $1 change in final demand.[8]

Output is less preferred as a metric because it counts transactions at all stages of production without including the value of goods and services in previous stages. Value added is preferable because it eliminates this double counting, and is analogous to GDP for a local

[8] Employment multipliers are originally expressed as the change in jobs per $1 million change in final demand.

region. Earnings is a subset of value added, representing the portion that ends up as wages and salaries rather than as a return on investment.

A fifth measure of economic impacts is government tax revenue. Calculating tax revenues can be quite complex because tax rates depend on the finances of numerous individual companies. We have not attempted to estimate government tax revenues in this study.

The multipliers are provided by industry type and are specific to each region. The multipliers estimate the economic impact of *outside money* coming into a region; thus, we estimate the economic impact of changes in coastal recreation associated with the marine debris scenarios for visiting recreators only.

As described above, the NOAA (2012) NORES dataset provides spending per coastal recreation day by study area and expenditure type. Different sectors of the economy such as lodging and food service contribute to the regional economy to different degrees. The RIMS II multipliers are provided for 64 aggregated industries. We mapped the expenditure categories provided in the NOAA (2012) NORES dataset to the appropriate RIMS II industry. That is, each industry has a unique multiplier, which we apply to the corresponding expenditure category. The total economic impact is the sum of the multipliers applied to the corresponding expenditure. We include the expenditure by category and RIMS II industry in Appendix D. We have included the multipliers in Appendix D, Table D-2.

To illustrate the regional economic impact of a change in spending, we provide the following example for increased spending on lodging in Alabama: From the NORES data, we estimated the average expenditure per day for lodging to be $39.68 (see Appendix D, Table D-1). We then mapped the expenditure category "lodging" to the RIMS II industry "accommodation." The RIMS II final-demand multipliers (Appendix D, Table D-2) for the accommodation industry in Alabama (Baldwin and Mobile counties) are:

- Output: 1.6311
- Earnings: 0.4604
- Jobs: 0.0000173756
- Value added: 1.0023.

First, we multiply the accommodation output multiplier, 1.6311, by the average daily expenditure for lodging of $39.68. This yields output generated per recreation day from expenditures on lodging equal to $64.72. This is $25.39 more than the initial or direct expenditure of $39.68. This additional increase of $25.39 in output is the measure of the indirect and induced effects that result from the initial direct change in spending of $39.33 on lodging. This is because the initial increase in spending stimulates additional changes in spending, such as by hotel employees. These additional changes in spending then cause further changes in production, income, and employment in the region.

Next we estimate the value-added portion of this output, which is the value of gross output less intermediate inputs. Multiplying our value-added multiplier, 1.0023, estimates the value added of lodging expenditures equal to $39.77. The earnings portion of this value-added is $18.27 (0.4604 times $39.77). To estimate the expected change in jobs, we would next apply the jobs multiplier of 0.0000173756, but we skip this final calculation in our per-day example.

We conduct this step for each of the expenditure categories and industry combinations. The total economic impact is the sum of the product of each expenditure category/industry combination for each metric.

Finally, we conduct an additional step for retail industries in which we convert consumer value (i.e., the visitor expenditures) into producer value. This step is explained in Appendix D.

4.3. Regional Economic Impacts of Marine Debris

The results of this regional economic impact analysis show that marine debris can have profound effects on regional economies. A reduction of debris to almost none is estimated to contribute an additional $29 million in economic activity (measured as value-added) in Alabama; $27.8 million in Delaware and Maryland; $206.0 million in Ohio; and $137.8 million in Orange County, California (Table 10). Conversely, a doubling of debris is estimated to cost the local economies $96.3 million in Alabama; $203.2 million in Delaware and Maryland; $207.3 million in Ohio; and $304.5 million in Orange County, California (Table 10).

Table 10. Economic impacts of changes to debris levels at the four study areas (2018 dollars)

	Alabama	Delaware and Maryland	Ohio	Orange County, California
Recreation day expenditures	$139	$92	$97	$89
Output generated per recreation day	$169	$102	$130	$116
Earnings generated per recreation day	$50	$27	$34	$30
Jobs generated per recreation day	0.0022	0.0010	0.0013	0.0009
Value added generated per recreation day	$95	$58	$73	$66
Debris change to almost none				
Change in visitor days	308,365	478,410	2,823,268	2,092,920
Change in visitor spending	$42,724,928	$44,040,576	$272,761,000	$187,294,392
Change in output	$52,116,000	$48,879,000	$367,522,000	$241,994,000
Change in earnings	$15,547,000	$13,076,000	$96,026,000	$61,797,000
Change in jobs	672	464	3,703	1,925
Change in value added	$29,423,000	$27,834,000	$205,977,000	$137,830,000
Doubling of debris				
Change in visitor days	-1,009,130	-3,492,845	-2,840,854	-4,624,417
Change in visitor spending	-$113,427,000	-$254,086,000	-$274,460,000	-$413,837,000
Change in output	-$170,551,000	-$356,865,000	-$369,811,000	-$534,698,000
Change in earnings	-$50,877,000	-$95,467,000	-$96,624,000	-$136,543,000)
Change in jobs	-2,198	-3,386	-3,726	-4,254
Change in value added	-$96,288,000	-$203,211,000	-$207,260,000	-$304,542,000

4.4. Caveats and Uncertainties

There are several areas of uncertainty in this calculation of regional economic impacts. First, the NOAA (2012) NORES dataset reports all expenditures per trip, which is not limited to expenditures made for individual respondents. For example, the NORES data include hotel expenditures for a family. To align these expenditures with the increased number of trips estimated in Section 3, we assume that the trips referred to in the NORES data and the trips referred to in our marine debris surveys have the same average number of people.

Second, as noted previously, the NOAA (2012) NORES data estimate trip expenditures that occur in a larger region than our study areas. Thus, some of the trip expenditures (e.g., gas, transportation) in the larger NORES regions may occur outside of our study area. For this analysis, we applied the trip expenditures to our smaller study region, noting that this may overstate transportation spending in the regions.

Finally, we note that RIMS II multipliers measure the impact of employment using a count of jobs that include both full-time and part-time workers (Bess and Ambargis, 2011). Since the tourism industry may have a large portion of part-time and seasonal workers, the employment impact may be an upper-bound.

5. SUMMARY OF RESULTS

This study provides estimates of recreation value and regional economic impacts, including an evaluation of key features in the design of the study, the comparison of model output to external estimates, and potential implications for future research. Highlights of our study findings are described below.

- We adjusted mail-survey sampling weights to match key characteristics of respondents in the onsite survey, thereby taking advantage of the high response rate in the onsite survey (77%) and potentially improving representativeness of the mail survey data. The adjustments led to significant changes in the representation of certain demographic groups but only modest changes in the estimates of recreation value and economic impacts.
- We found a high correlation (0.87) between ratings of debris levels on beaches provided by survey respondents (where respondents rated debris levels on a 1-to-5 scale, with one referring to “almost no” debris and 5 referring to a “high amount” of debris) and actual debris amounts estimated from onsite measurements conducted by NOAA.
- The benefit function transfer based on the extensive *Deepwater Horizon* nationwide dataset resulted in a model that appeared to provide reliable estimates when applied to study areas in Alabama, Delaware/Maryland and Orange County, California. However, the model did not appear to fit well with Lake Erie beaches in Ohio, particularly for estimating changes in day use, and thus we only provide estimates of changes to multiple-day trips in Ohio.
- We compared our results in Orange County, California to recent estimates from a revealed-preference study in the same location (Leggett et al., 2018). Our estimates

of the impacts from debris on the value and number of trips were either comparable or somewhat higher than those of the previous study, depending on the scenario examined. However, an exact comparison was not possible because the Leggett et al. (2018) study focused exclusively on single-day trips, whereas our analysis included multiple-day trips.

- We used data from NORES that provides estimates of recreator expenditures during coastal recreation trips. We adjusted the expenditure values to account for differences with our study, which provided recently estimated expenditures for relevant recreation activities for each study area. The NORES data did not include the Great Lakes, however, so we used the national average for Lake Erie beaches in Ohio.

Table 11 provides a summary data table for all the results of this study. This section provides a summary of only selected data; additional information is included in subsequent sections and in the Executive Summary.

Table 11. Summary of study results

	Alabama	Delaware and Maryland	Ohio[a]	Orange County, California
Annual number of recreation days	4,552,112	24,014,592	8,155,158	27,143,415
Average debris ratings	1.7	1.6	2.3	2.1
Percent of recreation days from outside the local area (visitor recreation days)	83.7%	89.2%	97.7%	81.4%
Spending per day by non-local residents	$139	$92	$97	$89
Debris reduced to almost none				
Percent change in recreation days	8.1%	2.2%	35.4%	9.5%
Change in recreation days	368,525	536,341	2,889,191	2,571,725
Change in recreational value	$10,051,517	$19,741,209	$88,006,606	$129,689,616
Change in visitor recreation days	308,365	478,410	2,823,268	2,092,920
Change in employment	672	464	3,703	1,925
Change in value added[b]	$29,423,000	$27,834,000	$205,976,434	$137,830,000
Doubling of debris				
Percent change in recreation days	-26.5%	-16.3%	-35.6%	-20.9%
Change in recreation days	-1,206,006	-3,915,792	-2,907,188	-5,682,362
Change in recreational value	-$32,347,029	-$140,914,688	-$83,935,614	-$275,077,340
Change in visitor recreation days	-1,009,130	-3,492,845	-2,840,854	-4,624,417
Change in value added[b]	-$96,288,000	-$203,211,000	-$207,259,895	-$304,542,000

a. Estimates for Lake Erie beaches in Ohio account for multiple-day trips only.
b. Value added is net combined value of all goods and services, analogous to GDP for the local area.

Although the data collection and analysis was based on changes in recreation trips, we have expressed the results in units of recreation days to reflect the relative importance of multiple-day trips in influencing economic value. Based on a benefits function transfer from the *Deepwater Horizon* dataset, the estimated annual number of recreation days is 4.5 million on beaches in Alabama, 24.0 million on beaches in Delaware and Maryland, and 27.1 million on beaches in Orange County, California (Table 11). We also estimate there are 8.2 million recreation days taken annually on Lake Erie beaches in Ohio, accounting for multiple-day trips only. As noted previously, estimates for Ohio account only for multiple-day trips

because of limitations in how the nationwide recreation model could be applied to Lake Erie beaches.

All estimates include uncertainties that reflect not only statistical error, but also potential error from the combined use of data collected at different times between 2012 and 2018 and from a benefit-transfer analysis that relies on reasonable but imperfect assumptions about similarities in recreation behavior across different coastal locations.

Respondents' ratings of the amount of debris on beaches on a 1-to-5 scale ranged from 1.6 for Delaware/Maryland beaches to 2.3 for Lake Erie beaches in Ohio. The effect of changes in debris on recreation was lowest in Delaware/Maryland, where respondents said the beaches had less debris, and highest in Ohio, where respondents said that beaches have more debris. These results are consistent because the debris scenarios presented in the survey involved changes in debris that are proportionate to current debris levels. Estimates of the percentage increase in trips from reducing debris to "almost none" (defined in the survey as one piece of debris per 500 square feet) are 8.1% in Alabama; 2.2% in Delaware/Maryland; 35.4% in Ohio; and 9.5% in Orange County, California (Table 11).

The effect of a doubling of current debris levels is a decrease in trips and is a larger percentage change compared to debris reduction in all areas. The specific estimates of decreasing trips are 26.5% in Alabama, 16.3% in Delaware/Maryland, 35.6% in Ohio, and 20.9% in Orange County, California (Table 11).

Table 11 also shows changes in the value of recreation, reflecting the public's enjoyment of area beaches. If marine debris were reduced to almost none, the estimated annual increase in recreation value is $10.1 million in Alabama, $19.8 million in Delaware/Maryland, $88.0 in Ohio (multiple-day trips only), and $129.7 million in Orange County, California (Table 11). If the amount of marine debris on beaches were to double, the estimated annual decrease in recreational value is $32.3 million in Alabama, $140.9 million in Delaware/Maryland, $83.9 million in Ohio (multiple-day trips only), and $275.1 million in Orange County, California (Table 11).The differences in dollar amounts among study areas reflect differences in the baseline number and value of trips in each area in addition to the percentage changes in trips. All recreation values were adjusted for the 7.9% Consumer Price Index (CPI) inflation (Federal Reserve Bank of St. Louis, 2019) from 2013 (when the *Deepwater Horizon* data collection was completed) to 2018.

Reductions or increases in marine debris leads to changes in spending by non-local beach visitors that has a significant impact on the regional economies of beach communities. In all four of our study areas, the portion of recreation days that come from outside the local area is greater than 80% (Table 11). Based on NORES data (NOAA, 2012), spending by non-local visitors ranges from $89 per day in Delaware/Maryland to $139 per day in Alabama (Table 11).

Direct spending by visitors leads to "multiplier effects," which include additional spending for supplies by local business and additional spending by new employees hired by local businesses. Multiplier effects also account for the portion of visitor and additional spending that leaves the local economy, called "leakages" (Bess and Ambargis, 2011).

We expressed the regional economic impacts using two key metrics:

- *Value added*: The value of gross output less intermediate inputs. The value of this metric is equal to the sum of compensation of employees, taxes on production and imports less subsidies, and gross operating surplus. Value added is the net combined value of all goods and services and is analogous to GDP for the local area.
- *Employment*: Number of full- and part-time jobs (including proprietors' jobs).

For a reduction in debris to almost none, the increase in employment ranges from 464 additional jobs in the Delaware/Maryland study area to 3,703 additional jobs in Ohio (Table 11; as above, accounting only for multiple-day trips in Ohio). Under this scenario, value added ranges from an increase of $27.8 million in the Delaware/Maryland study area to an increase of $206.0 million in Ohio (Table 11).

For a doubling of debris, there is a decrease in employment ranging from 2,198 jobs in the Alabama study area to 4,254 jobs in Orange County, California (Table 1). Under this scenario, the decrease in value added ranges from $96.3 million in the Alabama study area to $304.5 million in Orange County, California (Table 11).

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APPENDIX A: EXAMPLE SURVEYS

Onsite Recruitment Survey Form
Beach Recreation Surveys

Alabama
Delaware and Maryland
Lake Erie, Ohio
Orange County

For any selected party that did not give an interview, go to Sheet REFUSALS to record Reason, Respondent Gender, Age, and Party Size		
Number recruited	0	Do not change this cell
Hello my name is _____. I am doing research on beach recreation for the federal government. I have just a few questions. May I begin?____		

Interview number	Interviewer initials	Beach	City	Date	Time	AM/PM
ex1	CW	Huntington Beach	Huntington Beach	7/11/2018	10	AM
ex2	MD	Doheny State Beach	Dana Point	7/11/2018	10	AM
1						
2						
3						
4						
299						
300						

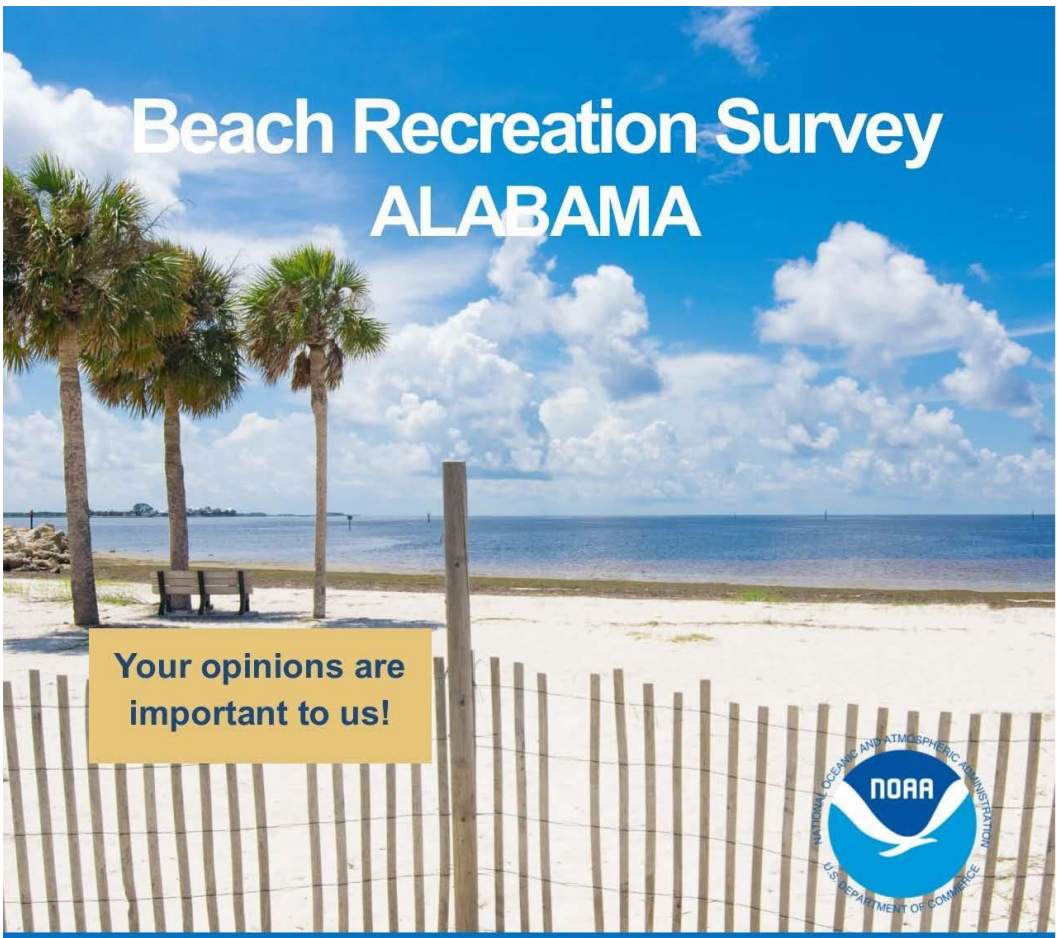
1. Is your visit here today part of a trip away from home lasting more than one day?						**2. How many hours in total will you spend at the beach today?**
No ***Indicate with "x". Skip to Q2 or end interview***	**Yes** ***Indicate with "x".***	**1a. How many days will your trip last in total?**	**1b. How many of those days will you spend time at the beach?**	**1c. How many nights will you pay for lodging during your trip?**	**1d. Is going to the beach the main reason for your trip?** ***Indicate with "y" or "n"***	
x		n/a	n/a	n/a	n/a	4
	x	7	6	5	y	2

3. What year were you born?	**4. What is the highest degree or level of school you have completed?** ***Indicate with "x", select one***					**5. Including yourself, how many adults and children live in your household?**		***6. Record respondent gender. Indicate with "x"***	
	Less than high school graduate	**High school graduate (includes GED)**	**Some college or Associate's degree**	**Bachelor's degree**	**Graduate or professional degree**	**Adults (18 and older)**	**Children (Under 18)**	**Male**	**Female**
1982					x	2	2		x
1998		x				1	0	x	

	8. Would you be willing to take part in a short mail survey that we will send to your home?				
7. Record number of people in party including respondent	**YES: Could I get your name and address? It will be used only to mail this survey and will be deleted immediately after.** ***Record address and confirm verbally, including spelling of cities or streets if necessary. Provide this info:*** **The survey will be mailed to you in early September. It will come in a large envelope from the National Oceanic and Atmospheric Administration. Please complete it and mail it back as soon as you are able. There are 20 questions, and the survey should take around 10 minutes to complete. We greatly appreciate your participation in this research!"**				
	First and last name	**Street address**	**City**	**State**	**ZIP code**
4	Jane Doe	1881 Ninth St. Suite 805	Boulder	CO	80302
2	John Smith	20 Main St.	Atlanta	GA	80303

NO: Could I get your zip code ***(if refused, type " no")***	**9. Lastly, please tell me how important the following characteristics are to you when you decide which beaches to visit. On a scale of 1 to 5, where 1 is Not Important and 5 is Very Important:** ***(Read each characteristic and enter the response as a number 1 - 5)***			
	Parking is free or inexpensive	**Not crowded**	**No garbage or manmade debris on the beach**	**10. Do you think garbage or manmade debris is a problem on Orange County beaches?** ***Y or N***
n/a	3	3	3	Y
n/a	2	5	1	N

Beach Recreation Survey - Alabama



Paperwork Reduction Act Statement

The National Oceanic and Atmospheric Administration (NOAA) is authorized by 33 U.S.C. 1951 et seq. to conduct this survey. The information collected will be used by NOAA to estimate economic impacts associated with marine debris on beaches.

Public reporting burden for this collection of information is estimated to average 10 minutes per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other suggestions for reducing this burden to Amy V. Uhrin, NOAA NOS, 1305 East-West Hwy, SSMC4, Room10240, Silver Spring, MD 20910.

The questionnaire has an identification number for mailing purposes only. Your name and address will be deleted after we receive your completed questionnaire. Notwithstanding any other provisions of the law, no person is required to respond to, nor shall any person be subjected to a penalty for failure to comply with, a collection of information subject to the requirements of the Paperwork Reduction Act, unless that collection of information displays a currently valid OMB Control Number.OMB Control Number 0648-0756 I Current Expiration Date: 08/31/2020

Privacy Act Statement

Authority: The collection of this information is authorized under 33 U.S.C. 1853 et seq, the Marine Debris Research, Prevention and Reduction Act, which, along with the Marine Debris Amendments of 2012, established the NOAA Marine Debris Program to "identify, determine sources of, assess, prevent, reduce, and remove marine debris and address the adverse impacts of marine debris on the economy of the United States, the marine environment, and navigation safety."

Purpose: The information will be used to estimate economic impacts associated with marine debris on beaches.

NOAA Routine Uses: The survey data will be combined with a national model of coastal recreation, which relies on data collected for the Deepwater Horizon oil spill assessment, to estimate the economic impacts of marine debris on tourism-dependent communities. Disclosure of this information is permitted under the Privacy Act of 1974 (5 U.S.C. Section 552a) to be shared among NOAA staff for work-related purposes. Disclosure of this information is also subject to all of the published routine uses as identified in the Privacy Act System of Records Notice Commerce/NOAA-11, Contact Information for Members of the Public Requesting or Providing Information Related to NOAA's Mission.

Disclosure: Furnishing this information is voluntary; the only consequence of failure to provide accurate information is thatyour responses will not contribute to the successof this research.

Thank you for speaking with us a few weeks ago at the beach.
This mail survey is the final step in our study. We appreciate your participation!

Coastal beaches are vital to the area's economy and quality of life. Your answers to this survey will help inform decisions about improving and protecting coastal resources. We want to hear from everyone about things people want to experience when they visit the beach. Your response is important – please complete this *voluntary* survey.

Our questions are about ocean beaches in Alabama, shown in the map below.

1. In the list below, please circle the names of any beaches you went to between September 1, 2017 and August 31, 2018. If you don't know the name of a beach you went to or it is not on the list, please circle the name of a nearby beach.

1. Dauphin Island West End Beach
2. Dauphin Island Public Beach
3. Dauphin Island East End Beach
4. Fort Morgan Public Beach
5. Bon Secour National Wildlife Refuge
6. Gulf Shores Public Beach
7. Gulf State Park Pier
8. Gulf State Park Pavilion
9. Cotton Bayou/Orange Beach
10. Alabama Point/Florida Point

Now we would like to ask you about the number of day trips and overnight trips you took to ocean beaches in Alabama. A **day** trip is any time you went to the beach and returned home the same day. An overnight trip is when you spent at least one night away from home.

2. Between September 1, 2017 and August 31, 2018, did you take any *day* trips to ocean beaches in Alabama? Please check ☒ one box.

   ☐ No ☐ Yes →

   How many day trips? ☐ day trips

3. Between September 1, 2017 and August 31, 2018, did you take any *overnight* trips where the main purpose was visiting ocean beaches in Alabama? Please check ☒ one box.

   ☐ No ☐ Yes →

   How many overnight trips? ☐ overnight trips

The next question is about beach characteristics.

4. Please tell us how important the following characteristics are to you when you decide which beaches to visit. Please check ☒ one box in each row.

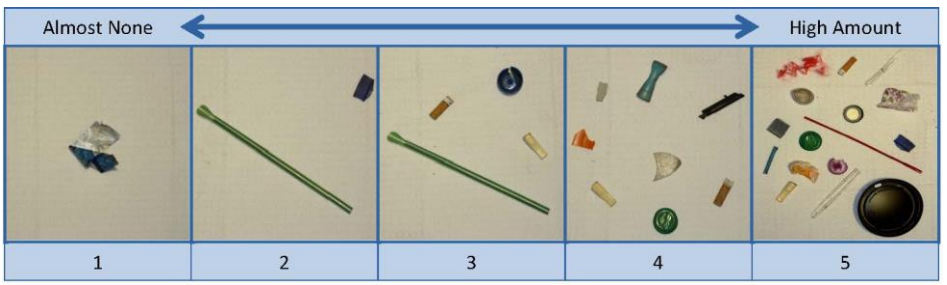
	Not Important	**Somewhat Important**			**Very Important**
	1	**2**	**3**	**4**	**5**
Scenic beauty or view	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Good water quality	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Close to home	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Parking is convenient	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Parking is free or inexpensive	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Good surfing available	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Sandy (rather than rocky)	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Not crowded	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Long enough to go for a walk/run	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Bike path available	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Fishing opportunities available	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
No garbage or manmade debris on the beach	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
No natural debris like kelp or seaweed on the beach	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

Garbage or Manmade Debris You May See on Beaches

Different beaches can have different amounts of garbage or manmade debris. Garbage or manmade debris refers to items like bottles, wrappers, straws, plastic fragments, or cigarettes. It does not include twigs or seaweed.

The pictures below illustrate the amount of debris commonly found on United States beaches. Imagine you are picking up debris over an area of 500 square feet or approximately the area of three parking spaces, outlined in red below.

If you walked back and forth in this area and picked up all the debris, you might find different amounts ranging from "almost none" to a "high amount." As the pictures below show, different levels of debris on the beach can be given a score from 1 to 5. Higher scores mean more debris.



On the next page, we will ask you to use the above scale to estimate the amount of garbage or manmade debris you saw on ocean beaches you have been to in Alabama.

5. In the table below, please write the names of ocean beaches in Alabama that you went to between September 1, 2017 and August 31, 2018. You may want to refer back to the map at the beginning of this survey.
   To the right of each beach you went to, use the debris scale from the previous page and write a number between 1 and 5, indicating the amount of garbage or manmade debris you saw on the beach. Writing a "1" indicates you saw almost none, while writing a "5" indicates you saw a high amount of garbage or manmade debris. For any beach where you don't recall the amount of debris, please write "don't recall" in place of a number.

Beach Name	**How Much Garbage or Manmade Debris Did You See on the Beach? (1 = Almost None) (5 = High Amount) (Don't Recall)**
Orange Beach	*4*
Dauphin Island Beach (Example)	2

6. Between September 1, 2017 and August 31, 2018, if there had been *almost no* garbage or manmade debris on ocean beaches in Alabama, would you have gone to the beach more often or the same number of times? Please check ☒ one box.

☐ **More often → Please answer the two questions below.**

Between September 1, 2017 and August 31, 2018, how many more day trips would you have taken if there were almost no garbage or manmade debris on ocean beaches in Alabama? ☐ more day trips

Between September 1, 2017 and August 31, 2018, how many more overnight trips would you have taken if there were almost no garbage or manmade debris on ocean beaches in Alabama? ☐ more overnight trips

☐ The same number of times

7. Between September 1, 2017 and August 31, 2018, if there had been *twice as much* garbage or manmade debris on ocean beaches in Alabama, would you have gone to the beach less often or the same number of times? Please check ☒ one box.

☐ **Less often → Please answer the two questions below.**

Between September 1, 2017 and August 31, 2018, how many fewer day trips would you have taken if there had been twice as much garbage or manmade debris on ocean beaches in Alabama? ☐ fewer day trips

Between September 1, 2017 and August 31, 2018, how many more overnight trips would you have taken if there were almost no garbage or manmade debris on ocean beaches in Alabama? ☐ fewer overnight trips

☐ The same number of times

The next few questions ask about your experiences with debris on beaches.

8. How concerned would you be to see the following types of garbage or manmade debris while visiting a beach? Please check ☒ one box in each row.

	Not At All Concerned	**Somewhat Concerned**			**Very Concerned**
	1	**2**	**3**	**4**	**5**
Plastic items or bottles	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Styrofoam	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Paper products	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Wooden items	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Metal items or cans	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Glass	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Rubber items	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Cloth or clothing	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Cigarette butts	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Fishing gear	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Medical waste	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Animal waste	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

9. Please look at the list below and check ☒ the box next to all the types of garbage or manmade debris that you have *actually seen* on ocean beaches in Alabama.

☐ Plastic items or bottles
☐ Styrofoam
☐ Paper products
☐ Wooden items
☐ Metal items or cans
☐ Glass
☐ Rubber items
☐ Cloth or clothing
☐ Cigarette butts
☐ Fishing gear
☐ Medical waste
☐ Animal waste
☐ Other (please specify)
_____

10. Do you think garbage or manmade debris is a problem on ocean beaches in Alabama? Please check ☒ one box.

☐ Yes ☐ No ☐ Not sure

11. To the best of your knowledge, what do you think is the largest source of garbage or manmade debris found on ocean beaches in Alabama? Please check ☒ one box.

☐ Left by beach visitors

☐ Blown to the beach from nearby areas on land

☐ Washed ashore from the ocean

☐ Washed ashore from nearby rivers or storm drains

☐ Other (please specify)_____

Finally, we have just a few questions about you and your household. These questions are a way to make sure that we understand the values and opinions of all types of people visiting beaches in Alabama.

12. Have you participated in any beach cleanups within the last three years? Please check ☒ one box.

☐ No ☐ Yes

13. How many adults and children live in your household?

_____ Adults (18 and older) _____ Children (under 18)

14. What is your gender? Please check ☒ one box.

☐ Male ☐ Female

15. In what year were you born?

_____ Year

16. Are you of Hispanic, Latino, or Spanish origin? Please check ☒ one box.

☐ No ☐ Yes

17. What is your race? Select all that apply.

☐ American Indian or Alaskan Native

☐ Asian

☐ Black or African American

☐ Native Hawaiian or other Pacific Islander

☐ White

☐ Other (please specify)_____

18. What is the highest degree or level of school you have completed? Please check ☒ one box.

☐ Less than high school graduate

☐ Some college or Associate's degree

☐ High school graduate (includes GED)

☐ Bachelor's degree

☐ Graduate or professional degree, beyond a bachelor's degree

19. Which of the following income categories best describes your total household income last year, before taxes? Please check ☒ one box.

☐ Less than $15,000

☐ $15,000 to $24,999

☐ $25,000 to $34,999

☐ $35,000 to $49,999

☐ $50,000 to $74,999

☐ $75,000 to $99,999

☐ $100,000 to $150,000

☐ More than $150,000

Thank you for participating!
Please return your survey in the enclosed Business Reply Mail envelope.

Beach Recreation Survey – Delaware and Maryland

Paperwork Reduction Act Statement

The National Oceanic and Atmospheric Administration (NOAA) is authorized by 33 U.S.C. 1951 et seq. to conduct this survey. The information collected will be used by NOAA to estimate economic impacts associated with marine debris on beaches.

Public reporting burden for this collection of information is estimated to average 10 minutes per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other suggestions for reducing this burden to Amy V. Uhrin, NOAA NOS, 1305 East-West Hwy, SSMC4, Room10240, Silver Spring, MD 20910.

The questionnaire has an identification number for mailing purposes only. Your name and address will be deleted after we receive your completed questionnaire. Notwithstanding any other provisions of the law, no person is required to respond to, nor shall any person be subjected to a penalty for failure to comply with, a collection of information subject to the requirements of the Paperwork Reduction Act, unless that collection of information displays a currently valid OMB Control Number.OMB Control Number 0648-0756 I Current Expiration Date: 08/31/2020

Privacy Act Statement

Authority: The collection of this information is authorized under 33 U.S.C. 1853 et seq, the Marine Debris Research, Prevention and Reduction Act, which, along with the Marine Debris Amendments of 2012, established the NOAA Marine Debris Program to "identify, determine sources of, assess, prevent, reduce, and remove marine debris and address the adverse impacts of marine debris on the economy of the United States, the marine environment, and navigation safety."

Purpose: The information will be used to estimate economic impacts associated with marine debris on beaches.

NOAA Routine Uses: The survey data will be combined with a national model of coastal recreation, which relies on data collected for the Deepwater Horizon oil spill assessment, to estimate the economic impacts of marine debris on tourism-dependent communities. Disclosure of this information is permitted under the Privacy Act of 1974 (5 U.S.C. Section 552a) to be shared among NOAA staff for work-related purposes. Disclosure of this information is also subject to all of the published routine uses as identified in the Privacy Act System of Records Notice Commerce/NOAA-11, Contact Information for Members of the Public Requesting or Providing Information Related to NOAA's Mission.

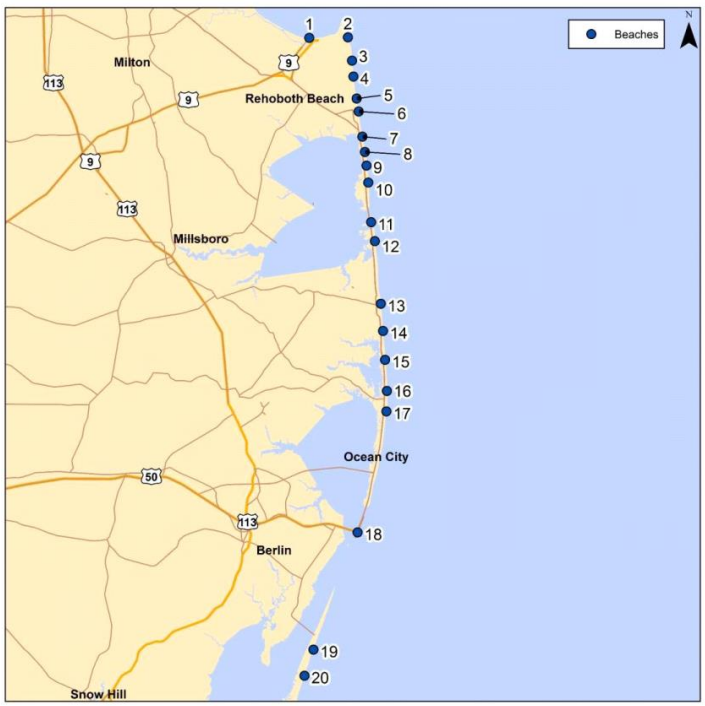
Disclosure: Furnishing this information is voluntary; the only consequence of failure to provide accurate information is thatyour responses will not contribute to the successof this research.

Thank you for speaking with us a few weeks ago at the beach.
This mail survey is the final step in our study. We appreciate your participation!

Coastal beaches are vital to the area's economy and quality of life. Your answers to this survey will help inform decisions about improving and protecting coastal resources. We want to hear from everyone about things people want to experience when they visit the beach. Your response is important – please complete this **voluntary** survey.

Our questions are about ocean beaches in Delaware and Maryland, shown in the map below.

1. In the list below, please circle the names of any beaches you went to between September 1, 2017 and August 31, 2018. If you don't know the name of a beach you went to or it is not on the list, please circle the name of a nearby beach.



1. Lewes Beach
2. Cape Henlopen Beach
3. Herring Point Beach
4. Gordon's Pond Beach
5. Deauville Beach
6. Rehoboth Beach
7. Dewey Beach
8. Towers Beach
9. Keybox Road Beach
10. Conquest Road Beach
11. South Indian River Inlet Beach
12. 3 R's Road Beach
13. Bethany Beach
14. South Bethany Beach
15. Fenwick Island State Park Beach
16. Town of Fenwick Island Beach
17. Ocean City (140th Street)
18. Ocean City (Boardwalk)
19. Assateague State Park
20. Assateague Island National Seashore

Now we would like to ask you about the number of day trips and overnight trips you took to ocean beaches in Delaware and Maryland. A **day** trip is any time you went to the beach and returned home the same day. An **overnight** trip is when you spent at least one night away from home.

2. Between September 1, 2017 and August 31, 2018, did you take any day trips to ocean beaches in Delaware and Maryland? Please check ☒ one box.

   ☐ No ☐ Yes →

   How many day trips? ☐ day trips

3. Between September 1, 2017 and August 31, 2018, did you take any *overnight* trips where the main purpose was visiting ocean beaches in Delaware and Maryland? Please check ☒ one box.

   ☐ No ☐ Yes →

   How many overnight trips? ☐ overnight trips

The next question is about beach characteristics.

4. Please tell us how important the following characteristics are to you when you decide which beaches to visit. Please check ☒ one box in each row.

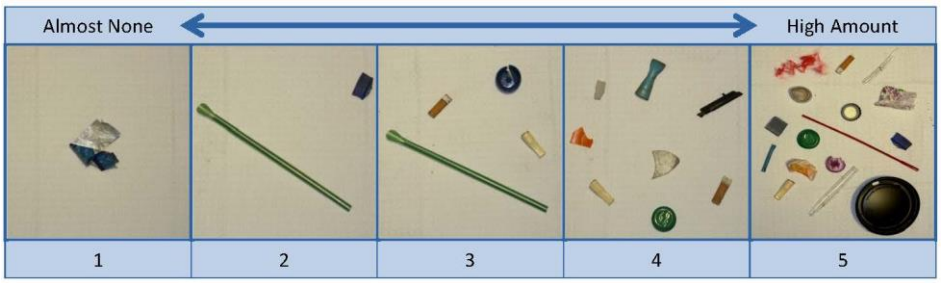
	Not Important	**Somewhat Important**			**Very Important**
	1	**2**	**3**	**4**	**5**
Scenic beauty or view	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Good water quality	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Close to home	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Parking is convenient	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Parking is free or inexpensive	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Good surfing available	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Sandy (rather than rocky)	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Not crowded	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Long enough to go for a walk/run	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Bike path available	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Fishing opportunities available	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
No garbage or manmade debris on the beach	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
No natural debris like kelp or seaweed on the beach	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

Garbage or Manmade Debris You May See on Beaches

Different beaches can have different amounts of garbage or manmade debris. Garbage or manmade debris refers to items like bottles, wrappers, straws, plastic fragments, or cigarettes. It does not include twigs or seaweed.

The pictures below illustrate the amount of debris commonly found on United States beaches. Imagine you are picking up debris over an area of 500 square feet or approximately the area of three parking spaces, outlined in red below.

If you walked back and forth in this area and picked up all the debris, you might find different amounts ranging from "almost none" to a "high amount." As the pictures below show, different levels of debris on the beach can be given a score from 1 to 5. Higher scores mean more debris.



On the next page, we will ask you to use the above scale to estimate the amount of garbage or manmade debris you saw on ocean beaches you have been to in Delaware and Maryland.

5. In the table below, please write the names of ocean beaches in Delaware and Maryland that you went to between September 1, 2017 and August 31, 2018. You may want to refer back to the map at the beginning of this survey.
   To the right of each beach you went to, use the debris scale from the previous page and write a number between 1 and 5, indicating the amount of garbage or manmade debris you saw on the beach. Writing a "1" indicates you saw almost none, while writing a "5" indicates you saw a high amount of garbage or manmade debris. For any beach where you don't recall the amount of debris, please write "don't recall" in place of a number.

Beach Name	How Much Garbage or Manmade Debris Did You See on the Beach? (1 = Almost None) (5 = High Amount) (Don't Recall)
Rehoboth Beach Example	*4*
Lewes Beach	2

6. Between September 1, 2017 and August 31, 2018, if there had been *almost no* garbage or manmade debris on ocean beaches in Delaware and Maryland, would you have gone to the beach more often or the same number of times? Please check ☒ one box.

☐ More often → **Please answer the two questions below.**

Between September 1, 2017 and August 31, 2018, how many more day trips would you have taken if there were almost no garbage or manmade debris on ocean beaches in Delaware and Maryland?

☐ more day trips

Between September 1, 2017 and August 31, 2018, how many more overnight trips would you have taken if there were almost no garbage or manmade debris on ocean beaches in Delaware and Maryland?

☐ more overnight trips

☐ The same number of times

7. Between September 1, 2017 and August 31, 2018, if there had been *twice as much* garbage or manmade debris on ocean beaches in Delaware and Maryland, would you have gone to the beach less often or the same number of times? Please check ☒ one box.

☐ Less often → **Please answer the two questions below.**

Between September 1, 2017 and August 31, 2018, how many fewer day trips would you have taken if there had been twice as much garbage or manmade debris on ocean beaches in Delaware and Maryland?

☐ fewer day trips

Between September 1, 2017 and August 31, 2018, how many fewer overnight trips would you have taken if there had been twice as much garbage or manmade debris on ocean beaches in Delaware and Maryland?

☐ fewer overnight trips

☐ The same number of times

The next few questions ask about your experiences with debris on beaches.

8. How concerned would you be to see the following types of garbage or manmade debris while visiting a beach? Please check ☒ one box in each row.

	Not At All Concerned	**Somewhat Concerned**			**Very Concerned**
	1	**2**	**3**	**4**	**5**
Plastic items or bottles	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Styrofoam	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Paper products	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Wooden items	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Metal items or cans	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Glass	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Rubber items	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Cloth or clothing	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Cigarette butts	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Fishing gear	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Medical waste	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Animal waste	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

9. Please look at the list below and check ☒ the box next to all the types of garbage or manmade debris that you have *actually seen* on ocean beaches in Delaware and Maryland.

☐ Plastic items or bottles
☐ Styrofoam
☐ Paper products
☐ Wooden items
☐ Metal items or cans
☐ Glass
☐ Cloth or clothing
☐ Cigarette butts
☐ Fishing gear
☐ Medical waste
☐ Animal waste
☐ Other (please specify)

☐ Rubber items _____

10. Do you think garbage or manmade debris is a problem on ocean beaches in Delaware and Maryland? Please check ☒ one box.

☐ Yes ☐ No ☐ Not sure

11. To the best of your knowledge, what do you think is the *largest* source of garbage or manmade debris found on ocean beaches in Delaware and Maryland? Please check ☒ one box.

☐ Left by beach visitors

☐ Blown to the beach from nearby areas on land

☐ Washed ashore from the ocean

☐ Washed ashore from nearby rivers or storm drains

☐ Other (please specify) _____

Finally, we have just a few questions about you and your household. These questions are a way to make sure that we understand the values and opinions of all types of people visiting ocean beaches in Delaware and Maryland.

12. Have you participated in any beach cleanups within the last three years? Please check ☒ one box.

☐ No ☐ Yes

13. How many adults and children live in your household?

_____ Adults (18 and older) _____Children (under 18)

14. What is your gender? Please check ☒ one box.

☐ Male ☐ Female

15. In what year were you born?

_____Year

16. Are you of Hispanic, Latino, or Spanish origin? Please check ☒ one box.

☐ No ☐ Yes

17. What is your race? Select all that apply.

☐ American Indian or Alaskan Native
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☐ Black or African American
☐ Native Hawaiian or other Pacific Islander
☐ White
☐ Other (please specify)

18. What is the highest degree or level of school you have completed? Please check ☒ one box.

☐ Less than high school graduate
☐ Some college or Associate's degree
☐ High school graduate (includes GED)
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19. Which of the following income categories best describes your total household income last year, before taxes? Please check ☒ one box.

☐ Less than $15,000
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☐ $50,000 to $74,999
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☐ $100,000 to $150,000
☐ More than $150,000

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Beach Recreation Survey – Lake Erie, Ohio

Paperwork Reduction Act Statement

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The questionnaire has an identification number for mailing purposes only. Your name and address will be deleted after we receive your completed questionnaire. Notwithstanding any other provisions of the law, no person is required to respond to, nor shall any person be subjected to a penalty for failure to comply with, a collection of information subject to the requirements of the Paperwork Reduction Act, unless that collection of information displays a currently valid OMB Control Number.OMB Control Number 0648-0756 I Current Expiration Date: 08/31/2020

Privacy Act Statement

Authority: The collection of this information is authorized under 33 U.S.C. 1853 et seq, the Marine Debris Research, Prevention and Reduction Act, which, along with the Marine Debris Amendments of 2012, established the NOAA Marine Debris Program to "identify, determine sources of, assess, prevent, reduce, and remove marine debris and address the adverse impacts of marine debris on the economy of the United States, the marine environment, and navigation safety."

Purpose: The information will be used to estimate economic impacts associated with marine debris on beaches.

NOAA Routine Uses: The survey data will be combined with a national model of coastal recreation, which relies on data collected for the Deepwater Horizon oil spill assessment, to estimate the economic impacts of marine debris on tourism-dependent communities. Disclosure of this information is permitted under the Privacy Act of 1974 (5 U.S.C. Section 552a) to be shared among NOAA staff for work-related purposes. Disclosure of this information is also subject to all of the published routine uses as identified in the Privacy Act System of Records Notice Commerce/NOAA-11, Contact Information for Members of the Public Requesting or Providing Information Related to NOAA's Mission.

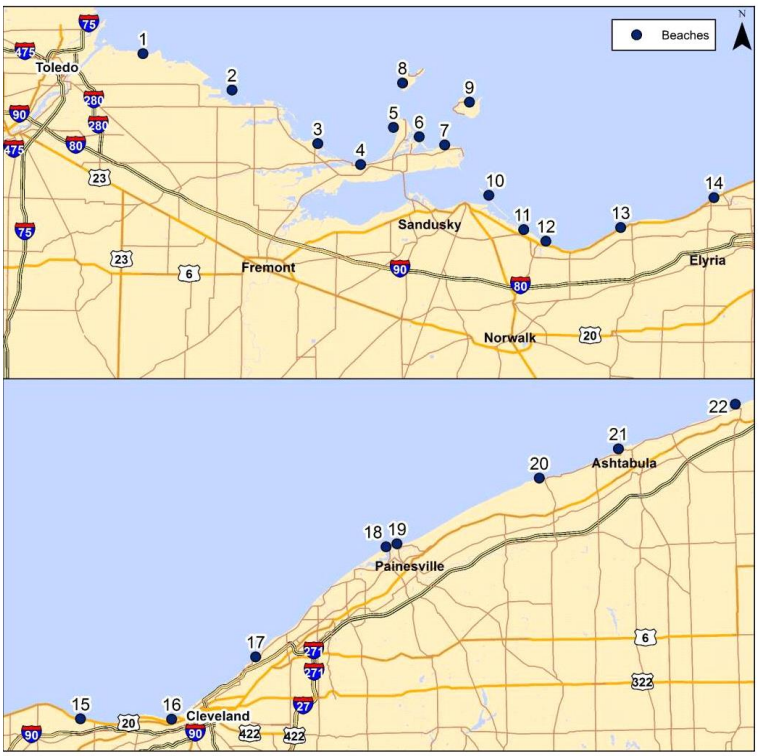
Disclosure: Furnishing this information is voluntary; the only consequence of failure to provide accurate information is thatyour responses will not contribute to the successof this research.

Thank you for speaking with us a few weeks ago at the beach.
This mail survey is the final step in our study. We appreciate your participation!

Coastal beaches are vital to the area's economy and quality of life. Your answers to this survey will help inform decisions about improving and protecting coastal resources. We want to hear from everyone about things people want to experience when they visit the beach. Your response is important – please complete this **voluntary** survey.

Our questions are about Lake Erie beaches in Ohio, shown in the map below.

1. In the list below, please circle the names of any beaches you went to between September 1, 2017 and August 31, 2018. If you don't know the name of a beach you went to or it is not on the list, please circle the name of a nearby beach.



1. Maumee Bay State Park
2. Crane Creek State Park
3. Camp Perry Beach
4. Port Clinton City Beach
5. Catawba Island State Park
6. East Harbor State Park
7. Lakeside Beach
8. South Bass Island State Park
9. Kelleys Island State Park
10. Cedar Point Beach
11. Sawmill Creek Beach
12. Nickel Plate Beach
13. Sherod Park Beach
14. Lakeview Park Beach
15. Huntington Beach
16. Edgewater Park Beach
17. Euclid Beach Park
18. Headlands Beach State Park
19. Fairport Harbor Lakefront Park
20. Geneva State Park
21. Walnut Beach Park
22. Conneaut Township Park

Now we would like to ask you about the number of day trips and overnight trips you took to Lake Erie beaches in Ohio. A **day** trip is any time you went to the beach and returned home the same day. An overnight trip is when you spent at least one night away from home.

2. Between September 1, 2017 and August 31, 2018, did you take any *day* trips to Lake Erie beaches in Ohio? Please check ☒ one box.

   ☐ No ☐ Yes→

   How many overnight trips? ☐ day trips

3. Between September 1, 2017 and August 31, 2018, did you take any *overnight* trips where the main purpose was visiting Lake Erie beaches in Ohio? Please check ☒ one box.

   ☐ No ☐ Yes→

   How many overnight trips? ☐ overnight trips

The next question is about beach characteristics.

4. Please tell us how important the following characteristics are to you when you decide which beaches to visit. Please check ☒ one box in each row.

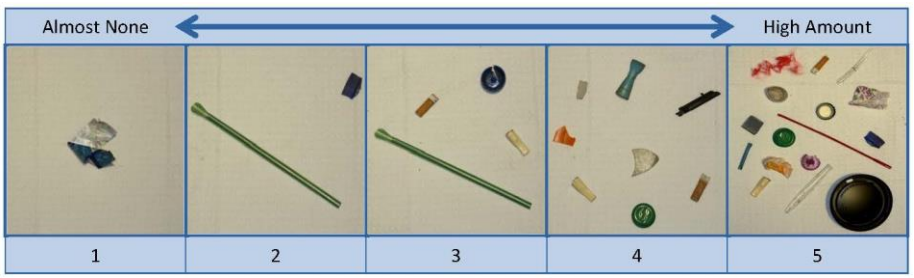
	Not Important	**Somewhat Important**			**Very Important**
	1	**2**	**3**	**4**	**5**
Scenic beauty or view	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Good water quality	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Close to home	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Parking is convenient	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Parking is free or inexpensive	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Good surfing available	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Sandy (rather than rocky)	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Not crowded	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Long enough to go for a walk/run	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Bike path available	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Fishing opportunities available	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
No garbage or manmade debris on the beach	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
No natural debris like kelp or seaweed on the beach	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

Garbage or Manmade Debris You May See on Beaches

Different beaches can have different amounts of garbage or manmade debris. Garbage or manmade debris refers to items like bottles, wrappers, straws, plastic fragments, or cigarettes. It does not include twigs or seaweed.

The pictures below illustrate the amount of debris commonly found on United States beaches. Imagine you are picking up debris over an area of 500 square feet or approximately the area of three parking spaces, outlined in red below.

If you walked back and forth in this area and picked up all the debris, you might find different amounts ranging from "almost none" to a "high amount." As the pictures below show, different levels of debris on the beach can be given a score from 1 to 5. Higher scores mean more debris.



On the next page, we will ask you to use the above scale to estimate the amount of garbage or manmade debris you saw on Lake Erie beaches you have been to in Ohio.

5. In the table below, please write the names of Lake Erie beaches in Ohio that you went to between September 1, 2017 and August 31, 2018. You may want to refer back to the map at the beginning of this survey.
   To the right of each beach you went to, use the debris scale from the previous page and write a number between 1 and 5, indicating the amount of garbage or manmade debris you saw on the beach. Writing a "1" indicates you saw almost none, while writing a "5" indicates you saw a high amount of garbage or manmade debris. For any beach where you don't recall the amount of debris, please write "don't recall" in place of a number.

Beach Name	**How Much Garbage or Manmade Debris Did You See on the Beach? (1 = Almost None) (5 = High Amount) (Don't Recall)**
Lakeside Beach	*4*
Huntington Beach Example	2

6. Between September 1, 2017 and August 31, 2018, if there had been *almost no* garbage or manmade debris on Lake Erie beaches in Ohio, would you have gone to the beach more often or the same number of times? Please check ☒ one box.

☐ More often → **Please answer the two questions below.**

Between September 1, 2017 and August 31, 2018, how many more day trips would you have taken if there were almost no garbage or manmade debris on Lake Erie beaches in Ohio? ☐ more day trips

Between September 1, 2017 and August 31, 2018, how many more overnight trips would you have taken if there were almost no garbage or manmade debris on Lake Erie beaches in Ohio? ☐ more overnight trips

☐ The same number of times

7. Between September 1, 2017 and August 31, 2018, if there had been *twice as much* garbage or manmade debris on Lake Erie beaches in Ohio, would you have gone to the beach less often or the same number of times? Please check ☒ one box.

☐ Less often → **Please answer the two questions below.**

Between September 1, 2017 and August 31, 2018, how many fewer day trips would you have taken if there had been twice as much garbage or manmade debris on Lake Erie beaches in Ohio? ☐ fewer day trips

Between September 1, 2017 and August 31, 2018, how many fewer overnight trips would you have taken if there had been twice as much garbage or manmade debris on Lake Erie beaches in Ohio? ☐ fewer overnight trips

☐ The same number of times

The next few questions ask about your experiences with debris on beaches.

8. How concerned would you be to see the following types of garbage or manmade debris while visiting a beach? Please check ☒ one box in each row.

	Not At All Concerned	**Somewhat Concerned**			**Very Concerned**
	1	**2**	**3**	**4**	**5**
Plastic items or bottles	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Styrofoam	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Paper products	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Wooden items	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Metal items or cans	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Glass	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Rubber items	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Cloth or clothing	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Cigarette butts	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Fishing gear	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Medical waste	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Animal waste	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

9. Please look at the list below and check ☒ the box next to all the types of garbage or manmade debris that you have *actually seen* on Lake Erie beaches in Ohio.

☐ Plastic items or bottles
☐ Styrofoam
☐ Paper products
☐ Wooden items
☐ Metal items or cans
☐ Glass
☐ Rubber items
☐ Cloth or clothing
☐ Cigarette butts
☐ Fishing gear
☐ Medical waste
☐ Animal waste
☐ Other (please specify)
_____

10. Do you think garbage or manmade debris is a problem on Lake Erie beaches in Ohio? Please check ☒ one box.

☐ Yes ☐ No ☐ Not sure

11. To the best of your knowledge, what do you think is the *largest* source of garbage or manmade debris found on Lake Erie beaches in Ohio? Please check ☒ one box.

☐ Left by beach visitors

☐ Blown to the beach from nearby areas on land

☐ Washed ashore from the ocean

☐ Washed ashore from nearby rivers or storm drains

☐ Other (please specify) _____

Finally, we have just a few questions about you and your household. These questions are a way to make sure that we understand the values and opinions of all types of people visiting Lake Erie beaches in Ohio.

12. Have you participated in any beach cleanups within the last three years? Please check ☒ one box.

☐ No ☐ Yes

13. How many adults and children live in your household?

_____ Adults (18 and older)

_____ Children (under 18)

14. What is your gender? Please check ☒ one box.

☐ Male ☐ Female

15. In what year were you born?

_____Year

16. Are you of Hispanic, Latino, or Spanish origin? Please check ☒ one box.

☐ No ☐ Yes

17. What is your race? Select all that apply.

☐ American Indian or Alaskan Native

☐ Asian

☐ Black or African American

☐ Native Hawaiian or other Pacific Islander

☐ White

☐ Other (please specify)

18. What is the highest degree or level of school you have completed? Please check ☒ one box.

☐ Less than high school graduate

☐ Some college or Associate's degree

☐ High school graduate (includes GED)

☐ Bachelor's degree

☐ Graduate or professional degree, beyond a bachelor's degree

19. Which of the following income categories best describes your total household income last year, before taxes? Please check ☒ one box.

☐ Less than $15,000

☐ $15,000 to $24,999

☐ $25,000 to $34,999

☐ $35,000 to $49,999

☐ $50,000 to $74,999

☐ $75,000 to $99,999

☐ $100,000 to $150,000

☐ More than $150,000

Thank you for participating!
Please return your survey in the enclosed Business Reply Mail envelope.

Beach Recreation Survey – Orange County, California

Paperwork Reduction Act Statement

The National Oceanic and Atmospheric Administration (NOAA) is authorized by 33 U.S.C. 1951 et seq. to conduct this survey. The information collected will be used by NOAA to estimate economic impacts associated with marine debris on beaches.

Public reporting burden for this collection of information is estimated to average 10 minutes per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other suggestions for reducing this burden to Amy V. Uhrin, NOAA NOS, 1305 East-West Hwy, SSMC4, Room 10240, Silver Spring, MD 20910.

The questionnaire has an identification number for mailing purposes only. Your name and address will be deleted after we receive your completed questionnaire. Notwithstanding any other provisions of the law, no person is required to respond to, nor shall any person be subjected to a penalty for failure to comply with, a collection of information subject to the requirements of the Paperwork Reduction Act, unless that collection of information displays a currently valid OMB Control Number.
OMB Control Number 0648-0756 I Current Expiration Date: 08/312020

Privacy Act Statement

Authority: The collection of this information is authorized under 33 U.S.C. 1853 et seq. the Marine Debris Research, Prevention and Reduction Act, which, along with the Marine Debris Amendments of 2012, established the NOAA Marine Debris Program to "identify, determine sources of, assess, prevent, reduce, and remove marine debris and address the adverse impacts of marine debris on the economy of the United States, the marine environment, and navigation safety."

Purpose: The information will be used to estimate economic impacts associated with marine debris on beaches.

NOAA Routine Uses: The survey data will be combined with a national model of coastal recreation, which relies on data collected for the Deepwater Horizon oil spill assessment, to estimate the economic impacts of marine debris on tourism-dependent communities. Disclosure of this information is permitted under the Privacy Act of 1974 (5 U.S.C. Section 552a) to be shared among NOAA staff for work-related purposes. Disclosure of this information is also subject to all of the published routine uses as identified in the Privacy Act System of Records Notice Commerce/NOAA-11, Contact Information for Members of the Public Requesting or Providing Information Related to NOAA's Mission.

Disclosure: Furnishing this information is voluntary; the only consequence of failure to provide accurate information is that your responses will not contribute to the success of this research.

Thank you for speaking with us a few weeks ago at the beach.
This mail survey is the final step in our study. We appreciate your participation!

Coastal beaches are vital to the area's economy and quality of life. Your answers to this survey will help inform decisions about improving and protecting coastal resources. We want to hear from everyone about things people want to experience when they visit the beach. Your response is important – please complete this voluntary survey.

Our questions are about ocean beaches in Orange County, shown in the map below.

1. In the list below, please circle the names of any beaches you went to between September 1, 2017 and August 31, 2018. If you don't know the name of a beach you went to or it is not on the list, please circle the name of a nearby beach.

1. Seal Beach
2. Surfside Beach
3. Sunset Beach
4. Bolsa Chica
5. Huntington City Beach
6. Huntington State Beach
7. Santa Ana River County Beach
8. Newport Beach
9. Balboa Beach
10. Corona del Mar State Beach
11. Crystal Cove State Park Beach
12. Emerald Bay Beach
13. Laguna Beach
14. Aliso Beach
15. Salt Creek Beach
16. Monarch Beach
17. Dana Point Headlands Beach
18. Doheny State Beach
19. Capistrano Beach Park
20. Poche County Beach
21. San Clemente City Beach
22. San Clemente State Beach

Now we would like to ask you about the number of day trips and overnight trips you took to beaches in Orange County. A **day** trip is any time you went to the beach and returned home the same day. An overnight trip is when you spent at least one night away from home.

2. Between September 1, 2017 and August 31, 2018, did you take any *day* trips to beaches in Orange County? Please check ☒ one box.

   ☐ No ☐ Yes →

   How many day trips? ☐ day trips

3. Between September 1, 2017 and August 31, 2018, did you take any *overnight* trips where the main purpose was visiting beaches in Orange County? Please check ☒ one box.

   ☐ No ☐ Yes →

   How many overnight trips? ☐ overnight trips

The next question is about beach characteristics.

4. Please tell us how important the following characteristics are to you when you decide which beaches to visit. Please check ☒ one box in each row.

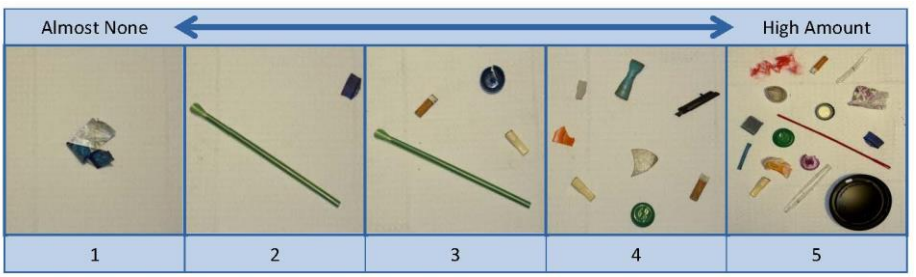
	Not Important	**Somewhat Important**			**Very Important**
	1	**2**	**3**	**4**	**5**
Scenic beauty or view	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Good water quality	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Close to home	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Parking is convenient	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Good surfing available	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Sandy (rather than rocky)	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Not crowded	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Long enough to go for a walk/run	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Bike path available	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Fishing opportunities available	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
No garbage or manmade debris on the beach	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
No natural debris like kelp or seaweed on the beach	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

Garbage or Manmade Debris You May See on Beaches

Different beaches can have different amounts of garbage or manmade debris. Garbage or manmade debris refers to items like bottles, wrappers, straws, plastic fragments, or cigarettes. It does not include twigs or seaweed.

The pictures below illustrate the amount of debris commonly found on United States beaches. Imagine you are picking up debris over an area of 500 square feet or approximately the area of three parking spaces, outlined in red below.

If you walked back and forth in this area and picked up all the debris, you might find different amounts ranging from "almost none" to a "high amount." As the pictures below show, different levels of debris on the beach can be given a score from 1 to 5. Higher scores mean more debris.



On the next page, we will ask you to use the above scale to estimate the amount of garbage or manmade debris you saw on beaches you have been to in Orange County.

5. In the table below, please write the names of beaches in Orange County that you went to between September 1, 2017 and August 31, 2018. You may want to refer back to the map at the beginning of this survey.
   To the right of each beach you went to, use the debris scale from the previous page and write a number between 1 and 5, indicating the amount of garbage or manmade debris you saw on the beach. Writing a "1" indicates you saw almost none, while writing a "5" indicates you saw a high amount of garbage or manmade debris. For any beach where you don't recall the amount of debris, please write "don't recall" in place of a number.

Beach Name	**How Much Garbage or Manmade Debris Did You See on the Beach? (1 = Almost None) (5 = High Amount) (Don't Recall)**
Surfside Beach	*4*
Monarch Beach Example	2

6. Between September 1, 2017 and August 31, 2018, if there had been almost no garbage or manmade debris on beaches in Orange County, would you have gone to the beach more often or the same number of times? Please check ☒ one box.

☐ More often → **Please answer the two questions below.**

Between September 1, 2017 and August 31, 2018, how many more day trips would you have taken if there were almost no garbage or manmade debris on Orange County beaches? ☐ more day trips

Between September 1, 2017 and August 31, 2018, how many more overnight trips would you have taken if there were almost no garbage or manmade debris on Orange County beaches? ☐ more overnight trips

☐ The same number of times

7. Between September 1, 2017 and August 31, 2018, if there had been twice as much garbage or manmade debris on beaches in Orange County, would you have gone to the beach less often or the same number of times? Please check ☒ one box.

☐ Less often → **Please answer the two questions below.**

Between September 1, 2017 and August 31, 2018, how many fewer day trips would you have taken if there had been twice as much garbage or manmade debris on Orange County beaches? ☐ fewer day trips

Between September 1, 2017 and August 31, 2018, how many fewer overnight trips would you have taken if there had been twice as much garbage or manmade debris on Orange County beaches? ☐ fewer overnight trips

☐ The same number of times

The next few questions ask about your experiences with debris on beaches.

8. How concerned would you be to see the following types of garbage or manmade debris while visiting a beach? Please check ☒ one box in each row.

	Not At All Concerned	**Somewhat Concerned**			**Very Concerned**
	1	**2**	**3**	**4**	**5**
Plastic items or bottles	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Styrofoam	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Paper products	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Wooden items	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Metal items or cans	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Glass	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Rubber items	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Cloth or clothing	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Cigarette butts	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Fishing gear	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Medical waste	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
Animal waste	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

9. Please look at the list below and check ☒ the box next to all the types of garbage or manmade debris that you have *actually seen* on beaches in Orange County.

☐ Plastic items or bottles
☐ Styrofoam
☐ Paper products
☐ Wooden items
☐ Metal items or cans
☐ Glass
☐ Other (please specify) _____

☐ Cloth or clothing
☐ Cigarette butts
☐ Fishing gear
☐ Medical waste
☐ Animal waste
☐ Rubber items

10. Do you think garbage or manmade debris is a problem on Orange County beaches? Please check ☒ one box.

☐ Yes ☐ No ☐ Not sure

11. To the best of your knowledge, what do you think is the *largest* source of garbage or manmade debris found on beaches in Orange County? Please check ☒ one box.

☐ Left by beach visitors

☐ Blown to the beach from nearby areas on land

☐ Washed ashore from the ocean

☐ Washed ashore from nearby rivers or storm drains

☐ Other (please specify) _____

Finally, we have just a few questions about you and your household. These questions are a way to make sure that we understand the values and opinions of all types of people visiting beaches in Orange County.

12. Have you participated in any beach cleanups within the last three years? Please check ☒ one box.

☐ No ☐ Yes

13. How many adults and children live in your household?

_____ Adults (18 and older)
_____ Children (under 18)

14. What is your gender? Please check ☒ one box.

☐ Male ☐ Female

15. In what year were you born?

_____Year

16. Are you of Hispanic, Latino, or Spanish origin? Please check ☒ one box.

☐ No ☐ Yes

17. What is your race? Select all that apply.

- ☐ American Indian or Alaskan Native
- ☐ Asian
- ☐ Black or African American
- ☐ Native Hawaiian or other Pacific Islander
- ☐ White
- ☐ Other (please specify) _____

18. What is the highest degree or level of school you have completed? Please check ☒ one box.

- ☐ Less than high school graduate
- ☐ Some college or Associate's degree
- ☐ High school graduate (includes GED)
- ☐ Bachelor's degree
- ☐ Graduate or professional degree, beyond a bachelor's degree

19. Which of the following income categories best describes your household income last year, before taxes? Please check ☒ one box.

- ☐ Less than $15,000
- ☐ $15,000 to $24,999
- ☐ $25,000 to $34,999
- ☐ $35,000 to $49,999
- ☐ $50,000 to $74,999
- ☐ $75,000 to $99,999
- ☐ $100,000 to $150,000
- ☐ More than $150,000

Thank you for participating!
Please return your survey in the enclosed Business Reply Mail envelope.

APPENDIX B: MAIL SURVEY SUMMARY STATISTICS

Table B-1. Variables in STATA survey data. The variables refer to the question numbers in the mail survey (Appendix A). Additional statistics for each question (variable) are provided in the subsequent tables in this appendix

Variable	Number of Obs	Mean (arithmetic)	Standard Deviation
q4a	323	4.5368	0.7475567
q4b	322	4.683071	0.6270579
q4c	319	3.372723	1.189056
q4d	322	4.033061	0.9473828
q4e	318	3.932792	1.07588
q4f	319	1.583717	1.092779
q4g	326	4.040598	1.000017
q4h	322	3.656785	0.8938503
q4i	324	3.359708	1.21757
q4j	323	2.102542	1.14559
q4k	324	2.013652	1.217244
q4l	325	4.716321	0.5838761
q4m	327	2.68117	1.286081
q8a	322	4.276305	0.9278153
q8b	324	4.335596	0.8825632
q8c	322	3.715351	1.140694
q8d	322	3.398923	1.17883
q8e	323	4.500011	0.7438369
q8f	323	4.697954	0.6825499
q8g	323	4.307	0.8572738
q8h	323	3.60704	0.9968901
q8i	325	4.139015	0.9070701
q8j	322	4.15308	0.9713461
q8k	324	4.971814	0.2278477
q8l	324	4.213526	1.129816
q9a[a]	275	1	0
q9b[a]	151	1	0
q9c[a]	213	1	0
q9d[a]	119	1	0
q9e[a]	158	1	0
q9f[a]	113	1	0
q9g[a]	81	1	0
q9h[a]	126	1	0
q9i[a]	262	1	0
q9ja	99	1	0
q9k[a]	29	1	0
q9l[a]	94	1	0
q9m[a]	26	1	0
q10	327	1.922661	0.8546131
q11	278	1.360309	0.9004653
q12	328	1.205919	0.4049896
q13_adults	319	2.130537	0.7557102
q13_children	153	1.514315	1.635995
q14	323	1.580502	0.4942426
q15	323	1968.255	13.93063
q16	321	1.0171	0.1298465

Variable	Number of Obs	Mean (arithmetic)	Standard Deviation
q17a[a]	0		
q17b[a]	3	1	0
q17c[a]	1	1	
q17d[a]	0		
q17e[a]	298	1	0
q17f[a]	13	1	0
q18	316	3.790975	0.9911346
q19	295	5.790274	1.777295

a. Denotes a dummy variable.

Q4a	Freq.	Percent	Cumulative Percent
1	0.465270436	0.14	0.14
2	6.44418623	1.96	2.1
3	28.1870333	8.57	10.67
4	73.5298536	22.35	33.02
5	217.57721	66.13	99.15
Missing	2.796446	0.85	100
Total	*329*	*100*	

Q4b	Freq.	Percent	Cumulative Percent
2	3.5523906	1.08	1.08
3	18.0294583	5.48	6.56
4	56.8946862	17.29	23.85
5	248.444812	75.52	99.37
Missing	2.07865269	0.63	100
Total	*329*	*100*	

Q4c	Freq.	Percent	Cumulative Percent
1	30.8056405	9.36	9.36
2	30.996049	9.42	18.78
3	106.60122	32.4	51.19
4	85.4165294	25.96	77.15
5	62.555489	19.01	96.16
Missing	12.6250721	3.84	100
Total	*329*	*100*	

Q4d	Freq.	Percent	Cumulative Percent
1	3.69250807	1.12	1.12
2	13.31390398	4.05	5.17
3	77.7193064	23.62	28.79
4	103.240732	31.38	60.17
5	126.139939	38.34	98.51
Missing	4.89361112	1.49	100
Total	*329*	*100*	

Q4e	Freq.	Percent	Cumulative Percent
1	10.5836478	3.22	3.22
2	22.2386043	6.76	9.98
3	58.3728326	17.74	27.72
4	102.6387679	31.2	58.92
5	113.917606	34.63	93.54
Missing	21.2485416	6.46	100
Total	*329*	*100*	

Q4f	Freq.	Percent	Cumulative Percent
1	223.636678	67.97	67.97
2	42.9462081	13.05	81.03
3	24.1138373	7.33	88.36
4	9.69906202	2.95	91.31
5	16.121357	4.9	96.21
Missing	12.4828579	3.79	100
Total	*329*	*100*	

Q4g	Freq.	Percent	Cumulative Percent
1	14.5699689	4.43	4.43
2	3.909818293	1.19	5.62
3	57.1293851	17.36	22.98
4	128.7410869	39.13	62.11
5	121.904106	37.05	99.17
Missing	2.74563449	0.83	100
Total	*329*	*100*	

Q4h	Freq.	Percent	Cumulative Percent
1	0.378126	0.11	0.11
2	22.11331	6.72	6.84
3	135.5761	41.21	48.04
4	97.20097	29.54	77.59
5	69.47877	21.12	98.71
Missing	4.25273	1.29	100
Total	*329*	*100*	

Q4i	Freq.	Percent	Cumulative Percent
1	34.89423	10.61	10.61
2	36.81815	11.19	21.8
3	93.31505	28.36	50.16
4	99.42796	30.22	80.38
5	62.37014	18.96	99.34
Missing	2.174457	0.66	100
Total	*329*	*100*	

Q4j	Freq.	Percent	Cumulative Percent
1	130.8723	39.78	39.78
2	77.60353	23.59	63.37
3	72.95473	22.17	85.54
4	26.69719	8.11	93.66
5	12.46578	3.79	97.44
Missing	8.406503	2.56	100
Total	*329*	*100*	

Q4k	Freq.	Percent	Cumulative Percent
1	163.5065	49.7	49.7
2	56.16639	17.07	66.77
3	57.32418	17.42	84.19
4	35.72227	10.86	95.05
5	13.06174	3.97	99.02
Missing	3.218888	0.98	100
Total	*329*	*100*	

Q4l	Freq.	Percent	Cumulative Percent
1	0.797679	0.24	0.24
2	1.771307	0.54	0.78
3	11.93473	3.63	4.41
4	59.10748	17.97	22.37
5	248.8711	75.64	98.02
Missing	6.517668	1.98	100
Total	*329*	*100*	

Q4m	Freq.	Percent	Cumulative Percent
1	78.13972	23.75	23.75
2	74.598	22.67	46.42
3	78.29913	23.8	70.22
4	66.60926	20.25	90.47
5	29.91576	9.09	99.56
Missing	1.438135	0.44	100
Total	*329*	*100*	

Q8a	Freq.	Percent	Cumulative Percent
1	3.788065	1.15	1.15
2	6.709796	2.04	3.19
3	64.68349	19.66	22.85
4	71.83337	21.83	44.69
5	179.7556	54.64	99.32
Missing	2.229706	0.68	100
Total	*329*	*100*	

Q8b	Freq.	Percent	Cumulative Percent
1	3.880809	1.18	1.18
2	5.846718	1.78	2.96
3	49.79547	15.14	18.09
4	84.88491	25.8	43.89
5	183.0123	55.63	99.52
Missing	1.579805	0.48	100
Total	*329*	*100*	

Q8c	Freq.	Percent	Cumulative Percent
1	8.50777	2.59	2.59
2	42.68206	12.97	15.56
3	88.68301	26.96	42.51
4	74.3102	22.59	65.1
5	107.8922	32.79	97.9
Missing	6.924797	2.1	100
Total	*329*	*100*	

Q8d	Freq.	Percent	Cumulative Percent
1	17.35829	5.28	5.28
2	56.32591	17.12	22.4
3	110.2657	33.52	55.91
4	63.98914	19.45	75.36
5	78.67321	23.91	99.27
Missing	2.387715	0.73	100
Total	*329*	*100*	

Q8e	Freq.	Percent	Cumulative Percent
1	2.157791	0.66	0.66
2	0.418666	0.13	0.78
3	35.07331	10.66	11.44
4	83.35541	25.34	36.78
5	205.7804	62.55	99.33
Missing	2.214463	0.67	100
Total	*329*	*100*	

Q8f	Freq.	Percent	Cumulative Percent
1	2.88934615	0.88	0.88
2	4.45316302	1.35	2.23
3	10.6898452	3.25	5.48
4	52.2793732	15.89	21.37
5	256.048805	77.83	99.2
Missing	2.63946719	0.8	100
Total	*329*	*100*	

Q8g	Freq.	Percent	Cumulative Percent
1	1.88567992	0.57	0.57
2	8.74995417	2.66	3.23
3	47.473652	14.43	17.66
4	97.9651042	29.78	47.44
5	171.061221	51.99	99.43
Missing	1.86438909	0.57	100
Total	*329*	*100*	

Q8h	Freq.	Percent	Cumulative Percent
1	3.576701	1.09	1.09
2	37.64481	11.44	12.53
3	116.6838	35.47	48
4	94.16568	28.62	76.62
5	74.4097	22.62	99.23
Missing	2.519295	0.77	100
Total	*329*	*100*	

Q8i	Freq.	Percent	Cumulative Percent
1	0.395085	0.12	0.12
2	14.29139	4.34	4.46
3	69.23019	21.04	25.51
4	98.46537	29.93	55.44
5	144.4301	43.9	99.33
Missing	2.187868	0.67	100
Total	*329*	*100*	

Q8j	Freq.	Percent	Cumulative Percent
1	5.191183	1.58	1.58
2	15.04663	4.57	6.15
3	54.76564	16.65	22.8
4	98.29962	29.88	52.68
5	149.91	45.57	98.24
Missing	5.78689	1.76	100
Total	*329*	*100*	

Q8k	Freq.	Percent	Cumulative Percent
1	0.49919	0.15	0.15
3	0.993103263	0.3	0.45
4	5.24560921	1.59	2.05
5	320.673051	97.47	99.52
Missing	1.589046488	0.48	100
Total	*329*	*100*	

Q8l	Freq.	Percent	Cumulative Percent
1	9.945969	3.02	3.02
2	25.2586	7.68	10.7
3	44.90734	13.65	24.35
4	51.45356	15.64	39.99
5	194.9905	59.27	99.26
Missing	2.444054	0.74	100
Total	*329*	*100*	

Q9a	Freq.	Percent	Cumulative Percent
1	273.5497	83.15	83.15
Missing	55.45026	16.85	100
Total	*329*	*100*	

Q9b	Freq.	Percent	Cumulative Percent
1	159.938	48.61	48.61
Missing	169.062	51.39	100
Total	*329*	*100*	

Q9c	Freq.	Percent	Cumulative Percent
1	202.6654	61.6	61.6
Missing	126.3346	38.4	100
Total	*329*	*100*	

Q9d	Freq.	Percent	Cumulative Percent
1	87.66652	26.65	26.65
Missing	241.3335	73.35	100
Total	*329*	*100*	

Q9e	Freq.	Percent	Cumulative Percent
1	147.6069	44.87	44.87
Missing	181.3931	55.13	100
Total	*329*	*100*	

Q9f	Freq.	Percent	Cumulative Percent
1	117.5638	35.73	35.73
Missing	211.4362	64.27	100
Total	*329*	*100*	

Q9g	Freq.	Percent	Cumulative Percent
1	61.27066	18.62	18.62
Missing	267.7293	81.38	100
Total	*329*	*100*	

Q9h	Freq.	Percent	Cumulative Percent
1	103.6768	31.51	31.51
Missing	225.3232	68.49	100
Total	*329*	*100*	

Q9i	Freq.	Percent	Cumulative Percent
1	268.5817	81.64	81.64
Missing	60.41826	18.36	100
Total	*329*	*100*	

Q9j	Freq.	Percent	Cumulative Percent
1	119.6409	36.37	36.37
Missing	209.3591	63.63	100
Total	*329*	*100*	

Q9k	Freq.	Percent	Cumulative Percent
1	22.5913	6.87	6.87
Missing	306.4087	93.13	100
Total	*329*	*100*	

Q9l	Freq.	Percent	Cumulative Percent
1	101.4602	30.84	30.84
Missing	227.5398	69.16	100
Total	*329*	*100*	

Q9m	Freq.	Percent	Cumulative Percent
1	29.94231	9.1	9.1
Missing	299.0577	90.9	100
Total	*329*	*100*	
	301.911902	91.77	91.77
Balloons & Ribbons			
Shotgun Shell Casing	0.077010125	0.02	91.79
Balloons/Balloon Strings	0.290259182	0.09	91.88
Bird Feathers – Very Common	3.55020999	1.08	92.96
Bucket	0.944605824	0.29	93.24
Campfire Debris	0.337247609	0.1	93.35
Condoms	0.629406093	0.19	93.54
Condoms, Tampons	0.139868021	0.04	93.58
Dead Bird	0.184361383	0.06	93.64
Dead Fish Decaying	0.620689509	0.19	93.83
Diapers	0.147608906	0.04	93.87
Diapers/ Tampons	4.43776225	1.35	95.22
Dirty Diapers!	0.021963626	0.01	95.23
Dog-Not On Leash!	0.024418583	0.01	95.23
Don't Really Remember	2.19378471	0.67	95.9
Fire Remnants	0.084700189	0.03	95.93

Q9m	Freq.	Percent	Cumulative Percent
Fireworks	1.6551721	0.5	96.43
I Do Think They Have Become Much Cleaner	5.83357857	1.77	98.2
None	0.570842145	0.17	98.38
Plastics	0.087626762	0.03	98.4
Shipwreck	0.488371669	0.15	98.55
Small, Dead Jellyfish Occasionally	1.93506109	0.59	99.14
Tennis Balls	0.708081839	0.22	99.35
Trash from Ships,Plastic Caps, Balloon	0.011237546	0	99.36
Very Little Garbage	1.90280711	0.58	99.94
Wrappers	0.211423024	0.06	100
Total	*329*	*100*	

Q10	Freq.	Percent	Cumulative Percent
1	132.764391	40.35	40.35
2	87.00607479	26.45	66.8
3	107.456936	32.66	99.46
Missing	1.77259856	0.54	100
Total	*329*	*100*	

Q11	Freq.	Percent	Cumulative Percent
1	240.093478	72.98	72.98
2	7.43887217	2.26	75.24
3	12.8481432	3.91	79.14
4	22.9884512	6.99	86.13
Missing	45.6310558	13.87	100
Total	*329*	*100*	

Q12	Freq.	Percent	Cumulative Percent
1	261.238622	79.4	79.4
2	67.7438075	20.59	99.99
Missing	0.017570794	0.01	100
Total	*329*	*100*	

Q13_adult	Freq.	Percent	Cumulative Percent
0	0.488313442	0.15	0.15
1	44.7352698	13.6	13.75
2	210.2766934	63.91	77.66
3	48.0574236	14.61	92.27
4	16.146302	4.91	97.17
5	0.044918481	0.01	97.19
6	1.80025634	0.55	97.74
Missing	7.45082288	2.26	100
Total	*329*	*100*	

Q13_children	Freq.	Percent	Cumulative Percent
0	58.5541417	17.8	17.8
1	29.109033	8.85	26.65
2	52.6726524	16.01	42.66
3	18.5743402	5.65	48.3
4	8.687789412	2.64	50.94
5	0.553287415	0.17	51.11
7	0.066206887	0.02	51.13
9	3.55020999	1.08	52.21
Missing	157.232339	47.79	100
Total	*329*	*100*	

Q14	Freq.	Percent	Cumulative Percent
1	135.686246	41.24	41.24
2	187.762502	57.07	98.31
Missing	5.55125187	1.69	100
Total	*329*	*100*	

Q15	Freq.	Percent	Cumulative Percent
1934	0.074870951	0.02	0.02
1938	0.591800906	0.18	0.2
1941	0.639178557	0.19	0.4
1942	5.83357857	1.77	2.17
1943	1.90077471	0.58	2.75
1944	1.107090939	0.34	3.08
1945	0.084505647	0.03	3.11
1946	3.04781218	0.93	4.04
1947	1.3777715	0.42	4.46
1948	12.7418869	3.87	8.33
1949	9.30144942	2.83	11.16
1950	4.84057041	1.47	12.63
1951	8.96105836	2.72	15.35
1952	0.550644875	0.17	15.52
1953	1.98817121	0.6	16.12
1954	11.4541054	3.48	19.6
1955	2.53151116	0.77	20.37
1956	6.42728621	1.95	22.33
1957	8.14833361	2.48	24.8
1958	1.46830603	0.45	25.25
1959	1.29674611	0.39	25.64
1960	10.0863543	3.07	28.71
1961	4.03392168	1.23	29.94
1962	7.60492488	2.31	32.25
1963	10.54472139	3.21	35.45
1964	12.2118266	3.71	39.16
1965	14.30379091	4.35	43.51
1966	7.65839568	2.33	45.84
1967	22.1889382	6.74	52.58
1968	10.198818	3.1	55.68
1969	1.11117051	0.34	56.02
1970	12.00261659	3.65	59.67
1971	2.22521773	0.68	60.35
1972	2.75979206	0.84	61.18
1973	12.05605129	3.66	64.85

1974	3.40031766	1.03	65.88
1975	10.4544393	3.18	69.06
1976	2.76773814	0.84	69.9
1977	5.06276673	1.54	71.44
1978	9.774746662	2.97	74.41
1979	3.85749973	1.17	75.58
1980	11.1293337	3.38	78.97
1981	1.704603674	0.52	79.48
1982	4.385268823	1.33	80.82
1983	2.78065366	0.85	81.66
1984	13.4307986	4.08	85.75
1985	0.6845865	0.21	85.95
1986	2.32661944	0.71	86.66
1987	5.98357601	1.82	88.48
1988	2.29331421	0.7	89.18
1989	8.12466529	2.47	91.65
1990	0.98396444	0.3	91.94
1991	0.962745574	0.29	92.24
1992	0.629406093	0.19	92.43
1993	3.80622767	1.16	93.59
1994	7.68842692	2.34	95.92
1995	0.29518932	0.09	96.01
1996	0.859969613	0.26	96.27
1997	6.14318834	1.87	98.14
1998	1.36330092	0.41	98.56
2001	0.290259182	0.09	98.64
Missing	4.46240036	1.36	100
Total	*329*	*100*	

Q16	**Freq.**	**Percent**	**Cumulative Percent**
1	313.670578	95.34	95.34
2	5.45708684	1.66	97
Missing	9.8723348	3	100
Total	*329*	*100*	

Q17a	**Freq.**	**Percent**	**Cumulative Percent**
Missing	329	100	100
Total	*329*	*100*	

Q17b	**Freq.**	**Percent**	**Cumulative Percent**
1	5.77040758	1.75	1.75
Missing	323.229592	98.25	100
Total	*329*	*100*	

Q17c	**Freq.**	**Percent**	**Cumulative Percent**
1	1.69057619	0.51	0.51
Missing	327.309424	99.49	100
Total	*329*	*100*	

Q17d	**Freq.**	**Percent**	**Cumulative Percent**
Missing	329	100	100
Total	*329*	*100*	

Q17e	Freq.	Percent	Cumulative Percent
1	301.1354	91.53	91.53
Missing	27.86457	8.47	100
Total	*329*	*100*	

Q17f	Freq.	Percent	Cumulative Percent
1	4.63993341	1.41	1.41
Missing	324.360067	98.59	100
Total	*329*	*100*	
Q17_specify	**Freq.**	**Percent**	**Cumulative Percent**
	326.5538513	99.26	99.26
American/NYOB	0.050585009	0.02	99.27
Doesn't Matter	0.067449517	0.02	99.29
European/ American	0.006150371	0	99.31
Hispanic	0.893990544	0.27	99.59
Humans, People, Earthlings	0.08692009	0.03	99.61
Italian/Southern European	0.570842145	0.17	99.79
Middle Eastern	0.04957331	0.02	99.8
Polish, Irish,	0.620689509	0.19	99.99
Slovenian			
Why Does It Matter	0.033741321	0.01	100
Total	*329*	*100*	

Q18	Freq.	Percent	Cumulative Percent
1	4.97862701	1.51	1.51
2	20.3752626	6.19	7.71
3	103.557481	31.48	39.18
4	93.7122933	28.48	67.67
5	93.2239724	28.34	96
Missing	13.1523635	4	100
Total	*329*	*100*	

Q19	Freq.	Percent	Cumulative Percent
1	13.9969713	4.25	4.25
2	5.72833636	1.74	6
3	18.1299508	5.51	11.51
4	20.4721055	6.22	17.73
5	42.765065	13	30.73
6	81.42122586	24.75	55.48
7	82.4757779	25.07	80.54
8	42.0623832	12.78	93.33
Missing	21.948184	6.67	100
Total	*329*	*100*	

APPENDIX C: METHODS FOR REWEIGHTING MAIL RESPONDENTS

The first step in reweighting methods was to identify key variables that influence people's recreation response to marine debris on beaches. To estimate the relationship between explanatory variables and the effect of marine debris on an individual's recreation trips, we began with a logistic demand equation that is frequently used in recreation applications (Train, 2003). Individual *i*'s trips y_i are given by:

$$y_i = K \times 1/\left(1 + e^{\theta_i}\right) \tag{C1}$$

K is a scalar that converts the logistic function that follows it from a number between zero and one into a demand function that can describe any quantity of trips less than *K*. The constant *e* generates the exponential function. θ_i is the variable the determines the value of the logit expression and will later be defined in specific terms. We set *K* to equal 500 so that it was comfortably larger than the highest demand of 365 trips for any respondent in our data that further increases in *K* did not change the results. Since each respondent's baseline number of trips, *ti*, is known from the survey, we can rearrange the demand equation to show that under baseline beach conditions, with no change in debris:

$$e^{\theta_i} = (K - t_i)/t_i \tag{C2}$$

We can then represent an individual's demand after a change in beach quality as:

$$y_{i\Delta} = K \times e^{\Delta_i}/(e^{\Delta_i} + (K - t_i)/t_i) \tag{C3}$$

Here e^{θ_i} has been replaced with $(K - t_i)/t_i$, and Δ_i has been introduced to represent the perceived quality change to individual *i* from a change in debris. We specify Δ_i for a reduction in debris to almost none (*n*) as:

$$\Delta_{in} = \beta_{a_i n} + \beta_x x_i \tag{C4}$$

The term $\beta_{a_i n}$ is a constant representing the average effect on demand of a reduction to almost no debris, and is specific to the study area *a* where individual *i* was intercepted at the beach. The term $\beta_x x_i$ represents individual-specific deviations from the average, in which β_x are coefficients and x_i are characteristics of individual *i*. We represent individual *i*'s perceived quality change for a doubling of debris (*d*) as:

$$\Delta_{id} = \beta_{a_i d} - \beta_x x_i \tag{C5}$$

These expressions allow us to interpret a positive β_{an} as the average perceived increase in quality from a reduction of debris in area *a* and a negative β_{ad} as the average perceived

decrease in quality from a doubling of debris in area *a*. Given the addition in the first expression and subtraction in the second expression, it is also possible to consistently interpret a positive coefficient β_x to mean that *x* is positively associated with a concern for marine debris. This is because in each case a positive coefficient augments the size of the estimated constant. For example, if the starting point for Δ_{in} is a positive constant β_{an}, then Δ_{in} becomes more positive when β_x is positive and x_i is increasing. Likewise, if the starting point for Δ_{id} is a negative constant β_{ad}, then Δ_{id} becomes more negative if β_x is positive and x_i is increasing.

The final demand equations used in estimation are:

$$y_{in} = K \frac{e^{\beta a_i n + \beta_x x_i}}{e^{\beta a_i n + \beta_x x_i + (K - t_i)/t_i}} \tag{C6}$$

$$y_{id} = K \frac{e^{\beta a_i d - \beta_x x_i}}{e^{\beta a_i d - \beta_x x_i + (K - t_i)/t_i}} \tag{C7}$$

Using a Poisson estimator and defining t_{in} and t_{id} to be *i*'s trips with almost no debris and a doubling of debris, respectively, the likelihood function is:

$$L(\beta; x, t) = \prod_i \left(\frac{e^{-y_{in}} {y_{in}}^{t_{in}}}{t_{in}!} \frac{e^{-y_{id}} {y_{id}}^{t_{id}}}{t_{id}!} \right)^{w_{ib}} \tag{C8}$$

The base weights w_{ib} are the mail-survey base weights derived in Section 2.3.1.

There were eight attitudinal or demographic variables included in *xi*. Since the goal was to detect the presence of an effect for each variable rather than a detailed characterization of the effect, all eight variables were expressed in a simple binary form for the purpose of model estimation. The eight variables were:

- Age = 1 if the respondent was 50 years old or older
- Education = 1 if the respondent had a bachelor's degree or higher
- Children in household = 1 if there were children in the respondent's household
- Female = 1 if the respondent was female
- Debris is a problem = 1 if the respondent answered "yes" to the question whether debris was a problem on local beaches
- Parking = 1 if the respondent gave a rating of "4" or "5" for the importance of free or inexpensive parking
- Not crowded = 1 if the respondent gave a rating of "4" or "5" for the importance of beaches not being crowded
- No debris = 1 if the respondent gave a rating of "5" for the importance of no debris on beaches.

Different break points were tested for each variable to find the binary specification with the greatest effect. The model was estimated using the maximum likelihood procedure in Aptech Gauss 12 software.

Table C-1 shows the results of the contingent behavior model. The sample includes all 329 completed mail surveys, with a breakout by region as presented in Table 1 of the report. The model coefficients have no intuitive interpretation apart from the way the sign and magnitude of the coefficients affect predicted demand. The constants representing the average effect of the two scenarios show the expected sign for all four study areas, with positive constants indicating an increase in trips when debris is reduced to almost none and negative constants indicating a decrease in trips when debris is doubled. The constants are larger for a doubling of debris than for a reduction in debris, consistent with the larger change in debris in the first scenario than in the second. Three of the four constants for a reduction in debris are not statistically significant at the 5% level, with *p* values greater than 0.05. However, we are not using the model to estimate the effects of the scenarios; the sampling-based estimates for the effect of the changes in debris reported in Table 4 of the report in fact have quite narrow confidence intervals.

Table C-1. Results of the contingent behavior model

Variable	Coefficient	Standard error	*p* value
Debris reduced to almost none			
Constant – Alabama	0.174	0.104	0.094
Constant – Delaware/Maryland	0.174	0.110	0.113
Constant – Ohio	0.381	0.093	0.000
Constant – Orange County, California	0.156	0.098	0.110
Doubling of debris			
Constant – Alabama	-0.465	0.110	0.000
Constant – Delaware/Maryland	-0.316	0.113	0.005
Constant – Ohio	-0.524	0.100	0.000
Constant – Orange County, California	-0.325	0.102	0.001
Demographic and attitudinal variables[a]			
Age ≥ 50	-0.230	0.046	0.000
Education ≥ bachelor's	0.101	0.039	0.010
Children in household	-0.080	0.049	0.104
Female	-0.055	0.041	0.183
Debris is a problem on local beaches	0.129	0.040	0.001
Importance of "parking is free or inexpensive"	-0.017	0.046	0.711
Importance of "not crowded"	-0.008	0.044	0.863
Importance of "no debris"	-0.017	0.046	0.713

a. Coefficients apply to both scenarios but with opposite signs, so that positive coefficients indicate a positive association with effects in both scenarios: a larger increase in trips for a reduction of debris to almost none, and a larger reduction in trips for a doubling of debris.

To help evaluate the performance of the contingent behavior model, we provide the total percentage change in trips implied by the model for each scenario in each study area, which are similar to the final estimates in Table 4. The model estimates for a decline in debris to almost none are 8.8% in Alabama, 6.6% in Delaware/Maryland, 38.8% in Ohio, and 8.2% in Orange County, California. The model estimates for a doubling of debris are -29.3% in Alabama; -17.1% in Delaware/Maryland; -36.5% in Ohio; and -19.6% in Orange County, California.

The purpose of the contingent behavior model is to identify the characteristics or attitudinal variables that most influence people's answers to the debris scenarios. Only three of the explanatory variables are statistically significant: age, education, and the response to the question of whether marine debris is a problem on beaches in a given study area. In the case of age, those at least 50 years old had a lower than average response to debris, reflected in the negative coefficient of -0.230. Those with a bachelor's degree or higher had a higher than average response to debris, reflected in the positive coefficient of 0.101. Those who answered "yes" to the question whether marine debris is a problem on local beaches had a higher than average response to debris, indicated by the positive coefficient of 0.129.

Adjusting Sampling Weights Using Key Variables

The overall response rate for the onsite survey was 76.7%. Some onsite respondents did not provide their address, and some who provided their address did not return the mail survey that was sent to them. As a result, the overall response rate for the mail survey, which accounts for nonresponse at every stage of the study, was 19.0%. A common way to test and correct for potential nonresponse bias is to reweight the final mail-survey observations so that the proportions of reweighted mail-survey respondents with given key demographic characteristics match the proportions for the analogous groups of respondents in the onsite survey. This is a form of post-stratification described in Little (1993).

Table C-2 shows the onsite and mail survey percent frequencies for the four demographic variables that were included in both the onsite survey and the mail survey: age, education, whether there are children in the respondent's household, and the respondent's gender. Table C-2 also shows the percent frequencies for the question about whether debris is a problem on beaches in the area. Two attitudinal variables that were asked in both the onsite and mail surveys about the importance of crowding and free parking at beaches, are not shown in Table C-2 because they were not found to be associated with a response to marine debris.

In Table C-2, age is grouped into 6 categories, divided at age 25, 35, 45, 55, and 65. Education is divided into five categories, including those with less than a high school degree, those with a high school degree, those with some college but no bachelor's degree, those with a bachelor's degree, and those with a graduate degree. The categories for whether a household has children are "yes" and "no," and the categories for gender are "male" and "female." The categories for whether debris is a problem are "yes" and "no" for the onsite survey and "yes," "no," and "not sure" for the mail survey. Given the option of "not sure" included in the mail survey, we do not view the "no" response category as comparable in the two surveys. They are therefore separated into different columns in the table. The frequencies for the onsite survey and mail survey are both weighted using the onsite weights, so that measured differences between the two surveys reflect differences in representation of demographic groups rather than differences in sampling weights.

Cells shaded in light gray show under-representation in the mail survey relative to the onsite survey by at least five percentage points for the three key variables. This threshold was chosen as a reasonable way to highlight the most significant areas of divergence between the two surveys. Cells shaded in dark gray show over-representation by at least five percentage points.

Table C-2. Onsite and mail survey percent frequencies showing mail survey over- and under-representation[a]

Alabama								
Age	Value	18 to 25	26 to 35	36 to 45	46 to 55	56 to 65	65+	Missing
	Onsite	16%	22%	15%	20%	19%	8%	1%
	Mail	6%	9%	8%	27%	36%	14%	1%
	Value	< H.S.	H.S.	Some Col.	Bach.	Grad.		Missing
Education	Onsite	1%	24%	33%	28%	14%		0%
	Mail	2%	10%	27%	36%	24%		1%
Children in household	Value	No	Yes					Missing
	Onsite	60%	34%					7%
	Mail	87%	13%					0%
Gender	Value	Male	Female					Missing
	Onsite	43%	56%					0%
	Mail	41%	59%					1%
Debris a problem[b]	Value	No	Yes	No	Not sure			Missing
	Onsite	60%	40%					0%
	Mail		36%	45%	19%			0%
Delaware and Maryland								
Age	Value	18 to 25	26 to 35	36 to 45	46 to 55	56 to 65	65+	Missing
	Onsite	7%	21%	22%	28%	11%	11%	0%
	Mail	5%	6%	17%	25%	16%	28%	1%
Education	Value	< H.S.	H.S.	Some Col.	Bach.	Grad.		Missing
	Onsite	0%	9%	33%	43%	11%		4%
	Mail	3%	16%	28%	19%	33%		2%
Children in household	Value	No	Yes					Missing
	Onsite	78%	22%					0%
	Mail	69%	31%					0%
Gender	Value	Male	Female					Missing
	Onsite	47%	51%					2%
	Mail	29%	70%					1%
Debris a problem[b]	Value	No	Yes	No	Not sure			Missing
	Onsite	68%	18%					6%
	Mail		33%	41%	25%			0%
Ohio								
Age	Value	18 to 25	26 to 35	36 to 45	46 to 55	56 to 65	65+	Missing
	Onsite	8%	10%	27%	21%	23%	11%	0%
	Mail	2%	9%	23%	15%	26%	22%	4%
Education	Value	< H.S.	H.S.	Some Col.	Bach.	Grad.		Missing
	Onsite	1%	20%	25%	36%	18%		1%
	Mail	0%	7%	41%	29%	20%		4%
Children in household	Value	No	Yes					Missing
	Onsite	39%	53%					4%
	Mail	65%	35%					0%

Table C-2. (Continued)

Gender	Value	Male	Female					Missing
	Onsite	45%	54%					1%
	Mail	53%	44%					3%
Debris a problem[b]	Value	No	Yes	No	Not sure			Missing
	Onsite	36%	64%					0%
	Mail		64%	15%	21%			0%
Orange County, California								
Age	Value	18 to 25	26 to 35	36 to 45	46 to 55	56 to 65	65+	Missing
	Onsite	6%	20%	26%	23%	17%	7%	1%
	Mail	2%	17%	16%	15%	35%	14%	0%
Education	Value	< H.S.	H.S.	Some Col.	Bach.	Grad.		Missing
	Onsite	0%	13%	35%	28%	24%		0%
	Mail	0%	7%	28%	20%	44%		2%
Children in household	Value	No	Yes					Missing
	Onsite	46%	52%					0%
	Mail	66%	34%					0%
Gender	Value	Male	Female					Missing
	Onsite	50%	46%					4%
	Mail	58%	42%					0%
Debris a problem[b]	Value	No	Yes	No	Not sure			Missing
	Onsite	51%	49%					0%
	Mail		55%	20%	23%			2%

a. Dark gray and light gray show, respectively, over-representation and under-representation by at least five percentage points in mail survey responses relative to onsite survey responses. Attitudinal variables involving crowded beaches and inexpensive parking are available from both the onsite and mail surveys but are excluded from the table because they are not related to marine debris and were not found to be associated with a response to marine debris in the contingent behavior model. For the demographic variables "children in household" and "gender," over- and under-representation are not highlighted because these variables were not found to be associated with a response to marine debris in the contingent behavior model.

b. The variable "debris a problem" refers to the question, "Do you think garbage or marine debris is a problem [on beaches in your local area]?" The mail survey elicited one of three responses: "yes," "no," or "not sure." The onsite survey elicited only a "yes" or "no" response.

To illustrate the selection of categories for reweighting, we use as examples age and education in Alabama. The first 3 age categories, including respondents aged less than or equal to 25, respondents aged 26 to 35, and respondents aged 36 to 45, are under-represented in the mail survey by at least five percentage points. The remaining three categories, including those aged 46 to 55, 56 to 65, and older than 65, are all over-represented by at least five percentage points. Representativeness could be improved by increasing the sampling weights for the first 3 groups and decreasing the sampling weights for the last three groups. For reweighting, we therefore combine the first 3 groups into a single category of those aged 45 or under and we combine the last 3 groups into a single category of those aged 46 or older. Aggregating into just two categories helps reduce variation in the size of the weighting adjustments and in the variance of the final weights.

In the case of education, Alabama respondents with a high school diploma or with some college but not a bachelor's degree are under-represented in the mail survey by at least five percentage points. Those with a bachelor's degree or a graduate degree are over-represented

by at least five percentage points. We therefore combine the first two groups and the second two groups, respectively. The remaining group, those without a high school degree, is quite small and is unlikely to significantly affect the adjusted weights or final weighted results. For simplicity, we combined it with the two other groups without a bachelor's degree to form two weighting categories: those with less than a bachelor's degree and those with a bachelor's degree or higher.

Based on a similar review of all variables in Table C-2, we chose to reweight by three variables (age, education, and debris is a problem) in all four regions, with one exception: for Ohio, the percentage of people who viewed marine debris as a problem was the same in the onsite survey and mail survey, and remained nearly the same after reweighting by age and education. Although the discrepancy in Alabama for the debris question was less than 5 percentage points (Table C-2), this diverged to more than 5 percentage points after reweighting by age and education, so all three variables were ultimately used in the adjustment.

The selection of variables for reweighting leads to the definition of "cells" used in the reweighting. Each variable was divided into two categories so that there were two cells for *n* variables. For example, if age were the only variable used, and respondents were divided into those age 45 or younger and those over 45, then there would be two cells. If the mail survey proportions for these two cells are 0.5 and 0.5 and the onsite survey proportions are 0.25 and 0.75, then the base weights for mail respondents in the first cell would each be reweighted by a factor of 0.25/0.5 and the base weights for mail respondents in the second cell would each be reweighted by 0.75/0.5. If the education variable were also used, with a division into those without a bachelor's degree and those with a bachelor's degree, there would now be four cells: "age $\leq$ 45" and "education < bachelor's degree"; "age > 45" and "education < bachelor's degree"; "age $\leq$ 45" and "education $\geq$ bachelor's degree"; "age > 45" and "education $\geq$ bachelor's degree."

The final adjustments involved reweighting by eight cells in Alabama, Delaware/Maryland, and Orange County, California; and by four cells in Ohio as follows:

- In Alabama, every combination of "age $\leq$ 45" or "age > 45," "education < bachelor's degree" or "education $\geq$ bachelor's degree," and "problem = yes" or "problem $\neq$ yes" formed one of eight cells.
- In Delaware/Maryland, every combination of "age $\leq$ 55" or "age > 55," "education = either high school or graduate school" or "education $\neq$ either high school or graduate school," and "problem = yes" or "problem $\neq$ yes" formed one of eight cells.
- In Ohio, every combination of "age $\leq$ 55" or "age > 55" and "education = some college" or "education $\neq$ some college" formed one of four cells.
- In Orange County, California, every combination of "age $\leq$ 55" or "age > 55," "education < graduate degree" or "education = graduate degree," and "problem = yes" or "problem $\neq$ yes" formed one of eight cells.

Due to an oversight, the question asking whether marine debris was a problem at area beaches was not asked in exactly the same way in the onsite and mail surveys. The mail survey included the option to choose "not sure" while this option was not offered to onsite respondents. One might therefore expect that both the "yes" and "no" categories would be

overstated in the onsite survey relative to the mail survey, all else equal. This suggests that upward reweighting of "yes" responses in the mail survey to match "yes" responses in the onsite survey could lead to bias and would not be appropriate. In all cases described above, the proportion of respondents answering "yes" in the mail survey was weighted down to match the onsite survey. Although "yes" responses for the mail survey in Alabama initially appeared low, as shown in Table C-2, once the responses were reweighted by age and education, the reweighted frequency of "yes" responses was 45%, or five percentage points higher than the onsite frequency. This means that including the debris variable in the reweighting procedures for Alabama was appropriate, because it ultimately led to a downward reweighting of the variable relative to a procedure that only used age and education.

APPENDIX D: DETAILED METHODS OF ECONOMIC IMPACTS MODEL

In this appendix, we present additional technical details of the economic impacts model.

D.1. Converting Visitor Spending into Producer Value

We used type II final demand multipliers from the U.S. Bureau of Economic Analysis's Regional Input-Output Modeling system (RIMS II). Type II multipliers are used to measure the economic impact of industry and household expenditures. These multipliers estimate the economic input using the *producer's value,* which excludes distribution costs such as transportation costs and wholesale and retail trade margins, but includes excise taxes collected and paid by producers (U.S. BEA, 2018). Producer values are calculated from expenditures using ratios from the RIMS II national distribution cost tables (Rebecca Bess, BEA, personal communication, August 20, 2009). Our analysis includes two expenditure categories that map to industries where we convert the consumer value (i.e., the visitor expenditures) into a producer value:

- Auto fuel costs are mapped into industry "petroleum and coal products manufacturing"
- Grocery and convenience stores are mapped into industry "food and beverage and tobacco product manufacturing."

Applying the ratios from the national accounts table, we assume 10.7% of the expenditures on auto fuel and 15.6% of expenditures on grocery and convenience stores flow into the local economy. These ratios are multiplied by the RIMS II multiplier for each of these industries.

D.2. Expenditure and Multiplier Tables

In Table D-1, we present the expenditures by category included in our analysis as final demand changes. We report the expenses by category for marine-debris related activities, weighted by participation in each activity. Table D-2 presents final RIMS multipliers used in this study.

Table D-1. Daily visitor expenditures by category[a]

.		Daily expenditures			
Expenditure category	RIMS II industry	Alabama	Delaware and Maryland	Ohio	Orange County, California
Auto fuel cost	24 Petroleum and coal products manufacturing	$26.15	$19.31	$19.89	$18.69
Auto rental cost	36 Transit and ground passenger transportation	$3.77	$0.55	$2.48	$3.00
Bus, taxi, etc.	36 Transit and ground passenger transportation	$5.09	$1.40	$3.55	$4.50
Parking, beach fees, etc.	63 Other services[a]	$1.48	$2.35	$1.71	$1.54
Lodging	61 Accommodation	$39.33	$27.77	$27.07	$24.86
Vacation package	½ accommodation – ½ amusements	$0.69	$1.01	$0.85	$0.57
Restaurants, bars, etc.	62 Food services and drinking places	$45.32	$31.71	$30.95	$26.13
Grocery, convenience stores	19 Food and beverage and tobacco product manufacturing	$16.54	$7.87	$9.69	$8.71
Viewing whale, wildlife watching boat fees	38 Other transportation and support activities[a]	$0.14	$0.07	$0.20	$0.38
Viewing rental fees for sailboat, etc.	34 Water transportation	$0.04	$0.00	$0.17	$0.15
Water contact rented equipment, gear for snorkeling, etc.	60 Amusements, gambling, and recreation	$0.00	$0.00	$0.00	$0.33
Water contact rented equipment, gear for surfing, etc.	60 Amusements, gambling, and recreation	$0.00	$0.00	$0.00	$0.24
Water contact rented equipment, gear for kayaking, etc.	60 Amusements, gambling, and recreation	$0.00	$0.00	$0.00	$0.32
Outdoor rented equipment, gear for activities like biking, hiking, etc.	60 Amusements, gambling, and recreation	$0.00	$0.02	$0.04	$0.07
Outdoor rented equipment, gear for games like volleyball, frisbee, etc.	60 Amusements, gambling, and recreation	$0.00	$0.00	$0.00	$0.00
Outdoor horseback riding fees	60 Amusements, gambling, and recreation	$0.00	$0.00	$0.00	$0.00
Total daily expenditures		**$138.55**	**$92.06**	**$96.61**	**$89.49**

a. Source: NOAA (2012), using average expenditures for all recreators (see Section 4.1). Average expenditures are weighted by participation in four marine-debris impacted recreation activities (see Section 4.1).

Table D-2. RIMS II final demand multipliers[a]

RIMS II industry/Economic sector	Output multiplier	Earnings multiplier	Jobs multiplier (for $1 change in final demand)	Value added multiplier
Alabama				
24 Petroleum and coal products manufacturing	1.4877	0.268	0.0000051560	0.5459
29 Food and beverage stores (retail)	1.7498	0.5798	0.0000231406	1.1119
34 Water transportation	1.969	0.3968	0.0000092139	0.8125
36 Transit and ground passenger transportation	2.0576	0.7027	0.0000323946	1.0328
38 Other transportation and support activities	1.96	0.6468	0.0000163615	1.1381
60 Amusements, gambling, and recreation	1.7889	0.5289	0.0000253024	1.0243
61 Accommodation	1.6311	0.4604	0.0000173756	1.0023
62 Food services and drinking places	1.7268	0.528	0.0000251495	0.9466
63 Other services	1.8773	0.6108	0.0000188710	1.0651
Delaware and Maryland				
24 Petroleum and coal products manufacturing	1.26	0.1855	0.0000026361	2.6361
29 Food and beverage stores (retail)	1.5187	0.4403	0.0000144831	0.9824
34 Water transportation	1.4677	0.2614	0.0000047682	0.5756
36 Transit and ground passenger transportation	1.5729	0.5601	0.0000298866	0.7994
38 Other transportation and support activities	1.5734	0.5612	0.0000125574	0.9317
60 Amusements, gambling, and recreation	1.4961	0.3305	0.0000114617	0.8561
61 Accommodation	1.4534	0.3625	0.0000102803	0.9024
62 Food services and drinking places	1.5902	0.4425	0.0000177161	0.8637
63 Other services	1.5851	0.5198	0.0000146296	0.9137
Ohio				
24 Petroleum and coal products manufacturing	1.2881	0.183	0.0000027883	0.4114
29 Food and beverage stores (retail)	1.8588	0.5221	0.0000199355	1.1662
34 Water transportation	2.0162	0.3485	0.0000075218	0.834
36 Transit and ground passenger transportation	2.1424	0.6069	0.0000286231	1.0656
38 Other transportation and support activities	1.9707	0.5665	0.0000128953	1.1291
60 Amusements, gambling, and recreation	1.823	0.4023	0.0000138182	1.0336
61 Accommodation	1.783	0.4491	0.0000147213	1.0835
62 Food services and drinking places	1.9485	0.5206	0.0000219974	1.0616
63 Other services	2.013	0.5873	0.0000168018	1.1316
Orange County, California				
24 Petroleum and coal products manufacturing	1.2504	0.1718	0.0000021136	0.4067
29 Food and beverage stores (retail)	1.7644	0.4838	0.0000145975	1.1273
34 Water transportation	1.7721	0.2929	0.0000051666	0.7484
36 Transit and ground passenger transportation	1.9323	0.5405	0.0000206913	1.0001
38 Other transportation and support activities	1.7736	0.4772	0.0000097029	1.0415
60 Amusements, gambling, and recreation	1.6984	0.3996	0.0000132521	0.9679
61 Accommodation	1.7498	0.427	0.0000107093	1.0762
62 Food services and drinking places	1.8616	0.4874	0.0000170229	1.0307
63 Other services	1.9649	0.5613	0.0000135477	1.1214

a. Data source: U.S. BEA, 2019.

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Chapter 112

GREAT LAKES LAND-BASED MARINE DEBRIS ACTION PLAN ACCOMPLISHMENTS REPORT*

National Oceanic and Atmospheric Administration

LIST OF ACRONYMS

ACC	American Chemistry Council
BMP	Best management practices
ECCC	Environment and Climate Change Canada
EPA	United States Environmental Protection Agency
FEMA	Federal Emergency Management Agency
GCSC	Great Canadian Shoreline Cleanup
GLSGN	Great Lakes Sea Grant Network
IAGLR	International Association of Great Lakes Research
ICC	International Coastal Cleanup
IJC	International Joint Commission
IL	Illinois
IN	Indiana
LS NERR	Lake Superior National Estuarine Research Reserve
MI	Michigan
MN	Minnesota
NERR	National Estuarine Research Reserve
NOAA	National Oceanic and Atmospheric Administration
NOAA MDP	National Oceanic and Atmospheric Administration Marine Debris Program
NY	New York
OH	Ohio
OSG	Ohio Sea Grant
OSU	The Ohio State University

* This is an edited, reformatted and augmented version of a U.S. Department of Commerce on 2014-2019 Accomplishments Report, dated May 2014-May 2019.

OWC NERR	Old Woman Creek National Estuarine Research Reserve
PA	Pennsylvania
PWG	Plastic Working Group
SUNY	The State University of New York
WI	Wisconsin

INTRODUCTION

The following document details accomplishments of the Great Lakes Land-based Marine Debris Action Plan. This Action Plan consisted of 53 actions, which were to be completed within five years (May 2014–May 2019). It is through the commitment, efforts, and care of the Action Plan's partners that there has been great progress made on the issue of marine debris in our community. Of the 53 actions in the Action Plan, contributors to the Great Lakes Land-based Marine Debris Action Plan successfully completed 34 actions, and are currently working on 17 actions. One action has not yet started, and another was removed during review.

The first workshop in the planning and development of the Action Plan brought the Great Lakes marine debris community together for the first time. Participants recognized the need and value of an action plan to promote collaboration, coordination, and raise awareness of each other's efforts. Through the process, multiple aspects of the marine debris issue were synthesized into shared goals that participants could work on together. Participants established diverse partnerships due to shared interests and networked across sectors. This collaborative approach has served as a model for other regional action plans around the United States.

Collectively, the group has helped to raise awareness of the marine debris issue in the Great Lakes through clear and consistent messaging. These developed partnerships amplified the voice and the role of freshwater in what has been largely considered an ocean problem. As a result, there has been increased attention and interest in research, policy, and management of marine debris within the Great Lakes.

The Great Lakes marine debris community made great strides to address the issue over the past five years. This includes completing research and publishing scientific articles, engaging with the policy and management community, educating the public and students, and removing debris from the environment. In total, 180,062 people were educated on the topic during 711 events and approximately 306,665 pounds of debris was removed by volunteers.

Semi-annual check-ins for the Action Plan were held via webinar in May and November of each year. During the webinars, participants heard and shared summaries of the progress of actions to date. Specifically, summary information was presented for each of the four goals in the Action Plan, completed actions were highlighted in presentations, and other high-level updates were given from action coordinators. The webinars ended with an open discussion of feedback on reporting, communications, and challenges. The majority of these webinars were recorded, and archived on the Great Lakes Marine Debris Collaborative Portal.

Action Plan participants reconvened in August 2019 to review these accomplishments, evaluate the Action Plan, discuss challenges, and plan for the future of marine debris efforts

in the Great Lakes. While much progress has been made in addressing the issue, more work remains to see the Great Lakes free from the impacts of marine debris.

ACTION PLAN PURPOSE

The overall purpose of the Great Lakes Land-based Marine Debris Action Plan is to establish a comprehensive framework for strategic action to ensure that the Great Lakes, its coasts, people, and wildlife are free from the impacts of marine debris.

ACTION PLAN STATUS

The 2014-2019 Great Lakes Land-based Marine Debris Action Plan was completed in May 2019. Upon its completion, participants provided feedback on improvements to the Action Plan scope, structure, implementation, monitoring, and communication. Participants expressed an interest to build on successes and develop a new five-year Action Plan. The new 2020-2025 Great Lakes Land-based Marine Debris Action Plan will soon be publically available.

MARINE DEBRIS GOALS, OBJECTIVES AND ACTIONS

The Tables below are the core of the Action Plan. They list goals, objectives, and actions that contribute to achieving the Action Plan's goals.

GOALS

- Goal 1: Research & Monitoring

Knowledge gaps are identified and filled through research and monitoring of land-based marine debris.

- Goal 2: Science-based Approaches & Management

A science-based and strategic approach is used to guide land-based marine debris policy and management decisions in the Great Lakes.

- Goal 3: Prevention & Education

Land-based marine debris is prevented and reduced through an educated and involved community.

- Goal 4: Removal & Tracking

The impacts of land-based marine debris are reduced through removal and tracking efforts.

Objectives

In the context of this Action Plan, the objectives define how each goal will be achieved. Typically, there are several objectives per goal.

Actions

Actions are projects and activities supporting an objective, undertaken to achieve the associated goal.

In this Action Plan, Coordinators and Partners are entities that have volunteered to carry out a given action, pending the availability of resources (funding, staff, time, materials, etc.). Coordinators/Co-Coordinators were responsible for undertaking activities that fulfilled the action strategy and reported on the progress, challenges, and completion of the action. Partners were responsible for supporting and undertaking activities that fulfilled the action strategy and provided input on progress.

Vision

The Great Lakes, its coasts, people, and wildlife are free from the impacts of marine debris.

Mission

The Great Lakes will be free from marine debris through an increased understanding of the problem, preventative actions, reductions in impacts, and collaborative efforts of diverse groups.

GOAL 1. RESEARCH AND MONITORING

Knowledge gaps are identified and filled through research and monitoring of land-based marine debris.

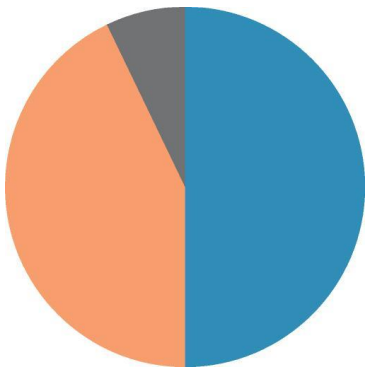
Action Status

- 7 Complete
- 6 In Progress
- 1 Removed.

Goal Accomplishments

Great Lakes Marine Debris Collaborative Portal (Objective 1.1)

The NOAA Marine Debris Program, in cooperation with input from regional partners, developed a regional web-platform, the Great Lakes Marine Debris Collaborative Portal. Broader than a research platform, this tool provides the opportunity for all of those engaged in the regional marine debris community to share resources and ideas. Stakeholders submitted ideas on function and format for the platform, and provided input on the prioritization of features. The Collaborative Portal is also accessible to the public to serve as a tool for regional marine debris information. The site is available at https://greatlakes-mdc.diver.orr.noaa.gov/. This tool is currently being replicated in other regions around the country.



Convened Researchers to Foster Collaboration (Objective 1.2)

Marine debris researchers from around the region met at each of the last five annual conferences of the International Association of Great Lakes Research (IAGLR). Marine debris sessions at each of these conferences focused on microplastics in the Great Lakes environments and their sources, fates, and impacts. Each year, approximately 50 people attend the sessions, which generated discussions on the issue, as well as provided an opportunity for researchers to network and forge new collaborative opportunities.

Completed a Research Summary and Gap Analysis (Objective 1.3)

A synthesis paper, entitled "Plastic debris in the Laurentian Great Lakes: A review," was published in the March 2015 issue of the Journal of Great Lakes Research and authored by the research group at the University of Waterloo (doi:10.1016/j.jglr.2014.12.020). This work provided key information for attendees at an International Joint Commission (IJC) microplastics workshop in April 2016 and informed the identification of research gaps and recommendations to guide future research efforts. The IJC recommendations include:

- Communicate results of research to share information with the public of all ages and decision makers, through the development of Great Lakes focused educational materials.
- Encourage prevention of plastic marine debris through changing behavior by using education, outreach, policy, and market-based instruments.

- Assess the impacts of ecological and potential human health impacts using an ecological risk assessment framework (exposure/hazard).
- Compare and analyze existing programs and policies for the reduction and prevention of plastic marine debris and promote those that are good models for plastics management.
- Invest in solution-based research, including innovative product development and water infrastructure improvements.
- Conduct modelling to determine the sources and fate of microplastics in the Great Lakes.
- Enable the plastic industry, its clients and other Great Lakes stakeholders to enhance effective management and implementation of reduce, reuse, and recycle programs.
- Promote improved waste management and debris removal.
- Develop and strengthen binational Great Lakes linkages to support sharing of research, education and outreach programs, and best management practices.
- Develop and/or adopt standardized sampling and analytical methods for microplastics.

The IJC produced a final workshop report, which provided additional information on these recommendations. An updated research review is now in process from the University of Toronto.

Management Community Connections (Objective 1.4)

Conversations have begun to connect Great Lakes marine debris researchers with management agencies in an effort to foster collaborative research on relevant marine debris topics. This work is being done through the Plastic Working Group (PWG) in Cleveland, Ohio, as well as through science symposiums that were organized by ECCC.

Goal 1. Knowledge gaps are identified and filled through research and monitoring of land-based marine debris

Objective 1.1: By the end of 2016, develop a platform for long-term collaboration on Great Lakes marine debris research.			
Action Strategies	Coordinating & Partner Organizations	Status	Progress Description
1.1.1. Convene a small working group to discuss research community needs, timeline, scale, and intent for a future platform.	Coordinator: NOAA MDP Partners: University of Waterloo, Clean Water Action Participants: All research organizations	Complete	The NOAA MDP finished the development of a regional web-platform for the Great Lakes marine debris community. Stakeholders submitted ideas/desires for the platform, and provided input on the prioritization of features. The Great Lakes Marine Debris Collaborative Portal site is now available.
1.1.2. Once the needs analysis is complete, examine existing platforms to meet those needs.	Coordinator: NOAA MDP	Complete	The NOAA MDP finished the development of a regional web-platform for the Great Lakes marine debris community. The NOAA MDP met with the developers and manager of NOAA Data Integration Visualization Exploration and Reporting (DIVER) and have concluded that this platform was a good fit based on desires/requirements of regional community that were identified in action 1.1.1. The Great Lakes Marine Debris Collaborative Portal site is now available.

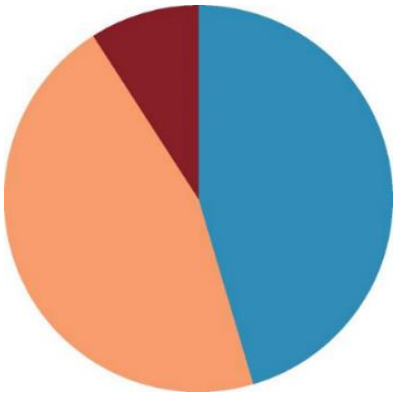
Action Strategies	Coordinating & Partner Organizations	Status	Progress Description
Objective 1.2: By 2016, convene researchers to foster partnerships and collaboration.			
1.2.1. Share current venue opportunities and identify invitees. Invitees should include those researchers who are currently involved in, or have an interest in, land-based marine debris research in the region.	Coordinator: Alliance for the Great Lakes Partners: SUNY Fredonia, NOAA MDP, University of Wisconsin – Superior	Complete	The Alliance for the Great Lakes was one of the partners for the first ever Great Lakes Day on August 20, 2016 coordinated by the eXXpedition. As a partner, they connected the Inland Seas Education Association with participants, and as a result, Inland Seas helped lead the large sampling effort. In addition, the eXXpedition planners connected Inland Seas with an educational program through Toronto Brigantine Inc., which also participated in Great Lakes Day and used Inland Seas'shipboard curriculum for microbeads.
1.2.2. Host a research panel or session at a regional conference.	Coordinator: University of Michigan Partners: University of Wisconsin – Superior, SUNY Fredonia, Western University, Ontario Ministry of the Environment, IL - IN Sea Grant, NOAA MDP	Complete	The IAGLR 2014 microplastics session was completed. Approximately 40-50 people attended the session and generated discussion around the research and possible ways to prevent microplastics. Researchers also gathered again at IAGLR in 2015, 2016, 2017, and 2018 for a session ^{on}marine debris.
Objective 1.3: Over the next five years, create summary documents of existing research on land-based marine debris and identify key data gaps for the research community.			
1.3.1. Publish a synthesis paper on plastic marine debris research in the Great Lakes.	Coordinator: University of Waterloo Partner: ECCC, University of Toronto	Complete	The synthesis paper, entitled "Plastic debris in the Laurentian Great Lakes: A review," was published in the March 2015 issue of the Journal of Great Lakes Research (doi:10.1016/j.jglr.2014.12.020). ECCC recently hired Dr. Chelsea Rochman at the University of Toronto to synthesize available research in order to provide a better understanding of the contamination, sources, and effects of plastic pollution on our Great Lakes. This will allow for the identification of outstanding science gaps and updated science priorities. In addition, she will explore the policy implications of the findings to inform decision making on actions to reduce plastic pollution in the Great Lakes.
1.3.2. Based on results of the synthesis paper, perform a gap analysis to identify research needs in the region.	Coordinator: NOAA MDP Partners: University of Waterloo, University of Western Ontario, University of Michigan, Loyola University, SUNY Fredonia, University of Wisconsin – Superior, ECCC	Complete	The IJC held a microplastics workshop in April 2016. At this workshop, research gaps were identified for this particular debris type. Additional analysis will be needed to identify research gaps in other types of land- based marine debris. The IJC produced a final workshop report, which includes prioritized research needs.
Objective 1.4: Through 2019, engage Great Lakes management communities to prioritize and foster implementation of relevant research topics.			
1.4.1. Identify relevant management stakeholders and establish communication channels.	Coordinator: Great Lakes Sea Grant Network Partners: NERRs, Alliance for the Great Lakes, Keep America Beautiful	In Progress	Ohio Sea Grant is participating in the PWG in Cleveland. Through the PWG, Ohio Sea Grant has broadened the stakeholders it has worked with on the issue of plastic debris and shared education ideas. Barrier to Completion: Many groups are now working on the topic of marine debris, especially plastic pollution. Unfortunately, this has led to a more competitive and less collaborative atmosphere in certain areas of the region.
1.4.2. Through established communication channels, work with management stakeholders to develop a list of research questions that are of interest to the Great Lakes management community.	Coordinator: Great Lakes Sea Grant Network Partners: NERRs, Alliance for the Great Lakes, Keep America Beautiful	In Progress	There is a need for additional information on the use of plastic bags and the effectiveness of reminder strategies for reusable bags. This was identified as a result from the social marketing campaign conducted in Cleveland, OH (see actions 3.2.2 and 3.3.2). Ohio Sea Grant received funding from the NOAA MDP to complete a study on reminder strategies. Data collection began in May 2019. ECCC hosted a science symposium to identify research gaps related to the development of a Canadian science agenda on plastics. This event engaged domestic stakeholders, particularly intergovernmental departments and academia, to determine domestic research gaps and prioritize those of high importance.

Goal 1. (Continued)

Action Strategies	Coordinating & Partner Organizations	Status	Progress Description
Objective 1.5: Within the next five years, the Great Lakes research community will address knowledge gaps on land-based marine debris by completing and publishing a minimum of two studies.			
1.5.1. Based on the completed synthesis paper and gap analyses (Objective 1.3), develop a list of prioritized questions that need to be addressed and share it with the regional research community.	Coordinator: NOAA MDP Partners: Researchers, Alliance for the Great Lakes, ECCC, Inland Seas Education Association	Complete	The IJC held a microplastics workshop in April 2016 where research gaps were identified for this particular debris type. Additional analysis will be needed to identify gaps in other types of land-based marine debris. IJC produced a final workshop report, which includes prioritized research needs.
1.5.2. Identify funding sources for land-based marine debris research, both current and future.	Coordinators: University of Waterloo, University of Michigan Partners: Great Lakes Sea Grant Network, University of Wisconsin – Superior	In Progress	No Report for 2017, 2018, or 2019 No new progress. University of Michigan and other colleagues took part in a Sea Grant training workshop on microplastics at the NOAA Great Lakes Environmental Research Lab in April 2016. Sea Grant affiliates will request microplastics be stated as a priority research item in the next Sea Grant request for proposals. Sea Grant is identified as a potential funding source.
1.5.3. Create and send a bi-national letter to inform potential funding officials of regional research needs for land-based marine debris.	Coordinators: University of Waterloo, University of Michigan	In Progress	No Report for 2017, 2018, or 2019 No new progress. University of Michigan and University of Waterloo staff, along with other colleagues, took part in an IJC workshop on microplastics. The goal of the workshop was to establish research gaps and priorities, which was accomplished. The IJC will now formalize these statements to present to the United States and Canadian governments to encourage funding basic research. This report could also serve as a template for further dissemination.
Objective 1.6: Through 2019, ensure data collection consistency and quality assurance by multiple users.			
1.6.1. Establish standardized metadata on land-based debris for the following mediums: Open and nearshore water Shoreline Deep and nearshore sediments Fisheries Water infrastructure (wastewater systems, stormwater, etc.)	Coordinators: SUNY Fredonia, University of Michigan Partners (corresponding to mediums listed): SUNY Fredonia, NOAA MDP, University of MI, University of Wisconsin – Superior, Inland Seas Education Association Loyola University, University of Waterloo, Alliance for the Great Lakes, NOAA MDP, Inland Seas Education Association University of Western Ontario, NOAA MDP State/Provincial Departments of Natural Resources SUNY Fredonia	In Progress	No Report for 2018 or 2019 No new progress. The first 2 steps in the list have been completed. Significant progress has been made on step 3, while the others have not been started. Compile methods (complete) Review methods and identify the steps in the protocols where metadata collection is crucial (complete) Develop optimized/recommended metadata for each sampling media modeled after the Minimum Information about a Genome/ Metagenome Sequence (MIGS/MIMS) specification, especially the metadata requirements for environmental sampling component Share drafts of optimized/recommended metadata collection forms for various media types. Request various research personnel use the form to "report" some of their samples to ensure that all crucial metadata is accounted for Revise/amend metadata forms based on feedback from researchers Submit final recommendations for metadata collection to Action Plan
1.6.2. Establish opportunities to share standardized meta- data across the regional organizations. This may be completed in conjunction with Objective 1.2.	Coordinator: None Partners: SUNY Fredonia, NOAA MDP, University of Wisconsin – Superior, NERRs	In Progress	No Report for 2017, 2018, or 2019 No progress without a coordinator to lead the action. Dependent on completion of action 1.6.1.

GOAL 2. SCIENCE-BASED APPROACHES AND MANAGEMENT

A science-based and strategic approach is used to guide land-based marine debris policy and management decisions in the Great Lakes.



Action Status

- 5 Complete
- 5 In Progress
- 1 Not Started.

Goal Accomplishments

Summary of Existing Marine Debris Policies (Objective 2.1)

After reviewing policies from other regions, the Alliance for the Great Lakes and Keep America Beautiful created a list of categories and model examples of existing policies and best management practices. Based on this list, outreach began in 2017 across the Great Lakes basin to find relevant regional examples. The idea of a broad survey was eliminated due to logistical and practical concerns, and instead targeted outreach through the Great Lakes Land-based Marine Debris Action Plan list serve and other Great Lakes coalitions (such as the Healing Our Waters coalition) led to the creation of a list of communities that have implemented policies. Examples of these policies included bag bans/fees, bans on the use of single-use plastics in government facilities, public outreach campaigns, and social marketing campaigns. To date, few examples of local, county, or state level policies aimed at reducing marine debris appear to exist in the Great Lakes.

Based on the results of the outreach in 2017, it was determined that a toolkit for volunteer advocates would be the best way to encourage policies and best management practices in more communities. The toolkit, "Plastic-Free Great Lakes: An Advocacy Toolkit to Make a Difference in Your Community" was released by the Alliance for the Great Lakes on November 8, 2018 in conjunction with a public webinar. The toolkit features brief examples

of plastic pollution reduction policies and programs in the Great Lakes region and tools for volunteers to use in their advocacy to local and state policy makers.

Dissemination of Action Plan to Policy and Management Officials (Objective 2.2, Actions 2.2.1 and 2.2.2)

Throughout the past five years, the Action Plan was presented 39 times, reaching approximately 1,796 policy and management representatives. Additionally, in 2014, the NOAA MDP, in partnership with state management programs (Coastal Zone Management Offices, Departments of Natural Resources or Environmental Quality), the Environmental Protection Agency, the Great Lakes Sea Grant Network, and the National Estuarine Research Reserves developed a contact list of relevant policy and management representatives from around the region. These contacts received the Great Lakes Land-based Marine Debris Action Plan via email on August 5, 2014.

Goal 2. A science-based and strategic approach is used to guide land-based marine debris policy and management decisions in the Great Lakes

Objective 2.1: By the end of 2017, create a summary document of existing land-based marine debris policies and management systems in the Great Lakes.			
Action Strategies	Coordinating & Partner Organizations	Status	Progress Description
2.1.1. Inventory and create a summary document on existing policies and best management practices related to land-based marine debris in the Great Lakes.	Coordinators: Alliance for the Great Lakes, Keep America Beautiful Partners: ACC, NERRs	Complete	After reviewing policies from other regions, a list of categories and examples of policies was created. Based on this list, outreach began across the Great Lakes basin to find examples. After the idea of a board survey was eliminated due to logistical and practical concerns, targeted outreach through the Action Plan list serve and other Great Lakes coalitions (such as the Healing Our Waters coalition) led to the creation of a list of communities that have implemented policies. Examples of these policies included bag bans/fees, bans on the use of single-use plastics in government facilities, public outreach campaigns, and social marketing campaigns. To date, few examples of local, county, or state level policies aimed at reducing marine debris appear to exist in the Great Lakes. Information gathering from regional policy research was incorporated into "Plastic-Free Great Lakes: An Advocacy Toolkit to Make a Difference in Your Community." This toolkit features several examples of policies and best management practices.
2.1.2. Once the needs analysis is complete, examine existing platforms to meet those needs.	Coordinators: Alliance for the Great Lakes, Keep America Beautiful Partners: ACC, NERRs	Complete	Based on the results of outreach in 2017, it was determined that a toolkit for volunteer advocates would be the best way to encourage policies and best practices in more communities. The toolkit, "Plastic-Free Great Lakes: An Advocacy Toolkit to Make a Difference in Your Community" was released on Thursday, November 8, 2018 in conjunction with a public webinar.
2.1.3. Share the developed summary with policy makers in the Great Lakes region and make recommendations for policy improvements.	Coordinators: Alliance for the Great Lakes, Keep America Beautiful Partners: ACC, NERRs	Complete	The volunteer advocacy toolkit was released on Thursday November 8, 2018. This toolkit features brief examples of plastic pollution reduction policies and programs in the Great Lakes region, and tools for volunteers to use in their advocacy to local and state policy makers.
Objective 2.2: Share the developed land-based marine debris Action Plan with the policy and management communities upon its completion.			
2.2.1. Present the finalized Action Plan at relevant local, regional, and national conferences and/or meetings.	Coordinator: None – captured in annual reporting. Partners: Everyone	Complete	Throughout the last five years, the Action Plan was presented 39 times, reaching approximately 1,796 policy and management representatives.

Action Strategies	Coordinating & Partner Organizations	Status	Progress Description
2.2.2. Develop a contact list of policy and management representatives for distribution of the final Action Plan.	Coordinator: NOAA MDP Partners: State/ Provincial management programs (Coastal Zone Management Offices, Departments of Natural Resources), EPA, Great Lakes Sea Grant Network, NERRs	Complete	The NOAA Marine Debris Program, in partnership with state management programs (Coastal Zone Management Offices, Departments of Natural Resources or Environmental Quality), the EPA, the Great Lakes Sea Grant Network, and the National Estuarine Research Reserves developed a contact list of relevant policy and management representatives from around the region. These contacts received the Great Lakes Land-based Marine Debris Action Plan via email on August 5, 2014. Partners continue to follow-up with these individuals.
2.2.3. Develop a summarized Action Plan with brief talking points.	Coordinator: Great Lakes Sea Grant Network Partners: Michigan Department of Environmental Quality, City of Cleveland Office of Sustainability, Wisconsin Coastal Management, Ohio Coastal Management	In Progress	A fact sheet of a summarized Action Plan was previously drafted by Ohio Coastal Management and sent to partners for feedback. The fact sheet has been forwarded to Sarah Zack at IL-IN Sea Grant, who will now be seeing it through to completion. This project was delayed due to time constraints. Given that the best use of this tool would have been at the beginning of the Action Plan, IL-IN Sea Grant is proposing to instead develop a fact sheet and information on success stories from the Action Plan. This will be discussed at the next Great Lakes Sea Grant Network Meeting in 2019.
2.2.4. Create a general presentation or summary slide on the final Action Plan and share with Great Lakes marine debris community for use in outreach to the policy and management communities.	Coordinator: Great Lakes Sea Grant Network Partners: Michigan Department of Environmental Quality, City of Cleveland Office of Sustainability, Wisconsin Coastal Management, Ohio Coastal Management	In Progress	The presentation that was previously created by Ohio Coastal Management and sent to partners for feedback has been forwarded to Sarah Zack at IL-IN Sea Grant, who will now be seeing it through to completion. This project was delayed due to time constraints until Spring 2018. Given that the best use of this tool would have been at the beginning of the Action Plan, IL-IN Sea Grant is proposing to develop information on success stories from the Action Plan. This will be discussed at the next Great Lakes Sea Grant Network Meeting in 2019.
2.2.5. Work with states, municipalities, and other key constituencies throughout the Great Lakes region to fully understand their trash prevention goals, programs, and perceived needs and barriers to success, and develop collaborative regional projects to address those factors.	Coordinator: EPA	Not Started	No progress to report due to a lack of staffing support to implement the EPA Trash Free Waters Program in the Great Lakes.
Objective 2.3: Review and prioritize storm water control practices at five municipal districts in the Great Lakes by 2019.			
2.3.1. Engage storm water professionals and the Great Lakes Storm Water Management Institute on land-based marine debris issues in the region.	Coordinator: NERRs Partners: Great Lakes Sea Grant Network, Erie Soil & Water Conservation District, Cleveland Mayor's Office of Sustainability, Alliance for the Great Lakes, City of Cleveland Water Pollution Control	In Progress	The LS NERR has recently acquired a new Coastal Training Program Coordinator. OWC NERR is hoping to strengthen ties and marine debris collaboration, and recently met with LS NERR staff at the 2018 NERRS Annual Meeting, which was hosted by the LS NERR. While the staff capacity of the LS NERR has limited their availability to this topic, new research opportunities relating to oil residue attaching itself to microplastics in Lake Superior and educational endeavors, indicate they may be able to coordinate again soon. OWC NERR is continuing to collaborate with the Erie Conservation District on the Clean Water Contractor Program, which targets the construction industry and provides trainings on stormwater best management practices, and the threat of storm water-driven land-based marine debris. The 2019 Clean Water Contractor Sediment & Erosion Control Expo took place on February 28, 2019.
2.3.2. Identify existing storm water control practices related to land-based marine debris and identify gaps in programs and/ or policy.	Coordinator: NERRs Partners: Alliance for the Great Lakes, Keep America Beautiful, ACC	In Progress	LS NERR staff limitations have put on hold a potentially planned call with NERR partners to identify existing marine debris efforts, create a strategy, and allocate assignments to move forward on the progress of this action item. Keep America Beautiful, Alliance for the Great Lakes, and ACC agreed to help contribute to this action item via their assessment of practices that is an outcome of action item 2.1.1.

Goal 2. (Continued)

Action Strategies	Coordinating & Partner Organizations	Status	Progress Description
2.3.3. Implement tools and incentives to leverage action by various groups.	Coordinator: NERRs Partner: Keep America Beautiful	In Progress	OWC NERR is working with local stakeholders (e.g., community representatives) to install and track cigarette butt recycling receptacles and monofilament fishing line recycling receptacles. Technical assistance was provided to the Huron Yacht Club and City of Sandusky. OWC NERR also hosted two workshops focused on plastic reduction strategies in July 2018. The workshops were hosted with support from Talkin' Trash, Ohio Sea Grant College Program, the Ohio Clean Marinas and Clean Boaters Programs, the NOAA Marine Debris Program, Lake Erie Nature & Science Center, Bowling Green State University Firelands, and the Cleveland Office of Sustainability. The workshops involved outlining the issue of marine debris in the Great Lakes, highlighted several regional and local awareness campaigns, directing participants on monofilament fishing line receptacle construction and painting, and sharing the NOAA MDP Monitoring and Assessent Project protocol. OWC NERR continued to circulate designs from Recycled Runway 2018, which involved multiple action groups' representation to confront the issue of land-based marine debris, and raise funds for conservation projects along Ohio's Lake Erie coast. Venues include the Milan Historical Museum, Ohio State Fair, Stone Laboratory on Gibraltar Island, and the Greater Cleveland Aquarium.

GOAL 3. PREVENTION AND EDUCATION

Land-based marine debris is prevented and reduced through an educated and involved community.

Action Status

- 15 Complete
- 0 In Progress
- 0 Not Started.

Goal Accomplishments

All 15 of the actions outlined in Goal 3 were completed. Specific accomplishment highlights are below.

Education Needs Assessment (Objective 3.1)

In 2015, the Alliance for the Great Lakes interviewed Action Plan partners to identify a target audience for education efforts in the region. Based on feedback, it was determined that for this particular objective, the focus was on formal education, or education that takes place in a classroom setting. The Alliance for the Great Lakes compiled formal educational materials from partners around the region and attempted to review them for potential

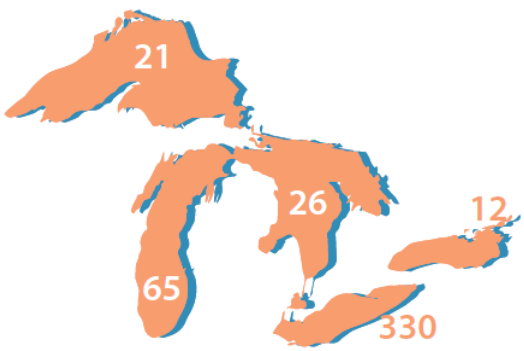
education gaps. However, they found it challenging to gather partners to analyze materials. Limited analysis has found many ocean-focused materials can simply be adapted for the Great Lakes region, but partners face time and document accessibility challenges, and difficulties adapting curricula that they do not own. Therefore, it was recommended that the adaptation of materials be the responsibility of owners.

Awareness Campaign in Cleveland, OH (Objective 3.2)

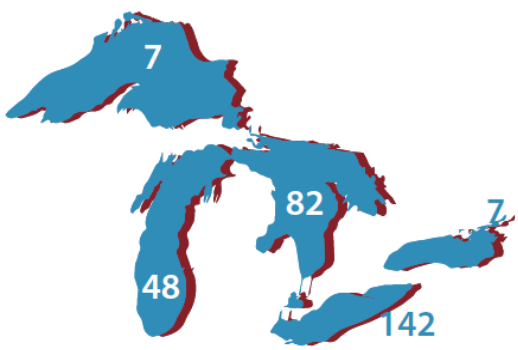
The City of Cleveland Mayor's Office of Sustainability, with the support of a NOAA Marine Debris Program Prevention Grant, identified barriers to the reduction of plastic marine debris and developed a community-based social marketing campaign to overcome these barriers. The goal of this project was to achieve lasting behavior change in Cleveland residents that would result in the reduction of littering, reduce the use of plastics, and increase proper disposal of plastics, which would ultimately reduce marine debris.

The pilot social marketing campaign, Don't Break the Lake, utilized various communication tools and methods, including signage at water refill stations and in grocery stores, an insert in mailed water bills, outreach through social media, and the purchase and giveaways of reusable bags and bottles. Preliminary grocery store results indicate that the number of reusable bags used in the store increased as a result of the campaign.

Number of informal education activities on land-based marine debris per lake, with the public:



Number of informal education activities on land-based marine debris per lake, with targeted audiences.



Outreach Highlights

Gave 386 Presentations to 149,764 People

Participated in 325 Educational Events with 30,298 Students

Created 61 Displays

Goal 3. Land-based marine debris is prevented and reduced through an educated and involved community

Objective 3.1: By the beginning of 2016, conduct a needs assessment for future education efforts on land-based marine debris in the Great Lakes.			
Action Strategies	Coordinating & Partner Organizations	Status	Progress Description
3.1.1. Identify target audiences and potential new partners for future education efforts.	Coordinator: Alliance for the Great Lakes	Complete	In March and April of 2015, the Alliance for the Great Lakes interviewed all partners listed in Goal 3, with the exception of those that did not respond to outreach. Through that initial outreach, the Alliance determined the target audiences that partners want to reach varied between formal classroom education and informal public education. To narrow the focus of this objective it was changed to formal education, while informal public education became the focus of Objective 3.2.
3.1.2. Inventory educational resources that are currently available and identify gaps in content and style/type.	Coordinator: Alliance for the Great Lakes Partners: Great Lakes Sea Grant Network, NOAA MDP, NERRs, Coastal Training Program, City of Cleveland Office of Sustainability, Inland Seas Education Association, Shedd Aquarium	Complete	Educational materials have been complied and reviewed by partners and the initial steps for identifying gaps have been taken.

Action Strategies	Coordinating & Partner Organizations	Status	Progress Description
3.1.3. Create new or adapt existing educational materials to fill gaps identified in action 3.1.2.	Coordinator: Alliance for the Great Lakes Partners: Great Lakes Sea Grant Network, NOAA MDP, NERRs, Wisconsin Coastal Management Program	Complete	See action 3.1.2. The action team has found many ocean-focused materials that can simply be adapted for the Great Lakes region, but partners face both time and document accessibility challenges, and difficulties adapting curricula that they do not own. Adaptation of materials is now the responsibility of owners. Five organizations, including Ocean Wise, Ocean School, Students on Ice, Sea Smart, and WE Charity, have teamed up with support from ECCC to provide teachers with new educational tools focused on plastic pollution and how it affects our environment. The Ocean Plastic Education kit featured materials and classroom resources to help students learn about how to reduce plastic use and waste. It related the plastic material to current curriculum, so that it can easily be incorporated into teacher's lesson plans. The materials supported this action by providing custom curriculum to fill educational gaps related to marine plastic waste for Ontario, Canada.
Objective 3.2: By the end of 2016, launch at least one awareness campaign for Great Lakes communities regarding land-based marine debris.			
3.2.1. Identify and review existing awareness campaigns that could be leveraged.	Coordinator: Keep America Beautiful Partners: Alliance for the Great Lakes, Inland Seas Education Association, Hubbub (UK-based litter prevention organization)	Complete	Keep America Beautiful completed a local pilot in Erie, PA to test the effectiveness of the Hubbub Ballot Bin for reducing cigarette litter. The Erie affiliate placed two bins in their community, one by a library and the second by a park. Overall results were an 88.5% reduction in cigarette litter over the months of the pilot, which compared preliminary results to final litter scans.
3.2.2. Engage a marketing expert to assist in the development of a campaign.	Coordinator: City of Cleveland Partners: Alliance for the Great Lakes, ACC, Great Lakes Sea Grant Network, Keep America Beautiful, Hi-Cone, Inland Seas Education Association	Complete	The pilot campaign "Don't Break the Lake" is continuing beyond the original project. This includes a reusable bag campaign with Zagara's Marketplace in Cleveland Heights, OH, encouraging reusable water bottle usage through educational signage and social media, and continued research into plastic cigar tip disposal behaviors.
Objective 3.3: By 2019, develop a social marketing plan, including 10 communication products to address land-based marine debris in the Great Lakes.			
3.3.1. Identify and review effectiveness and impact of existing social marketing programs on littering behavior.	Coordinators: Keep America Beautiful, Alliance for the Great Lakes Partners: Great Lakes Sea Grant Network, Clean Water Action, NERRs	Complete	A list of social marketing campaigns was compiled for review. The partners will continue to add to and refine this list, seeking additional input from relevant contacts, affiliates and others, particularly those connected with water.
3.3.2. Create a brand with localized and targeted messages based on social science research on barriers and benefits. Social science research may include public-opinion focus groups and value identification.	Coordinators: City of Cleveland, Alliance for the Great Lakes Partners: OSG	Complete	The City of Cleveland contracted with a local marketing firm, thunder::tech, to develop a social marketing campaign for three plastic marine debris items, including plastic grocery bags, plastic disposable water bottles, and plastic cigar tips. Thunder::tech subcontracted with Ohio Sea Grant (OSG) to conduct an online survey focused on identifying the barriers to desired behaviors for these three items. The survey was distributed via multiple channels. Within the five county regions of concern, 982 respondents finished the survey. A focus group was convened to discuss disposal methods of plastic cigar tips. OSG presented the results of their research to thunder: tech in March 2017. In general, the results showed that the greatest barrier was that respondents forget to bring their reusable bags and bottles, while the barriers to the proper disposal of cigar tips are both material and social. The results were shared with the local marketing firm for the development of the campaign and have been published on the Great Lakes Marine Debris Collaborative Portal.
Objective 3.4: Conduct five informal education activities on land-based marine debris per year, per lake, to the public.			
3.4.1. Engage the Beach Ambassador Program as well as museums and/or aquariums on educational activities related to land-based marine debris in the Great Lakes.	Coordinator: Alliance for the Great Lakes Partner: OWC NERR	Complete	The Alliance for the Great Lakes launched a new pilot initiative in Chicago called "My Beach Your Beach," through which multiple social and cleanup activities were hosted at Oak Street Beach throughout the summer of 2016. These events included placing small flags on the beach during cleanups as items were removed from the beach, a voting box in which individuals could vote with cigarette butts, and various cleanup events. Over the next several months, the Alliance will be assessing the impacts of this work. In addition, the Alliance is working with the State of Michigan on a Clean Beaches Initiative, bringing together agencies at the state level to share information on marine debris in Michigan coastal areas, and looking for opportunities to collaborate to reduce debris.

Goal 3. (Continued)

Objective 3.4: Conduct five informal education activities on land-based marine debris per year, per lake, to the public.			
Action Strategies	Coordinating & Partner Organizations	Status	Progress Description
3.4.2. Organize an annual Boat Float in Lake Erie.	Coordinator: City of Cleveland Office of Sustainability Partners: Cleveland Metroparks, Cleveland Museum of Natural History, Alliance for the Great Lakes, Drink Local Drink Tap, RES Polyflow	Complete	The 10th and final Annual Great Lake Erie Boat Float was held at Edgewater Park Beach on Saturday, September 8, 2018. Eight boats participated in the event, and despite the less than ideal weather conditions, all boats got in the water. Photos from the event may be found at www.ldempsey.com. Dr. Marcus Eriksen from The 5 Gyres Institute was the guest judge for the event and gave an inspirational and positive speech. Four previous boat floats were organized from 2014-2017 as a part of this Action Plan.
3.4.3. Outreach to the general public, including presentations to coastal communities during field research expeditions.	Coordinator: None – captured in annual reporting. Partners: All educators	Complete	Over the past five years, 386 events and presentations were conducted, reaching 149,764 people. Additionally, there was a Canada-wide public consultation on plastic waste and plastic pollution and a #BePlasticWise campaign launched in Canada.
3.4.4. Create land-based marine debris educational displays at public venues (i.e., state parks, museums, marinas, universities, etc.). The displays may be mobile in nature to be easily shared across the region.	Coordinator: None – captured in annual reporting. Partners: Everyone	Complete	Sixty-one displays were developed within the last five years.
3.4.5. Encourage events (e.g., conferences, etc.) to be "zero-waste" or "reduced-waste."	Coordinators: Keep America Beautiful, City of Cleveland Office of Sustainability Partners: SC2019 ZeroWasteNEO Working Group, Graduates of the Cuyahoga County Solid Waste District's Master Recyclers class	Complete	Requests for assistance with zero or reduced-waste events have increased over the past few years. The Sustainable Cleveland ZeroWasteNEO (Northeast Ohio) Working Group provides guidance to event planners by offering workshops, an Event Planning Guide, and additional resources. The Cleveland Office of Sustainability also has signs available for loan. With many events planned in June 2019 for the City-wide celebration of the 50th anniversary of the last burning of the Cuyahoga River, the Working Group held several workshops in April and May 2019 and updated the Guide. In addition, for some events, Cathi Lehn serves as the Waste and Recycling Coordinator and attends all planning meetings and was on site for the day of the event. Over the past five years, the City of Cleveland Office of Sustainability has aided 28 events to be zero or reduced waste.
Objective 3.5: Conduct five formal education activities on land-based marine debris per year, per lake, to targeted audiences.			
3.5.1. Host a regional webinar on land-based marine debris for education/outreach professionals.	Coordinators: NOAA MDP, Alliance for the Great Lakes Partner: Great Lakes Sea Grant Network	Complete	The NOAA MDP, in partnership with OSG, hosted a Great Lakes Education Webinar on April 3, 2019. Thirty-one educators learned about marine debris, received a list of resources, and viewed an example lesson. A recording of the webinar is available Great Lakes Marine Debris Collaborative Portal.
3.5.2. Host a teacher workshop or class on land-based marine debris.	Coordinators: Great Lakes Sea Grant Network, NOAA MDP Partners: Alliance for the Great Lakes, OWC NERR, Regional Science Consortium at the Tom Ridge Environmental Center	Complete	In 2015, through a partnership with the OSU Stone Lab on Lake Erie, the NOAA MDP and OSG held a free three-day workshop on marine debris for teachers and informal educators on June 8-10, 2015. Thirteen educators experienced a mix of classroom curriculum and field activities, including instruction on the use of manta net trawls and sample analysis with Dr. Rios Mendoza, as well as a beach cleanup with the Alliance for the Great Lakes. Pennsylvania Sea Grant and the Center for Great Lakes Literacy also held a teacher training for ten teachers at Tom Ridge Environmental Center in Erie, PA. Throughout 2016, an additional 33 teachers participated in workshops in PA and OH. In 2017, 67 teachers were engaged in marine debris workshops in PA and NY. On October 20, 2018, twenty-seven teachers learned about the impacts of plastic pollution on water quality, wildlife, and potentially humans, as part of a Center for Great Lakes Literacy Resources and Curriculum workshop in Erie, PA. New York Sea Grant also held two educator workshops in the summer of 2018, reaching 38 teachers.
3.5.3. Conduct land-based marine debris educational activities with students of all ages in the Great Lakes.	Coordinator: None – captured in annual reporting. Partners: Everyone	Complete	During the past five years, 30,298 students of various ages were engaged through 325 educational events.

GOAL 4. REMOVAL AND TRACKING

The impacts of land-based marine debris are reduced through removal and tracking efforts.

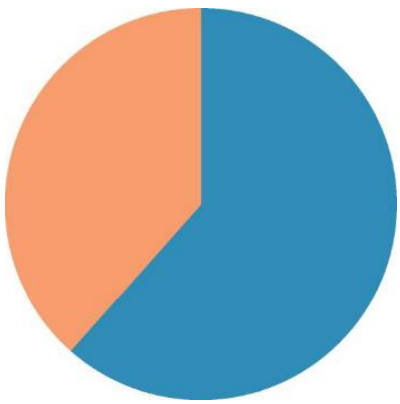
Action Status

- 8 Complete
- 5 In Progress
- 0 Not Started.

Goal Accomplishments

Bi-Nationally Remove Marine Debris (Objective 4.1)

The Alliance for the Great Lakes, the Great Canadian Shoreline Cleanup, the Ocean Conservancy, Partners for Clean Streams, and other volunteer cleanup organizations have annually reported the amount of debris removed from Great Lakes shorelines. Over the past five years approximately 306,665 pounds (153.3 tons) of land-based marine debris was removed from the environment by volunteers. This included collaborating with industry and manufacturers, such as Dow Chemical and Amcor, on volunteer employee cleanups. Additional cleanup opportunities are found on the Great Lakes Marine Debris Collaborative Portal.



Best Management Practices for Removing and Preventing Debris (Objective 4.2)

The Ohio Clean Marinas Program adapted marine debris removal best management practices (BMPs) for the marina and boating community and incorporated these BMPs into the Program's outreach materials. In spring 2018, a revised Clean Marina Program Tiered Checklist was completed, with the following required BMPs specifically addressing land-based marine debris removal:

- Cigarette disposal containers are available for patrons and staff.
- Collection bins for solid recyclables are available throughout the marina.

- Trash cans are emptied and litter pick-ups are conducted within the marina and along the shoreline daily.
- Derelict vessels are removed from the property.
- Shrink wrap is recycled or dry rack storage is available for winterization of boats.
- All storm drains are labelled to notify patrons of outfall points (i.e., "No Dumping, Drains to Lake").
- Boaters are required to sign an environmental commitment pledge.

In summer 2018, staff began certifying marinas using this revised checklist, and collaborated with Cuyahoga and Summit County Soil and Water Conservation Districts to provide Clean Boater BMPs as part of their outreach materials and social media posts.

Additionally, Old Woman Creek National Estuarine Research Reserve (OWC NERR) has begun to construct, paint, disseminate, and track monofilament fishing line recycling receptacles along Ohio's Lake Erie coastline. These recycling bins are placed at prominent fishing piers, marinas, beaches, and parks. Line is collected by volunteers and shipped to Berkley, Inc. to be recycled into other fishing products. OWC NERR has hosted workshops to educate adopters on the benefits of removing gear from the environment, and how to care for a bin.

Goal 4. The impacts of land-based marine debris are reduced through removal and tracking efforts

Objective 4.1: By 2019, bi-nationally record and report count and weight (in tons) of land-based marine debris removed from Great Lakes shorelines and watersheds by volunteer programs.			
Action Strategies	Coordinating & Partner Organizations	Status	Progress Description
4.1.1. Investigate Canadian volunteer program reporting mechanisms and see how they align with U.S. efforts.	Coordinator: Alliance for the Great Lakes Partners: Surfrider Foundation, Ocean Conservancy	Complete	GCSC has a similar system to the Alliance for the Great Lakes for tracking volunteer cleanups through an online database tool. The GCSC is now also encouraging year-round cleanups in addition to the traditional ICC event. They use the same data collection card as the Alliance and the other groups in the Great Lakes hosting the ICC event.
4.1.2. Identify a centralized location that will direct people to sources of volunteer coastal cleanup data.	Coordinator: Alliance for the Great Lakes	Complete	The Great Lakes Marine Debris Collaborative Portal provides links to cleanup opportunities, and sources of cleanup data. Partners, like the Alliance for the Great Lakes, continue to host their Adopt-a-Beach data on their own websites and databases, which are linked to on the portal.
Objective 4.2: By 2019, develop and publish recommendations for best practices for removing land-based marine debris.			
4.2.1. Investigate existing removal methods for land-based marine debris and analyze for similarities, as well as benefits and challenges.	Coordinator: NERRs Partner: Great Lakes Sea Grant Network	Complete	OWC NERR has worked with partners on plastics reduction strategies workshops in the northern Ohio area. These involve the construction, painting & dissemination of monofilament fishing line recycling receptacles along Ohio's Lake Erie coast at prominent fishing piers, marinas, beaches and parks, and include a literacy component. OWC NERR has also installed one cigarette butt recycling receptacle at their visitor's center, and plans to install another at their beach/boat house, as well as a mobile receptacle for off-site trainings. The OWC NERR Coastal Training Program Coordinator would still like to survey other coastal entities' stormwater control measures/policies and methods of removal regarding land-based marine debris. OWC and LS NERRs have also discussed the potential for surveying other NERRs outside of the Great Lakes to gain insight on different marine debris removal tactics (e.g., removal of derelict crab pots, disposal/recycling of monofilament fishing line). OWC NERR would like to disseminate a survey to the other NERRS despite LS NERR's recent inactivity in the plan.

Objective 4.2: By 2019, develop and publish recommendations for best practices for removing land-based marine debris.			
Action Strategies	Coordinating & Partner Organizations	Status	Progress Description
4.2.2. Develop or adapt BMPs to target specific environments or debris types.	Coordinator: Great Lakes Sea Grant Network	In Progress	OSG is working with the Toledo Tennis Club and Mentor Heisley Racquet Club to obtain and distribute personal monofilament recycling containers to anglers. OSG received 400 bins from the Toledo Tennis Club through Partners for Clean Streams, and three bins from Heisley Racquet Club. They are in the process of writing a grant to purchase the other items needed to complete the personal monofilament bins. OSG has seen great success having students or children write letters to the angler who will receive the personal monofilament bins, thanking them for removing their waste, protecting wildlife, humans, and the environment. Additionally, many youth volunteers from Cleveland Metroparks have helped to construct the personal monofilament bins, and in the process, have learned more about marine debris while receiving volunteer hours for school. OSG will also work with senior citizen groups in Lake County to help put together the personal monofilament containers. Cleveland Metroparks has requested a building session to incorporate into one of their public events, as well.
4.2.3. Incorporate land-based marine debris BMPs into existing guides (e.g., Clean Marina).	Coordinator: Great Lakes Sea Grant Network Partner: Wisconsin Coastal Management Program	Complete	The Ohio Clean Marinas Program Tiered Checklist has been completed and is being used to certify marinas for implementing environmental BMPs across Ohio. The checklist includes seven items related to marine debris: Cigarette disposal containers are available for patrons and staff. Collection bins for solid recyclables are available throughout the marina. Trash cans are emptied and litter pick-ups are conducted within the marina and along the shoreline daily. Derelict vessels are removed from the property. Shrink wrap is recycled or dry rack storage is available for winterization of boats. All storm drains are labelled to notify patrons of outfall points (i.e., "No Dumping, Drains to Lake"). Boaters are required to sign an environmental commitment pledge. The Ohio Clean Marinas Program also partnered with the Ohio Department of Natural Resources and Cleveland Metroparks to create a video on how to prepare boat shrink wrap for recycling. The link to the video and more information about the recycling program are available on a draft fact sheet, to be formally published in 2020.
4.2.4. Share BMP recommendations with stakeholders.	Coordinator: Great Lakes Sea Grant Network	In Progress	At outreach events, OSG and the Ohio Clean Marinas/Clean Boater Programs educate stakeholders on marine debris and share current research to create an informed public. Over the past five years, numerous presentations were given to resource and watershed management groups.
Objective 4.3: Within five years, target removal efforts towards land-based marine debris items that are expected to have the greatest impact or which are most abundant in number.			
4.3.1. Analyze existing cleanup data to identify land-based marine debris types, quantities by type, and impact. Reports may be separated by geography due to variations in data.	Coordinator: Alliance for the Great Lakes Partner: Ocean Conservancy, University of Michigan, Loyola University	In Progress	In 2017, the Alliance for the Great Lakes shared Adopt-a-Beach data with multiple research partners across the Great Lakes basin. In January of 2017, Adopt-a-Beach data was featured in an article in Science of the Total Environment Vol. 577, entitled "Citizen science datasets reveal drivers of spatial and temporal variation for anthropogenic litter on Great Lakes beaches." Dr. Timothy Hoellein from Loyola University was the lead author on the paper with several Alliance for the Great Lakes staff members. Additionally, the Alliance for the Great Lakes has shared Adopt-a-Beach data with other partners working on analysis, such as the Shedd Aquarium.
4.3.2. Partner with industry, manufacturers, and packaging companies to participate in and support removal efforts.	Coordinator: Ocean Conservancy	Complete	Each year during the ICC, the Ocean Conservancy usually hosts at least one cleanup in the region, often in partnership with Alliance for the Great Lakes, which engages companies and their employees at cleanups. For example, in September 2018, an Ocean Conservancy team hosted a cleanup for Dow Chemical employees in Saginaw and Midland, MI, and removed 7,074 lbs of land-based debris. As another example, Amcor, a member of the Trash Free Seas Alliance, leads their own cleanup events and completed one on Belle Isle, MI during the 2018 ICC.

Goal 4. (Continued)

Objective 4.4: Create a natural disaster preparedness plan (by state or lake) for land-based marine debris within five years.			
Action Strategies	Coordinating & Partner Organizations	Status	Progress Description
4.4.1. Review lessons-learned from Hurricane Sandy response.	Coordinator: NOAA MDP Partner: Alliance for the Great Lakes, Ohio Department of Natural Resources/Ohio Emergency Management Association, ECCC	Complete	In the fall of 2012, marinas in the Great Lakes found themselves dealing with towering waves of up to 20 feet, and winds of over 60 miles per hour as a result of Hurricane Sandy. After the event, OSG pursued and obtained a NOAA Coastal Storms Grant in partnership with the OSU School of Environment and Natural Resources, Pennsylvania Sea Grant, and Wisconsin Sea Grant to understand how marina owners in the Great Lakes prepare for these natural hazards and to develop tools to help marinas now, and in the future. The Preparation, Adaptation and Response Tool (PART), is complete.
4.4.2. Review preparedness plans from other regions and industry partners.	Coordinator: NOAA MDP Partner: ECCC	Complete	The following resources were reviewed: Great Lakes Clean Marina Network Best Management Practices Regarding Increasing Resilience, Boat U.S. Hurricane Preparedness for Boaters, NOAA MDP Storm Preparedness Fact Sheet, and other NOAA MDP response guides from other states.
4.4.3. Engage the U.S. Coast Guard, FEMA, and the Canadian equivalents on land-based marine debris issues in the region, as well as the creation of the preparedness plans.	Coordinator: NOAA MDP Partner: ECCC	In Progress	NOAA MDP staff are participating in trainings and networking with the U.S. Coast Guard and FEMA.
Objective 4.5: By 2019, remove 200 tons of land-based marine debris from Great Lakes environments.			
4.5.1. Understand the current baseline of land-based marine debris removed by volunteers in the region. Report on these values.	Coordinator: Alliance for the Great Lakes Partner: Ocean Conservancy, GCSC	In Progress	From 2014 to 2018, volunteers in the U.S. and Canada have removed approximately 306,665 pounds of marine debris from the Great Lakes watershed.
4.5.2. Identify funding opportunities and potential land-based marine debris removal projects throughout the region.	Coordinator: None – captured in annual reporting Partner: Everyone	Complete	Funding opportunities are now available on the Great Lakes Marine Debris Collaborative Portal.

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Chapter 113

STATEMENT OF ANNE KINSINGER, ASSOCIATE DIRECTOR FOR ECOSYSTEMS*

Chair McCollum, Ranking Member Joyce, and members of the Subcommittee, thank you for the opportunity to testify today regarding marine debris work conducted by the United States Geological Survey (USGS). The USGS conducts research and monitoring related to marine debris. Although we currently do not have an extensive program in this area, marine debris is becoming an increasingly important issue in the management of the Department of the Interior (DOI) trust lands and species, and we anticipate increased programmatic focus in future years.

My testimony today will focus on large debris and the breakdown products of that debris.

ROLE OF THE U.S. GEOLOGICAL SURVEY

The USGS is the science agency of the DOI with a mission to provide unbiased scientific information to Federal and non-Federal resource managers and planners, emergency response officials, and the public. Debris, and the breakdown products of debris, are receiving increased attention due to possible impacts on fish and wildlife, particularly in the marine environment. The DOI has land management and administrative responsibilities for numerous coastal and estuarine units of the national park system, national wildlife refuges, public lands, Indian lands, U.S. territorial islands, and other insular areas that have documented or potential impact from marine debris. In addition, the DOI has management responsibilities for migratory birds, interjurisdictional fish, federally listed species, and marine mammals that may be impacted by marine debris outside DOI managed lands.

The Department of Commerce, through the National Oceanic and Atmospheric Administration's Marine Debris Program (MDP), leads national efforts to research, prevent, and reduce the impacts of marine debris. The MDP is authorized by Congress as the Federal lead to work on marine debris through the Marine Debris Act, and last October was reauthorized for an additional five years.

* This is an edited, reformatted and augmented version of U.S. Geological Survey, Department of The Interior Before The Subcommittee on Interior, Environment, and Related Agencies House Committee on Appropriations on Marine Debris Publication, Dated September 19, 2019.

Over the past 10 years, the USGS has published marine debris research in the following three areas:

Coastal Surveys: This research comprised the design, conducting, or analysis of shoreline and benthic surveys for marine debris in Hawaii, Alaska, Caribbean, Gulf of Mexico, Mediterranean, and Pacific Islands, including Midway Atoll. These studies were designed to establish baseline conditions on specific lands important to the DOI; understand trends in marine debris accumulation over larger geographic areas; or characterize composition and transport of marine debris with potential to impact lands or species of concern. Many of these surveys involve the use of volunteers through citizen science programs.

Species Impacts: These studies focused on the incidence of ingested marine debris by sea turtles and seabirds, including black-footed albatross chicks. The purpose of these studies was to quantify the occurrence of marine debris in the digestive tract of species that utilize marine food sources in order to begin understanding if marine debris ingestion impacts survival of individuals or persistence of populations among species of concern.

Contaminants: This analysis examined the incidence of contaminants in ingested marine plastics, including those sorbed onto the plastics and those leached from the plastics. Endocrine disrupting compounds are one example. The purpose of these studies was to begin understanding if marine debris, particularly fragmented plastics, is a source of contaminants such as endocrine disruptors that can harm species directly or through decreased reproduction when ingested.

Although USGS and other science organizations have conducted research in these areas and other areas of marine debris, many unanswered questions remain related to the potential for marine debris to pose harm to lands or species managed by the DOI. This list is partially based on expert opinion of leading researchers from around the world that was published in 2014,[1] as well as a growing body of recent journal review articles identifying key knowledge gaps.

1. What are the impacts of marine debris on important coastal and marine habitats?
2. What are the impacts of marine debris on critical food webs?
3. Which species are most susceptible to marine debris impacts at the individual or population level?
4. What are the main sources of marine debris and what factors drive transport and deposition?
5. Does marine debris carry or concentrate contaminants of concern to wildlife when ingested?
6. What are standard approaches for the quantification of marine debris in coastal habitats?
7. How does marine debris contribute to the transport of invasive species and wildlife disease?
8. Are impacts of debris the same in freshwater systems and the Great Lakes as they are in marine environments?

[1] Vegter, et al. 2014. Global research priorities to mitigate plastic pollution impacts on marine wildlife. Endangered Species Research 25: 225-247.

USGS CAPABILITIES

The USGS maintains extensive capability to conduct physical and biological research and monitoring in support of natural resource management by DOI bureaus and other Federal, State, and tribal partners. Capabilities available to help resolve questions related to marine debris include population assessments to determine changes in species numbers over time, trophic analysis for determining disruptions in food webs, physical assessments and remote sensing capabilities to determine habitat condition and changes due to environmental stressors, and contaminants research for determining the source, transport, fate, exposure pathways, and impact of compounds on species and their habitats. In addition, the USGS maintains specialized facilities in fish and wildlife disease diagnostics to determine the cause of animal fatalities and advanced genetics and genomics capability to detect and track new species and wildlife diseases using environmental DNA (eDNA). The USGS is a national leader in design and analysis of scientifically defensible surveys, including effective use of citizen science to engage the public in gathering scientific data. Finally, the USGS has invested significant resources in developing invasive species detection, tracking, and control technologies that may be important in understanding and managing secondary effects of marine transport in global ecosystems.

Thank you for this opportunity to testify today. I would be happy to answer any questions you may have.

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Chapter 114

STATEMENT OF STEPHEN GUERTIN, DEPUTY DIRECTOR U.S. FISH AND WILDLIFE SERVICE, DEPARTMENT OF THE INTERIOR*

Good morning Chair McCollum, Ranking Member Joyce, and members of the Subcommittee. Thank you for the opportunity to discuss marine debris and its impact on wildlife. My testimony will focus on the U.S. Fish and Wildlife Service's (Service) role in addressing the threat of marine debris to our ocean and coastal areas.

MARINE DEBRIS OVERVIEW

Marine debris is one of the most pervasive global threats to the health of the world's coastal areas, oceans, and waterways. It is an issue of local, regional, national, and international concern. Marine debris can injure or kill marine and coastal wildlife; damage and degrade habitats; interfere with navigational safety; cause economic loss to fishing and maritime industries; degrade the quality of life in coastal communities; and threaten human health and safety. The Service works collaboratively with Federal and non-Federal partners to address marine debris and its impacts on wildlife.

Marine debris is defined as "any persistent solid material that is manufactured or processed and directly or indirectly, intentionally or unintentionally, disposed of or abandoned into the marine environment or Great Lakes" (33 U.S.C. 1951 et seq., as amended by in 2012 by Title VI of Public Law 112-213 and 2018 by Public Law 115-265). Anything man-made – such as fishing gear, plastic bags, beverage bottles, balloons, food wrappers, and even vessels – can become marine debris through dumping, improper waste management, litter that is blown or washed out to sea through storm drains, and extreme natural events which can transport both small and large items into the ocean. Major marine debris events caused by natural disasters, such as the 2017 hurricanes Harvey, Irma and Maria and 2018

* This is an edited, reformatted and augmented version of Statement Before the Subcommittee on Interior, Environment, and Related Agencies House Committee on Appropriations on Marine Debris , dated September 19, 2019.

typhoon Yutu, continue to bring national and international attention to the marine debris issue.

It is believed that at least 8 million tons of plastic end up in our oceans every year, and make up 80 percent of all marine debris from surface waters to deep-sea sediments. The National Oceanic and Atmospheric Administration (NOAA) estimates there are somewhere between 20 million and 1.8 billion pieces of plastic along the coastline of the United States, with the number likely at the upper end of this range. Marine debris can be found even in the deepest parts of our ocean.

For example, a plastic bag was documented in 2016 by the Okeanos Explorer submersible at Enigma Seamount in the Arc of Fire National Wildlife Refuge, part of the Marianas Trench Marine National Monument.

There are three main types of marine debris that impact wildlife: plastics, derelict fishing gear, and abandoned and derelict vessels. Each is discussed briefly below.

Plastics and Microplastics

Plastics are one of the most extensive types of marine debris. They are commonly used in many items, and as society has developed new uses for them the variety and quantity of plastic items found in the marine environment has increased dramatically. Plastics are now known to break down into smaller components, called microplastics. Microplastics and their associated toxic chemical components contribute to human and wildlife health risks as the toxic microplastics are ingested and move through the marine food web.

The impact of these plastics isn't limited to our oceans. Research in 2014 discovered that plastic particle counts reached one million plastic particle parts per square mile in Lake Erie, with higher counts found in Lake Ontario;[1] The Great Lakes have among the highest densities of microplastics recorded. Microfibers, a type of microplastic, are also pervasive in the marine environment, and while our knowledge of the impacts of microfibers is limited, they make up the majority of microplastics, and can even be found in the seafood we consume.

Plastics such as bottle caps, balloons, and lighters are mistaken for food or the debris item may have been attached and ingested accidentally with other food by wildlife such as sea turtles, seabirds, and marine mammals. Plastic ingestion leads to loss of nutrition, internal injury, intestinal blockage, starvation, and death in wildlife. Seabirds are especially vulnerable to plastic pollution; a recent study found plastic in 90 percent of seabirds.[2] In addition to plastic ingestion, other debris such as packing bands, balloon strings, rubber bands, six-pack rings, and mesh bags can lead to entanglement. Entanglement affects many different types of animals: 44 sea bird species, nine cetacean species, 11 pinniped species, 31 invertebrate species/taxa, and six sea turtle species reported entangled in marine debris in the United States.[3] Recovery Plans for Endangered Species Act listed loggerhead, leatherback,

[1] Mason, S.A., Eriksen, M., and Edwards, W.J. (2014). "Great Lakes Plastic Pollution Survey," 57th Annual Conference on Great Lakes Research (IAGLR 2014), Hamilton, Ontario.

[2] Wilcox, C., E. V. Sebille, and B. D. Hardesty. 2015. Plastic in seabirds is pervasive and increasing. Proceedings of the National Academy of Sciences 201502108; DOI: 10.1073/pnas.1502108112.

[3] National Oceanic and Atmospheric Administration Marine Debris Program. 2014 Report on the Entanglement of Marine Species in Marine Debris with an Emphasis on Species in the United States. Silver Spring, MD. 28 pp.

green, and Kemp's Ridley sea turtles all list marine debris as one of the highest priority threats facing these protected species.

Derelict Fishing Gear

A second highly visible and impactful form of marine debris is derelict fishing gear (DFG). DFG has numerous impacts on the environment, including: damaging marine habitats, entangling marine species including seabirds and marine mammals, creating hazards to navigation, and ghost fishing of commercially important species resulting in lost catch opportunities and economic losses for fishermen. One of the most notable types of impacts from this type of marine debris is wildlife entanglement. Derelict nets, ropes, line, or other fishing gear can wrap around marine life. Entanglement has led to injury, illness, suffocation, starvation, and death.

Sea turtles are at great risk for entanglement in marine debris, and this has caused injuries and in many cases death for a variety of sea turtle species. One study found that between 1997–2009, over 1,000 sea turtles were found stranded in Florida due to entanglement in fishing gear.[4] Some of these entanglements resulted from netting and monofilament line that accumulated on both artificial and natural reefs. These areas are often heavily fished, resulting in snagging of hooks and discarding of lines. Turtles foraging and/or resting in these areas can become entangled and drown.

Bird species become entangled when swimming in bodies of water where line and netting is carelessly discarded, and sometimes use fishing line and netting fragments as nesting material, which can lead to entanglement of both the parents and chicks. Easy solutions for responsible fishers include proper disposal of monofilament line and awareness of safe procedures for de- hooking and disentangling entrapped birds.

Abandoned and Derelict Vessels

Abandoned and derelict vessels (ADVs) are a third highly visible type of marine debris with thousands littering our nation's coastal waters. ADVs threaten oceans, coasts, and waterways by obstructing navigational channels, causing harm to the environment, and diminishing commercial and recreational activities. ADVs may pose an immediate or future threat to wildlife and wildlife habitat from the release of hazardous substances to surrounding areas. ADVs may occur in large numbers, following a natural disaster such as a hurricane, or as a single vessel.

Following the 2017 hurricanes, the Service and its partners removed damaged vessels from Florida, Puerto Rico, U.S. Virgin Islands National Wildlife Refuges and adjacent communities.

ADVs occurring in remote locations are difficult to reach for cleanup and assessment of the impacts. Removal of such ADVs is very expensive and technical. As an example, in 1993

[4] Adimey, N.A, C.A. Hudak, J.R. Powell, K. Bassos-Hull, A. Foley, N.A. Farmer, L. White, and K. Minch. Fishery gear interactions from stranded bottlenose dolphins, Florida manatees and sea turtles in Florida, U.S.A. Marine Pollution Bulletin 81: 103-115.

Jin Shiang Fa wrecked at Rose Atoll National Wildlife Refuge in American Samoa. Although the sum of the wreck was removed initially by the responsible party, over 215 tons of debris were left behind on the perimeter of the reef and lagoon when funds ran out. Iron debris from the wreck caused an overgrowth of cyanobacteria, which the Service and partners continue to monitor. The Service has also removed nearly one million pounds of shipwrecks at Palmyra Atoll and Kingman Reef National Wildlife Refuges, which fueled outbreaks of algae and invasive corallimorphs.

EXAMPLES FROM ACROSS THE NATIONAL WILDLIFE REFUGE SYSTEM

The National Wildlife Refuge System (Refuge System), managed by the Service, is the world's premier network of public lands devoted to conserving wildlife and habitat for the benefit of future generations. There are over 850 million acres of land and water in the Refuge System, which preserves a diverse array of terrestrial, wetland, and ocean ecosystems. The Refuge System plays an essential role in providing outdoor recreation opportunities to the American public and the associated economic benefits to local communities. The Refuge System includes more than 180 refuges and over 750 million acres that protect ocean, coastal or Great Lakes habitats. Spanning from above the Arctic Circle to south of the Equator, the Refuge System protects an incredible diversity of marine and coastal ecosystems, including salt marshes, rocky shorelines, tide pools, sandy beaches, kelp forests, mangroves, seagrass meadows, barrier islands, estuaries, lagoons, tidal creeks, tropical coral atolls, as well as open ocean. Marine debris is an issue that impacts coastal and island refuges across the country. Examples include:

Northwestern Hawaiian Islands and Midway Atoll National Wildlife Refuges

The Northwestern Hawaiian Islands are made up of dozens of tiny islands, atolls and shoals, spanning 1,200 nautical miles of the world's largest ocean. These remote islands and atolls are home to an ecosystem that hosts more than 7,000 species, including marine mammals, fishes, sea turtles, birds, and invertebrates. At least one quarter are endemic and found nowhere else on Earth. These remote islands are rarely visited, and often considered to be some of the most pristine places on earth. However, plastic from the "Great Pacific Garbage Patch," an enormous area where high concentrations of litter – the majority of which are microplastics – accumulates here.

Annual accumulation of marine debris within the Papahānaumokuākea Marine National Monument, which includes the Midway Atoll and Northwestern Hawaiian Islands National Wildlife Refuges, is estimated at 52 metric tons per year.[5] Much of this debris is comprised of derelict fishing gear: 848 metric tons (more than 900 tons or 1.9 million pounds) have been recovered since 1996. The Northwestern Hawaiian Islands are home to approximately 70 percent of the tropical, shallow-water coral reef habitat in the nation. Derelict fishing gear

[5] O. J. Damero, M. Parke, M. A. Albins, and R. Brainard. 2007. Marine debris accumulation in the Northwestern Hawaiian Islands: An examination of rates and processes. Marine Pollution Bulletin 54:4, p. 423-433.

threatens these reefs, as well as the threatened and endangered turtles, dolphins, seals, and other wildlife that inhabit them.

Tern Island is an important nesting site for Hawaiian green sea turtles, a monk seal pupping site, and home to many bird species. There, storms have eroded the islands' shores, exposing debris that entraps monk seals and sea turtles. Midway Atoll is home to nearly three million birds, including endangered Laysan ducks, the world's largest albatross colony, and 19 other seabird species. The oldest known wild bird continues to nest at Midway, a Laysan albatross named Wisdom that is at least 68 years old and currently raising another chick. Albatross scour thousands of ocean miles in search of food for their young. Along with fish, they scoop up plastic debris. The stomachs of nearly all dead albatross chicks contain plastic (e.g. cigarette lighters, parts of toys, and fishing gear) all fed to them by their parents. It is estimated that albatross carry over 5 tons of plastic to Midway each year to feed their chicks. Another 5-10 tons of marine debris washes up on Midway beaches each year. Recent studies indicate that there is a 20.4 percent chance of lifetime mortality for seabirds ingesting a single debris item, rising to 100 percent after consuming 93 items.[6]

The Service partners with NOAA and the U.S. Coast Guard to remove much of the marine debris on Midway and in the Northwestern Hawaiian Islands. The nets and plastics are brought back to Honolulu aboard the NOAA ship or charter vessel. A key partner in the recycling process, Schnitzer Steel (a metal recycling company) chops the fishing nets into small pieces. The net pieces are then transported to the City and County of Honolulu's H-Power Plant (a Covanta Energy Corporation facility), where they are safely incinerated to produce electricity for the island of Oahu.

Maine Coastal Island National Wildlife Refuge Complex

The Maine Coastal Islands National Wildlife Refuge Complex consists of five individual refuges that span the coast of Maine and support an incredible diversity of habitats, including coastal islands, forested headlands, estuaries, and freshwater wetlands. All totaled, the Refuge includes approximately 8,238 acres of diverse coastal Maine habitats, including forested and non-forested offshore islands, coastal salt marsh, open field, and upland mature spruce-fir forest. The majority of the islands within the refuge are considered nationally significant nesting islands and support endangered and threatened species, colonial nesting seabirds, wading birds, and waterfowl.

The islands and Refuge shoreline accumulate marine debris throughout the year, but especially during seasonal storms and high tides when debris, including derelict lobster traps, washes ashore. This derelict gear poses threats to the breeding birds that use the islands for nesting.

Cleanups held by the Refuge in past years have collected over 19,200 pounds of marine debris from a 0.66 mile section of shoreline. The Refuge continues to work with the community and partners, such as Maine Audubon, to support cleanups. Planning cleanup efforts on Refuge islands is logistically challenging as it requires calm seas, and volunteers and transportation to/from the cleanup site, as well as transfer of the debris to the mainland.

[6] L. Roman, B.D. Hardesty, M. A. Hindell, and C. Wilcox. 2019. A quantitative analysis linking seabird mortality and marine debris ingestion Scientific Reports v. 9, Article number: 3202.

Florida Keys National Wildlife Refuge Complex

The Florida Keys National Wildlife Refuges Complex includes Key West National Wildlife Refuge, Great White Heron National Wildlife Refuge, and Crocodile Lake National Wildlife Refuge. Key West Refuge is among the first refuges established in the United States. President Roosevelt created the Refuge in 1908 as a preserve and breeding ground for colonial nesting birds and other wildlife. The Refuge encompasses 208,308 acres of land and water, only one percent of which (2,019 acres) is land. It contains vital feeding, breeding areas, and nesting beaches for both green and loggerhead sea turtles.

In 2017, Hurricane Irma delivered a significant blow to the communities and wildlife of the Florida Keys. The storm broke up fragile coral reefs, uprooted sponges, and blanketed the seafloor with sediment. Irma also left in its wake a significant amount of marine debris, which may float subsurface, making navigation challenging, or become submerged, causing damage to reef resources. An estimated 1,800 vessels sunk due to the hurricane, many of which were in the Keys, and Florida lobstermen estimated losing over 150,000 traps. Refuge beaches, important for nesting sea turtles and surrounded by seagrass habitats where turtles forage, were heavily impacted during the height of nesting season. The traps, ropes and buoys created dangerous obstacles for sea turtles and other wildlife. Following the hurricane, Refuge staff engaged in a complex exercise alongside Federal, State, and local partners to clean up Refuge lands and waters. Today, the Refuge continues to repair/restore infrastructure and habitats impacted by the storm, and to study impacts of resulting marine debris on wildlife resources.

CONCLUSION

The scope of marine debris and its impacts on wildlife is serious. The effects of marine debris can be observed at most of the Service's coastal and island refuges across the country. It also affects many species, including threatened and endangered plants and animals that the Service is working to recover. Preventing and cleaning up marine debris can be addressed by ensuring a comprehensive approach that is local in scale and global in scope, directed primarily at source prevention and education. Investing in prevention and education will reduce the threat of marine debris to wildlife and habitats, and future conservation efforts are likely to be less costly, more flexible, and more successful over time.

Addressing marine debris is a complex task that requires the engagement of multiple Federal agencies, State and local governments, Tribes, industry, and the international community. To that end, the Service participates in the Interagency Marine Debris Coordinating Committee (IMDCC), a multi-agency group tasked with ensuring that this comprehensive approach is implemented. The unanimous passage and 2018 signing of the Save our Seas Act, which extended NOAA's marine debris program for five years and focused efforts on addressing the waste stream at an international level, was also an important step towards addressing the problem of marine debris.

Marine debris is preventable through increased public awareness, changing individuals' behaviors, and movement towards an economy of reduced pollution and waste. The Service works to educate visitors to National Wildlife Refuges on the impacts of marine debris to humans and wildlife, and benefits from the commitment of hundreds of volunteers who

participate in marine debris cleanups and citizen science on our National Wildlife Refuges. We are also working with Federal and non-Federal partners to better understand the causes and effects of marine debris, identify strategies to address this issue, and educate the public on ways they can be a part of the solution.

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Chapter 115

TESTIMONY OF CHRISTY LEAVITT, CAMPAIGN DIRECTOR, OCEANA*

Good morning. Thank you, Chairwoman McCollum, Ranking Member Joyce and members of the committee for the opportunity to testify on marine debris and plastic pollution. My name is Christy Leavitt, and I am the plastics campaign director with Oceana. Oceana is the largest international advocacy organization dedicated solely to ocean conservation. We work in North America, South America, Europe and the Philippines to advocate for science-based policies that will restore the ocean's abundance and biodiversity. Oceana has protected more than 4.5 million square miles of ocean habitat and won 200 victories to stop overfishing, habitat destruction, pollution and killing of threatened species.

OCEANS ARE CRITICAL TO LIFE ON EARTH

Oceans cover more than 70% of the Earth's surface and are critical to life on the planet. Over 50% of the Earth's oxygen comes from the oceans, supplying us with the air we breathe. More than 3 billion people worldwide depend on the ocean for their livelihoods. In the United States specifically, over half of the population lives in coastal communities, and the country derives $280 billion a year from the ocean economy with three-quarters of U.S. trade being marine-based.

Oceans hold 97% of the world's water and also deliver some vital fresh water to land in the form of rain derived from ocean water evaporation. Oceans are home to a million species, according to Census of Marine Life, many of which have yet to be described. The powerful circulation of the atmosphere and the regulation of the climate are driven by the heat generated in equatorial seas, which is transported by currents to the Earth's poles. The climate crisis would be far worse if the oceans did not absorb and store 25% of the planet's carbon dioxide emissions.

* This is an edited, reformatted and augmented version of Testimony before House Committee on Appropriations Subcommittee on Interior, Environment, and Related Agencies, dated September 19, 2019.

Despite the ocean's importance to life on Earth and the livelihoods of billions of people, humanity has altered or destroyed marine ecosystems and driven marine species to the brink of extinction. And now, as one scientist put it, the oceans are literally spitting plastic back at us with every wave.[1]

PLASTIC POLLUTION IS A GROWING PROBLEM FOR OCEAN HEALTH

Plastic pollution is a growing threat to the world's oceans, as well as our food, health and climate. Each year, an estimated 17.6 billion pounds of plastic enters the marine environment. This is roughly equivalent to a garbage truck full of plastic being dumped into the oceans every minute.[2] Freshwater lakes also suffer from plastic pollution. The Great Lakes, for example, are flooded with 22 million pounds of plastic annually.[3]

As of 2015, 8.3 billion metric tons of plastic have been produced, and 79% of that became waste, accumulating in landfills, on the ground or in the ocean.[4] None of that plastic in the environment ever goes away; it simply breaks up into smaller and smaller pieces.

More than 40% of all plastic produced is for packaging, most of which is used once and thrown away.[5] Single-use plastic is profoundly flawed by design. Bottles, grocery bags, utensils and other products are made from a material created to last forever even though these products are designed to be thrown away after one use.

Plastic pollution is everywhere. Scientists have found it around the globe, floating on the surface of the ocean, washing up on the world's most remote coastlines, melting in Arctic sea ice and even sitting at the deepest part of the ocean floor nearly 7 miles beneath the surface.[6,7,8] Plastic also has been found in rain in the Rocky Mountains and in the air above the secluded French Pyrenees mountains.[9,10]

As plastics continue to flood into the oceans, the list of marine species affected by plastic debris expands. A piece of plastic can look like food to a fish, turtle, marine mammal or bird.

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[1] J. Jambeck, Feb. 15, 2019; American Association for the Advancement of Science Annual Meeting, Washington D.C.

[2] Jambeck JR, Geyer R, Wilcox C, *et al.* (2015) Plastic waste inputs from land into the ocean. *Science* 347: 768-771. doi: 10.1126/science.1260352.

[3] Baldwin AK, Corsi SR and Mason SA (2016) Plastic Debris in 29 Great Lakes Tributaries: Relations to Watershed Attributes and Hydrology. *Environmental Science & Technology* 50: 10377–10385. doi: 10.1021/acs.est.6b02917.

[4] Geyer R, Jambeck JR and Law KL (2017) Production, use, and fate of all plastics ever made. *Science Advances* 3. doi: 10.1126/sciadv.1700782.

[5] Ibid.

[6] Lavers JL and Bond JL (2017) Exceptional and rapid accumulation of anthropogenic debris on one of the world's most remote and pristine islands. *Proceedings of the National Academy of Sciences* 114: 6052-6055. doi: 10.1073/pnas.1619818114.

[7] Chiba S, Saito H, Fletcher R, *et al.* (2018) Human footprint in the abyss: 30 year records of deep-sea plastic debris. *Marine Policy* 96: 204-212. doi: 10.1016/j.marpol.2018.03.022.

[8] Peeken I, Primpke S, Beyer B, *et al.* (2018) Arctic sea ice is an important temporal sink and means of transport for microplastic. *Nature Communications* 9 doi: 10.1038/s41467-018-03825-5.

[9] Wetherbee G, Baldwin A and Ranville J (2019) It is raining plastic: Open-File Report 2019-1048. *United States Geological Survey*. doi: 10.3133/ofr20191048.

[10] Allen S, Allen D, Phoenix VR, *et al.* (2019) Atmospheric transport and deposition of microplastics in a remote mountain catchment. *Nature Geoscience* doi: 10.1038/s41561-019-0335.

We are seeing increasing reports of dead whales beached with bellies full of plastic debris.[11] Tens of thousands of individual marine organisms — almost 700 species — have been observed suffering from entanglement or ingestion of plastic permeating the marine environment.[12] Plastic ingestion and entanglements can lead to death by starvation or suffocation. Ingested plastic may also cause ulcers or punctures and impair feeding, growth, mobility, reproduction and behavior.[13,14] Scientists estimate that 90% of seabird species have ingested plastic, and a 2019 study investigating plastic in 102 sea turtles found plastic in every individual.[15,16] Even zooplankton, tiny marine organisms that form the base of the ocean food chain, are eating plastic, which can then accumulate in larger ocean predators like fish.

Ultimately plastic breaks up into microplastics, which are pieces of plastic smaller than 5 millimeters. Microplastics act as magnets for harmful chemical pollutants.[17] When eaten by fish and shellfish, some of those contaminants from microplastics may work their way into our food supply.[18] Everything from salt to water to beer has been found to contain microplastics.[19] Scientists are still studying how humans might be affected by the plastics that are making their way into our food,water and air.

While we begin to realize the extent of plastic pollution's effects on the oceans, plastic production is increasing at a rapid rate. The petrochemical industry is investing significantly in the future of plastic production. Global production of plastics has outpaced all other bulk materials — including steel, aluminum and cement — and has nearly doubled since the start of the millennium.[20] It's now projected to increase at least fourfold between 2014 and 2050.[21] As plastic production increases, so will the amount of plastic entering the ocean. Projections show a threefold increase of the amount of plastic in the ocean between 2015 and 2025.[22]

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[11] Irfan U (2019) The alarming trend of beached whales filled with plastic, explained. In: *Vox*. Available: https://www.vox.com/2019/5/24/18635543/plastic-bags-whale-stomach-beached. Accessed Jun 25, 2019.

[12] Gall SC and Thompson RC (2015) The impact of debris on marine life. *Marine Pollution Bulletin*. 92: 170–179. doi: 10.1016/j.marpolbul.2014.12.041.

[13] Cole M, Lindeque P, Fileman E, Halsband C and Galloway TS (2015) The Impact of Polystyrene Microplastics on Feeding, Function and Fecundity in the Marine Copepod *Calanus helgolandicus*. *Environmental Science & Technology* 49: 1130–1137. doi: 10.1021/es504525u.

[14] Watts AJR, Urbina MA, Corr S, Lewis C and Galloway TS (2015) Ingestion of Plastic Microfibers by the Crab *Carcinus maenas* and Its Effect on Food Consumption and Energy Balance. *Environmental Science & Technology* 49: 14597–14604. doi: 10.1021/ acs.est.5b04026.

[15] Wilcox C, Van Sebille E and Hardesty BD (2015) Threat of plastic pollution to seabirds is global, pervasive, and increasing. *Proceedings of the National Academy of Sciences* 112: 11899–11904. doi: 10.1073/pnas. 1502108112.

[16] Duncan, EM, Broderick, AC, Fuller, WJ, et al. Microplastic ingestion ubiquitous in marine turtles. Glob Change Biol. 2019; 25: 744– 752. https://doi.org/10.1111/gcb.14519.

[17] Cole M, Lindeque P, Fileman E, *et al.* (2013) Microplastic Ingestion by Zooplankton. *Environmental Science & Technology* 47: 6646-6655. doi: 10.1021/es400663f.

[18] Rochman CM (2015) The Complex Mixture, Fate and Toxicity of Chemicals Associated with Plastic Debris in the Marine Environment. In: Bergmann M, Gutow L, Klages M, editors In: *Marine Anthropogenic Litter*. Cham: Springer International Publishing.

[19] Kosuth M, Mason SA and Wattenberg EV (2018) Anthropogenic contamination of tap water, beer, and sea salt. *PLOS ONE* 13. doi: 10.1371/journal.pone.0194970.

[20] -- (2018) The Future of Petrochemicals: Towards More Sustainable Plastics and Fertilisers. Executive summary. International Energy Agency and Organisation for Economic Cooperation and Development. 11p.

[21] -- (2016) The New Plastics Economy: Rethinking the future of plastics. World Economic Forum. 36p.

[22] Jambeck JR, Geyer R, Wilcox C, *et al.* (2015) Plastic waste inputs from land into the ocean.

SOLUTION: REDUCE PLASTIC POLLUTION AT THE SOURCE

Recycling is not enough to solve the plastic pollution crisis. Waste-management solutions have not adequately dealt with plastic pollution in the past and cannot realistically keep up with the rising rates of plastic production. Only 9% of all the plastic ever produced has been recycled.[23] Unless companies change course, more plastic will inevitably end up in the ocean.

The solution to the plastic pollution problem is clear: Reduce the amount of plastic produced.

Oceana is focused on stopping plastic pollution from entering the oceans by reducing the amount of single-use plastic being produced at the source. We're calling on companies to significantly reduce the amount of plastic they are putting on the market and offer consumers plastic-free choices for their products. Whether it's a hotel room, supermarket aisle or restaurant, the onus should be on the manufacturers and retailers responsible for creating and selling throwaway plastics – not the consumers. Oceana is also calling for policy change at the local, state and national levels that will require companies to reduce plastic production and use.

GOVERNMENTS PLAY A CRITICAL ROLE IN REDUCING PLASTIC POLLUTION

While the responsibility to reverse the plastic pollution crisis should primarily fall on companies, governments play a critical role in moving society away from plastic, especially when companies are not motivated to change their ways. Policies governing the production and use of plastic can be effective in curbing the plastic pollution problem.

The United States has often been an international leader on environmental issues, but unfortunately it's falling behind other countries on addressing the plastics crisis on the national level. U.S. cities, towns, counties and states are not waiting for federal action and are instead leading the way in regulating single-use plastics. Around the world, other countries are announcing and implementing policies to reduce plastic pollution. The federal government should learn from these actions and implement national policies to stem the flow of plastic into our environment.

Local, state and national policies often focus on the plastic waste most commonly found on beaches, including plastic beverage bottles, grocery bags, straws, stirrers, lids, cups, foam and other containers. Policies like bans, fees, taxes, deposit return systems and extended producer responsibility can reduce plastic production, use and pollution.

U.S. cities, towns, counties and states that have taken action on plastics include:

- More than 450 cities and counties and seven states that have passed bans or put fees on plastic bags;
- *Washington, D.C.*, which passed a 5-cent plastic- and paper-bag fee in 2009 that resulted in more than a 60% reduction in single-use carryout bag consumption and reduced plastic bag litter in D.C.'s Anacostia River;[24]

[23] Geyer R, Jambeck JR and Law KL (2017) Production, use, and fate of all plastics ever made.

[24] D.C. Department of Energy and Environment & Alice Ferguson Foundation (2013) D.C. Resident and Business Bag Use Surveys. Opinion Works. 18p.

- *Chicago, Illinois,* which passed a 7-cent tax on both paper and plastic single-use bags at all stores, effective February 2017.[25] The bag tax resulted in a 42% reduction in the number of single-use bags consumers used per trip to the store;[26]
- More than 200 cities and counties that have passed polystyrene, or foam, bans;
- *Maine, Maryland and Vermont,* which passed statewide polystyrene bans in 2019;
- *St. Paul, Minnesota*, which passed a law in 2019 requiring restaurants and stores to use compostable or recyclable packaging for to-go and takeout containers. The law will go into effect in 2021;[27]
- Michigan, which implemented a bottle-deposit law in 1978, requiring a 10-cent deposit on certain containers under 1 gallon in size.[28] In 2017, Michigan's redemption rate was 91.2%.;[29] and
- Berkeley, California, which passed a comprehensive foodware ordinance in 2019 that ultimately will require food vendors to use only reusable foodware, with a few compostable exceptions.[30]

Other countries and regions have taken action to reduce single-use plastics, including:

- *The European Union* — In 2018, the European Union announced a phaseout of single- use plastics by 2021. The Single-Use Plastics Directive bans single-use plastic products, including plates, cutlery, polystyrene food and beverage containers, and other items that are estimated to represent 85% of single-use plastic found on beaches in the Union.[31]
- *Canada* — In June 2019, Canada announced plans to develop a strategy to address and reduce priority single-use plastics by the end of 2021.[32]
- *Peru* — In December 2018, Peru passed a law regulating throwaway plastics, which bans single-use plastics from sensitive areas like beaches and national parks; taxes plastics bags; and limits the manufacture and use of a broad swath of disposable plastics.[33]

The United States should join with the rest of the world and follow the lead of cities and states around the country to regulate the production and use of single-use plastic. Federal policies must enable states and localities to continue to lead the way and implement stronger regulations on plastics.

Thank you again for the opportunity to testify on the plastic pollution crisis threatening the future of our oceans.

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[25] CHICAGO, ILL., MUN. CODE § 3-50 (2019).

[26] -- (2017) Preliminary study suggests Chicago's bag tax reduces disposable bag use by over 40 percent. New York University Wagner, University of Chicago UrbanLabs & ideas42. 4p.

[27] ST. PAUL, MINN., CODE OF ORDINANCES ch. 236 (2019).

[28] MICH. COMP. LAWS §§ 445.571–445.576 (2017).

[29] -- (2019) Michigan Bottle Deposit Law: Frequently Asked Questions. Michigan Department of Environmental Quality. 4p.

[30] BERKELEY, CAL., MUN. CODE ch. 11.64 (2019).

[31] Directive 2019/904 of the European Parliament and of the Council of 5 June 2019 On the Reduction of the Impact of Certain Plastic Products on the Environment, 2019 O.J. (L 155) 1–19 (EU).

[32] -- (2019) Canada-Wide Action Plan on Zero Plastic Waste: Phase 1. Canadian Council of Ministers of the Environment. 9p.

[33] Law No. 30884, Diciembre 7, 2018, DIARIO OFICIAL [D.O.] (Peru), available at http://www2.congreso.gob.pe/Sicr/TraDocEstProc/Expvirt_2011.nsf/Repexpvirt?OpenForm&Db=201602248&View.

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Chapter 116

WRITTEN TESTIMONY OF LINSEY E. HARAM, RESEARCH ECOLOGIST, SMITHSONIAN ENVIRONMENTAL RESEARCH CENTER*

Linsey E. Haram

INTRODUCTION

Thank you, Chair McCollum, Ranking Member Joyce, and members of the Subcommittee for the opportunity to come before you today to testify on behalf of the Smithsonian Institution. My name is Linsey Haram, and I am a marine ecologist from the Smithsonian Environmental Research Center, where I study how plastic marine debris alters the dispersal and distribution of marine organisms across oceans.

The Smithsonian Environmental Research Center, or SERC, is a world leader in environmental research, especially in coastal marine ecosystems. SERC scientists are based at core laboratories on the Chesapeake Bay and San Francisco Bay, and work with collaborators across a truly global network of sites. Primarily funded by grants and contracts through such agencies as the U.S. Coast Guard, the National Science Foundation, the National Oceanic and Atmospheric Administration, the Department of Defense, and many others; SERC research aims broadly to understand fundamental ecological processes and changes at the land-sea boundary.

One area of focus at our facility is the study of marine invasive species, which is my area of expertise. The Marine Invasions Research Lab at SERC is the largest research program in the nation on biological invasions in coastal marine waters. Biological invasions occur when species are transferred by human activities to new (unoccupied) areas, where they establish new populations and spread, sometimes resulting in unwanted economic and ecological

* This is an edited, reformatted and augmented version of Written Testimony of Linsey E. Haram, Research Ecologist, Smithsonian Environmental Research Center Plastic Marine Debris Research, House Sub-Committee on Appropriations: Interior, Environment, and Related Agencies, U.S. House of Representatives Publication, dated September 19, 2019.

impacts. My colleagues have researched the causes, effects, and dispersal of invasive species within coastal ecosystems for over 3 decades. My research elucidates a missing link in our understanding of these processes - the emerging problem of plastic marine debris as a major source of invasive species spread across oceans.

PLASTIC MARINE DEBRIS

Plastic production has rapidly increased since its industrialization in 1950, having grown from 2 million tons produced in 1950 to 380 million tons in 2015 – half of which was produced within the past 13 years.[1] From 1950 to 2015, a total of 7.8 billion tons has been produced, with 6.3 billion tons discarded; less than 10% of plastic waste in this same period was recycled.[1] A large portion of the waste produced annually occurs in coastal communities because human populations are concentrated on the coasts. In 2010 alone, over 35% of the 275 million tons of global plastic waste generation occurred in coastal communities.[2] In areas with mismanaged waste, plastic pollution can be directly delivered from the land to our oceans, with an estimated 8 million tons of debris entering the oceans from coastal communities each year.[2] Effluent from rivers[3] and fishing gear from offshore fisheries further contribute plastic waste to ocean ecosystems, though resolved estimates for these sources are needed.

We now know that plastic debris is widespread in our oceans and accumulates in high concentrations in gyres, which are areas of current convergence and low flow in the open ocean.[4] One of these areas is the North Pacific Subtropical Gyre (NPSG), commonly called the Great Pacific Garbage Patch, which has the greatest reported concentrations of plastics in the oceans.[5] While much of this plastic debris consists of small microplastics, larger meso- to megaplastics are also common and make up an estimated 75% of the total mass of plastics in the NPSG,[5] including:

- Ghost nets (massive conglomerations of derelict fishing nets)
- Associated fishing gear (buoys, fish crates, eel trap cones)
- Bottles, buckets, and jugs
- Miscellaneous household goods
- Plastic fragments

Once at sea, plastics are often buoyant and do not biodegrade, allowing them to persist for long periods of time, accumulate in gyres, and be carried by currents over great distances. This is unlike wood and other natural materials that degrade much more rapidly. The emergence and persistence of large plastics has created a novel and growing *floating ocean ecosystem*, where large plastics are colonized by a wide diversity of organisms and

[1] Geyer et al. 2017. Production, use, and fate of all plastics ever made. Science Advances, 3: e1700782.
[2] Jambeck et al. 2015. Plastic waste inputs from land into the ocean. Science, 347 (6223): 768-771.
[3] Lebreton et al. 2017. River plastic emissions to the world's oceans. Nature Communications, 8: 15611.
[4] Eriksen et al. 2014. Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. PLoS ONE, 9(12): e111913.
[5] Lebreton et al. 2018. Evidence that the Great Pacific Garbage Patch is rapidly accumulating plastic. Scientific Reports, 8:4666.

transported across oceans. The magnitude and spatial scale of this species rafting on plastics is unprecedented in history, creating a new mechanism for invasive species introductions to coastal ecosystems of the United States and elsewhere.

FLOATING PLASTICS AS VECTORS FOR INVASIVE SPECIES

Invasive species are animals, plants, and microbes that are introduced to a new ecosystem, usually through human activities, subsequently causing harm to their new ecosystems. When new predators, parasites, competitors, or pathogens are introduced, they can impact native biodiversity, fisheries, and water quality, impairing ecosystem services, local economies, and health.

Historically, the main drivers of invasive species introductions in coastal ecosystems are vessels, which deliver marine species from around the world in their ballast water and on their hulls.

Within the past decade, however, floating plastic debris has entered as a potential new source of coastal species introductions.

The scientific community became aware of the potential importance of plastics for invasion dynamics following the Eastern Japan Tsunami. On March 11, 2011, a devastating 9.0 magnitude earthquake struck off the coast of the Japan's Honshu region, causing a tsunami over 120 feet high in the hardest hit areas. As the wave receded, it carried millions of tons of debris with it into the Pacific Ocean. One year later, in the spring of 2012, Japanese tsunami debris began washing ashore along the coasts of western North America and Hawaii. In an international effort led by SERC and Williams College, scientists, natural resources managers, and volunteers collected 634 Japanese tsunami debris and surveyed them for the biology attached6. Over a 6- year period, 287 marine invertebrate species, 2 fish species, and 49 seaweed species from Japan arrived on North American beaches alive, and in some cases, reproductive; over 5,000 miles from their home shores of Japan.[6,7] This study, published in Science, marked the first time in history that scientists were able to observe the transport of non-native species to distant shores in real-time. The scale and duration of this event was unprecedented, due in part to the presence of highly durable man-made debris, particularly plastics and plastic-compound materials, which effectively acted as rafts for coastal species at sea.

CURRENT RESEARCH AND COLLABORATIONS

Though the tsunami debris research provided essential information about the ability of hundreds of coastal species to raft across the open ocean and successfully land on new shores, many questions remain. In a NASA funded, multi-institutional research project, titled *Floating Ocean Ecosystems* (FloatEco), my colleagues and I build upon the results of the

[6] Carlton et al. 2017. Tsunami-driven rafting: transoceanic species dispersal and implications for marine biogeography. Science, 357: 1402-1406.

[7] Hansen et al. 2018. Genetic identification of macroalgal species on Japanese tsunami marine debris and genetic comparisons with their wild populations. Marine Pollution Bulletin, 132: 74-81.

Japanese tsunami debris effort with the purpose of better understanding the movement of large floating plastic marine debris in open ocean gyres and the ecological communities attached.

FloatEco combines expertise from marine ecologists from SERC, Fisheries and Oceans Canada, and Williams College and oceanographers and engineers from University of Hawaii, Scripps Institute of Oceanography and University of Washington – Applied Physics Laboratory. Our interdisciplinary team also includes the ocean health non-profit, Ocean Voyages Institute.

Our research, which takes place in the area of highest plastic accumulation in the NPSG, aims to elucidate the dynamics of this floating ocean ecosystem of marine debris and especially its role in dispersal and distribution of coastal species. We seek to answer the following questions:

- Are coastal species common on floating plastic debris in the open ocean, gyre ecosystems? How many coastal species utilize this new habitat?
- How does the distribution and movement of debris affect survival, reproduction, and fate of coastal organisms in the ocean?
- How does debris type influence community composition and origin of both coastal and open ocean species?
- How do different types of floating plastic debris drift through gyre environments? Can we predict their trajectories based on debris type, size, and oceanographic conditions?

These are large scale questions that can only be answered through the synthesis of effort by multiple stakeholders. Thus, the FloatEco team works closely with external collaborators to maximize our capacity for data collection. Below, we outline our research on floating ocean ecosystems in three primary efforts: *1) Ecological community surveys, 2) Large floating debris movement, and 3) Marine invertebrate colonization.*

1. Ecological Community Surveys

Through collaboration with non-profits and citizen scientists, we collect debris from our target study area in the NPSG. Oceanographers from the University of Hawaii model distributions of floating debris in the region and direct the collaborators to projected areas of highest plastic debris accumulation. During their journeys, our external collaborators either collect debris or they sample the marine invertebrates and seaweeds attached to the large floating debris in the field. All samples are sent to SERC and analyzed by FloatEco biologists.

Once in the lab, these samples are analyzed for the following characteristics:

- Species present (identified to lowest taxonomic level possible)
- Natural species distribution – coastal vs. pelagic
- Signs of reproduction
- Size class structure

All species present are characterized as those with natural distributions in the open ocean or coastal ecosystems. To determine if populations are sustaining at sea, we also analyze the marine invertebrates for evidence of reproduction and presence of multiple generations through age structure.

During the past year, we collected over 150 floating plastic debris items with the help of five external collaborators.

2. Large Floating Debris Movement

Though we know that plastic debris concentrates in gyres, there are gaps in the scientific knowledge regarding how large floating plastics respond to wind and oceanographic dynamics in these unique systems. Additionally, we do not know how differently floating debris responds based on type and size. To address these unknowns, we deploy Langrangian drifters, which measure ocean currents and conditions, and tag debris with GPS tracking buoys within the NPSG.

Lagrangian Drifters. FloatEco oceanographers from the Scripps Institute of Oceanography's Langrangian Drifter Lab and University of Hawaii, construct drifters of different shapes and depths within the water column to better determine how these factors affect the paths of floating debris.

GPS-Tagged Debris. Through our collaboration with Ocean Voyages Institute, we track large floating plastic debris in real-time within the NPSG. To do so, our external collaborators attach specially manufactured GPS tracking buoys to drifting derelict fishing nets (i.e. ghost nets). Once tagged, the locations of the ghost nets are tracked continuously via satellite GPS.

The trajectories of both the Langrangian drifters and the GPS-tracked ghost nets inform oceanographic models created to more accurately predict the seasonal distribution and movement of debris.

3. Marine Invertebrate Colonization of New Debris

In addition to establishing the presence of coastal species on debris in the NPSG, we also need to know if coastal species can colonize new debris when it enters the open ocean. If coastal species do colonize new plastics, coastal species populations may be able to persist and spread within gyres, making these novel floating ecosystems a source of non-native species.

Colonization Arrays. To investigate the colonization of new debris by coastal species, we have adapted methods used by the SERC Marine Invasions Laboratory for monitoring invasive marine invertebrates along coastlines. In our current design, four PVC discs stacked 0.5 inches apart along a central axis create a complex structure meant to mimic plastic debris. The colonization arrays are attached to both drifters and GPS-tagged debris. After several months, the colonization arrays will be recovered, and we will analyze each PVC disc for community composition and abundance. As in our *Ecological Community Surveys*, we will also measure reproductive state and size class structure.

By combining what we learn from our above research efforts, we will better understand how floating plastic debris disperses coastal species and the extent to which it may contribute to the spread of marine invasive species.

4. Education and Outreach

At SERC, as a complement to our research, we prioritize professional development of the next generation of scientists as well as effective science communication and education for the public.

The issue of plastic marine debris is truly global and one that requires buy-in from all stakeholders involved. Thus, in SERC's tradition, we prioritize outreach and education as a deliverable of our plastic marine debris research. Examples of our efforts include:

- A booth about floating plastic ecosystems at the World's Ocean Day event in the National Museum of Natural History.
- SERC Education department curriculum about plastic ingestion by albatrosses.
- Lectures and presentations at local community centers.
- Coverage in science journalism pieces and other media.
- Creation of a citizen science project for collection of plastic marine debris.
- Inclusion of young scientists in research activities.

From our participation in these activities, it is clear that more education about the societal and environmental context of our plastic pollution issue is needed; and in order to effectively communicate the issue to the public, we need to prioritize more cross-disciplinary research that identifies our current unknowns.

CONCLUSION

The research presented above highlights an under-investigated aspect of plastic marine debris as a vector of future marine species invasions. Through this work, we aim to clarify the role of plastic marine debris in creating a novel open ocean ecosystem and in transporting species across oceans in historically unprecedented ways. Beyond this research, there are still many unknowns about plastic pollution's sources, sinks, impacts, and solutions. We are in the early stages of understanding the extent of our global plastic pollution issue. Given the scale of the plastic marine debris problem and the scope of the unknowns, greater prioritization and standardization of research is necessary to ensure the future health of our oceans.

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Chapter 117

WRITTEN TESTIMONY OF DR. CHELSEA ROCHMAN, HOUSE APPROPRIATIONS SUBCOMMITTEE ON INTERIOR, ENVIRONMENT AND RELATED AGENCIES HEARING ON ECOSYSTEM IMPACTS OF MARINE DEBRIS/OCEAN PLASTIC POLLUTION*

Chelsea Rochman

Dear Chair McCollum and Ranking Member Joyce,

Thank you for holding this hearing on such an important topic. Plastic pollution presents a momentous challenge and it is heartening to see so much interest in finding solutions to tackle the issue on Capitol Hill. I'm thrilled to have the opportunity to share my expertise with you on this important issue and to help facilitate the use of science and evidence in informing policy.

I am Dr. Chelsea Rochman, an American citizen working abroad as a professor in Ecology at the University of Toronto. My expertise is in marine ecology, environmental chemistry and toxicology, I have been researching this issue for more than a decade. I currently run a research program where we investigate the sources, transport and effects of plastic pollution in our environment. I also serve as a Science Advisor to Ocean Conservancy, a nonprofit organization which works to create science-based solutions to the challenges facing our ocean. Ocean Conservancy is a leader in the global fight to address the proliferation of mismanaged plastic waste that has entered our oceans. In my capacity as a Science Advisor, I work with the organization to develop scientific research priorities and distill scientific findings into actionable information that can be used to provoke change, both here in the U.S. and abroad.

With more than a decade of experience researching plastic pollution, I have a vast knowledge base on this issue. I have published many papers about the topic and have advised

* This is an edited, reformatted and augmented version of Written Testimony before the Department of Ecology and Evolutionary Biology, University of Toronto, dated September 19, 2019.

managers and policy-makers in several countries. For example, I presented at the 2016 Our Ocean Conference at the U.S. State Department and in front of the UN General Assembly in 2017. I have also traveled to Washington, D.C. for one-on-one discussions with the offices of several Members of Congress.

My work in this field began in the middle of the ocean, aboard the first scientific expedition to the Great Pacific Garbage Patch with the Scripps Institution of Oceanography in 2009. Every four hours we dropped our net in the water to quantify plastic at the surface and 24 hours a day we had observers on deck looking for large debris. Day after day, we were not seeing much in terms of an island of garbage in the middle of the Pacific. Then, on the fourth day, the observers called us all up for assistance. On the bow of the ship were two rulers being used by observers to count debris as it passed. Up to that point, they had counted a buoy, a drink tray, a fishing net here and there. But then, all of a sudden there were too many pieces of plastic to count and the two observers needed the eyes of many. Looking over the bow of the ship were thousands of little pieces of plastic smaller than a pencil eraser. This was not a garbage patch, this was a soup of microplastic (plastic particles < 5 mm in size). At that moment, I knew that this small plastic material could infiltrate every level of the food web. I also knew that this was not just an issue of cleanup – but also one of prevention.

Coming back to land, we analyzed the samples that were collected on the expedition. We found that there was plastic in every single one. This finding, that the majority of plastic pieces in the garbage patches were microplastics, demonstrated a need to shift the conversation in terms of the way we were thinking about mitigation by putting a stronger focus on preventing plastic from leaking into the environment in the first place. It also demonstrated a need for more science to better quantify the magnitude of the problem, including sources, transport, and impacts of microplastics in the marine environment.

Since this expedition, I have witnessed our scientific field grow globally and expand from oceans to freshwater to land. We've learned that microplastics are not just an ocean contaminant, but a global contaminant. We've learned that they are found in the stomachs of animals big and small and that this contamination extends beyond our environment - into seafood, salt and drinking water.

As a pioneer in this field, my research program is globally known for doing work on method development, contamination in the environment and effects to wildlife. We study microplastics across the United States, including in the San Francisco Bay, the Chesapeake Bay, the Great Lakes, and the Arctic. We also measure contamination in animals, exposure to humans via drinking water and seafood consumption, and the effects of plastic pollution to animals and ecosystems. The topic my lab focuses on the most is impacts to wildlife and their ecosystems.

Today, there is no doubt that anthropogenic debris of all shapes and sizes litters our oceans and freshwater ecosystems. This debris is found in hundreds of species of wildlife, including in the species we consider seafood. It is also found in our drinking water. We know that plastic pollution harms individual organisms, wildlife populations and communities. These impacts, combined with evidence for accelerating plastic production and leakage into the environment, suggest the international community should come together to limit future environmental leakage of anthropogenic litter now, before they transform ecosystems irreparably. Below, I will speak specifically to microplastics followed by plastic pollution in general.

MICROPLASTICS

My research mainly focuses on microplastics (plastics 5mm in size or less) and demonstrates that microplastics are ubiquitous in the environment, including in seafood and waters extracted for drinking water. My research has also shown that microplastics are associated with a cocktail of toxins, including 78% of those we currently consider priority pollutants under the Clean Water Act. It also demonstrates that microplastics can be toxic to fish and invertebrates.

Although we often think of microbeads when we think of microplastics, the term microplastic incorporates a large diversity of plastic types, including those that were produced as microplastics (e.g., microbeads, pre-production pellets often referred to as "nurdles") and those that are literally degraded bits of larger plastic products (e.g., tire dust, microfibers and fragments of bottles, bags and film). The former is called primary microplastics and the latter is referred to as secondary microplastics. Secondary microplastics are the most common type of microplastic waste found at sea. Still, we must not forget the primary sources of microplastics as well as the sources that emit secondary microplastics into the oceans (e.g., microfibers). These particles, specifically microfibers, are some of the most common microplastic types found in global ecosystems.

Researchers estimate that there are between 15 and 51 trillion microplastic particles floating around in our oceans (van Sebille et al., 2015), reaching from the poles to the equator. Microplastic particles are found in large concentrations in Arctic sea ice (Obbard et al., 2014) and are also present in sediments (Browne et al., 2011) and wildlife from the deepest parts of the ocean (Woodall et al., 2014). Consequently, this widespread contamination has led to the contamination of hundreds of species of wildlife across all trophic levels (GESAMP, 2016). In our own work, for example, we find microplastics in several species of Great Lakes fish, including white sucker, brown bullhead, lake trout, shiners and minnow – and sometimes at concentrations of more than 100 pieces per fish sampled.

For microplastics, there have been many studies testing the effects on organisms. Although the results are variable, there is irrefutable evidence that microplastics can impact organisms. In laboratory studies, microplastics have been shown to cause a variety of biological effects including: changes in gene expression (e.g., Paul-Pont et al., 2016), inflammation (e.g., von Moos, Burkhardt-Holm, & Köhler, 2012), disruption of feeding behaviour (e.g., Cole, Lindeque, Fileman, Halsband, & Galloway, 2015), decreases in growth (e.g. Au, Bruce, Bridges, & Klaine, 2015), decreases in reproductive success (e.g., Au et al., 2015; Sussarellu et al., 2016), changes in larval development (e.g., Nobre et al., 2015), reduced filtration and respiration rates (e.g., Paul-Pont et al., 2016), and decreased survival (e.g., Au et al., 2015; Cui, Kim, & An, 2017). In my own work, we have seen deformities in larval fish (Figure 2) and tumor promotion in the liver of adult fish (Figure 3; Rochman et al., 2013) from exposure to microplastic debris. Although we do not yet understand how they affect human health, we know we are exposed via sea salt, seafood and drinking water – and thus relevant research is necessary.

Although policies that mitigate large plastic debris also reduce microplastic debris, we need to make sure we consider microplastics when we consider all of the policy options for plastic pollution. Policies specific to microplastics may include, but are not limited to, leakage

standards for microplastics (e.g., from washing machine effluent, wastewater, stormwater, etc. …), filters on washing machines to trap microfibers, bioretention cells (or rain gardens) on stormdrains, increasing industry participation in the voluntary initiative to reduce pellet loss (Operation Clean Sweep) and extend this model to textiles, material innovation, and banning microbeads.

The above mitigation strategies are simple solutions to combat some sources of microplastics. Still, when it comes to plastic pollution, we know the least about sources, fate and effects of microplastics. As such, while we begin implementing policies now related to known sources of microplastics, we must continue to put resources into research that helps us better understand what some other sources of microplastics are and which may be prioritized for policy based on contamination and risk.

By weight, large plastic debris such as fishing nets, make up the largest percentage of plastic floating in our oceans. However, as discussed above, microplastics are ubiquitous and infiltrate every level of the food web. As we develop policies aimed at plastic pollution, we must be mindful that sources of plastic pollution are diverse, and the policies to address them must therefore include unique considerations for microplastics and macroplastics. We cannot make the mistake of assuming that one policy intervention will fix all aspects of the problem.

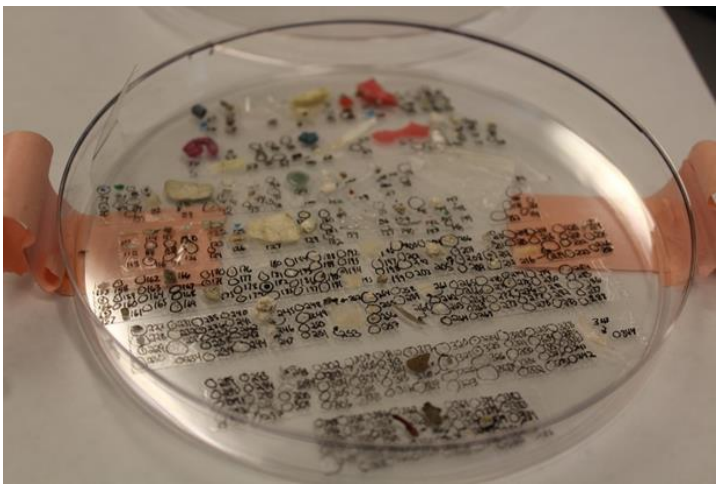

Figure 1. Microplastics picked from surface water samples in the San Francisco Bay, California, USA.

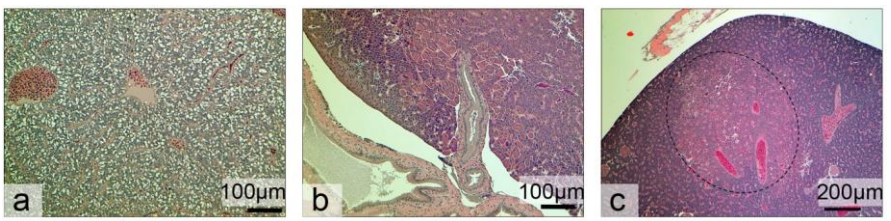

Figure 2. Livers of fish taken under a microscope after exposure to different treatments in a laboratory experiment. The liver on the left is from a fish exposed to a no-plastic control and is healthy, the liver in the middle is from a fish exposed to virgin polyethylene and has an abnormal proliferation of cells, and the liver on the right is from a fish exposed to polyethylene that was soaking in the San Diego Bay, California for a 3-month period. The image of the liver on the right is zoomed out to highlight a tumor comprising 25% of the liver. (Rochman et al., 2013)

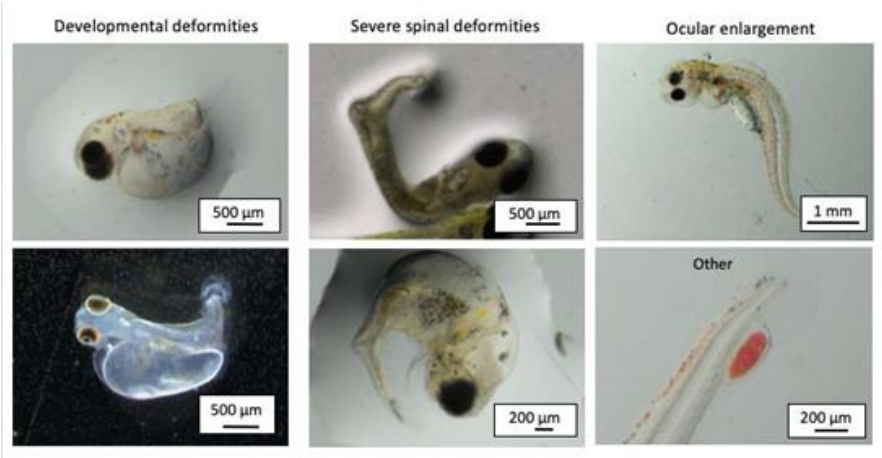

Figure 3. Larval fish with deformities taken under a microscope after exposure to microplastic debris collected from the shorelines of Lake Ontario. (Bucci et al., unpublished work).

MACROPLASTICS

When it comes to large plastic debris, there is no doubt that plastic pollution can have an impact on wildlife, and there is compelling evidence suggesting macroplastics are already impacting marine populations, species, and ecosystems. Studies have reported contamination via entanglement or ingestion in hundreds of species of wildlife. This contamination can lead to laceration of the tissues, mortality of an individual organism, declines in population size, and/or changes in the assemblages of species. A recent study published in *Science* found that plastic debris was correlated with disease in coral reefs (Lamb et al., 2018). In a recent systematic review, we found reports of adverse effects in 23 species of marine mammals, 4 species of turtles, 11 species of birds, 4 species of fish, many species of invertebrates, and one species of algae. The weight of evidence for how microplastic debris impacts wildlife suggests that the time to act is now.

Researchers estimate that about eight million metric tons of plastic enters the oceans annually from land (Jambeck et al., 2015). If we continue business as usual, this number is expected to increase by an order of magnitude by 2050.

I hope my words have expressed to you that the issue is large - and urgent. This issue is complex. The sources of plastics into the environment are diverse. The types of plastics we produce, sell and find in nature are diverse. The ecosystems and organisms this pollution contaminates are diverse. As a consequence, the solutions need to be diverse. As you know, there is no one size fits all solution. Instead, we need a toolbox of solutions that include plastic reduction, the building of a circular economy, improved waste management systems, innovation of new materials and technologies for prevention, cleanup, outreach and education. We also need everyone working together, including the plastics industry, waste managers, communities, scientists and all levels of government.

In the U.S., I think we have an opportunity to lead in this space. The U.S. can and should be a large part of the solution, and show other countries that reducing leakage of plastic is possible. I envision diverse policies that work in tandem to reduce our plastic leakage. For

example, we may adopt container deposit schemes to improve recycling rates, implement standards that increase the use of recycled content in new products, eliminate the use of some single-use plastic items that are unnecessary and/or replaceable (e.g., microbeads, straws), improve waste collection and recycling infrastructure, and agree to market only plastics that are recyclable and/or reusable in their region. We should also consider providing aid to build new infrastructure for waste collection and recycling in emerging economies abroad and also here at home. I welcome the chance to sit down with any of you and discuss the state of the science and how it might inform policy around this important issue both nationally and internationally.

Thank you for this opportunity to speak with you and I'd be very happy to answer any questions today or in the future.

Many thanks for your time. Sincerely,
Chelsea M. Rochman Assistant Professor

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Chapter 118

MUSSELS AS SENTINEL ORGANISMS IN METAL AND METALLOID CONTAMINATION SCENARIOS: ENVIRONMENTAL AND PUBLIC HEALTH RISK BIOINDICATORS

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ABSTRACT

Among environmental pollutants, metals and metalloids are of particular concern due to their potential toxic effects and the ability to bioaccumulate throughout the aquatic trophic web. However, the simple measurement of chemicals levels present in the environment is often not enough to reveal the actual adverse effects of the contamination, making it necessary to also assess the biological effects of contamination on living organisms, applying bioindicators and biomarker measurements. Mussels have been recognized as one of the best sentinel organisms regarding aquatic environments and have been increasingly used in many worldwide environmental monitoring programs. This chapter will discuss the role of several mussel species in environmental monitoring programs, with emphasis on metal contamination, including metal bioaccumulation and biomarker investigations. In addition, the fact that these organisms may be an important contaminant transfer link to the human population and pose important public health risks will also be discussed.

Keywords: mussels, sentinel organism, metal contamination, biomarkers, oxidative stress, public health risks.

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1. INTRODUCTION

One of the major damaging effects in aquatic environments due to anthropic activities is contamination by chemical pollutants. These contaminants continuously enter the aquatic environment through inadequate waste disposal, incomplete removal by sewage treatment plants, and sewage dumped into water bodies without adequate collection and treatment. Among the many different environmental contaminants present in these ecosystems, those of greatest concern present environmental persistence, bioavailability, a tendency to bioaccumulate in the food chain and the ability to cause toxic effects, such as persistent organic pollutants (Jones and de Voogt, 1999), polycyclic aromatic hydrocarbons (Abdel-Shafy and Mansour, 2016), metals (Singh et al., 2011) and, more recently, emerging contaminants originating from substances used by the human population in daily life, such as pharmaceutical formulations and personal products (Burkhardt-Holm, 2010).

Among environmental pollutants, metals and metalloids are of particular concern due to their potential toxic effects and the ability to bioaccumulate throughout the aquatic trophic web (Censi et al., 2006; MacFarlane and Burchett, 2000; Miller et al., 2002), thus being the subject of many biomonitoring efforts.

Metal and metalloids enter aquatic systems through both natural and man-made processes. The natural processes that most contribute to their presence in aquatic environments are rock weathering and the erosion of soils rich in metals and metalloids, while the main anthropic sources are industrial and mining activities, domestic effluents, surface water runoff from areas cultivated in the presence of chemical fertilizers and agricultural pesticides, and atmospheric precipitation of ash or small particles from fires (Esteves, 1988; Jordão et al., 1999; Mortatti and Probst, 1998; Nriagu and Pacyna, 1988; Ramalho et al., 2000; Yabe and Oliveira, 1998).

In the aquatic environment, metals and metalloids are distributed in both the aqueous (water column and interstitial water) and the solid (suspended and sedimented) phase, as well as in aquatic organisms, with a dynamic exchange between these phases, varying from one ecosystem to another (Conceição, 2004). Aquatic organisms assimilate metals and metalloids from both the aqueous and solid phases, namely water and sediments and, especially, food. These elements may then accumulate in tissues in several orders of magnitude above concentrations found in the water column. Therefore, aquatic organisms are responsible for much of the dynamics of these pollutants in the aquatic environment. It is important to note that the effects of these pollutants can be toxic and/or lethal to all components of the biota, such as phytoplankton, benthos, fish, birds, and, finally, humans that feed on contaminated organisms (Canli and Atli, 2003; Fowler, 1982; Junior et al., 2002; Pfeiffer et al., 1985), through bioaccumulation and bio magnification processes.

2. METAL AND METALLOID TOXICITY

Metals and metalloids can be classified as essential, non-essential and toxic elements. Essential elements, when present in low concentrations, play an important role in organism metabolism. Non-essential elements and toxic elements, however, do not play any known biological functions. Increasing essential element concentrations, up to a certain range, lead to

improvements in organism development and metabolism. Above this range, these elements become toxic and, beyond a certain limit, lethal. Non-essential elements, on the other hand, are tolerated by the body to a certain extent and, if present above certain concentration limits, follow the same behavior described above, becoming toxic and then lethal to the organism. These differences are displayed in Figure 1.

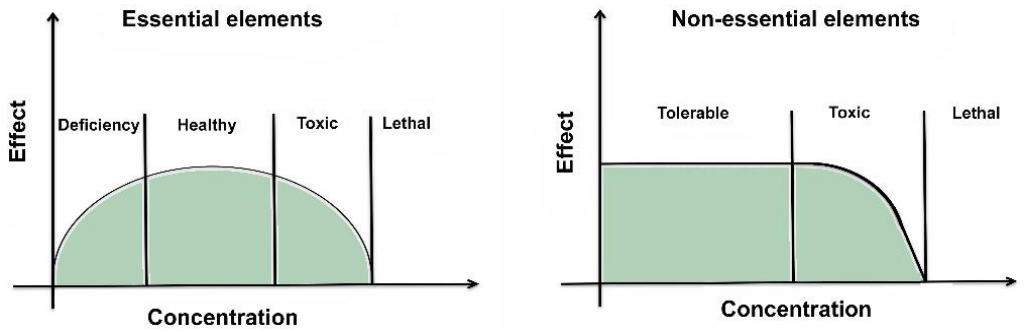

Figure 1. Graphical representation of the relationship between essential and non-essential concentrations and their effects on organisms. Adapted from Galvão (2003).

Element toxicity also depends on the chemical form present, i.e., the bioavailable fraction. Some metals and metalloids, such as Cd, Pb, Hg and As, for example, are non-toxic in the forms of free condensed elements, while, when present in their cation forms and when bonded to short carbon atom chains, become toxic. The amount absorbed by the organism also influences element toxicity, since even essential elements can be toxic when absorbed above limits tolerable for normal organism metabolism, as stated previously (Baird, 2002; Melo, 2003).

Different metal and metalloid toxicity mechanisms have been described, including (i) enzymatic inactivation due to the high reactivity of divalent transition metals with the amino and sulfhydryl groups of proteins; (ii) metal competition with essential elements and their replacement in the enzymatic metabolism - Pb, for example, can replace Zn in several proteins that function as transcription regulators - and (iii) the "cocktail effect," in which the excess of one element can cause the deficiency of another(s), such as the case of excess of Pb, that interferes in the absorption of Ca, Fe, Cu and Zn, for example (Förstner, 1989; Melo, 2003; Moreira and Moreira, 2004).

Thus, although the determination of metals and metalloids in tissues and organs of different aquatic organisms is an important method to evaluate metal contamination in aquatic ecosystems (Farkas et al., 2001), the simple measurement of metal levels present in the environment is often not enough to reveal the actual adverse effects of the contamination, making it necessary to also assess the biological effects of contamination on living organisms. This is performed through the use of bioindicators, also termed sentinel species, and biomarker measurements.

3. Mussels as Bioindicators Concerning Environmental Metal and Metalloid Contamination

Mussels are widely used as bioindicators for a number of aquatic contaminants, due to their sessile nature, which facilitates their sampling, their worldwide distribution, reasonable size, known biology and ecology, and filtering feeding habits, leading to the absorbance and retention of solid particles and, consequently, any contaminant adsorbed therein (Lamparelli, 1987; Rainbow, 1995; Resgalla Jr. et al., 2008; Wallner-Kersanach and Bianchini, 2008).

These organisms are able to concentrate contaminants in their tissues and survive, and even, thrive, in contaminated environments and are, therefore responsible for part of pollutant dynamics in the aquatic environment. In addition, they concentrate environmental contaminants proportionally to external (water) concentrations, thus reflecting the contamination history of the environment and, because they settle on shallow water substrates, they are exposed to environmental factors on a local scale, allowing for the comparison of environmental contamination between different locations (Chase et al., 2001; Chou et al., 2004; Dame, 1996; Rainbow, 1995; Storelli et al., 2000; Sun et al., 2004).

Because of these characteristics, mussels have been recognized as one of the best sentinel organisms regarding aquatic environments and have been increasingly used in national and international environmental monitoring programs (Bellotto et al., 2005) for both freshwater and marine ecosystems. For example, the United States National Oceanic and Atmospheric Administration Mussel Watch Program, the nation's longest running continuous coastal contaminant monitoring program, has applied several *Mytilus* species, such as *Mytilus edulis*, *Mytilus californianus*, *Mytilus galloprovincialis* and *Mytilus trossulusto*, to evaluate the contamination status of US coasts (Binelli et al., 2015), while many other species have been applied as bioindicators for metal and metalloid contamination scenarios worldwide.

Several biomarkers have been routinely investigated in these contamination scenarios, in order to evaluate the potentially deleterious effects of metal and metalloids, which shall be discussed further ahead in this chapter. First, however, it is important to understand the subcellular and molecular effects of metal and metalloid exposure in living organisms.

4. Subcellular and Molecular Effects of Metal and Metalloid Exposure: Reactive Oxygen Species and Oxidative Stress

One of the main deleterious effects of metals and metalloids are alterations in cellular redox regulation or oxidative reaction cycles, producing reactive oxygen species (ROS) and certain non-radicals derived from oxygen capable of generating free radicals, such as hydrogen peroxide and hypochlorous acid, which, in turn, may lead to oxidative stress (Buonocore and Groenendaal, 2007; Halliwell and Gutteridge, 2007).

Free radicals, like ROS, are small molecules with a short half-life that possess one or more unpaired electrons, characterized by high reactivity, sequestering electrons from neighboring molecules in order to become stable (Buonocore and Groenendaal, 2007; Halliwell and Gutteridge, 2007). The main ROS species are usually formed by the main oxygen metabolism route in the body, which is completely reduced to water, integrating four

electrons at the end of the electron transport chain inside the mitochondria. The main ROS, superoxide anion, hydrogen peroxide and hydroxyl radical, are produced when the oxygen molecule is reduced, along with a smaller number of electrons along the respiratory chain.

The body, however, possesses natural ROS elimination systems, known as intracellular antioxidant defenses, which are substances that prevent, delay or remove oxidative damage from a target molecule (Cogo et al., 2009; Halliwell and Gutteridge, 2007). When these antioxidant substances are present in amounts equivalent to ROS concentrations, the metabolism and cellular functioning of living organisms remain unchanged. However, situations where excessive ROS are being produced, and/or cases of failure of antioxidant defenses may lead to oxidative stress, caused by the imbalance between the formation rate of oxidant molecules and antioxidants in the body (Halliwell and Gutteridge, 2007). Oxidative stress may then lead to protein carbonylation, a process by which proteins can undergo reversible and irreversible oxidation and covalent modifications in their amino acids, modifying them into aldehyde or ketone groups, which are harmful to proteins, causing their aggregation, inactivation and degradation, and the produced ROS may interact with lipids, proteins or nucleic acids, resulting in various biochemical or genetic damages (Costa et al., 2002; Ghezzi and Bonetto, 2003; Levine et al., 1990).

The presence of metals and metalloid in biological systems in an uncomplexed form, not as protein co-factor or any other type of protective metal complex, can significantly increase oxidative stress levels (Dalle-Donne et al., 2006). In this context, certain redox-active metals, that undergo redox-cycling reactions, such as Cr, V, Co and Cr, are known to induce oxidative stress, causing damage to mitochondrial respiration (He et al., 2008; He et al., 2007; Valko et al., 2005), while other metals, such as Fe and Cu, are known to produce ROS by the Haber-Weiss (Equation 1) and Fenton (Equation 2) reactions (Barreiros et al., 2006).

$$O_2 + H_2O_2 \rightarrow \cdot OH + OH^+ + O_2 \quad (1)$$

$$Fe^{2+} / Cu^+ + H_2O_2 \rightarrow Fe^{3+} / Cu^{2+} + \cdot OH^+ + OH^- \quad (2)$$

The Fenton reaction occurs in the presence of metal ions and hydrogen peroxide, forming the hydroxyl radical, hydroxyl anion and leading to metal oxidation. In the Haber-Weiss reaction, metals can react with the superoxide anion through its reduction, generating molecular oxygen, and the reduced metal the reacts react with the hydrogen peroxide, generating the hydroxyl radical in the Fenton reaction (Leonard et al., 2004).

The generation of these radicals may lead to negative effects if organism defenses are overwhelmed. In this context, biomarker responses, meaning measurable alterations of any physiological, biochemical or behavioral steady state induced by an environmental change, against metal- and metalloid-induced oxidative stress are applied as early warning signs of the possible deleterious effects of these contaminants.

5. MUSSEL BIOMARKER RESPONSES AGAINST METAL - AND METALLOID-INDUCED OXIDATIVE STRESS

Mussel gills, digestive gland and haemolymph are regarded as excellent candidates for assessing toxic impacts of metal and metalloid exposure, due to their direct contact with the environment (water medium) and the fact that they are the center of metabolic regulations and metal transfer pathways to detoxifying tissues (Chandurvelan et al., 2013). Thus, biomarkers against metal- and metalloid-induced oxidative stress in these tissues are investigated in order to detect early negative effects and allow for decision-making processes regarding these environmental contaminants.

In this regard, one of the main biomarkers established by monitoring programs developed and implemented in the European community to investigate contamination by metals and metalloids is the metalloprotein metallothionein (MT) (Bebianno et al., 1993; Garrigues et al., 2002; Sarkars et al., 2006).

Metallothioneins are a group of low molecular weight (6 to 7 KDa) metalloproteins (proteins that bind to metals), rich in cysteines and presenting heat stability. They display a high affinity for metal ions and play an important role in several biological functions, such as essential metal homeostasis and detoxification of both non-essential elements and essential elements present in excess in the organism (Freire et al., 2008; Monserrat et al., 2007; Motta, 2012; Sarkars et al., 2006; Viarengo et al., 2007). Their synthesis is induced through the presence of metal and metalloid cations in the body, and the thiol group (-SH) present in this metalloprotein's structure allows binding with these elements, restricting their bioavailability (Freire et al., 2008; Linde et al., 1999; Monserrat et al., 2007; Nicholson and Lam, 2005; Viarengo et al., 2007).

Usually, these proteins bind to 7 equivalents of divalent metal ions, through the formation of metal-sulfhydryl clusters (Figure 2) (Melendez et al., 2012; Motta, 2012), in which the order of affinity is usually Cd>Pb>Cu>Hg>Zn>Ag>Ni>Co (Waalkes et al., 1984), although this may vary according to the bioavailability of the metals in the cell.

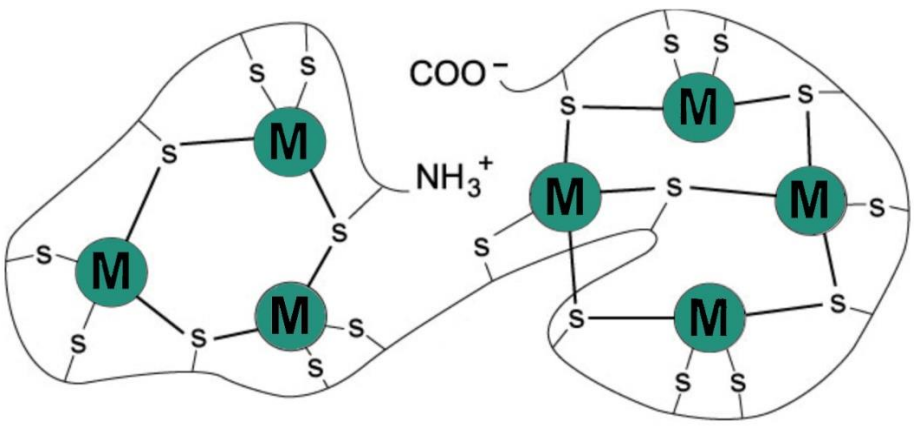

Figure 2. Metal binding sites present in one metallothionein molecule. "M" indicates metal ion binding sites. Adapted from UVED (2015).

Several metals have been shown to induce MT synthesis in mussels. For example, Mourgaud et al. (2002) observed that As, Cd, Cr, Cu, Hg, Ni, Pb and Zn concentrations were significantly correlated to MT in the mussel species *Mytilus galloprovincialis* mussels, while Purina et al. (2013) observed the same for Cd, Pb, Hg, Cu and Zn and MT in the freshwater mussel *Anodonta* spp. In another study, an increase in MTs in *Dreissena polymorpha* was shown to occur at relatively low aqueous Pd concentrations indicating that there is the need for Pd detoxification in these mussel, with significant correlations between MT and Pd associated with adverse effects on the mussels (Frank et al., 2008), while significant correlations were determined between MT induction and metal contamination in the green-lipped mussel, *Perna canaliculus*, indicating that different sampling sites were clearly distinguishable based on the metal contamination profiles and this biomarker response (Chandurvelan et al., 2015).

Tissue-specific responses have been reported for this biomarker, such as in the study conducted by Lavradas and collaborators with *Perna perna* mussels sampled from 3 contaminated areas in an extremely contaminated bay and compared to a reference site in southeastern Brazil, where the digestive gland was shown to be a better bioindicator for metal and metalloid contamination compared to muscle tissue (Lavradas et al., 2016). This was also observed in a study that investigated investigated Cd and MT distribution different tissues of the freshwater mussel *Anodonta woodiana* following Cd exposure. In this case, the authors reports that Cd was distributed in all tissues in the concentration order of gills>mantle>foot>visceral mass>digestive gland, and that the highest Cd accumulation was found in the digestive gland, while MT levels in the gills and mantle increased significantly and were positively correlated to Cd accumulation in the different tissues (Li et al., 2015).

However, it is important to note that variations in MT isoforms may be present, in which one or more may contribute to metal detoxification, while others may not display a function in this regard, or may even be inhibited following metal or metalloid exposure. For example, in one study, *Mytilus edulis* specimens were exposed to Cd and Hg for 21 days and the gills, mantle and digestive gland were evaluated regarding MT isoforms. Size exclusion chromatography showed the presence of an MT monomer and a dimer in gills, of respective apparent molecular weight of about 12 kDa and 20 kDa, which were then resolved into five components by anion exchange chromatography in the gills of control or Hg-treated mussels, whereas a sixth isoform was isolated in the gills of Cd-exposed mussels. In the mantle of mussels, exposed or not, five isoforms were separated, and in the digestive gland of mussels exposed or not, six isoforms were separated (Geret and Cosson, 2002). In another study, different MT isoforms were observed in the digestive gland of control and experimentally Cd-exposed mussels in *Mytilus edulis* (200 μg L^{-1} Cd^{2+} and 400 μg L^{-1} Cd^{2+}; 20 days), where an induction of the dimeric form MT20 II was detected in the 400 μg L^{-1} exposed mussels and an inhibition of the monomeric form MT10 IV was verified (Ciocan and Rotchell, 2004).

It is thus, important to investigate MT expressions in mussels through analytical techniques that allow for isoform differentiation, since different MT isoforms may contribute to different metal and metalloid detoxification mechanisms.

The glutathione metabolism is also extremely important in conferring protection against metal and metalloid toxicity.

Glutathione (γ-L-glutamyl-L-cysteinylglycine) (Figure 3). is a tripeptide with strong antioxidant capacity, determined by the reactive grouping of its cysteine, the thiol group (-SH).

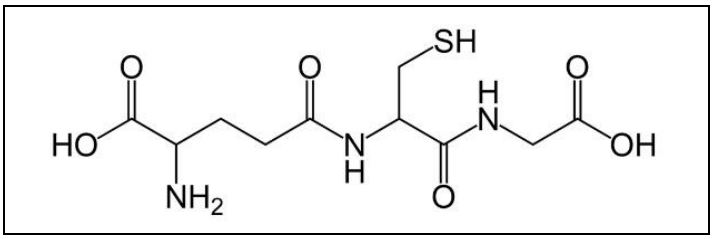

Figure 3. γ-L-glutamyl-L-cysteinylglycine.

This tripeptide acts directly or indirectly in many biological processes, such as in metabolic and transport processes, acting against the production of free radicals, in thiol homeostasis, maintenance of the redox balance of the cell, defense against xenobiotics and playing a central role in the biotransformation and elimination of electrophilic agents and protecting cells against the toxic effects of a variety of compounds. GSH is used as a substrate in conjugation reactions, catalyzed by glutathione S-transferase enzymes, but is also capable of participating in nonenzymatic conjugations with many xenobiotics (Aquilano et al., 2014).

Several studies demonstrate the induction of metal-GSH complexes in different organisms after exposure to these compounds, through the sulfhydryl group, such as Cu^{2+} and Fe^{3+}, that readily catalyze GSH oxidation, producing several free radical species (Rabestein et al., 1985; Stohs and Bagchi, 1995), and the Cu^{2+} reduction by GSH, that produces a stable Cu^{1+}–SG complex that impedes further redox cycling and leads to the generation of free radicals (Corazza et al., 1996). Thus, it has been proposed that GSH is able to complex with metals and detoxify organisms soon after the entry of these elements into the cell, representing a first line of defense against metal cytotoxicity (Canesi et al., 1999).

The glutathione metabolism also includes several glutathione-based enzymes, such as glutathione S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR), which have also been implicated in metal and metalloid biomarker responses.

In this regard, research has investigated glutathione metabolism proteins and enzymes in mussels and their responses after metal and metalloid exposure. For example, several studies indicate impairment of the antioxidant system and decreases in GR activity and GSH levels when *Perna perna* mussels were exposed to zinc (Franco et al., 2006; Trevisan et al., 2014). Exposure to sublethal copper concentrations in *Mytilus galloproincialis* also led to alterations (decreases) in total GSH content in both gills and the digestive gland, while sublethal mercury concentrations in the same species decreased tissue glutathione content when exposure was conducted with the organic mercury form (methylmercury), but did not significantly affect GSH content and metabolism when present in the inorganic form (Canesi et al., 1999), thus indicating that the chemical form of the contaminant is also of importance when evaluating metal and metalloid effects in these organisms. Other studies conducted with environmentally exposed mussels, such as the freshwater *Lasmigona costata* inhabiting a metal gradient, indicate that several biomarkers involving the SH metabolism, namely GSH, SH protein, total SH, GR and GST, were significantly correlated to gill metal concentrations (Machado et al., 2014), while alterations in GR and GPx were detected in the brown mussel *Perna perna* after exposure to 30 and 100 μmol L^{-1} zinc (Franco et al., 2006).

Thus, it is a consensus that the glutathione metabolism plays an essential role in metal- and metalloid-induced responses in these organisms.

Several other enzymes are important in this regard, since they participate in the biotransformation process of xenobiotics, which occurs in two stages, Phase I and Phase II.

Phase I reactions are responsible for exposing or introducing functional chemical groups into xenobiotic molecules leading to an increase in their polarity, which can lead to the loss of toxic potential and their excretion, resulting in detoxification. At this stage reduction reactions catalyzed by the cytochrome P450 enzymes occur, as well as hydrolysis reactions, catalyzed by esterases and amylases, and oxidation reactions. Metabolites resulting from the metabolism of this first phase may subsequently undergo detoxifying during biotransformation in Phase II. During this second phase, conjugation reactions catalyzed by sulfotransferases, glucuronosyltransferases and GST occur, promoting the conjugation of a variety of toxic molecules with endogenous molecules. The conjugates, mostly soluble and often inactive, are then excreted.

However, the mechanism by which metals and metalloids alter the activity of these enzymes is still unknown. Thus, some hypotheses that attempt to explain this phenomenon have been postulated, as follows: (i) Metals and metalloids cause protein denaturation because they alter the ionic interactions existing between the amino acid side chains, causing changes in enzyme conformation, leading to loss of catalytic activity; (ii) Metals and metalloids may bind to an amino acid residue, for example, cysteines, containing the sulfhydryl group and displaying affinity for various metals and metalloids, close to the active site of the enzyme, making it impossible for the substrate to fit into the enzyme for the enzymatic catalysis process (Bainy et al., 2006; Goncalves et al., 2010; Romani et al., 2003; Sant'anna et al., 2011). It is important to emphasize that studies on the effect of metals and metalloids on enzyme activities are discrepant since certain parameters, such as exposure time and test concentrations, can stimulate, diminish or even alter the activity of this group of enzymes in exposed organisms (Bainy et al., 2006; Barata et al., 2004; Najimi et al., 1997; Richetti et al., 2011; Vioque-Fernández et al., 2007).

Several of these enzymatic biomarkers have been evaluated in mussels regarding metal exposure scenarios. In one study, the mussel species *Mytilus galloprovincialis* from the Saronikos Gulf of Greece was applied to monitor metal pollution in three contaminated sites and the results were compared to unpolluted site. Seasonal variations in superoxide dismutase (SOD) and catalase (CAT) activities were observed over three years in relation to concentrations of trace metals mussel gills and mantle, with SOD activity increasing at least 2-fold at the polluted sites, while CAT activity increased 2-3 times at the polluted sites, both compared to the control site (Vlahogianni et al., 2007). Another study investigated the toxic effects of zinc on antioxidant status and stress proteins in the gills of the brown mussel *Perna perna*, and indicated significant induction of CAT activities (Franco et al., 2006).

However, discrepant results in the literature are frequent regarding metal-induced alterations in these organisms. For example, in a study that conducted both a transplantation experiment of these organisms to a contaminated site and laboratory-exposure study to copper with *Mytilus galloprovincialis* polluted organisms (native, transplanted and Cu-exposed) showed significantly lower levels of glutathione and higher activities of glyoxalases (which detoxify reactive α-ketoaldehydes formed in cellular oxidative processes). However, native mussels from both the polluted and control populations exhibited similar GR, GPx, catalase, SOD and alkaline phosphatase activities, whereas, in both transplanted and Cu-exposed mussels, these enzymes showed significant variations. The authors postulate that these findings could suggest the occurrence of an adaptation or compensatory mechanism in

chronically polluted organisms. In addition, they state that no clear results were obtained regarding GST, and that other esterases (arylesterases and cholinesterases) appeared not to be affected by metal pollution (Regoli and Principato, 1995).

6. MUSSEL RESPONSES TO NANOPARTICLE EXPOSURE

Nanotechnology applications have significantly expanded in the last decades, leading to the inevitable release of nanoparticles, mainly metal nanoparticles, into aquatic systems, resulting in growing environmental and human health concerns (Rocha et al., 2015b). Although very small, nanoparticles exhibit a high surface/volume ratio, providing them with a high reactivity potential and unique physical and chemical properties that differ from conventional forms (Oberdörster et al., 2005).

In this context, research regarding these metal contaminants has increased in the last decades. Most studies expose mussels to both a metal nanoparticle and the dissolved form of the metal, in order to compare mechanisms of action and ecotoxicological effects. For example, *Mytilus galloprovincialis* specimens were exposed to 10 μg L^{-1} of Ag nanoparticles (Ag NPs) and ionic silver (Ag^{+}) for 15 days. Results indicate that both Ag forms, the NPs and ionic form, accumulated in both gills and digestive glands of the organisms. Regarding antioxidant enzymes, activities of superoxide dismutase, catalase and glutathione peroxidase were activated by both Ag NPs and Ag^{+}, and displayed different antioxidant patterns in both gills and digestive glands. Moreover, metallothionein was inducted in gills, directly related to Ag accumulation, while in the digestive glands only a small fraction of Ag seems to be associated with this protein. Thus, the authors indicate that Ag NPs and Ag^{+} cause oxidative stress with distinct modes of action and it is not clear if for Ag NPs the observed effects are due to the free Ag^{+} ions associated with the nanoparticle effect (Gomes et al., 2014).

In another study conducted with the same species, *Mytilus galloprovincialis* specimens were exposed to 10 μg $Cu{\cdot}L^{-1}$ copper nanoparticles (CuO NPs) and Cu^{2+} in gills for 15 days. Results indicate that mussels accumulated copper in gills and responded differently to both copper forms, suggesting distinct modes of action. The CuO NPs induced oxidative stress by overwhelming the antioxidant defense system in gills, while Cu^{2+} exposure led to either unchanged or increased enzymatic activities. MT induction was also observed during both types of exposure, while neurotoxic effects reflected as AChE inhibition were only detected at the end of the exposure period for both copper forms (Gomes et al., 2011).

Tissue-specific responses related to alterations in the antioxidant defense system induced by CdTe quantum dots, in comparison with its dissolved counterpart, using the marine mussel *Mytilus galloprovincialis* have also been conducted. Results indicate that both Cd forms altered mussel antioxidant responses with distinct modes of action, tissue- and time-dependent differences in the biochemical responses to each Cd form, wherein QDs were more pro-oxidant when compared to dissolved Cd. Regarding tissue, the gills were observed as being the main tissue affected by QDs, with effects related to the increase of SOD, GST and GP*x* activities, while dissolved Cd was associated to increases in CAT activity, Cd accumulation and exposure time. The authors report that the digestive gland was the main tissue for accumulation of both Cd forms, but changes in antioxidant enzyme activities were noted as being lower than in gills. The authors conducted a multivariate analysis, that

revealed that the observed antioxidant patterns are tissue dependent, indicating nano-specific effects possibly associated to oxidative stress and changes in redox homeostasis (Rocha et al., 2015a).

Another study on *Mytilus galloprovincialis* evaluated short-term effects on antioxidant enzyme activities and long-term genotoxic and carcinogenic potential of CuO NPs (NPs) in comparison to bulk CuO and ionic copper after 21 days exposure to 10 µg Cu L^{-1}. After exposure, mussels were kept for up to 122 days in clean water. Results indicate that Cu accumulation depended on the form of the metal and on the exposure time, and that the three Cu forms produced different effects on antioxidant enzyme activities in digestive glands, overall increasing antioxidant activities. In addition, the authors report that CuO NPs significantly induced both catalase and superoxide dismutase activities, with fewer effects observed in gills (Ruiz et al., 2015).

However, nanoparticle studies have mostly been conducted on seawater species, with *Mytilus* genus as the main investigated taxa used as a model system (Rocha et al., 2015b). Thus, scarce literature is available on other species, indicating the need for further research in this regard in order to increase knowledge on the ecotoxicological effects of these emerging contaminants.

Some of the few examples found in the literature conducted with other species include a recent study on the toxicological perturbations resulting from zinc oxide nanoparticle exposure in the *Coelatura aegyptiaca* mussel, where the organisms were exposed to ZnO NPs (2, 10 and 50 mg L^{-1}) for 6 consecutive days. Exposure at 10 and 50 mg L^{-1} induced a significant increase in malondialdehyde, superoxide dismutase and nitric oxide, with a concomitant decrease in reduced glutathione, glutathione-S-transferase and catalase levels in the haemolymph, digestive glands and gills of the treated mussels, indicating significant oxidative stress mechanisms of action (Fahmy and Sayed, 2017).

Sublethal effects of nanoparticle exposure in the freshwater mussel *Elliptio complanata* have also been evaluated. On study investigated the effects of CdTe quantum (1.6–8 mg L^{-1}) dot exposure in this species, while another, by the same research group, investigated the effects of increasing concentrations of 20-nm and 80-nm silver nanoparticles, as well as dissolved Ag^+, for 48 h at 15°C. The former observed significant effects on hemocyte immuno competence (phagocytic activity, viability and cell lysis potential), as well as oxidative stress in gills and digestive glands, while a significant decrease in the number of DNA strand breaks was also observed at 4 and 8 mg L^{-1}, indicating inhibition of the DNA repair activity (Gagné et al., 2008). The latter observed that the response pattern of 80 nm nAg was more closely related to ionic Ag^+ than 20 nm Ag, suggesting higher release of dissolved Ag from the 80 nm nAg. The authors, however, also report that all forms of Ag increased MT and LPO levels, suggesting that the presence of ionic Ag^+ leads to oxidative stress. The nanoparticles also induced alterations in other proteins, such as ubiquitin, actinomyosin-ATPase, MT, and DNA strand breaks in the digestive gland in a manner different from Ag^+, while LPO was closely associated with DNA strand breaks in the digestive gland and was not entirely explained by induction of MT, suggesting another type of toxic interaction. It was concluded that the presence of nAg not only increases the toxic loadings of released Ag ions but also generates other and perhaps cumulative effects of nanoparticle-induced toxicity related to size and surface properties (Gagné et al., 2013).

Overall, mussels display significant oxidative stress effects after nanoparticle exposure, and it seems that metals in the nanoparticle form show different mechanisms of action than

those in free form, thus creating increased concerns regarding the effects of these emerging contaminants in the aquatic environment.

7. Necessary Considerations When Applying Mussels as Bioindicators for Metal and Metalloid Exposure in Aquatic Ecosystems

Seasonal metal accumulation variations may be due to several abiotic factors. For example, increased precipitation in the summer may be responsible for pollutant dilution of pollutants and decreased metal concentrations in the water and, consequently, in mussels, while differences in water temperature between summer and winter, which may affect mussel physiology and cause changes in metal concentrations in certain tissues (Szefer et al., 2004). In this regard, several factors have been postulated as the cause of the seasonal variations observed for biomarkers in mussels (Petes et al., 2008), including hyperthermia, hyposmotic stress, temperature and dissolved oxygen (Gourgou et al., 2010; Hamer et al., 2008; Jarque et al., 2014), as well as reproductive events, food availability and water temperature, among other factors (Amiard and Berthet, 1996; Mubiana et al., 2005; Wang and Dei, 1999).

In this context, significant seasonal variations have been reported for several antioxidant enzymes in mussel species, and conflicting results have ensued. For example, a study on the green-lipped mussel *Perna viridis* from Goa indicated that, although the oxidative stress status of gills and digestive gland of *P. viridis* expressed in terms of LPX and H_2O_2 were lowest in February, their levels were maximal in gills and digestive gland during May and November, respectively. On the other hand, SOD and GPX activities were low in August, and CAT and GR activities were low in February. GST activity in gills although remained high in February, while elevated values in the digestive gland were recorded in August and November (Verlecar et al., 2008). in another study, *Mytilus galloprovincialis* from the Gulf of Greece displayed seasonal variations in superoxide dismutase (SOD) and catalase (CAT) activities, as well as lipid peroxidation (LP). SOD activity displayed higher activity in spring, whereas CAT activity was highest activity in winter and spring. LP concentration was twice higher at the polluted sites, following the same seasonal pattern. However, trace metal showed only moderate variations along the months, with a winter maximum followed by a summer pre-spawning minimum (Vlahogianni et al., 2007).

Reproductive efforts, in particular, are known to lead to increases in metabolic activity, ROS and, consequently oxidative stress, as a result of reduced energy available for antioxidant defenses (Petes et al., 2008). Therefore, sampling should be avoided during the reproductive period, which is well documented for several commercial species (Mourgaud et al., 2002).

In addition, mussel development stage may also be a confounding factor. For example, when comparing adult and juvenile *Mytilus edulis* after cadmium exposure for 20 days, juveniles expressed increased GSTpi expression compared to the adults, revealing variations in the expression of this GST isoform among control and Cd^{2+}-exposed mussels of different ages (Ciocan and Rotchell, 2004).

In view of these factors, environmental monitoring studies should take care to conduct sampling efforts under standardized conditions (as much as possible), in order to avoid misinterpretation of biomarker results.

8. MUSSELS AND PUBLIC HEALTH

Bivalve shellfish culture and production for commercial purposes has been noted as a substantial contribution to the economic development of many countries (Santos et al., 2017).

However, the quality of mussels as food is closely related to the sanitary conditions of the marine environment where these bivalves are found (Oliveira et al., 2016), and public health risks associated with metal concentrations in these organisms have been increasingly reported for several species and geographical areas (Jović and Stanković, 2014). For example, in a study conducted with *Mytilus galloprovincialis* mussels sampled along the marine coast of Boka Kotorska Bay, Montenegro, in the Adriatic sea, Cd, Co and Pb levels were recognized as limiting factors for mussel consumption by humans for some Adriatic areas in relation to the provisional tolerable weekly intake prescribed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and the target hazard quotient (THQ) for average and high level mussels consumers in order to evaluate possible alert regarding adverse health effects that may be caused by trace metals individually (Jović and Stanković, 2014). A study conducted on *Perna perna* mussels in southern Brazil compared metal concentrations from 3 contaminated areas from the contaminated Guanabara Bay to a reference site, Ilha Grande Bay, and concluded that mussels from all sampling sites were improper for human consumption due to metal contamination (As, Se, Zn, Pb) according to both Brazilian and international standards, including Ilha Grande Bay, which was previously considered a reference site (Lavradas et al., 2016). Another study indicated that the invasive Indo-Pacific green mussel, *Perna viridis* (Linnaeus, 1758) in Jamaica accumulates significantly high levels of Cr and Cd, and that risks to public health through consumption of these mussels from Kingston Harbour are significant, exceeding the European Standards and Guidelines for Trace Metals in fish and shellfish. The authors suggest that depuration of the mussels prior to consumption should be carried out (Buddo et al., 2012).

The presence of metals in seafood depends on several factors including geographic location, species, size, feeding patterns and metal concentrations in the environment, which, in turn, are affected by many abiotic factors, such as solubility, temperature, salinity, dissolved oxygen and pH, among others (Stankovic and Jovic, 2012). Thus, it is clear that biomonitoring applying mussels as bioindicatores is vital and directly linked to risk assessments regarding human population health, since these animals are a staple diet for many coastal populations, and, in recent years, have become commercially important seafood species worldwide (Stankovic and Jovic, 2012).

It is very important to note that many reports in the literature indicate no risk to human consumers due to low metal and metalloid levels in the digestive gland or gills of certain mussel species. However, these organisms are almost always ingested whole, so levels in this tissue only cannot be used to indicate public health risks, since total levels would be those present in muscle tissue, digestive gland and gills, summed. Thus, care should be taken in this regard when calculating possible public health risks.

CONCLUSION

Mussels are one of the most important bioindicator species applied to date to investigate the effects of metals and metalloids in the environment. Biomarker responses, such as protein and enzymatic alterations, to metal and metalloid exposure are routinely evaluated in several marine and freshwater species, although research on emerging contaminants, such as metal nanoparticles, is still recent. Public health risks associated with metal concentrations in these organisms have been increasingly reported for several species and geographical areas, since metal contamination in aquatic ecosystems has increased in the last decades, and thus, environmental monitoring efforts applying these organisms is paramount to attempt public decision-making processes regarding environmental metal and metalloid contamination.

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Chapter 119

MUSSEL SHELLS' THERMAL VALORISATION AND ODOUR EMISSIONS

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ABSTRACT

Seafood processing industries in the NW coast of Spain generate significant amounts of shell waste. Recent regulations and strategies on waste open up new opportunities to sustainable development through the management and the treatment of aquaculture materials, previously considered as waste. They encourage the recovery of waste and their use as raw materials in such a way that the consumption of resources is reduced and the sources of pollution can be minimised. This paper presents a plant dealing with mussel shell waste as input in its valorisation process, which is considered *a priori* an eco-friendly solution to the disposal of these products. It is a clear procedure to turn waste into a high value-added product like lime, a secondary but sustainable raw material with a market potential to be used in different applications. However, as a result of the thermal treatment of the shell waste, odour emissions to the atmosphere become a serious problem. The pollution caused by these odours produces an environmental and social conflict between the industry and the population of the surrounding area. Accordingly, our work aims at identifying the sources of pollution to seek solutions for the odorous emissions from the installation.

Keywords: seafood processing industry, waste management, odour prevention and control, shell wastes, calcium carbonate

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1. INTRODUCTION

In 2015, Spanish aquaculture generated 293,000 tons of marine products, including 277,000 tons from the cultivation of marine species (94.5% of the total production), and the rest from continental aquaculture (5.5% of the total production). The highest production of Spanish aquaculture corresponds to mussel farming (mainly *Mytilus galloprovincialis*), which in 2015 represented 77% of the total national production with 225,000 tons (FAO, 2017).

Spanish aquaculture stands out in Europe and worldwide for the breeding of bivalve molluscs. In 2015, the production of molluscs in the world was 16,473,112 of tons, which 3.8% corresponded to the European Union (APROMAR, 2017). Galicia, in the north-western coast of Spain, is the world leader in the cultivation of mussels for human consumption, producing more than 212,000 tons in 2010 (Bello Bugallo et al. 2012).

Mussels farmed in floating nurseries are hence the most important product of the sector, requiring neither input of nutrients nor control over its reproduction cycle thanks to the special conditions of the warm water temperatures and high primary production of the estuaries.

Therefore, agro-food industries encompass a group of industrial activities aimed at transforming, manufacturing, preserving and canning foodstuffs, generally from raw vegetal or animal-natured materials. These industrial activities, as any other productive processes, show several features that have a great bearing on the environmental impacts they cause, as follows (Álvarez-Campana et al. 2004):

- The exploitation of raw materials never achieves the 100%.
- The necessary auxiliary materials in the manufacturing process, which are not incorporated in the final product, turn them into waste when they do not gather the essential specifications for their usage.
- The specific operations of a productive process generate emissions.

This is the case of the marine aquaculture and seafood-processing industries that fulfil the above conditions by generating a huge amount of shell waste that could be environmentally sound recovered.

Since the mid-1970s, the EU's environmental policy has been articulated around action programs that establish priority objectives for a period of several years. The current program, which is the seventh of its kind, was approved by the European Parliament and the Council of the European Union in November 2013 and covers the period up to 2020 (EC, 2013). The Program has helped to move towards a policy based on sustainable consumption and production. This highlights the importance of the Thematic Strategies on the Sustainable Use of Natural Resources and on the Prevention and Recycling of Residues (EC, 2013), regulated through the Directive 2008/98/EC (EC, 2008) on waste that includes specific obligations in this matter. Thus, the Member States would develop waste prevention programs with the ultimate objective of decoupling the waste generation from the economic growth. So, the kingdom of Spain has the obligation to have a Waste Prevention Program. On his behalf, Law 22/2011, of July 28, on waste and contaminated soils (Spanish Government, 2011), establishes in article 15 that public administrations, in their respective areas of competence, will approve waste prevention programs, in which the objectives of prevention and reduction

of the amount of generated waste will be established (MAPAMA, 2017). These regulations and strategies on waste point to new opportunities to sustainable development, since they promote the application of environmental technologies and the recovery of waste in such a way they could be exploited as resources (COM, 2005). However, this valorisation process is a source of odours owing to its inherent features. On the other hand, dimensions of nuisance odour provide a commonly accepted basis for the development of jurisdictional criteria of environmental odours (Brancher et al. 2017). Odours emitted by food and waste valorisation industries are even more worrying the nearer the installations are to the residential areas. Nuisance odours are the result of an industrial process where the biological or chemical reactions give rise to odorous VOC (Volatile Organic Compounds) and are frequently accelerated by thermal treatments such as drying or calcination. Generally, the odour threshold in the surrounding area and the sensations it causes are very subjective.

The Integrated Pollution Prevention and Control regulation, the so-called IPPC Directive (EC, 2010) that was updated in 2010 and transposed to the Spanish legal framework by the law 5/2013 on 11 of June (Spanish Government, 2013), has established a framework for assessing and regulating the most polluting industrial activities. The priority of this Directive is to achieve a high level of protection of the environment taken as a whole, especially by the prevention or, where not practicable, the reduction of pollutants, and thus, avoiding the transference from one medium to another (air, water and land). Specific processes that fall under the IPPC scope, like those carried out in plants for treating animal by-products, are required to determine their impact on several criteria, including odour impact. The industrial sectors involved are encouraged to define 'Best Available Techniques' (BAT) and 'Emission Limit Values' (ELV) at European level to achieve a greater efficiency in the environmental management and the performance of these facilities.

This work presents a plant dealing with mussel shell waste as input in its valorisation process, which is considered *a priori* an eco-friendly solution to the problem of disposal of this product. In fact, it is a clear procedure to turn waste into a high value-added product like lime, a secondary but sustainable raw material with a market potential to be used in different applications. However, as a result of the thermal treatment of the shell waste, odour emissions to the atmosphere become a serious problem. The pollution caused by these odours is creating an environmental and social conflict between the industry and the population of the surrounding area. Accordingly, this work aims to identify the sources of pollution to seek solutions and options for the prevention and control of odorous emissions from the installation. This is a new step in the greening process of the existing industrial activity.

1.1. Odour Management

The waste incineration activity in the EU is regulated by Directive 2010/75/UE (EC, 2010). It was transposed to the Spanish legal system by Royal Decree 815/2013 (Spanish Government, 2013), which establishes the design, equipment, building and exploitation conditions for the incineration installations. However, materials as specific as the by-products derived from the mussel industry (mainly mussel shells) are not included into the scope of these regulations. The whole animal carcasses and parts thereof can be considered as unprocessed animal by-products and must be incinerated or co-incinerated in accordance with Regulation (EC) nº 1069/2009 (EC, 2009). This law sets the health rules concerning animal

by-products not intended for human consumption. Should this waste be destined for incineration it will be regulated by law 22/2011 on waste, of July 28 (EC, 2013).

The shell, as part of an animal or product of animal origin not intended for human consumption, is considered a by-product, a category 3 material, as far as section j or section k (i) of article 10 is concerned according to Animal By-products Regulation (EC, 2009). It defines shells as the animal by-products of aquatic animals of establishments or plants that manufacture products for human consumption in section j or as a material of animal that does not present any sign of a disease transmissible to humans or he animals through said material, like mollusc shells stripped of soft tissue or meat. Consequently, their treatment has to comply with the specific provisions imposed by this law for this kind of material. Despite the extensive regulations regarding animal by-products processing, the already mentioned legislation does not include any measure concerning atmospheric emissions control, although waste treatment processes are known to produce both chemical and biological emissions that can be perceived as odour (Aatamila et al. 2011).

Odours have been ranked as the major generators of public complaints to regulatory agencies in North American and European communities (Leonardos, 1995), and it has even been reported that in some European countries between 13% and 20% of the population is bothered by environmental odours (Hudon et al. 2000). These complaints are mainly consequence of the negative effects that prolonged exposure to foul odours can generate on people, causing reactions that range from emotional stress to physical symptoms (National Research Council Committee on Odors, 1979). In these situations, in which an odour is proved to have substantial negative impacts on the quality of life of a community, the odour emissions are considered to be a nuisance (Duffee, 1995).

Currently, there is no specific legislation on odours either in the EU or in Spain. However, efforts towards regulations addressing odour control are being made. One example is the "Horizontal Guidance for Odour" (UKEPA, 2002) from the UK Environment Agency. This is used as a reference for environmental permits in Europe, and also establishes some reference limit values for odorous emissions. Another European example is the "Netherlands Emission Guidelines for Air" (*Nederlandse emissierichtlijn lucht*) (Netherlands Agency, 2004), which proposes the application of emission-abating measures by implementing Best Available Control Technology for reducing air pollution with regard to the ALARA principle (As Low As Reasonably Achievable).

Spain has no specific instruments to legislate on odours at federal level. Generally, odours are treated subjectively in federal environmental laws. The Spanish regulation is still incipient in this regard, and is based on municipal ordinances or activity licences (Brancher et al. 2017). For this, air quality is ruled by Law 34/2007 (Spanish Government, 2007) that in spite of not including any specific regulation about odours, it establishes the bases for preventing, monitoring and reducing atmospheric pollution in order to avoid, and when not possible lessen the damage they could cause to people and environment. The government of the Autonomous Community of Catalonia has been the first Spanish region to develop a draft (Generalitat de Catalunya, 2010) for a future law concerning odorous pollution. It started in 2005 and it is still being updated, as the latest draft version is from 2010. Although this does not fix any ELV, it establishes odour immission objectives for those areas requiring special protection, such as residential areas. These objective limits range between 3 and 7 ou_E (European odour units) calculated as 98th percentile. Areas exceeding these values are considered to be odorously polluted, whereas immission values over 10 ou_E cause nuisances

to people. The draft also proposes reducing odours by implementing techniques candidate to be BAT, as well as good practices over the individual focus.

Against this lack of regulation on odours, some authors develop strategies to control and mitigate them. That is the case of Nicell (2009), who has proposed a framework for the regulation of odours that states that facilities that are identified as sources of potentially offensive odours shall ensure odours concentrations below 1 ou 99,55% of the reference time, which is considered to be 10 minutes. Other authors carried out studies to analyse the origin of odours in order to reduce them. For instance Kim et al. (2008), measured emission concentrations of several compounds from various facilities located in a large industrial complex. They analyse their effect over odour pollution according to two major criteria, namely the industrial type and the source process unit. Another example is Cheng et al. (2009), who performed several odour sampling campaigns after implementing different odour control measures to reduce dimethyl sulphide emissions.

2. CASE STUDY

The shell is produced as a rejected material in different marine aquaculture activities and seafood processing industries such as production companies, mollusc purification plants and boiling facilities and canneries. For the particular case of the mussel, the shell roughly represents between 31-33% of the total weight for this kind of molluscs used in the canning and processing installations aforementioned (Conservas Isabel Galicia S. L., 2006; Barros et al. 2009a). The shell is a composite biomaterial, for which the mineral phase, calcium carbonate, accounts for 95 to 99% per weight, whilst the remaining 1-5% represents organic matter (Barros et al. 2007). This abundance of calcium carbonate in the shells can be exploited to be used for a wide range of applications.

The installation under study was a shell valorisation plant extracting calcium carbonate from mussel shells or other seashells by a thermal treatment to obtain commercial value-added products to be used in diverse applications. The plant, whose theoretical capacity was around 80,000 tons per year, was strategically located in Galicia, near the estuary of Arousa, in the north-western coast of Spain, to absorb the significant amounts of shell waste generated by the production centres located in the coastal areas of the region (Figure 1). It was placed in an industrial park taking up an area of 21,450 m^2. The built-up surface accounts for 5,120 m^2, where the production plant has 4,150 m^2 and eleven-meter high.

Since the beginning, the installation faced some general difficulties related to its environmental behaviour, but also specific problems related to odours, as the process uses as raw material an organic by-product, mussel shells, whose degradation results in odorous compounds among other pollutants. Aiming to improve the performance of the plant, the whole process was qualitatively analysed stage by stage, so that the potential Improvable Flows (IF) were identified and characterised. According to the results of this analysis, some techniques and good practices were proposed and implemented through a first five-year period aiming to "green the process." After this first period, as odour problems persisted, it was suggested a deeper study whose main objective was to qualitative and quantitatively identify and define the potential odour sources in order to select the most appropriate techniques to avoid odour emissions.

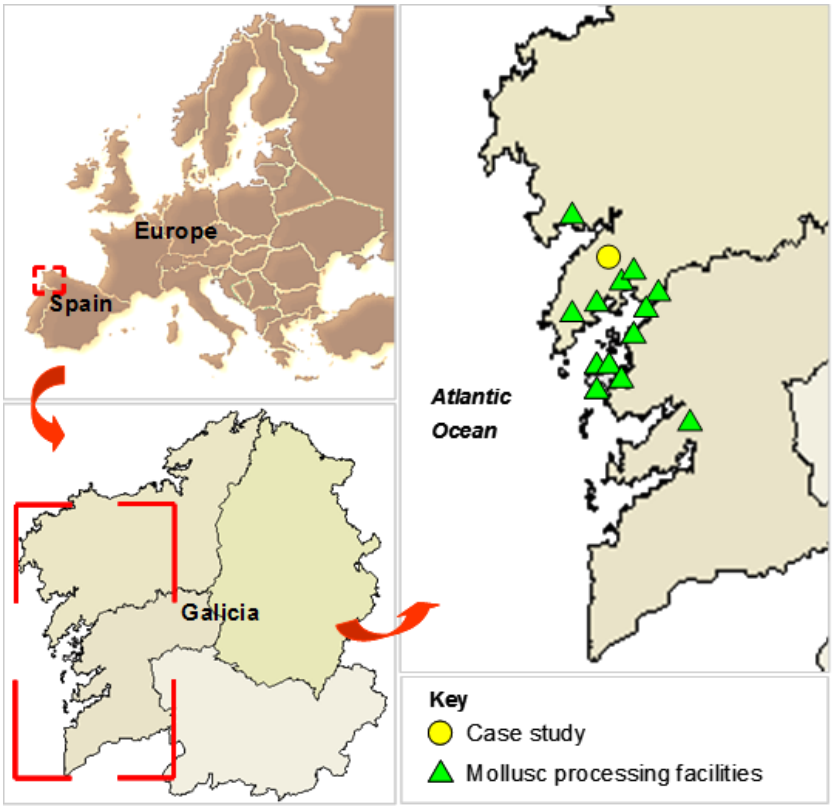

Figure 1. Geographical location of the installation under study and the shell production centres in Galicia.

3. METHODOLOGY

The method used in this work includes three main steps as indicated below.

- *1. Greening the process*. The whole plant was subjected to a greening process due to several facts: a) as a response to the first environmental problems detected in the plant, b) the growing awareness on the impact of emissions over the surrounding areas and, c) supported by the implementation of more restrictive regulations. It implied a deep qualitative analysis of the process following the methodology described by Barros et al (2009b). It continued with the proposal and implementation of several techniques and good practices addressed to improve the environmental performance of the installation. Key improvements were carried out during five years (first period) to reduce the polluting emissions and improve the working conditions in the plant. These results are explained in detail by Barros et al. (2009a).
- *2. Olfactometric analysis. This technique was proposed and implemented in 2007 owing to the persistence of the odour pollution in the plant. It includes three sub-stages:*

a) Qualitative analysis. Identification and justification of the potential odour sources of the plant, according to the process description included in the previous stage (Barros et al. 2009a).
b) Quantitative analysis. Sampling in the identified sources and analysis of the samples according to the European Standard EN 13725 (UNE, 2011). Odour and odorous compounds emissions, namely as VOC (Volatile Organic Compounds), NH_3, H_2S and R-SH, were quantified.
c) Immission analysis. Passive sampling in selected locations (inside the plant and in its surrounding area) to determine the immission concentrations of VOC and NH_3.

- *3. Results. Evaluation and discussion of the results of the olfactometric analysis. It includes two sub-stages.*
  a) Detection of improvable flows. According to the results obtained from the quantification of emissions, the most likely flows to be improved are identified and ranked according to their contribution to the total odour emissions of the plant.
  b) Identification of solutions. Techniques and good practices aiming to avoid, or at least reduce, odour emissions are proposed to each of the identified improvable flows.

3.1. Greening the Process

Greening the shell valorisation process solved several major problems that the plant was facing at that time (Barros et al. 2009a). During a first five-year period, 18 techniques and good practices aiming to improve the environmental performance of the plant were proposed and applied with quite satisfactory results since emissions of some pollutants (mainly dust, CO and VOC) were greatly reduced. Amongst the measures implemented during these years, five of them specifically referred to odour emissions:

- Use of fresh mussel shells as raw material, to reduce the grade of decomposition and minimise VOC releases.
- Hoppers with automatic doors permanently closed, except for loading and unloading. The material should be processed in the same day of its reception.
- Periodic water-cooling of the washed shells stored in the silos to avoid fermentation and VOC releases. Three days will be the maximum storage time in silos.
- Substituting the existing wet scrubber with a more efficient abatement technique. When inherently malodorous substances are produced during the calcination of the shell, the low intensity/high volume gases should go through some abatement equipment, such as a biofilter or an oxidiser, to clean the gas stream where odour prevention is not reasonably practicable. There are several devices to treat VOC emissions that should be considered to collect the waste gas stream by an extraction system prior to the end of pipe treatment. They are divided into recovery techniques, such as membrane separation, condensation, adsorption and wet scrubbers for gas removal and, abatement techniques such as biofiltration, bioscrubbing, biotrickling,

thermal or catalytic oxidation and flaring (EC, 2016). The technique chosen for the plant was a regenerative oxidiser (Figure 2), given the nature of the waste gas stream from the kiln. Besides its content in organic substances (carbon monoxide, VOC and others), the existence of ashes made necessary a bag filter prior to the abatement equipment to firstly scrub the exhaust gases. Thus, both equipments assure not only the removal of particulate matter from the gaseous stream (less than 10 mg/Nm^3), but also the destruction of any existing VOC at temperatures of 800-1000ºC inside the combustion chamber (efficiency higher than 95%).

Figure 2. Bag filter and regenerative thermal oxidiser.

- Monitor all sources of fugitive emissions. Despite the olfactory aggressiveness of some odorous emissions, their concentrations are usually below the detection level of conventional chemical analyses. This measure is deeply developed in the next stage of the methodology.

3.2. Olfactometric Analysis

Determining the odourous compounds is a complex process, as the odour potential of a compound depends on objective aspects such as volatility and solubility, and subjective ones such as physiological and psychological characteristics of the receptor (Bruno et al. 2007). Olfactometry is a technique for sampling and analysing odours, coupled with dispersion modelling, which allows evaluating the nuisance caused by odours and determining their origin. Olfactometric studies are a useful tool to control and reduce odours emitted by different types of sources as they do not only determine the degree of nuisance created in the environment, but also identify odour sources. This permits the adoption of effective elimination or reduction measures.

The olfactometric analysis performed at the installation includes three stages that will be discussed below, namely qualitative, quantitative and immission analysis.

3.2.1. Qualitative Analysis

A general flow sheet of the valorisation process is shown in Figure 3 as part of the mussel shell life cycle from de reception of de mussel shell to the exit of the $CaCO_3$. The valorisation process carried out in the plant can be divided in three main stages, such as preliminary operations, processing and auxiliary operations. Preliminary operations consists in the reception and unloading of the raw materials, in addition to those operations involving the elimination of salt and mud contained in the shells and the storage prior to their processing. Processing involves the thermal treatment of the shells and the subsequent cooling of the burnt material and, finally, auxiliary operations, which are additional operations such as milling and sorting, that prepare the final product for marketing. Other operations are the final product storage and its packing and shipment.

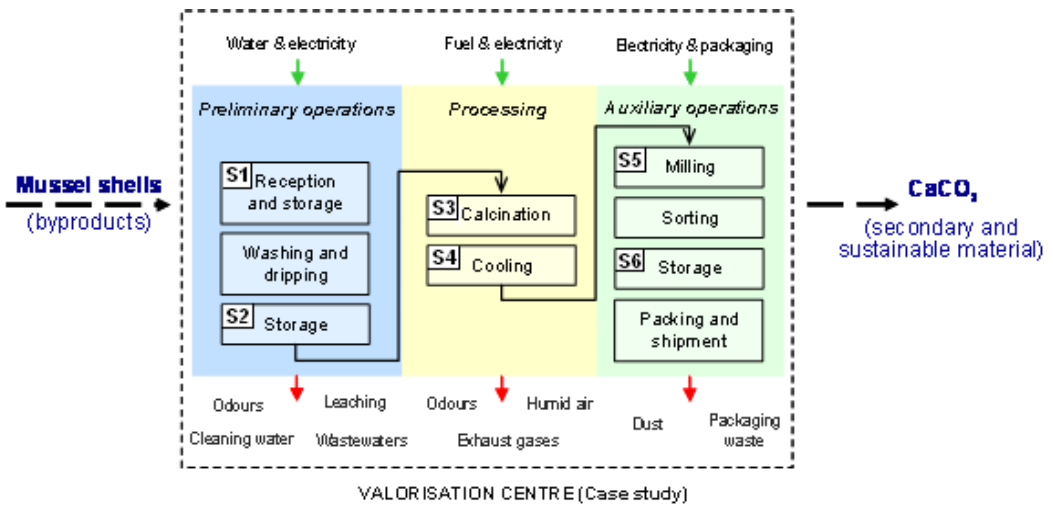

Figure 3. Flow sheet for the mussel shell valorisation process. S1 to S6 indicate the sampling points for quantitative olfactometric analysis in Table 2.

Considering the analysis of the process developed in a previous work (Barros et al. 2009a), the potential odour sources have been qualitatively identified. They mainly correspond to the storage of raw material prior to its processing and to thermal treatments, where VOC derived from the destruction of the organic fraction are released. The selected points, as well as their effect over the odorous emissions released by the plant, are described below:

- Reception and storage. Raw mussel shells are unloaded in two ground-level reception hoppers where they remain until they are processed (the same day they are received). The transport of raw materials from the production centres to the valorisation plant strongly influences the emission of odours. A key parameter to consider is the shell lifetime at the time of collecting, as it has a remarkable importance in the degradation state of the waste. The flesh attached to the shell is highly perishable and generally requires either rapid storage and containment or quick processing to avoid decomposition and, consequently, odour emission and dispersal of pathogens. Other parameters to take into account are the climatic conditions (sunny and hot weather

favours faster reactions), as well as the cleaning of the vehicles and the storage time of the shell in the production centres.

- Washing and dripping. This step consists in cleaning the shell with freshwater to reduce the salt content of the final product, avoid the wear of the equipment by corrosion and obtain a more concentrated product of $CaCO_3$. Therefore, it is very demanding on water and accounts for 80-90% of the total water consumption inside the installation. This activity is carried out in two rotary washing machines operating counter currently to increase the performance of the salt extraction. Afterwards, there is a shaker draining rack to remove the water dragged by the product. Then, wastewaters and mud waste go to the on-site wastewater treatment plant (WWTP) where they undergo a physicochemical treatment.
- Storage. After washing, clean mussel shells are stored while they complete their dripping in stainless steel silos that are loaded by gravity and unloaded by a vibrating system to favour the thermal treatment. The washed shells stay here at most for three days. The leachates generated here are channelled to the WWTP for its treatment. Odours released in this stage are mostly related to the dripping of the washed shells, which generates leachates containing some remains of the dirt and organic matter that embedded the raw mussel shells received in the plant.
- Calcination. Mussel shells are burnt in a counter-current rotary kiln that integrates two processes, drying and calcining, in a single piece of equipment. The kiln, whose production capacity is 18 tons per hour, is 17 meters long and 2.5/3.0 meters diameter, and it is divided in three sections (Figure 4). The residence time of the material depends on the spin velocity of the kiln, but it ranges between 20-30 minutes. Four thermocouples (T1-T4) register and monitor the temperature in different sections inside the kiln.

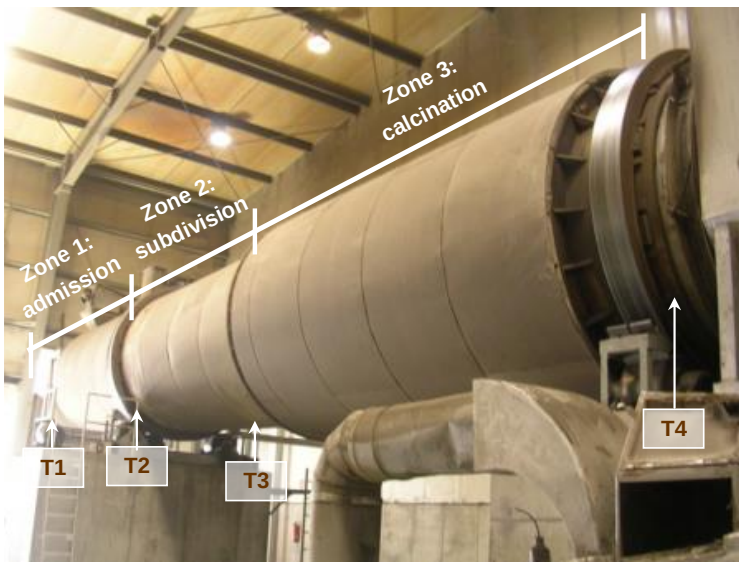

Figure 4. Rotary kiln. The four thermocouples indicate the following temperatures: T1 (125-250ºC), T2 (225-250ºC), T3 (300-325ºC) and T4 (475-500ºC).

Calcination is an important source of odours since its operating temperature ranges between 125-500ºC. This is not enough to destroy odorous compounds, as incineration processes with temperatures below 850ºC can result in odour emissions (EC, 2006). The gases emitted by the kiln during the analysed mussel shell valorisation process show the following features:

- Volume of flow: 14,000 Nm^3/h
- Temperature: 130-150ºC
- Moisture: 17% (vol.)
- VOC (as TOC) concentration: 650 mg/Nm^3

In spite of being hardly detectable concentrations of organic substances, the released gases are aggressive olfactory emissions of an intense and unpleasant smell and with high temperatures. As a result of the "greening the process" stage, an abatement technique for odorous compounds was implemented. This technique combines a bag filter, which assures the removal of particulate matter from the gas stream (<10 mg/Nm^3), with a regenerative oxidiser that destroys more than 95% of the VOC operating at temperatures that range between 800-1000ºC.

- Cooling. The burnt material is cooled down from 500-600ºC to 60ºC through a two-step process. It begins with the injection of thinly dispersed water to reduce temperature from 500-600ºC to 170-190ºC, and finishes with air refrigeration, that completes the reduction of the temperature from 170-190ºC to 60ºC.

Both the leachates from water injection and the refrigeration air could drag some organic compounds still stuck to the burnt shells that may result in odour emissions.

- Milling. Milling is aimed at crushing the shell to obtain products with purity levels of 90-95% in CaCO3 and different sizes of particle that will determine its industrial application. This stage includes diverse operations to obtain different particulate sizes (Table 1), namely grinding, screening and micronizing.

This process mainly generates dust and particulate matter emissions that are conducted to the dust abatement equipment that recovers dust and reincorporates it to the process. Besides dust, VOC can also be released during milling processes. Mostly because of some remains of organic compounds that were not totally destroyed during the thermal processes.

- Storage. After sorting the milled shells according to their particulate size, the sorted fractions of the final products are stored in three adjustable hoppers with regard to its grain size. From them, the products can be stored either in two enclosed warehouses or in silos depending on the final use of the product or the packaging format in which it is marketed.

Despite the high quality of the final product (90-95% of $CaCO_3$), some VOC can still be released during storage.

- Packing and shipment. The final products are packaged in bulk (tanks or containers) or big bags of 35 kg and 1 tons, and shipped off for sale.

Table 1. Particle size related to milling operations

Milling operations	Particle size (diameter)
Grinding	0-8 mm
Screening	<2 mm
	2-4 mm
	>4 mm
Micronizing	<63 μm

3.2.2. Quantitative Analysis

The quantitative olfactometric analysis was done according to the procedure described by the European Standard EN 1372:2004 ERRATIUM:2011, Air quality – Determination of odour concentration by dynamic olfactometry (UNE, 2011), which provides a reference method for sampling and assessing odours, whose concentrations are calculated and expressed in European odour units (ou_E/m^3).

The sampling points (S1 to S6) shown in Figure 3 correspond to the qualitatively identified potential odour sources (Table 2).

The samples taken from the identified sources were analysed by dynamic olfactometry, procedure PE-OLF/007 (UNE-EN 13.725), which resulted in odour concentrations for each sample in ou_E/m^3 (Table 3). From these results, the punctual odour emissions (ou_E/h) were calculated using data about the gas flows and the exposed area in each focus. Furthermore, for all the odour sources, sulphide, ammonia and mercaptan concentrations were assessed with Dräger colorimetric tubes. Ammonia results were confirmed by measurement with a specific electrochemical detector, Gas Alert Micro 5Pid (BW Technologies, Calgary, Canada).

Table 2. Process stage points sampled for the quantitative olfactometric analysis

Sample		Process stage
S.1		Reception and storage
S.2		Storage (in a silo)
S.3	S.3.1	Calcination (exhaust gases from the thermal oxidiser)
	S.3.2	
S.4		Cooling
S.5		Milling (shed)
S.6		Storage (shed)

Table 3. Odour emissions and odorous compounds emissions in the sampled points

Sample	Total flow (m^3/h)	Odour concentration (ou_E/m^3)	Punctual odour emissions (ou_E/h)	Concentration (mg/Nm^3)		
				NH_3	H_2S	R-SH
S.1	1,436	228	0.33	<0.25	<0.20	<0.50
S.2	1,044	512	0.50	4.00	<0.20	<0.50
S.3.1	15,751	96,755	1,524	634.00	<20.90	<52.30
S.3.2	16,272	89,276	1,453	714.00	<20.90	<52.30
S.4	15,906	<1,845	<29.30	<0.25	<0.20	<0.50
S.5	17,848	51	0.90	<0.25	<0.20	<0.50
S.6	41,324	181	7.50	2.00	<0.20	<0.50

For sample 3 (S.3.1/2), considering the odour concentrations obtained for each sample, an average odour concentration value is calculated, resulting 93,015 ou_E/m^3. The airflow is calculated considering the gases output velocity and the section area in the sampling point. The gases output temperature was 174.2ºC and 180.3ºC respectively, and the pressure 1,013 mbar. The average odour emission calculated using the data obtained for both samples is 1,488 ou_E/h.

The characteristics of the mussel shell drying/calcining gases depend on the nature of the VOC they contain. Thus the odours they release can be different in type and intensity. The gas stream resulting from the drying/calcination stage has been analysed to identify the most abundant and odorous VOC present in the gases released during the thermal treatments. The analysis focuses on the most odorous compounds shown in Table 4, where two different samples are compared:

- Sample A, taken and analysed in 2004, before implementing the abatement equipment for the gas stream of the kiln (bag filter – regenerative oxidiser). The sample corresponds to the gas stream emitted by the calcination kiln.
- Sample B (duplicated), as part of the present sampling, taken after implementing and operating the bag filter and the thermal oxidiser. The samples correspond to the gas stream emitted by the thermal oxidiser.

Table 4. VOC concentration in samples taken before and after implementing the thermal oxidizer

VOC	Concentration (mg/Nm^3)		
	Sample A	Sample B.1	Sample B.2
1,1-dichloroethene	-	3.27	6.38
Trans-1 ,2-dichloroethene	-	23.50	7.52
Benzene	4.07	5.93	5.00
1,2-dichloropropane	-	<0.10	1.370
Toluene	1.85	17.90	15.20
Tetrachloroethylene	0.036	<0.10	<0.10
Ethylbenzene	0.038	5.64	2.99
m,p-xylene	0.071	6.22	12.30
o-xylene	0.025	1.22	2.24
Styrene	0.017	0.64	0.38
Isopropylbenzene	-	0.12	1.06
1,2,3-trichloropropane	-	<0.10	0.58
n-propylbenzene	-	0.16	2.51
1,3,5-trimethylbenzene	0.005	0.22	0.84
Carbon disulfide	0.180	<0.10	<0.10
Ethyl acetate	<0.00005	<0.10	<0.10
Butyl acetate	<0.00005	<0.10	<0.10
1,2,4-trimethylbenzene	-	1.05	2.48
Dimethyl dilsulfide	1.57	<0.10	<0.10
α-pirene*	0.024	2.46	76.70
Hexane	0.128	-	-
C_4H_6 (probably 1,3-butadiene)	1.95	-	-
Acetone	3.87	-	-

* Semi-quantitatively analysed with respect to the areas corresponding to a pattern of benzene and chloroform (125 $\mu g/m^3$).

The gas output from the drying/calcination process in the case study is composed by a big amount of substances (mainly VOC) characterised by their odorous intensity, and with really low odour thresholds. The analysis results show quite high concentrations of some VOC, such as benzene, acetone, toluene and C_4H_6. For most of the compounds, the concentrations were found to be <0.10 mg/Nm^3, although for some other compounds the concentrations were quite higher (Table 4).

As it can be seen in Table 4, the observed differences in the results of VOC emissions in 2004 and 2007samples indicate malfunctions, not adequate techniques or abnormal operating conditions, among others.

3.2.3. Immission Analysis

Passive sampling was used to determine the immission concentrations of VOC and ammonia in different locations. The procedure used for sampling was UNE-EN 13528-1/2, ambient air quality: diffusive samplers for the determination of concentrations of gases and vapours (UNE, 2011). Twelve sampling points were selected from the plant and its perimeter to evaluate the effect of odorous compounds over the environment. The sampled points were:

P1: Truck unloading area
P2: Perimeter fence (close to entrance)
P3: Outer road
P4: Perimeter fence (close to fuel tank)
P5: Outflow of the air conditioner of the offices
P6: Reception
P7: Silos ship (6 m high)
P8: Silos ship (12 m high)
P9: Calcination ship
P11: Final product storage ship
P12: Sewage

Ammonia was determined by water sensor extraction according to the Radiello protocol followed by indophenols colorimetric analysis (Spectrophotometer Shimadzu UV-1603). On the other hand, VOC samples are taken with a graphitized carbon cartridge, which thermally desorbs using a thermo-desorption system directly coupled to a gas chromatography (Agilent 6890N) mass spectrometry detector (Agilent 5973).

The immission results (Table 5) show quite high NH_3 concentrations, especially in those sampling points where process equipment is located. The same applies to VOC emissions, although sampling point P4 shows quite big immission concentrations of certain VOC, owing to the fact that fuel is stored in that area. These results show that although VOC and NH3 concentrations may be quite high in the areas where the proper process takes place, they are reduced with the distance.

Table 5. Immission concentrations of VOC and NH_3

Compounds		Immission concentration (μg/m^3)										
		P1	P2	P3	P4	P5	P6	P7	P8	P9	P11	P12
VOC	Toluene	-	*	1.51	0.06	0.21	10.20	0.92	263.00	6.29	8.80	2.48
	Benzene	-	*	0.05	0.19	0.25	2.44	0.17	11.70	3.26	1.90	0.31
	Ethyl-benzene	-	*	1.27	16.00	0.47	3.67	5.54	195.00	1.47	3.08	2.37
	m,p-xylene	-	*	0.21	10.50	0.79	4.96	2.45	124.00	9.93	2.84	3.04
	o-xylene	-	*	0.92	10.90	0.43	3.07	8.56	232.00	7.67	1.38	4.23
	Styrene	-	*	0.47	0.99	1.25	25.70	0.32	3.27	49.10	15.20	0.84
NH_3		69.40	0.10	4.00	24.50	-	57.60	55.80	53.60	63.50	56.90	6.50

* Problems in the chromatograph injector made it impossible to analyse the sample.

3.3. Results

3.3.1. Detection of Improvable Flows

According to the results obtained from the olfactometric analysis (Table 3), the most important odour sources are the thermal treatments, as calcination has been pointed out the most odorous process by far. The storing areas are the next most odorous sources, being the storage of the raw materials more odorous than the storage of the final products. Finally, the analysed stage generating less odour emissions is milling. This ranking is supported by the results obtained from the analyses of NH_3, H_2S and R-SH concentrations, as calcination showed by far the highest concentration for all the compounds, whereas the rest of the sampled processes presented concentrations below the range in most of the cases. Consequently, calcination is the most important improvable flow detected in this process, as the odour concentration of its gas stream is more than fifty times bigger than the next most polluting source, namely cooling. However, in spite of these great differences between the sampled points, none of them are negligible when compared with the "relative offensiveness of odours" proposed in the Horizontal Guidance for Odour (UKEPA, 2002). Here it is suggested that offensive odours, such as the ones derived from activities involving putrescible waste or from processes involving animal of fish remains, can cause annoyances if they exceed 1.5 ou_E/m^3 98th percentile (hourly average concentration for odour measured at the nearest odour sensitive receptor beyond the facilities boundary), which is by far exceeded by all the analysed samples.

The stages likely to generate some leachates have been found to be quite odorous, as storage after washing (where the raw materials drips water), reception and storage of raw materials and cooling. According to this conclusion, the wastewater treatment plant should have been included as an out of process sampling point, as it may be a potential odour source. But its odour emissions have not been included into the scope of this paper because it is only focused on the mussel shell valorisation process itself. However, some techniques and good practices will be proposed to mitigate odour emissions from the wastewater treatment plant.

3.3.2. Identification of Solutions

Although diverse technologies to control odour nuisances have been developed or improved (EC, 2005), the best method to manage this type of emissions is the elimination of the odorous components before their formation, thus avoiding a further treatment of the waste gas stream. Nonetheless, most of the waste valorisation installations cannot have an exhaustive control over all the VOC emitted during their processes.

Considering the improvable flows detected after evaluating the results of the olfactometric analysis, some measures and alternatives aiming to prevent and/or limit the odours in the installation have been selected and proposed for their implementation in the plant. They are classified according to the process stage where they should be applied.

3.3.2.1. Reception and Storage of Raw Materials

- Collect the shells as soon as possible after their production. This measure can be easily implemented due to the strategic position of the objective plant with regard to

the production centres (Figure 1), so the shell arrives to the plant within 48 hours after its rejection.

- Raw materials should be transported in hermetically sealed containers to prevent the emission of liquid effluents. Waste should be saved dry whenever possible to avoid increasing odour problems.
- Containers, packaging and vehicles used for transporting unprocessed material must be cleaned in a specifically designed area.
- The floor in the storage facilities should be waterproof and extraction systems should be installed to collect residual gases for further treatment.
- Installations and equipment must be regularly cleaned and disinfected.
- Preventing anaerobic conditions by aeration, turning of waste and short timescales (UKEPA, 2002).
- Reduce temperature during storage by avoiding direct sunlight or otherwise reducing the water evaporation rate and the release of dissolved odorous chemicals (UKEPA, 2002)
- Minimise airflow over the surface of odour-releasing materials, which also reduces the rate of evaporation (UKEPA, 2002).
- The shells must be processed immediately after their arrival to the plant, otherwise stored properly until processed in enclosed buildings with sealed hoppers to avoid the emissions outside the facilities. In the plant, the maximum storage time in the hoppers is the same day they are received to avoid the decomposition of the remaining flesh attached to the shells.
- The shells are stored in silos up to three days after washing to avoid fermentation processes and the release of odours. But if fermentation occurred, the shells should be washed to control the achievable temperatures (up to 70ºC).

3.3.2.2. Thermal Treatment

- The shell must be placed straight in the kiln without any direct handling in order to prevent kiln opening during loading operations that favours the release of residual gases. The input of shell to the kiln is done and regulated by a dosing machine.
- Adjust the main parameters of the process, such as temperature, duration, pressure or venting, to reduce formation and/or release of odorous compounds (UKEPA, 2002).
- Contain odorous air within process machinery to avoid contaminating larger volumes of ventilation air within buildings, which can consequently reduce the cost of air treating (UKEPA, 2002).
- Enclosing odorous waste all the way to the thermal treatments stage (UKEPA, 2002).
- Drawing air from odorous areas at a rate that will ensure that odour is captured and feeding, as far as possible, odorous air into the combustion process (UKEPA, 2002).
- Control the temperature up to 850ºC ensuring that is maintained at all times during operation and as long as unburned material is inside.
- The gas resulting from the process is heated, in controlled conditions, to a temperature of 850ºC for two seconds. As previously explained, the temperatures inside the combustion chamber achieved this figure.

3.3.2.3. Final Product Management

- Avoid odours emissions by keeping windows and doors shut in process building, by implementing remotely operated roller doors that reduce the time that doors are open or by maintaining negative pressure within the process plant (UKEPA, 2002).
- Good housekeeping to avoid the appearance of malodorous materials and wastes except in designated (and appropriately managed) areas (UKEPA, 2002).
- Suitable and sufficient staff training with regard to odour control. Staff should also be aware of relevant licence conditions and emission limits in its work (UKEPA, 2002).
- Buffer zones in areas where odour is emitted at low level (UKEPA, 2002).

3.3.2.4. Waste Water Treatment Plant (WWTP)

- Prevent wastewater stagnation by laying the pipelines related to the WWTP with a certain inclination.
- Subject the leachates and the effluents to a biological treatment process, aerobic or anaerobic. At present, the treatment carried out in the plant consists on a physicochemical treatment including solid screening, homogenization and dissolved air flotation, where the flocculation-coagulation processes take place. Once the flocculates are separated, they are transferred to a sludge pit and then they are thickened and dewatered in a centrifuge. However these processes are not enough to comply with the emission values set by the Competent Authorities due to the high organic load in the wastewaters. For this reason, a biological stage of nitrification-denitrification is planned to be included in the WWTP for further treatment of the wastewaters. This stage is currently starting up and no data is available, but it is expected to meet the allowable discharge limits. Furthermore, water consumption in the most water-demanding step, the washing of shells, is minimised as cleaned water is fed back to this operation.

CONCLUSION

The case study of this work is a pioneering industrial activity on thermal valorisation of mussel shells reutilizing wastes as resources. This makes every unexpected technical fault is faced up to without any previous background. Therefore, the organisation is a source of new experiences regarding technological development.

Getting the environmental permit required to operate according to the provisions of the IPPC is a driving force to audit and monitor odour emissions, reduce their generation to the surrounding area by means of the aforementioned measures and best environmental practices and upgrade the operating conditions of the equipment and the facilities. Nevertheless, despite the efforts carried out over the years in order to improve the productive process, hard work is still going to be addressed to prevent and/or control the environmental impacts derived from shell valorisation.

In spite of the variety of prevention and abatement alternatives proposed, odour problems are better dealt with at source, by means of process design or modification, or even managing the activity differently (UKEPA, 2002). Therefore the best odour management program for the process analysed, mussel shell valorisation, should include a complete re-design of the process, taking into account the conclusions extracted from this work, which has pointed out thermal treatments as the most odorous processes, followed by raw materials storage. Operating procedures should also be updated to eliminate those practices that can affect the total odours emissions released by the plant.

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Chapter 120

DETERMINATION OF EMERGING METAL POLLUTANTS AND TOXIC METALS IN MUSSELS AND BIVALVE MOLLUSKS, VERY IMPORTANT FOOD AND ENVIRONMENTAL BIO-MONITORING SPECIES

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ABSTRACT

A quick and widespread diffusion of heavy metals as contaminants in all the environmental systems has called the attention to their determination. Indeed, heavy metals, together with pesticides, are very dangerous pollutants owing to their bioaccumulation and toxicity. It is, therefore, necessary to determine these metals at trace and ultra-trace level especially in aquatic ecosystems to establish reasonable water quality criteria.

Certain marine species, in particular mussels, clams, but also oysters accumulate toxic metals, being filtering organisms. It was verified that an adult organism is able to filter several liters per hours (also up to 4-5 L h^{-1}), depending on its weight.

This prerogative involves two important facts and consequences:

1. The ability to accumulate all harmful substances for humans, toxic metals, in particular, requires particular attention and inspections before being sold on the market.
2. In addition to this important and fundamental aspect of public health, the determination of toxic metals in mussels, clams and also oysters, that are not only filtering organisms but also sessile species, can be usefully employed for bio-monitoring campaigns, that evaluate the long-term trend of the pollution load of an aquatic ecosystem, information that evidently cannot be provided by punctual

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determinations. For completely mapping environmental pollution, the sampling duration and cadence are very important. However, it should be emphasized that the use of bio-monitors, just proposed by several authors, but certainly not scientifically supported, is possible only in the case of a long sampling plan.

In any case, the metal determination in mussels and bivalve mollusks evidently must be accurate, reproducible and especially it must show very low limits of detection.

The present work reports and discusses the different analytical methodologies for the determination of emerging metals pollutants, together with all toxic metals, in mussels, clams, and oysters.

Keywords: mussels, bivalve mollusks, toxic metals, instrumental determination.

INTRODUCTION

Metals are an integral part of the food chain, and everything is ingested by humans contains metals. Metals (that can have natural or artificial origins, the latter including also criminal acts) may become very dangerous in several cases, sometimes with effects irreversible for human life. Therefore, it is important to characterize and quantify these species also at trace and ultra-trace concentration levels, that is often enough for several metals to give high risks of toxicity to humans [1-3].

Moreover, toxic metals enter in the food chain by accumulation in certain marine species. In particular mussels, clams, and oysters, but also shrimps, algae and fishes, were found to sequestrate and concentrate metals from the aqueous environment. It is clear that these species, being part of the food chain, may be dangerous for human health. In particular mussels, clams and oysters are filtering organisms − an adult organism can filter up to 5 L h^{-1}, depending on weight, size, and water temperature −, thus they require special attention and surveys before placing them on the market.

Furthermore, it should be emphasized another aspect: mussels, clams, and oysters, which are also sessile species, may be employed as interesting bio-monitors of metal pollution in limited ecosystems [4-13]. Up today, it has not been described a rigorous correlation between pollution of the aquatic ecosystem and toxic metal content in bio-monitor species. Anyway, it is at least very close to the truth the statement "*high concentration of toxic metals in bio-monitor species most likely implies a strong pollution of their aquatic ecosystem*." The use of bio-monitors is probably the only possibility to obtain a long period environmental information and to evaluate the pollution trend of an aquatic ecosystem in the long-term; clearly, such information cannot be provided by punctual determinations: single samplings at different times to quantify metal contents in water can give only punctual information, without any information on the concentrations trend in the considered ecosystem. Instead, the pollutants determination in organisms living for a long period in a certain ecosystem, give more reliable information about the previous history of that ecosystem. In any case, it should be highlighted that, to obtain a complete environmental pollution information from bio-monitoring samplings, the sampling duration and cadence are extremely important.

The use of bio-monitors (mussels, clams, and oysters in the present case), proposed by many authors, is probably possible only with a long sampling plan. Obviously, this is a

disadvantage, but perhaps it is also the only way to get long period environmental information.

The determination of toxic metals in mussels, clams, and oysters, both to establish a food quality and to be employed for bio-monitoring, must certainly be accurate, with very low detection limits.

This review provides the results of a research line followed for several years in our laboratories, i.e., the determination of toxic metals in environmental matrices involved in the food chain including mussels, clams, and oysters.

For each metal or group of metals, all instrumental techniques used for their determination in filtering organisms are also reported.

Together with this purely instrumental part, the possibilities to test and monitor the pollution load from toxic metals of an aquatic ecosystem using these species as bio-monitor organisms will also be highlighted.

Finally, last but not least, the use of these species in bio-remediation processes of aquatic ecosystems will be underlined and discussed.

Moreover, a new section has been inserted. It regards the thallium, a polluting metal of new generation, poorly considered for a long time, but recently subject of great interest from many researchers, owing to its increasing diffusion in the environment and very high toxicity.

Of this metal will be reported the most significant works relevant to its determination in filtering organisms, and will be described in detail also the analytical procedure developed by our research group for its voltammetric and spectroscopic determination in mussels, clams, and oysters.

Our Proposals concerning voltammetric and spectroscopic determination of toxic metals in bivalve mollusks is the following.

From about two decades, our research group is interested in the development of analytical methods for voltammetric and spectroscopic determination of toxic metals in matrices involved in the food chain. Among these, certainly mussels, clams, and oysters are particularly important. For these matrices, and in an extremely concise way, the experimental conditions proposed by us and the results obtained [1, 14-29] are summarized in the following.

EXPERIMENTAL

Reagents, Reference Solutions, and Standard Reference Materials

All acids and chemicals were supra-pure grade (Merck, Germany). Acidic stock solutions of all the elements (1000 mg L^{-1}, Sigma-Aldrich, Germany) and Osmium tetroxide solution (4 wt% in water, Sigma-Aldrich, Germany) were employed in the preparation of reference solutions at varying concentrations, using for diluting water demineralized through a Milli-Q system.

Mussel Tissue BCR-CRM 278 (from Institute for Reference Materials and Measurements, European Commission, Joint Research Centre, Geel, Belgium) and Oyster Tissue NIST-SRM 1566a (from National Institute of Standards and Technology, Gaithersburg, MD, USA) were employed as Standard Reference Materials (SRMs).

Sampling Area and Sampling Sites

Mussels, clams, and oysters were sampled in the Goro Bay (Province of Ferrara, Italy; salinity: 2.7%; average temperature from June to September: 23-27°C). This is a very important area devoted to the fishing and breeding of mussels and clams for food, but with considerable pollution problems due to its location in proximity of the Po River mouth, which is the carrier towards the sea of a large amount of industrial and domestic wastewater.

Moreover, the waterways are also strongly influenced by atmospheric pollution load and then, they result to be also carriers of air pollutants, especially including those related to vehicular traffic as in particular the platinum group metals (PGMs).

In the Goro Bay flow three branches of the Po river delta mouth, i.e., Po of Volano, Canal Bianco and Po of Goro.

Samplings of bivalve mollusks were carried out in front of the points where such branches flow into the bay and in the central area of the bay itself, while an additional sampling, for eventual comparisons, was chosen at open sea.

All samplings were performed using a rubber boat without a motor to avoid any kind of pollution.

Sample Preparation before the Instrumental Determination

About *7-8 kg of Mytilus galloprovincialis* and of *Tapes philippinarum*, and 5-6 kg of *Crassostrea gigas* were collected in five sites (see section 2.1.2 “Sampling Area and Sampling Sites”), four within the mouth of the Po river (Goro Bay, Italy) and the fifth at open Adriatic Sea, taken to the laboratory and prepared for analyses.

They were opened with a plastic appliance and the organisms were carefully extracted and placed in polyethylene containers, previously treated with supra-pure HNO_3 diluted in 1:1 proportion with water and followed by repeated rinsing for 48 h with Milli-Q water in order to avoid any contamination. The samples were frozen and then lyophilized for 30 h. After that treatment, the samples were homogenized thoroughly in an agate mortar.

For all three kinds of real samples (mussels, clams, and oysters), the same sample mineralization procedure was carried out.

The sample preparation of Mussel Tissue BCR-CRM 278, Oyster Tissue NIST-SRM 1566a and of real samples of mussels, clams and oysters was the following: approximately 1.0 g, accurately weighed, was placed in a platinum crucible and dissolved in 5 mL 69%$_{w/w}$ HNO_3 + 4 mL 37%$_{w/w}$ HCl + 6 mL 98%$_{w/w}$ H_2SO_4 at 130-150°C. The mixture was evaporated to dryness and, after cooling, soluble salts were dissolved in 25 mL of the electrolyte employed in the determination of the different metals.

Voltammetric Procedure

All voltammetric curves were recorded by an Amel Model 433 multipolarograph, employing a conventional three-electrode cell. As for the working electrode, a hanging mercury drop electrode (HMDE) was employed for the determination of Cu(II), Pb(II), Cd(II) and Zn(II) [14-16, 20-22, 24-27], Sn(II) and Sb(III) [15, 16], As(III) and Se(IV) [16, 24, 26],

Pt(II), Pd(II), Rh(III) [16], Os(VIII) and Ru(III) [16, 17, 19], a gold electrode (GE) (surface area: 0.785 mm^2, AMEL, Milan) was employed for the determination of Hg(II) [14-16,20-22], and a glassy carbon electrode (GCE) (surface area: 7.065 mm^2, AMEL, Milan) was employed for the determination of Ir(III) [16, 18]. An $Ag \mid AgCl \mid Cl^-_{satd.}$ electrode and platinum wire were chosen as reference and auxiliary electrode, respectively.

The Teflon voltammetric cell was rinsed every day with supra-pure concentrated nitric acid, diluted 1:1, in order to prevent any contamination. Standard additions were made by disposable plastic tips.

Keeping the temperature at 20.0 ± 0.5°C, the solutions were deaerated by water-saturated pure nitrogen for 5 min prior to measurements, while a nitrogen blanket was maintained above the solution during the analysis. The solutions were deaerated after each standard addition for 1 min. In the electrolysis step, the solutions were stirred using a magnetic stirrer.

Spectroscopic Procedure

Atomic absorption spectrometric measurements were performed using a Perkin-Elmer Mod. A-Analyst 400 Atomic Absorption Spectrometer, equipped with a deuterium background corrector, Autosampler AS-72 and with HGA 800 graphite furnace. Single-element Lumina (Perkin-Elmer) hollow-cathode lamps were used. All measurements were carried out after studying the relevant ashing and atomization curves for each element [30], at the instrumental conditions suggested by manufacturer.

Except Hg(II), As(III), Se(IV) and Sb(III), which were determined by Cold Vapour Atomic Absorption Spectrometry (CV-AAS), all the elements were determined by electrothermal atomic absorption spectrometry (ET-AAS), employing argon flow at 300 mL min^{-1} in all steps except during atomization (60 mL min^{-1}).

Limits of Detection

In the aqueous reference solution, in the solutions obtained by digestion of the standard reference material and of mussels, clams, and oysters sampled in the Goro Bay, the limits of detection (LOD_s) for both techniques (voltammetry and spectroscopy) were obtained by the equation $LOD = K\, s_{y/x}/b$ [31], where $s_{y/x}$ and b are the estimated standard deviation and the slope of the analytical calibration function of each element, respectively, with a 99.7% (K = 3) confidence level [32].

In the case of voltammetric technique, since the analytical calibration functions were determined by standard addition method, it was possible to obtain the LODs directly also in the real matrix.

Metals of Interest

The present work will concern and discuss the most significant elements that are present in mussels, clams, and oysters mainly for environmental and anthropic causes, and the different analytical methodologies for their voltammetric and spectroscopic determination.

For each metal or group of metals, a brief introduction is reported. This to emphasize the most recent, but also the most significant and interesting works relevant to the various analytical procedures and techniques for determining the same metals in mussels, clams, and oysters.

Copper, Lead, Cadmium and Zinc

Cu(II), Pb(II), Cd(II) and Zn(II) are surely the most studied metals, and many works reported in the literature are devoted both to analytical procedures for the determination of these metals in bivalve mollusks and to the employment of such organisms as bio-monitors.

Among these elements, Pb(II) and Cd(II) are the most toxic and hazardous to human health.

The main sources of both Pb(II) and Cd(II) are vehicular traffic and industries. In gasoline, lead-tetraethyl has been added for a long time as anti-knock. Factories (paints, tires) and solid-waste management are the main sources of cadmium, while their emissions are correlated with the lead content in the air.

Cu(II) and Zn(II) are semi-essential metals: they are necessary for human beings and become toxic only at high concentrations. Their pollution is due to human activities as industries for the production of cosmetics, fungicides, and paints. Even if Cu (II) and Zn (II) are widespread in the environment (mostly in food), they become highly dangerous only in the presence of already existing physiological problems (altered regulatory mechanisms). On the contrary, poisoning due to Cd(II) and Pb(II) is not frequent but might appear also in healthy subjects.

For Cu(II), Pb(II), Cd(II) and Zn(II) determinations, the most widely used techniques are all the different types of spectroscopy [23, 28, 29, 33-39]. Other techniques as Neutron Activation Analysis (NAA) [40, 41] or Voltammetry [14-16, 21, 25-27, 29, 42-45] are seldom employed. Moreover, some authors proposed the use of Total Reflection X-Ray Fluorescence (TRXRF) [46, 47] for the determination of these four elements in mussels, clams, and oysters.

Finally, it is very interesting the work by Wu and coworkers [48], which proposes an "artificial mussel" for monitoring heavy metals in marine environments. In the field of artificial mussels, it is also interesting the work of Degger and coworkers [49], which takes into consideration Cu, Pb, Cd, Zn, and Cr. It is observed a significant spatial and temporal correlation for the majority of the analyzed elements. In any case, there is still much experimentation to be done for a definitive set-up.

For the study of these metals, mussels, clams, and oysters have also the advantage to be extremely versatile bio-monitors, because they can be used in all surface waters, like seas, rivers, lakes, and lacustrine areas.

In this context, the work of George and coworkers [50] should be highlighted, because it describes the use of mollusks to analyze the pollution of backwaters during the monsoon, post-monsoon and pre-monsoon periods. Bivalve mollusks are also used to bio-monitor intertidal areas [51-53], to identify sources of contamination in rivers [54, 55], coastal waters or sea [56-65].

Mercury

Every year, human activities, mainly industries, release in the ecosystem a large amount of mercury. This element has the ability to enter in the alimentary chain and to accumulate into organism tissues in progressively larger quantities. Thus, the dangerousness for human

health of this form of contamination comes mainly from food, especially in the case of marine filtering organisms like mussels, clams, and oysters.

Mercury can be introduced into the human body also by contact with the skin of eyes or inhalation. Although the percent of absorption is low, toxicity is severe. Hence the chemical risk is high. Particularly dangerous is the metal as it is, because it is liquid at atmospheric conditions, and its vapors are intensively absorbed [66]. Also, mercury compounds are very hazardous because they can be easily accumulated through food; its organic compounds are particularly dangerous.

Once absorbed, mercury can have harmful effects on nervous system and brain (it reduces the capacity of learning and memorizing, but causes also imbalances in personality, tremors, headache, diseases in viewing and hearing), chromosomes, and reproductive system, besides possible allergy onsets. The effect of intoxication depends on the assumed quantity.

For the mercury determination spectroscopic techniques [22, 23, 25, 27-29, 36, 39, 67-77] are mostly employed, sometimes also voltammetric techniques [22, 44, 78, 79]. There are also interesting works that quantify the inorganic mercury in mussel samples by Atomic Fluorescence Spectrometry (AFS) [80] and by NAA [81].

Until now, bio-monitoring of this element in environmental matrices seems not to be regularly used. In any case, some works concerning fresh- and sea-water, suggests mainly the use of *Mytilus galloprovincialis* as an important bio-monitoring organism [82]. Special attention was paid to coastal waters with nearby industries, that have released large amounts of mercury [83, 84].

Arsenic and Selenium

Arsenic and selenium are commonly present in coal. Therefore, the main sources of arsenic and selenium pollution are foundries and coal power plants. Furthermore, the coal is used also in cement plants, so these two elements can be commonly found in the air and in the areas around these implants, causing pollution of all the environmental matrices around them.

Toxicity of arsenic is mainly due to its compounds. Organic compounds have a relatively low toxicity because they are absorbed with water, but their water solubility is low. Moreover, they are easily eliminated by feces and urine [66].

On the contrary, inorganic arsenic compounds are very dangerous because they are very easily absorbed by the human organism and can be transported in all its parts, with irreversible consequences (up to death) for various systems: nervous, circulatory, hematopoietic, gastric. Moreover, inorganic arsenic easily passes placenta, causing malformations and fetal pathologies and it is carcinogenic to liver, kidneys, breast, skin, lungs, bladder [66].

Arsenic is naturally present in the environment, mostly in soils and rocks, that contain small quantities. However, human activities are the main source of pollution from this element. In particular, industries release arsenic as wastes and emissions, contaminating soil, water, and airborne particulate matter. There are also some fertilizers, herbicides, and pesticides that illegally contain arsenic, contributing to the diffusion of this pollutant.

Recently, a new excellent semiconductor, gallium arsenide (GaAs), has become particularly important. Therefore, it is massively used in integrated circuits, especially in photovoltaic panels, determining a certain environmental pollution due to incorrect disposal of them.

Unlike arsenic, which is highly toxic, selenium is a semi-essential element, since it is essential to the development of animal and vegetal life, but very toxic beyond certain levels.

Human organism needs a daily dose of selenium of about 60 μg, which is easily achieved through the normal diet (mostly from meat and seafood). Selenium produces seleno-derivatives of some amino acids (like cysteine and methionine) that are involved in many enzymes. Its main function is in muscles functionality, in the production of the thyroid hormone, and to prevent inflammation, cardiovascular pathologies, and arteriosclerosis. Moreover, seleno-enzymes are useful to prevent the Keshan cardiomyopathy, a disorder widely diffused in the Keshan region of China but also in Finland and in New Zealand, where the soil is very poor in selenium.

The main sources of selenium pollution are electronic industries, because this element has excellent photovoltaic and photoconductive properties, and is used to produce photocells and photovoltaic cells.

The determination of As(III) and Se(IV) in mussels, clams and oysters, is prevalently reported in the literature with spectroscopy [39, 54, 57, 85-94]. NAA [41, 56, 95] and voltammetry [24, 44] are less frequently employed, while interesting are the works using EDXRF [96, 97] and AFS [98, 99].

Arsenic is an element particularly investigated by bio-monitors.

Also for this element, the water component was monitored by mussels [63, 100-102] and clams [103].

Even if selenium is not considered to be toxic, it is generally investigated in biomonitoring studies. In the case of water, the literature reports an interesting work by Gay and Maher [104], who propose mollusks to bio-monitor selenium together copper, zinc, and cadmium.

Tin and Antimony

The mainly employing of tin is for the production of cans for the preservation of food. Antimony, instead, is used for the production of ceramics, rubbers, paint and glazes (as pigment), fungicides, and in the military industries. These industries are therefore the primary pollution source for these two metals. With regard to the toxicity, tin is a semi-essential element generally present in traces, but, in excessive amounts, it can interfere with the absorption of calcium and zinc. Antimony, on the contrary, is extremely toxic to human health.

Moreover, as in the case of mercury, these two elements to bio-accumulate in marine organisms. The determination at trace and ultra-trace level becomes, thus, very important to control the pollution due to these metals, especially in matrices that enter in the food chain, such as mussels, clams, and oysters.

Toxicity of tin is increased when it is involved in organic compounds, especially with two or three substituents, which are very dangerous for the immunologic system also at low concentration. Antimony compounds, on the other hand, may cause severe diseases to the human heart, respiratory system, stomach, intestine, and skin.

The environmental concentration of these two metals is generally low, but it varies considerably in space and time.

Most of the works on the Sn (II) and Sb (III) determination in mussels, clams, and oysters regard the investigation of the organic tin compounds, and in any case, they are not numerous. Spectroscopic [105-108], NAA [109], voltammetric [110] and AFS [111] techniques are the

most used, while Ferrarello and coworkers [112] employed size-exclusion chromatography and double focusing ICP-MS allowing the resolution of several spectral interferences that cannot be resolved by quadrupole ICP-MS.

Nickel and Cobalt

Nickel and cobalt are essential elements because they are used by some enzymes. For instance, cobalamin (Vitamin B12) contains Co and it is involved in iron metabolism and nucleic acid synthesis. Moreover, cobalt is also involved in the endocrine system (stimulating thyroid, pancreas, and adrenal) and in red-blood cells production (a long-time deficiency, although rare, may generate anemia and slowness in body development). However, high concentrations of nickel and cobalt cause some diseases: asthma, bronchitis, pulmonary embolism in the case of nickel, cardiac and vascular pathologies, migraine attacks, digestive diseases for cobalt.

The main sources of pollution from Ni (II) and Co (II) are the industries that use them: manufacture of alloy steels (they make alloys highly resistant to corrosion), rechargeable catalyst batteries, paint and pigment industry.

The determination of cobalt and nickel in bivalve organisms was performed mainly by spectroscopic techniques: electrothermal furnace (ET-AAS) or flame (FAAS) atomic absorption spectrometry [113-116], inductively coupled plasma-optical emission spectroscopy (ICP-OES) [117-119] and inductively coupled plasma-atomic emission spectroscopy (ICP-AES) [37, 120, 121].

Voltammetric techniques [43, 122] and neutron activation analysis [41,123] are less frequently used.

Concerning the determination of these two elements, some extremely interesting works should be reported. Three of these illustrate specific sample preparation methods: the dispersive liquid-liquid microextraction (DLLME) and subsequent determination of nickel and cobalt in mussels by ICP-OES [117], the ultrasonic bath-induced acid leaching [124] and rapid micro-assisted digestion and subsequent determination of nickel via ET-AAS [125].

Finally, two more works are of utmost interest.

The former concerns the comparison and development of two instrumental methods, the laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) and the micro-drill sampling and solution [126].

The latter illustrates a sensitive and low-cost flow injection method that combines acid extraction, pre-concentration and FAAS determination of nickel in mussels and clams [114].

Ni and Co were intensively monitored by biological species in all environmental components. In this context, as regards the water component, sea- and fresh-water, the most commonly used species are bivalve organisms: mussels in prevalence [127-130], but also clams and oysters [131-133].

Chromium

Chromium has two principal degrees of oxidation: Cr(III) and Cr(VI). Cr(III) is an essential element in low concentration (about 50-200 µg/day). Even if some studies gave contradictory results about its toxicity, its compounds are water soluble, hence they are not much dangerous for human health.

On the contrary, Cr(VI) is very dangerous: its toxicity and carcinogenicity have been demonstrated since a long time. Most of all, Cr(VI) affect respiratory system (asthma, bronchitis, and laryngitis), gastrointestinal tract, kidney, liver, and eyes. A recent research correlated this metal also with autism [134].

Moreover, Cr(VI) is produced by many urban and industrial activities, like the metallurgical field, production of enamels and paints, and industrial manufacturing of catalysts determining a huge release of this pollutant in all the environmental matrices.

Also in the case of chromium, for its determination in the filtering organisms, the literature reports works that employ prevalently as instrumental techniques all the spectroscopic methods: ET-AAS [135-138], ICP-OES [139, 140], and ICP-AES [37, 141].

The INAA is often used in biomonitoring studies [56, 63].

Finally, two very interesting works are reported. The former illustrates the comparison of three techniques for the determination of Cr, together with other elements in raw clams and commercial clam meat, i.e., ET-AAS, ICP-OES, and wavelength dispersive X-ray fluorescence (WD-XRF) [142].

The latter one proposes the flame atomic absorption spectroscopic determination of total chromium in mussel samples using a continuous ultrasound-assisted extraction system connected to an on-line flow injection manifold [143].

Precisely because of its high toxicity, chromium has always been considered in all environmental pollution studies, both by samplings and punctual determinations and by bio-monitoring.

As usual, mussels, clams, and oysters are the species predominantly used in the case of water. Indeed, they are the species perhaps considered indispensable in studies concerning the pollutant load from Cr and other elements in the lagoon ecosystems [102, 144-148], but also in the sea- [101, 149] and fresh-water [150, 151].

Bismuth

Bismuth is toxic only at high concentrations, while it is not dangerous at low levels. Damages to human health due to a long-time exposure to bismuth have been demonstrated Fowler and coworkers [152], which can affect the intestine, kidneys, and skin. Recent studies [152] indicate that bismuth is not much absorbed by the skin, however, no reliable quantitative results are yet available. For this reason, cosmetic products should be accurately checked for the bismuth content.

The main sources of pollution are industries using this metal. For example, it is used to prepare low melting alloys used in fuses, but also as a catalyst in the production of acrylic fibers. Moreover, bismuth has been recently used to produce low-temperature superconductors to be employed in medical diagnostic machinery, like Magnetic Resonance Tomography (MRT).

As for the determination of the bismuth in filtering organisms, of extreme interest is the work of Das and coworkers [153]. It illustrates all the techniques for its accurate and highly sensitive detection in solid environmental samples at extremely low concentration levels, discussing all steps of sample preparation prior to instrumental measurements such as pre-treatment, separation, and pre-concentration.

Although at least in our knowledge, the literature does not report specific works about the bio-monitoring of Bi in superficial waters employing filtering organisms, Das and coworkers [153] suggest the possibility of using mussels as biomonitors.

Really, aquatic plants rather than filtering organisms are used in the biomonitoring of aquatic ecosystems.

Indeed, bio-monitoring of bismuth together also with tin and antimony in surface waters is carried out by aquatic plants, as reported in the very interesting work by Luy and coworkers [154], who use *Posidonia oceanica (L.)* as biomonitor.

Vanadium

Vanadium is an essential element at low concentration, so it is required to assume it with diet. However, high amounts of vanadium may cause cardiovascular pathologies and stomach and intestines diseases, besides allergenic dermatitis.

Vanadium is widely used in metal alloys and as a catalyst for various industrial processes. Industries involved in this kind of processes are easily identifiable as the main sources of pollution.

Electrothermal atomic absorption spectroscopy is largely used in the determination of vanadium in bivalve organisms [87, 155-157], but also instrumental neutron activation analysis is often used by several authors [123, 158].

Finally, some interesting works have to be reported.

The first by Santos and Lemos [159] relates to a new method for pre-concentration and spectrophotometric determination of vanadium using dispersive liquid-liquid microextraction (DLLME) and optical sensors using 4-(5bromo-2pyridylazo)-5-(diethylamino)-phenol (Br-PADAP) as a complexing agent.

Lastly, three works in which vanadium is determined in mussels and clams by high-performance liquid chromatography [160-162].

Even if it is considered a semi-essential element, vanadium is often studied and taken into account in environmental bio-monitoring studies of water systems.

Concerning aquatic ecosystems, interesting are two works that use mussels [163] and oysters [131] as bio-monitor species.

Finally, the work by Tarique and coworkers [164] should be highlighted. It describes the use of the *Amiantis umborella* clams in monitoring metal contamination in coastal sediments, vanadium included.

Platinum Group Metals (PGMs)

Platinum group metals (PGMs) pollution is undoubtedly due mainly to vehicle emissions. The concentration of PGMs is constantly increasing in all the environmental matrices [165, 166] with great risk for human health [167, 168]. Indeed, PGMs have been massively used in the latest year for the production of autocatalytic converters. This resulted in a decrease of pollutants like carbon monoxide, nitrogen oxides, unburned hydrocarbons and also lead (present in exhaust gases of motor vehicles), but, at the same time, is increasing the concentration of fine particulate and dust at a high concentration of PGMs. This is mainly originated from deterioration or abrasion of the bulk catalysts due to the incorrect “stop and go” use of the vehicles

In order to try to solve this problem, instead of a massive use of platinum, palladium, and rhodium for autocatalytic converters, there has been a gradual reduction of these PGMs and a growing use of osmium, ruthenium and iridium.

Indeed, in recent years, alloys of these elements together with PGMs have been employed more and more frequently for the production of autocatalytic converters. These alloys have

the advantage to better withstand high temperatures and high wear, increasing the lifetime of the same converters.

Determination of PGMs, Part I: Pt(II), Pd(II) and Rh(III)

In the case of Pt(II), Pd(II) and Rh(III), spectroscopy is the technique most widely employed [169-173], while very interesting is the paper by Essumang and coworkers [174] who proposes NAA as instrumental technique.

As regards the voltammetric techniques, the literature reports prevalently the works of two research team: the former (Zimmermann and coworkers [175-180]) that develops the research on the use of mussels as biomonitors for Pt(II), Pd(II) and Rh(III), the latter (Locatelli and coworkers [181-194]) that proposes analytic voltammetric methods for the determination either of Pt(II), Pd(II), Rh(III) or of Os(VIII), Ru(III), Ir(III).

Indeed, in our laboratories since some years it is underway an important line of research on the development of analytical methods for the voltammetric determination of PGMs in all the environmental matrices, starting with those most investigated (platinum, palladium, rhodium) [181-187] to finish those generally considered, at least until a few years ago, less important from the environmental pollution point of view (osmium, ruthenium and iridium) [188-194].

Determination of PGMs, Part II: Os(VIII), Ru(III) and Ir(III)

As regards the determination of Os(VIII), Ru(III) and Ir(III) in mussels, clams, and oysters, the literature reports interesting reviews by Locatelli and Melucci [16-19, and therein references].

As regards the bio-monitoring aspect, only recently all the PGMs have attracted great attention and an increasing number of authors have begun to investigate the possibility to employ biological species to monitor the mentioned metals [16, and therein references].

In any case for such metals, the bio-monitoring path is only at the beginning. Certainly, in the near future, there will be important and interesting developments.

Thallium

Thallium salts are very toxic [195, 196] and must, therefore, be handled with extreme care. Thallium has the ability to replace the positive ions of the alkaline metals present in the body, mainly sodium and potassium. This replacement alters many of the normal cellular processes. Thallium is also a suspected carcinogen. The first symptoms of poisoning are nausea, vomiting, tachycardia, fever, diarrhea, abdominal pain. Over time, ataxia (loss of muscle coordination), alopecia (loss of hair and hair) appear, until death. Poisoning can occur through ingestion and inhalation (maximum permissible concentration of long-term exposure to thallium salts should not exceed 0.1 mg m^{-3}). Contact with the skin is also extremely dangerous.

Precisely because of its toxicity, the use of thallium salts as a rat poison has been banned in many countries. For example, since 1972 this use has been prohibited in the United States.

Much of thallium production is used in the electronics industry.

It is also used in the pharmaceutical industry, in glass production, and in the construction of infrared light detectors. Thallium-201 radioisotope is used in small amounts as a non-toxic

radiotracer in biomedical imaging examinations in nuclear medicine, particularly in myocardial scintigraphy.

According to International Organizations, in addition to the industries above mentioned, among anthropogenic sources of thallium environmental pollution, there are also gaseous emissions from cement factories, coal-fired power plants and metal sewers (underground ducts to drain water or waste material).

Also in the case of thallium, its determination in the filtering organisms is carried out prevalently by spectroscopic methods [197-199].

Electroanalytical methods [200] are less frequently used.

Finally, very interesting is the paper by Michalski and coworkers [201] that shows how the hyphenated techniques, in which separation method is coupled with multidimensional detectors, have become useful alternative in speciation analysis of thallium, together with arsenic and antimony, in environmental and food matrices

Analytical Procedure for the Voltammetric Determination of Tl(I)

0.5 mol L^{-1} ammonium citrate buffer pH 6.5 + 6.9 x 10^{-3} mol L^{-1} EDTA-Na_2 was employed as supporting electrolyte, which was the aqueous reference solution.

As regards the composition of the supporting electrolyte, it should be emphasized that the presence of EDTA-Na_2 is necessary to obtain better resolution for the Tl(I) in presence of Pb(II), Sn(II) and Sb(III).

Indeed, in the marine ecosystems Pb(II), Sn(II) and Sb(III) are always and inevitably present. Unfortunately, it shows to have a peak potential very close to those of Tl(I).

In the supporting electrolyte without the presence of EDTA-Na_2 the peak potentials E_p (V/ $Ag|AgCl|Cl^-_{satd.}$) are: $E_{p\ Tl(I)}$ = -0.509 ± 0.010 $E_{p\ Pb(II)}$ = -0.569 ± 0.015, $E_{p\ Sn(II)}$ = -0.677 ± 0.015 and $E_{p\ Sb(III)}$ = -0.783 ± 0.010.

The problem is decidedly important, considering that the same problem is also present in the standard reference material and in the real samples collected in the sampling sites. Also, in this case, our methodological procedure proposes the possibility to shift the interferent peaks towards more cathodic potential values by adding EDTA-Na_2. Indeed, the presence of EDTA-Na_2 shifts towards more cathodic potential the values of all the four elements: Tl(I): -0.523 ± 0.010; Pb(II): -0.657 ± 0.010; Sn(II): -0.796 ± 0.015 and Sb(III): -0.949 ± 0.015 V *vs.* $Ag|AgCl|Cl^-_{satd.}$. Evidently, the new peak position of the elements allows their resolution and then also their quantitative determination.

Evidently, the proposed analytical procedure, with the addition of EDTA-Na_2, allows determining beyond Tl(I) also Pb(II), Sn(II) and Sb(III).

10 mL sample aliquots of supporting electrolyte or of solutions obtained in the mineralization step of the standard reference material and of real samples (see section 2.1.3. "Sample Preparation before the Instrumental Determination") were pipetted into the voltammetric cell and deaerated for 5 min by bubbling water-saturated pure nitrogen. Square wave anodic stripping voltammetry was employed as voltammetric technique, using hanging mercury drop electrode (HMDE) as working electrode.

The voltammetric experimental conditions are the following.

Deposition potential E_d (V/Ag, AgCl, $Cl^-_{satd.}$): -1.150; initial potential E_i (V/Ag, AgCl, $Cl^-_{satd.}$): -1.150; final potential E_f (V/Ag, AgCl, $Cl^-_{satd.}$): -0300; electrodeposition time t_d (s): 420; delay time before the potential sweep t_r (s): 10; potential scan rate dE/dt (mV/s): 100;

superposed potential amplitude ΔE (mV): 50; sampling time τ (s): 0.010; wave period υ (s): 0.100; wave increment η (mV): 10; stirring rate u (r.p.m.): 600.

The experimental peak potentials E_p (V/ Ag | AgCl | $Cl^-_{satd.}$) of Tl(I) are reported in Table 1.

Table 1. Peak potentials (E_p, V/Ag | AgCl | $KCl_{sat.}$) in the reference solutions and in solutions obtained by digestion of mussels, clams, and oysters sampled in the Goro Bay. The determined values are the mean of 5 independent determinations. Confidence level: 95%

	Sampling sites [a]	Tl(I)
Supporting electrolyte		-0.523 ± 0.010
Solution obtained by digestion of Mussel Tissue BCR-CRM 278		-0.534 ± 0.015
Solution obtained by digestion of **Oyster Tissue** NIST-**SRM** 1566a		-0.516 ± 0.015
Solution obtained by digestion of **Mussels**	A	-0.515 ± 0.010
	B	-0.527 ± 0.015
	C	-0.519 ± 0.010
	D	-0.529 ± 0.015
	E	-0.532 ± 0.010
Solution obtained by digestion of **Clams**	A	-0.526 ± 0.010
	B	-0.517 ± 0.015
	C	-0.525 ± 0.015
	D	-0.518 ± 0.010
	E	-0.524 ± 0.015
Solution obtained by digestion of **Oysters**	A	-0.521 ± 0.015
	B	-0.530 ± 0.015
	C	-0.528 ± 0.015
	D	-0.531 ± 0.010
	E	lack of sample

[a] In the case of Oyster samplings in site E, the absence of element concentration data is due to lack of sample present in the same sampling site.

The spectroscopic experimental conditions are the following: Wave-length (nm): 276.8; Slit (nm): 0.7; Drying Temperature (°C): 105; Charring Temperature (°C): 750; Atomization Temperature (°C): 1850; Matrix Modifiers: 0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$.

RESULTS AND DISCUSSION

In this section, in a synthetic way, the fundamental parameters that characterize a correct analytical procedure — limits of detection, linear range, trueness, and precision — are reported.

Limits of Detection and Linear Range

In the aqueous reference solution, in the solutions obtained by digestion of the standard reference materials and of real samples, the LOD_s (Table 2) for both techniques were calculated as described in section “Limits of Detection.”

At the experimental conditions employed, the linear range for all the elements in the aqueous reference solutions is < LOD – 15.0 µg L^{-1}.

Quality Control and Quality Assessment

The method set up in aqueous reference solutions was applied to Mussel Tissue BCR-CRM 278 and Oyster Tissue NIST-SRM 1566a standard reference materials in order to confirm and verify the applicability of the analytical procedure (see section 8.2.2).

Trueness and precision for the voltammetric and spectroscopic measurements were determined and reported in Table 3.

Practical Application

Once the procedure for the Tl(I) determination was set up, the method was applied to mussels, clams, and oysters sampled in the Goro Bay (see section 2.1.2. "Sampling Area and Sampling Sites").

The experimental results relevant to both techniques are reported in Table 4.

Table 2. LOD_s [a] of Tl(I) determined in the supporting electrolyte (µg L^{-1}), in the solutions obtained by digestion of Mussel Tissue BCR-CRM 278 and Oyster Tissue NIST-SRM 1566a standard reference materials, and in the solutions obtained by digestion of real samples (calculated in µg L^{-1} and expressed in µg kg^{-1}). The determined values are the mean of 5 independent determinations; confidence level: 95%

	Sampling sites [b]	Tl(I)
Supporting electrolyte		0.13
Solution obtained by digestion of **Mussel** Tissue BCR-CRM 278		5.1
Solution obtained by digestion of **Oyster** Tissue NIST-**SRM** 1566a		5.3
Solution obtained by digestion of **Mussels**	A	4.9
	B	5.5
	C	4.7
	D	5.0
	E	5.2
Solution obtained by digestion of **Clams**	A	5.1
	B	4.6
	C	4.9
	D	5.1
	E	4.7
Solution obtained by digestion of **Oysters**	A	5.4
	B	5.5
	C	5.0
	D	4.9
	E	lack of sample

[a] In the case of spectroscopic measurements, the Tl(I) LOD in the aqueous reference solution is 0.69 µg L^{-1}. Considering a sample weight exactly equal to 1.0 g (see section 2.1.3. "Sample Preparation before the Instrumental Determination"), the same limit of detection is 26.9 µg kg^{-1}.

[b] In the case of Oyster samplings in site E, the absence of element concentration data is due to lack of sample present in the same sampling site.

Table 3. Accuracy of the analytical procedure for the determination of Tl(I). The determined values are the mean of 5 independent determinations. Confidence level: 95%. Concentrations in µg kg^{-1}

Mussel Tissue BCR-CRM 278

	Voltammetry			*Spectroscopy*		
Certified Values	Determined Values	*e* (%)	s_r (%)	Determined Values	*e* (%)	s_r (%)
96.7*	91.5 ± 5.7	-5.4	5.3	90.3 ± 6.9	-6.6	6.2

OysterTissue NIST-SRM 1566a

	Voltammetry			*Spectroscopy*		
Certified Values	Determined Values	*e* (%)	s_r (%)	Determined Values	*e* (%)	s_r (%)
96.7 *	102.3 ± 5.9	+5.8	5.7	89.5 ± 0.3	-7.4	6.1

* Spiked-sample concentration.

Table 4. Mean values of Tl(I) (µg kg^{-1}) relevant to oysters, mussels, and clams sampled in the Goro Bay. The determined values are the mean of 5 independent determinations. Confidence level: 95 %

	Sampling sites [a]	*Voltammetry*	*Spectroscopy*
Mussels	A	69.3 ± 3.5	65.9 ± 3.7
	B	53.7 ± 2.6	56.1 ± 2.7
	C	60.3 ± 3.4	57.2 ± 3.6
	D	62.5 ± 3.5	59.4 ± 3.4
	E	37.9 ± 2.0	36.0 ± 2.2
Clams	A	38.0 ± 2.0	39.8 ± 2.1
	B	30.1 ± 1.6	31.6 ± 1.7
	C	32.8 ± 2.2	34.9 ± 2.3
	D	34.7 ± 1.9	36.5 ± 2.0
	E	15.6 ± 0.2	< LOD
Oysters	A	49.6 ± 2.5	51.9 ± 2.7
	B	41.3 ± 2.3	43.5 ± 2.4
	C	47.4 ± 2.4	49.5 ± 2.5
	D	45.8 ± 2.7	48.1 ± 2.6
	E	lack of sample	lack of sample

[a] In the case of Oyster samplings in site E, the absence of element concentration data is due to lack of sample present in the same sampling site.

Bio-Remediation

This very short paragraph highlights another aspect of the use of biological organisms in the environmental field. This aspect concerns bio-remediation, i.e., the possibility of reducing the pollutant load of an ecosystem by the use of bio-accumulative organisms. These are biological organisms that can survive in the presence of pollutants accumulating in their tissues. Evidently, bio-remediation can be carried out in quite narrow ecosystems showing a few inbound and outbound paths.

In the case of limited polluted aquatic ecosystems, the organisms most often used in this type of procedure are predominantly vascular plants [202, 203]. However, it is a conviction of

the authors that also mussels and clams, organisms that require the water for their nourishment, can be successfully used in bioremediation processes.

It should be emphasized that for this procedure, the studies and subsequent feasibility checks are still in progress. In any case, the possibility of cleaning up, or at least limiting the pollution of an ecosystem with biological organisms, shows a very interesting path, to be developed and increased more and more in the future.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Chapter 121

HEMOCYTES OF THE RIBBED MUSSEL *AULACOMYA ATRA ATRA* FROM NUEVO GULF (CHUBUT) AS BIOMARKERS OF OXIDATIVE STRESS

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ABSTRACT

Bivalve mollusks exposed to a wide variety of natural and anthropogenic environmental changes are widely used as sentinels. These factors can cause an imbalance between the generation and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to an oxidative stress that is manifested by alterations of the antioxidant defense system and/or oxidative damage. The hemocytes constitute the immune system in bivalves, and cell death processes have been recently described as part of the mechanism of defense against various pathogens and contaminants. Previous studies showed a higher content of trace metals Fe and Cd in the gills and digestive gland of the *Aulacomya atra atra* from Folías Wreck (impacted area) than from Punta Cuevas (control area). In order to compare oxidative stress conditions in bivalves obtained from both sites, we evaluated the production of ROS and oxidative stress biomarkers in hemocytes from the *A. atra atra* during the month of September of 2015. The results obtained by flow cytometry, using MitoSox as probe, showed that superoxide anion was 58% higher in bivalve's hemocytes from Folías Wreck than in

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those from the reference place Punta Cuevas. The oxidation of the dye 2´7´dichlorofluorescein diacetate (DCFH-DA), as a general indicator of oxidative stress, showed a 14% increase in bivalve's hemocytes from Folías Wreck, as compared to hemocytes from Punta Cuevas. Thiobarbituric acid reactive substances (TBARS) content showed no differences between hemocytes from animals isolated from both locations. In addition, the content of lipid radical measured by Electron Spin Resonance increased 2.1 fold in the hemocytes from Folías Wreck samples as compared to the level obtained in hemocytes from bivalves collected from Punta Cuevas. The oxidized/depleted cardiolipin was 16% higher in samples obtained from Folías Wreck than in Punta Cuevas. Based on these results, it can be concluded that hemocytes from the ribbed mussel *A. atra atra* could be used as a model to evaluate oxidative stress induced by pollutants or other environmental stressors.

Keywords: bivalves, reactive oxygen species, hemocytes, superoxide anion, cardiolipin

INTRODUCTION

Marine invertebrates represent 95% of animal species, which ecological role is determinant in the evaluation of potential routes of exposure to the toxicity of metals. Bivalves have been employed as sentinel organisms in worldwide routine biomonitoring programs based on their wide geographic distribution, their straightforward availability in the field and through aquaculture, and their suitability for caging experiments along coast lines (Viarengo et al., 2007). The ribbed mussel *Aulacomya atra atra* (Molina 1782) is a filter feeder organism with the ability to accumulate a wide range of contaminants and a typical species of the rocky shores along the Patagonian coast of Argentina forming extensive beds (Zaixso, 1999). It is an important habitat-forming species for numerous species of algae and animals and it is also a food source for gastropods, asteroids and sea birds as well as for humans (Zaixso, 2003). It has been used as biomonitor to evaluate the recovery of normal cytogenetic records through micronucleus test after cessation of urban discharges in Puerto Madryn (Nuevo Gulf) (Machado-Schiaffino et al., 2009). In ribbed mussels from the same area, several biomarkers of oxidative stress have been measured for environment quality assessment (Giarratano et al., 2013; 2014).

The physico-chemical characteristics of seawater were within the range of the previously informed data (Gil, 2001; Solís, 1998; Giarratano et al., 2013; 2014). Previous studies have shown that the presence of a high concentration of metals such as Fe Al, Cu and Cd, from Folías Wreck seemed to be caused by the ship's metal corrosion (Giarratano et al., 2013; 2014).

Metabolic homeostasis in bivalves is performed at least partially upon cells circulating in hemolymph described as hemocytes, which are routinely used to understand the impact of pollutants with accurate precision (Ladhar-Chaabouni and Hamza-Chaffai, 2016). These cells are involved in different and numerous physiological functions as well as shell and tissue repair (Sparks and Morado, 1988; Mount et al., 2004). Hemocytes also mediate cellular internal defense mechanism through accumulation and detoxification of toxicants, phagocytosis and encapsulation of different biological toxins and inorganic material (Matozzo et al., 2001).

Reactive oxygen species (ROS) are molecules produced by hemocytes of different bivalve's species including oysters, mussels, scallops and clams (Donaghy et al., 2012). In order to protect themselves against the effect of toxic substances, marine organisms developed a variety of detoxification pathways including an important battery of antioxidant mechanisms against ROS, as observed in mammals. The presence of oxidative stress is quite common in marine organisms due to a wide variety of natural and anthropogenic environmental changes. Induction of the antioxidant system can be considered as an adaptation of the organisms to overcome an unsafe environment and to prevent toxicity. The use of biomarkers has been proposed as a sensitive tool for biological effects monitoring, playing an important role in the risk assessment of complex ecosystems (Monserrat et al., 2007). Since several pollutants can directly or indirectly modify the balance between the concentration of pro-oxidants and antioxidants, the determination of oxidative stress and/or antioxidant responses in aquatic species is commonly and successfully employed as a non-specific biomarker (Bainy et al., 1996).

OBJECTIVE

The production of ROS and oxidative stress biomarkers were evaluated in hemocytes of ribbed mussel *A. atra atra* obtained from two sites within Nuevo Gulf.

MATERIALS AND METHODS

Zones Selected

A. atra atra is present along the Pacific Ocean from El Callao, Peru, to the Strait of Magellan in Chile, extending along the Atlantic Ocean from the south of Argentina to the south of Brazil (Caza et al., 2016). Two sites within Nuevo Gulf were selected in order to obtain the ribbed mussel samples (Figure 1). Folías Wreck is a ship of 60 m in length located at 300 m of the shore of Paraná Beach, used for scuba diving activities. Punta Cuevas is a dive site that hosts fishes, crabs, sea stars, among other, and is located at the south end of the city. The last one was considered the reference place.

Animal Collection

In September 2015, 30 individuals of *A. atra atra* were collected by scuba diving at each sampling site. Following collection, mussels were placed in isolated plastic containers previously filled with water from the sampling site and transported to the laboratory within 2 hours of collection. After hemolymph collection from the posterior adductor muscle sinus with sterile hypodermic needles, the hemocytes were filtered and maintained on ice. Each experiment was performed after pooling hemolymph from 3-5 ribbed mussels to obtain enough volume and to minimize the individual variability.

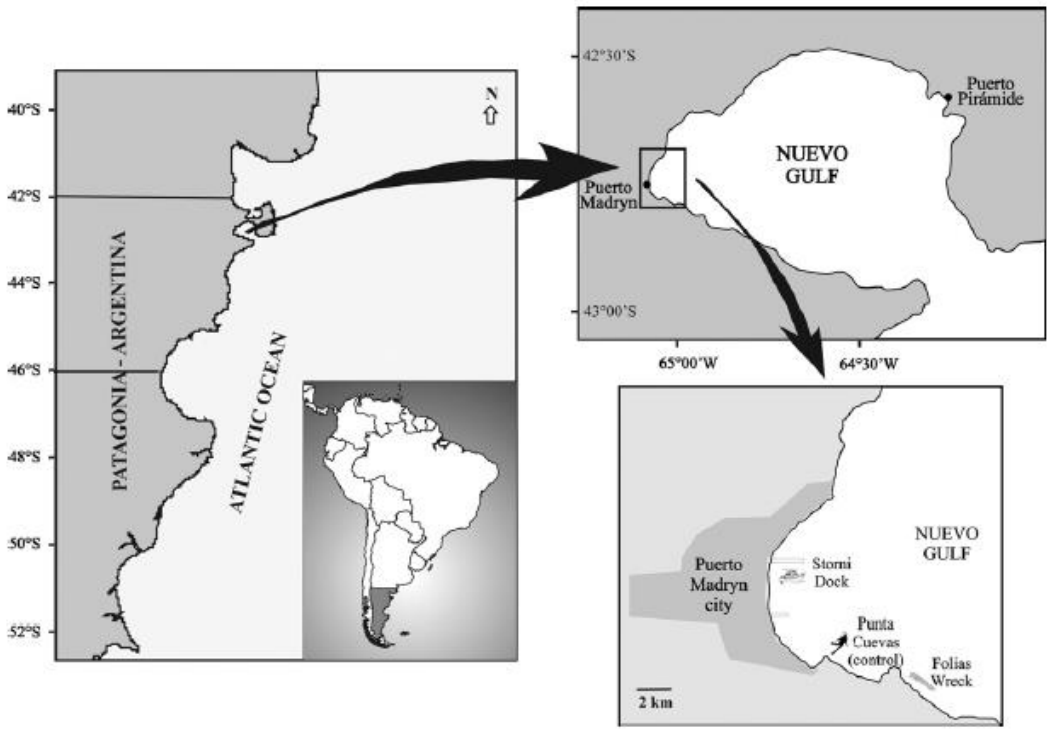


Figure 1. Map of the studied area.

Flow Cytometry Studies

Flow cytometry assays were performed in a FACS calibur (Becton-Dickinson, US) equipped with a 488 nm argon laser and a 615 nm red diode laser. The studied hemocytes population with high FSC (Forward Scatter) and high SSC (SideScatter) characteristics was chosen as the gate (R1) according to the typical cellular hemocyte size, calibrated for all the cytometric studies.

Superoxide Anion Production

Superoxide anion (O_2^-) production was determined in unstimulated isolated hemocytes. Cells suspended in 1ml PBS and incubated at 25°C for 20 min in the presence of 12.5µM MitoSox, a potentiometric probe that is used for direct measurement of mitochondrial superoxide by flow cytometry. Samples were protected from light until acquired by the cytometer FACS calibur (Becton-Dickinson, US (Bustamante et al., 2004)). After auto-fluorescence evaluation, MitoSox fluorescence was analyzed using the median value of the fluorescence events distribution from each treatment. Differences in MitoSox fluorescence histograms were evaluated in the gated R1 population from three independent experiments as the number of events that drop under a common marker. The results were expressed as the

percentage of control MitoSox fluorescence from hemocytes obtained from animals located in Punta Cuevas (reference place), which was considered as 100%.

DCFH-DA Oxidation Rate

The membrane-permeable non-fluorescent DCFH-DA oxidation has been used for detecting several ROS in biological media (McDowell et al., 2013). DCFH-DA was initially thought to be useful as a specific indicator for hydroperoxides. It was already demonstrated that DCFH is oxidized by different reactive molecules, including superoxide anion (O_2^-), hydroxyl radical, peroxyl, alkoxyl, hydroperoxyl and peroxynitrite which are products of normal metabolism (Halliwell and Gutteridge, 2007). DCFH-DA is a fluorogenic probe that passes through cell membranes and is cleaved by cellular esterases. During incubation, DCFH-DA is hydrolyzed by intracellular hydrolytic deacetylation to DCFH, which is trapped inside the cell due to its polarity. This substance is then rapidly oxidized to the highly fluorescent compound, DCF that allows the evaluation of oxidant cellular levels. Hemocytes were incubated *in vivo* in the dark for 30 min in 2 ml of 40 mM Tris-HCl buffer (pH 7.0) in the presence of 5 µM DCFH-DA at 27°C (Bass et al., 1983; Malanga et al., 2001). Fluorescence was measured with λ_{ex} = 498 nm and λ_{em} = 525 nm.

Content of Thiobarbituric Acid Reactive Substances (TBARS)

The hemocytes were homogenized in relation 1:3 of 0.1 M Tris buffer pH 7.8. The homogenates were treated with 30% (w/v) trichloroacetic acid and 50 mM potassium phosphate buffer (pH 7.0). After centrifugation, the content of TBARS was determined in the supernatant spectrophotometrically at λ = 535 nm, using the acid hydrolysis of 1, 1, 3, 3 tetraethoxy propane as standard, according to Malanga et al., (2004).

Quantification of Lipid Radicals by Electron Paramagnetic Resonance (EPR)-Spin Trapping

Lipid radical ($LR^{\bullet}$) content in hemocytes was detected by a spin trapping technique using N-t-butil-α-phenyl nitrone (PBN). A 40 mM PBN stock solution was prepared in DMSO immediately prior to use. The homogenates were prepared in DMSO-PBN (stock solution). EPR spectra were obtained at room temperature using a Bruker spectrometer ECS 106, operating at 9.81 GHz with 50 kHz modulation frequency. EPR instrument settings for the spin trapping experiments were: microwave power, 20 mW; modulation amplitude, 1.194 G; time constant, 81.92 ms; scans number, 5; center fields, 3480 G; modulation frequency, 50 kHz; and receiver gain, 2 104 (Lai et al., 1986). Quantification was performed according to Kotake et al., (1996).

Cardiolipin Content

Acridine orange 10-nonyl bromide (nonyl acridine orange, NAO, λ_{em} = 525 nm) binds specifically to cardiolipin providing a direct and reliable method for evaluating the cardiolipin content in mitochondria (McMillin and Dowhan, 2002; Petit et al., 1992; Wright et al., 2004). Nomura et al., (2000) reported that NAO is associated selectively with monolayer of cardiolipin. Also, they described that the binding of NAO to cardiolipin decreased by oxidation/depletion of cardiolipin; NAO is notable to bind to peroxidated cardiolipin (CL-OOH) producing a decreased NAO fluorescence. Previous observations indicate that low NAO fluorescence would be due to oxidation/depletion of cardiolipin (Nomura et al., 2000; Paradies et al., 2000; 2001). This can explained by the fact that free radicals-induced cardiolipin damage leads to decreased cardiolipin accessibility to NAO. After suspension of hemocytes in PBS, samples were loaded with 100 nM NAO during 20 min at 25°C in a dark room and data acquired by the cytometer as previously described. NAO relative fluorescence intensity (r.f.i.) of hemocytes evaluated in three independent experiments as the number of events, which drop under a common and specific marker were evaluated in the presence or absence of antimycin (0.5 μM). Quantification is shown by cardiolipin oxidation/depletion for each hemocyte sample. Hemocytes autofluorescence without probe was also evaluated.

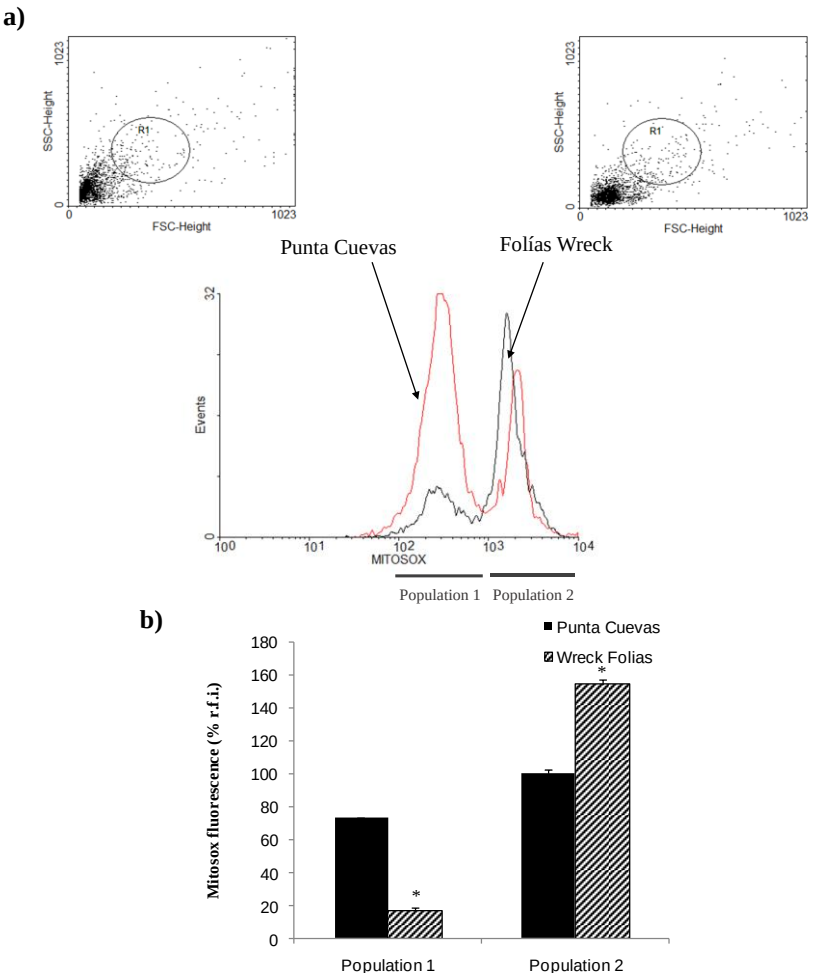
Statistical Analyses

Data in the text and tables were expressed as mean ± S.E.M. of three independent experiments, with two replicates in each experiment. Significant differences were tested by factorial analysis of variance ANOVA ($P < 0.05$).

RESULTS

Ribbed mussels *A. atra atra* from Punta Cuevas and Folías Wreck (Nuevo Gulf) were analyzed by flow cytometry using the probe MitoSox to measure their ability to produce O_2^-. Unstimulated hemocytes loaded with MitoSox were used for the flow cytometry assays. Typical histograms of FL-1MitoSox fluorescence for both samples are presented in Figure 2 a. Histograms under the respective markers showed two different O_2^- producing hemocytes populations with: low r.f.i (population 1) and high r.f.i. (population 2). Quantification of three different experiments was shown in the bar graph of Figure 2 b indicating a significant 58% increase ($P < 0.05$) in O_2^- level in Folías Wreck hemocytes as compared to Punta Cuevas hemocytes from population (2).

In addition, ROS were also determined using DCFH-DA. The results obtained by this procedure showed a high rate of DFC oxidation by hemocyte loading. As shown in Table 1, hemocytes from Folías Wreck samples showed a 14% increase as compared to hemocytes from Punta Cuevas in agreement with the results obtained by flow cytometry using MitoSox as a probe.



* Significant different as compared to values obtained in hemocytes of ribbed mussel collected at Punta Cuevas ($P < 0.05$).

Figure 2. Determination of superoxide anion levels in hemocytes of ribbed mussel *A. atra atra* from Punta Cuevas and Folías Wreck. a. Overlay of MitoSox fluorescence histograms from both hemocytes populations. b. Quantification of MitoSox fluorescence as a bars graph representing the mean ± SEM of 3 different experiments.

Table 1. DCF-DA oxidation rate, TBARS content and $LR^{\bullet}$ content in hemocytes isolated from ribbed mussels from Punta Cuevas and Folías Wreck

Zone	Punta Cuevas	Folías Wreck
DCF-DA oxidation rate (u.a./ min 10^6 cells)	148 ± 23	169 ± 15
TBARS content (nmol/ 10^6 cells)	29 ± 2	31.3 ± 0.9
$LR^{\bullet}$ content (nmol/ 10^6 cells)	0.27 ± 0.04	0.92 ± 0.01*

Values represent the mean ± S.E.M. of 3 different experiments.
*Significant different as compared to values obtained in hemocytes of ribbed mussel collected at Punta Cuevas values ($P < 0.05$).

The TBARS content showed in Table 1 was similar in hemocytes from Folías Wreck as compared to the values obtained in samples collected from Punta Cuevas.

Oxidative damage to lipid in the hemocytes of *A. atra atra* was also estimated in the present study, as the content of $LR^{\bullet}$ assessed by EPR. $LR^{\bullet}$ content in hemocytes combined with the spin trap PBN resulted in adducts that gave a characteristic EPR spectrum with hyperfine coupling constants of a_N = 15.8 G and a_H = 2.6 G, in concordance with computer spectral simulated signals obtained using the overall mentioned parameters (Figure 3). A solution of PBN and DMSO itself was examined and no PBN spin adduct was observed (Figure 3d). $LR^{\bullet}$ content showed significant differences between the zones (Table 1), being 70.6% higher in Folías Wreck samples than in hemocytes isolated from ribbed mussel collected in Punta Cuevas ($P < 0.05$).

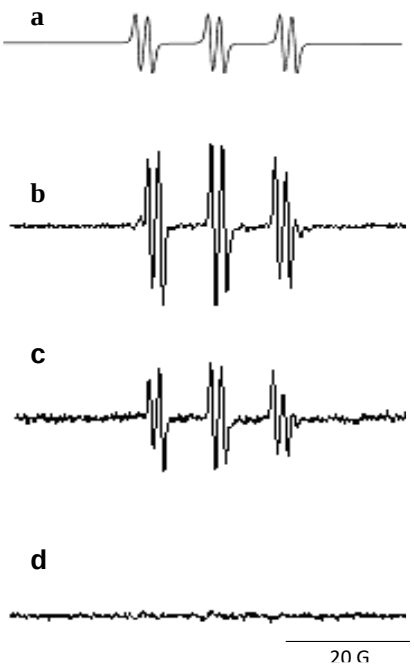
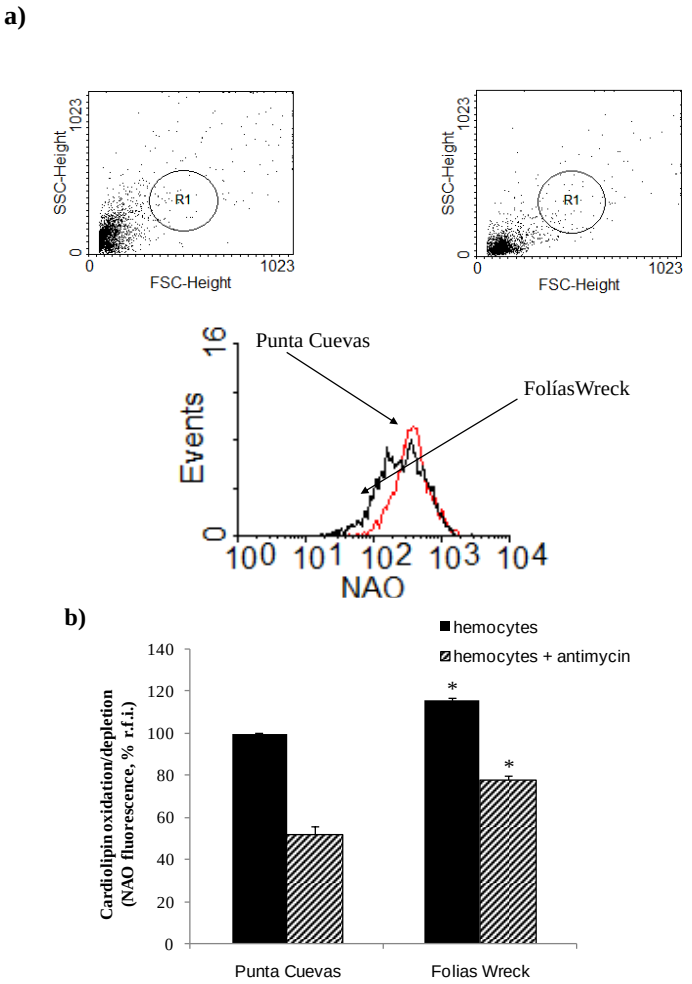


Figure 3. $LR^{\bullet}$ detection by EPR in hemocytes of the ribbed mussel *A. atra atra.* Spectra from: a. computer-simulated EPR spectra exhibiting hyperfine splittings that are characteristic of $PBN/LR^{\bullet}$, a_N = 15.8 G and a_H = 2.6 G, b. hemocytes from Folías Wreck, c. hemocytes from Punta Cuevas and d. EPR spectra of PBN itself, are shown.

Immediately after hemocytes isolation, they were stained with NAO to detect mitochondrial cardiolipin oxidation/depletion by flow cytometry as shown in Figure 4 a. We can observe in the overlay histograms that Folías Wreck samples presented higher amount of events with lower NAO-fluorescence indicating a higher rmitochondrial cardiolipin oxidation/depletion as compared with the samples collected in Punta Cuevas samples. In Figure 4 b the quantification of the cardiolipin histograms showed a significant increase ($P < 0.05$) of 16% in cardiolipin oxidation/depletion from Folías Wreck hemocytes as compared to hemocytes of ribbed mussel from Punta Cuevas. When hemocytes from both zones were stimulated with antimycin, the levels of mitochondrial cardiolipin oxidation/depletion were decreased in both samples.



*Significant different as compared to values obtained in hemocytes of ribbed mussel collected at Punta Cuevas ($P < 0.05$).

Figure 4. Determination of the cardiolipin content in hemocytes of the ribbed mussel *A. atra atra* from Punta Cuevas and Folías Wreck. a. Overlay of NAO fluorescence histograms. b. Quantification of the level of cardiolipin oxidation/depletion present in both hemocytes samples with and without antimycin. The bars represent the mean ± SEM of 3 different experiments.

DISCUSSION

Effective O_2^- detection has been performed using flow cytometry with a variety of different probes such as dihydroethidium (Ding et al., 2007) and MitoSox (Velez-Pardo et al., 2010; Bustamante et al., 2014). These fluorophores are readily oxidized by O_2^- but not by other ROS and specifically bind to the mitochondria, which is an important place of O_2^- generation. In this work, two different hemocytes populations were observed in agreement with previous studies where hemocytes after characterization by flow cytometry and morphological studies presented also two different groups of cells (Soares da Silva et al., 2002). Both hemocytes populations observed in our study presented different ability to

produce O_2^-. The results obtained showed that hemocytes from Folías Wreck (population 2) showed higher levels of FL-1 fluorescence as compared to the population 2 from Punta Cuevas. Interesting to note was the fact that hemocytes from the reference place Punta Cuevas presented the highest population (1) with low O_2^- production. Different types of radicals can mediate DCFH oxidation (Curtin et al., 2002; Rao et al., 1992); by reacting with different types of hydroperoxides after intracellular de-esterification and oxidation. Hemocytes from Folías Wreck showed a higher level of DCFH fluorescence than hemocytes isolated from samples collected in Punta Cuevas. These results indicated an enhanced hydroperoxide metabolism in *A atra atra*, potentially due to a higher exposure to metals such as Fe and Cd (Giarratano et al., 2013). Toxicological studies at cellular level have shown that metals such as Fe and Cd can affect mitochondrial metabolism and induce ROS production (Wang et al., 2004; Taze et al., 2016).

Although the measurement of TBARS is rather an unspecific test, it was used with other method to unequivocally characterize the lipid peroxidation process (Rice-Evans et al., 1991). Since endogenous lipid radicals show short half-lives and are present in low concentrations, detection is a difficult task. Spin trapping-EPR analysis overcomes the limit of sensitivity of endogenous radical content in biological systems, being the best method available to detect short-lived reactive free radicals generated in low concentrations in biological systems (Luo et al., 2006). Even though EPR detection of lipid radicals could be considered a fingerprint of radical presence, spin trapping studies cannot really distinguish among peroxyl, alcohoxyl and alkyl adducts owing to the similarity of the corresponding coupling constants (Jurkiewicz and Buettner, 1994). Coincidently with TBARS results, the EPR analysis showed higher $LR^{\bullet}$ content in hemocytes from Folías Wreck than in those from Punta Cuevas. This fact could be in agreement with observations of Giarratano et al., (2013, 2014), in which gills and digestive glands of bivalves presented higher $LR^{\bullet}$ by EPR procedure.

The high efficiency of the mitochondrial electron transport is mainly due to the presence of the phospholipid cardiolipin at the inner mitochondrial membrane, which is required for the proper structure and activity of mitochondrial respiratory chain complexes. NAO is a mitochondrial probe that binds specifically to cardiolipin. Cardiolipin content has been used as a parameter of mitochondrial function in marine bivalves (Barmo et al., 2013; Ciacci et al., 2012). This fluorescent dye binds tightly to the acidic phospholipid cardiolipin, which is found exclusively in inner mitochondrial and bacterial membranes (Petit et al., 1994). In this work the level of cardiolipin oxidation/depletion measured as low NAO fluorescence, was higher in Folías Wreck hemocytes than in Punta Cuevas organisms. Contrary to mammalian cells, incubation of ribbed mussel's hemocytes from Punta Cuevas and Folías Wreck with antimycin (complex III inhibitor) resulted in a decrease ROS production, coincident with the results reported for the Pacific oyster *Crassostrea gigas* (Donaghy et al., 2012). Antimycin effect in mitochondrial mammalians induces an increase in ROS, mediated by its ability to inhibit the respiratory chain at complex III between cytochrome b and cytochrome c1 (Alexandre and Lehninger, 1984; Maguire et al., 1992). The decrease in ROS production observed in bivalves could be due to the fact that in invertebrates the electron transport chain is branched with the presence of additional terminal oxidases (Buchner et al., 2001; Tschischka et al., 2000). The occurrence of these terminal oxidases and its genetic basis could indicate an alternative pathway for the electron transport, accepting electron directly from ubiquinol and reducing O_2 to H_2O (Hoffman and Brookes, 2009).

Monitoring of mixed beds containing *A. atra atra* has been proposed as an important opportunity to study the effect of climate change and pollution on the marine ecosystems in this area (Caza et al., 2016). In this sense, we can conclude that Folías Wreck bivalves seem to be exposed to higher stressing factors than those from Punta Cuevas, such as the high metal content that would induce an oxidative pressure. This fact could have a deleterious effect against organisms itself but could lead to important ecological alterations. Thus, we could conclude that hemocytes represent a sensitive model for the rapid screening of the cellular effects of different contaminants and pathogens. Furthermore, they could be useful to generate valuable tools for pollution monitoring and for establishing preventive measures that tend to protect the environment. Finally, we can state that this is the first work that indicates that two populations of *A. atra atra* hemocytes show different level of O_2^- content. It is interesting to note that the source of production is not already known and, requires future deeper studies.

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Chapter 122

BIOMARKER RESPONSES IN BIVALVES AFFECTED BY ENVIRONMENTAL STRESSORS ASSOCIATED WITH THE GLOBAL CLIMATE CHANGE

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ABSTRACT

The evaluation of oxidative stress in relation to the impact of global climate change on bivalves through the study of the organism's responses to mitigate the damage and control the generation of reactive oxygen species is analyzed in this chapter. Biomarkers are biological components that complement physico-chemical factors analysis in aquatic organisms by providing first biological signals of environmental situations. Since integration of individual biomarker responses into a set of tools and indices capable of detecting and monitoring the degradation in health of a particular organism is an actual challenge, we propose to evaluate the information on oxidative and physiological biomarkers triggered in bivalves in relation to the exposure to different environmental changes to obtain the best approach to it particular variation. We analyzed bivalves facing "natural stress conditions" (acidification, seasonality, temperature, oxygen partial pressure, salinity, biotoxins). From the revised researches, we found that some of the biomarkers seemed to respond in a better way than others depending on the environmental conditions faced by the bivalves. This chapter expands our understanding of multifactorial effects on the marine ecosystem, providing insight into the acclimation, adaptive and stress response processes of bivalves. Finally, such ecological and biogeochemical changes in the oceans could have profound consequences for marine biodiversity and seafood quality with deep implications for fishery industries in the upcoming decades.

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Keywords: biomarkers, natural environmental conditions, mussels, oxidative stress parameters

INTRODUCTION

Over the last decades, global warming has increased having substantial effects on acidification, salinity, temperature, dissolved oxygen (O_2), light penetration, nutrient conditions, biotoxins presence and metal inputs due to higher sediment run-off specially impacting on coastal and benthic ecosystems, between others (Dierssen et al., 2002; Quartino et al., 2013; Gutt et al., 2015; Sahade et al., 2015).

O_2 consumption is required by animals for the oxidation of food and the generation of energy. This partial reduction of O_2 results in the formation of reactive oxygen species (ROS), including superoxide anion radical (O_2^-), hydroxyl radical and hydrogen peroxide (H_2O_2). A basal amount of ROS is formed continuously in all aerobic cells, and the steady state concentration of ROS in each cell depends on the formation rate of these molecules, their reactivity and the concentration of available reaction partners. Then, when ROS formation exceeds antioxidant defense capability or disrupt redox signaling, affecting cell functionality, oxidative stress occurs (Jones, 2006). Regulated production of ROS and maintenance of "redox homeostasis" are essential for the physiological health of organisms (Ames et al.,1993). Antioxidant defenses include antioxidant molecules and enzymes which protect organisms from oxidative stress episodes. Essentially, pro- and anti-oxidant processes are the same in marine invertebrates and in mammalian systems (Livingstone et al., 1990; Livingstone, 1991; Winston & Di Giulio, 1991). However, the specific conditions for ROS formation are different and highly variable among invertebrate aquatic species (Lesser, 2006) where metabolic rates are much lower than in mammals (Abele & Puntarulo, 2004). Despite the presence of antioxidant defense systems, increased levels of oxidative damage generated by ROS to protein, lipid and DNA occurs in bivalves studied in laboratory and field (Livingstone, 2003).

Biomarkers are biological components that complement physico-chemical factors analysis in aquatic organisms by providing first biological signals of environmental situations. These components may be related to oxidative stress or to biochemical factors. Oxidative biomarkers, such as enzymatic antioxidants activities, non-enzymatic molecules, redox balances, oxidative indexes, and damage to lipid, protein and DNA can be considered. Also, other enzymes and biochemical components are involved in several metabolic pathways (Figure 1). Therefore, all of them can be taken as potentially indicators of the ecosystem fitness (Martinez-Haro et al., 2015). The balance between the mechanisms of damage and defense has ecological relevance since it keeps the ecosystem productivity and contributes to the regulation of potential climate change (Hernando et al., 2016). Even more, bivalves are considered key biomarkers organisms for environmental monitoring based on their economic importance, wide geographical distribution, abundance, sedentarism, tolerance to environmental alterations, their ability to concentrate pollutants, their levels of metabolizing

enzyme activities of organic contaminants, the nature of the populations, the life span, the body size, and the potential to survive in laboratory and field studies in cages.

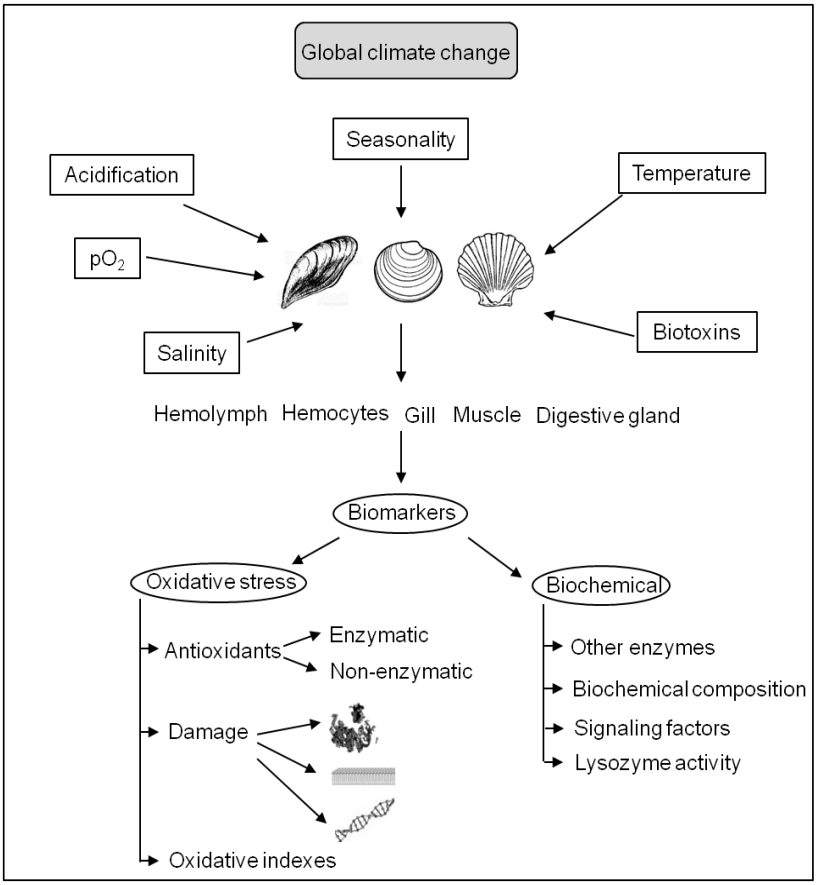

Figure 1. Diagram showing biomarkers commonly used to detect alterations related to natural factors modified by climate change on bivalves.

Actually, there is growing interest in the evaluation of oxidative stress in relation to the impact of global climate change on natural ecosystems in different trophic levels, through the study of the organism's responses to mitigate the damage and control the generation of ROS (Hernando et al., 2016). Since integration of individual biomarker responses into a set of tools and indices capable of detecting and monitoring the degradation in health of a particular organism is an actual challenge, we propose to evaluate the information on oxidative and physiological biomarkers triggered in bivalves in relation to the exposure to different environmental changes to obtain the best approach to it particular variation. We analyzed bivalves facing "natural stress conditions" (acidification, seasonality, temperature, O_2 partial pressure (pO_2), salinity and biotoxins).

ACIDIFICATION

Ocean acidification, a complex phenomenon that lowers seawater pH, is the net outcome of several contributions. They include the dissolution of increasing atmospheric carbon dioxide (CO_2) in addition with dissolved inorganic carbon generated upon mineralization of primary producers and dissolved organic matter (Mostofa et al., 2016). Marine organisms at low and high latitudes do not respond uniformly to ocean acidification (Hendriks et al., 2010; Toseland et al., 2013), and the expected effects can thus be stimulative, inhibitory, or neutral (Anthony et al., 2008; Gao et al., 2012; Hutchins et al., 2013). Facing ocean acidification marine organisms would express detrimental impacts which may derive from several processes that are closely interlinked: (i) acidification, (ii) synergistic effects of acidification and oxidative stress in surface seawater, (iii) low dissolved O_2 (hypoxia) and acidification in subsurface/deep seawater, and (iv) stress by algal or biotoxins and pathogens (Mostofa et al., 2016). Species that might be resilient to elevated CO_2 partial pressure (pCO_2) have been predicted to be metabolically active to compensate acid–base disturbances (Fabry et al., 2008; Widdicombe & Spicer, 2008). The capacity to compensate the reduction of the pH is related to the excretion of acid equivalents (e.g., H^+) or the increase in the intracellular buffering capacity via the import of HCO_3^- to compensate the intracellular acidosis.

Hemocytes of *Mytilus edulis* were analyzed under the effects of CO_2 or HCl-induced seawater acidification (pH 7.7 or 7.1 and control: pH 8.1) (Sun et al., 2017). It was observed that ROS production was significantly induced in both CO_2 and HCl treatments, and four antioxidant components, glutathione (GSH), glutsthione-S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx), had active responses to the acidification stress. Comparatively, CO_2 had more severe destructive effects on hemocytes than HCl at the same pH level, indicating that CO_2 stressed cells in other ways beyond the increasing H^+ concentration. One possible explanation was that seawater acidification induced ROS overproduction, which damaged the ultrastructure of hemocytes and decreased phagocytosis.

In gills and digestive glands of the mussel *Mytilus coruscus* three pH levels were studied (8.1, 7.7 and 7.3). It was detected that mussels exposed to pH 7.3 showed significantly higher activities for superoxide dismutase (SOD), catalase (CAT), GPx, alkaline phosphatase and glutamic-pyruvic transaminase, but a decrease in the acid phosphatase activity was observed when compared to those under higher pH condition (Hu et al., 2015). Tomanek et al. (2011) also showed that a decreased pH (pH 7.5) resulted in SOD upregulation in the mantle tissue of *Crassostrea virginica*. In that study, other proteins involved in antioxidant defence, such as peroxiredoxins, also showed upregulations, suggesting prooxidant conditions and potential oxidative stress.

Results obtained from Freitas et al. (2017) in *Mytilus galloprovincialis* showed that mussels exposed to seawater acidification, pH 7.3, presented decreased metabolic capacity that may have induced lower energy expenditure (observed in higher glycogen, protein and lipids content at this condition) compared to the control pH 7.8. Low pH condition induced the increase of carbonic anhydrase activity that was related to acid-base balance, while no significant activation of CAT and SOD activities were observed resulting in higher lipoperoxidation (LPO).

The clam *Scrobicularia plana* was able to develop mechanisms to prevent oxidative damage when was exposed to low pH 7.1 for 28 days compared to control pH 7.8. Freitas et

al. (2016) findings further demonstrated that the antioxidant enzymes were maintained (SOD activity) or significantly decreased (CAT activity); however, GST activity was increased when exposed to low pH. These results may be related to the higher LPO levels obtained, when *S. plana* was exposed to low pH compared to control. The results obtained revealed that after 28 days of exposure clams seem to have acquired the ability to adapt to pH 7.1 since mortality was recorded only up to 96 h of exposure. This is in concordance with the findings of Lindinger et al. (1984) which showed that decreases in intracellular pH appear to be almost fully compensated for after 24-48 h exposure in a wide range of marine invertebrates.

Priya et al. (2016) reported the investigation of the sublethal impacts of ocean acidification inducing hypercapnia (pH 7.5 and 7.0) and leakage of benthic CO_2 storage sites (pH 6.5-5.5) on the marine clam *Donax cuneatus*. The increase in LPO and CAT activities suggested that hypercapnia induced oxidative alterations. The decreasing pH also increased the activity of GSH and GST which act as detoxifying enzymes for hypercapnia induced toxicity. However, the hypercapnic conditions inhibited the levels of acetylcholine esterase, which indicates that the polluted environment induced high rate of mortality at low pH levels.

Bamber (1990) reported that the critical acid tolerances of marine bivalves, after 30 days exposure, could range from pH 6.6 in *M. edulis* to pH 6.0 in the oyster *Crassostrea gigas*. The impact of elevated pCO_2 in an organism will reflect the magnitude and duration of exposure and the physiological capacity of that organism to regulate tissue pCO_2 (Stumpp et al., 2012; Thomsen et al., 2013). Actually, whilst exposure to elevated pCO_2 has been reported to cause negative impacts in key marine invertebrates (Pörtner, 2008), other studies have shown that some species can succeed in the field in unfavorable pH environments (Thomsen et al., 2013). Maintenance of intracellular pH is essential for countless cellular functions and regulations (Putnam & Roos, 1997) and, in general, intracellular pH is regulated at 0.5–0.8 pH units below extracellular pH (Pörtner et al., 2004). Recent studies also confirmed that calcifying organisms can biologically control the environment of carbonate deposition by controlling the pH in extracellular fluid, or by controlling deposition in a regulated intracellular environment (Hendriks et al., 2015).

SEASONALITY

Abiotic and biotic environmental features such as temperature, O_2 consumption, availability of food, endogenous rhythms and algal blooms, may fluctuate according to the annual seasons. These factors might be potential stressors for aquatic organisms via induction of disbalance between the generation and elimination of ROS that would lead to damage to macromolecules (Abele & Puntarulo, 2004). Seasonal changes in oxidative metabolism have frequently been reported in marine ectotherms, including various bivalve species (Viarengo et al., 1991; Solé et al., 1995; Wilhelm Filho et al., 2001).

A decrease in the activity of antioxidant defenses in the digestive gland of *M. edulis* has been observed in winter with respect to summer, accompanied by an increase in the LPO (Viarengo et al., 1991); and also, the lipid content of these organisms changed significantly due to seasonal and metabolic factors (Okumuş & Stirling, 1998). Wilhelm Filho et al. (2001) reported in the brown mussels *Perna perna* an increase in O_2 consumption, endogenous antioxidants and lipid damage in summer compared to winter measurements. Lesser & Kruse

(2004) showed no seasonal-dependent changes in either SOD activity or protein levels in the subtidal horse mussels *Modiolus modiolus*, although respiration rates were high during summer with respect to winter. However, seasonal variability of antioxidant parameters evaluated in the digestive gland of the ribbed mussel *Aulacomya atra atra* showed that the antioxidant activities of both GST and CAT were induced in summer compared to winter, meanwhile SOD activity and ascorbyl radical content were parameters seasonally-independent (Giarratano et al., 2013). Giarratano et al. (2011) reported in the mussels *Mytilus edulis chilensis* a significant effect of seasonal-dependent variation on biological responses, in metal bioaccumulation and antioxidant enzymes activity. SOD activity was higher in winter than in summer organisms; meanwhile, CAT activity was not altered in *M. edulis chilensis*.

From these studies, it is notice that significant changes of antioxidant parameters in bivalves are closely correlated with the seasonal variations of temperature, dissolved O_2, chlorophyll a and many other changing factors. Seasonal variations arise from a complex interaction between exogenous and endogenous factors; consequently, it is difficult to draw unambiguous conclusions from such variations. However, these reports show the importance to include such variables in biomonitoring programs when analyzing data obtained from different periods of the year. Moreover, seasonal data may be useful to indicate the periods of the year more suitable for the marketing and consumption of commercial species. When the organisms have a best antioxidant protection during the year, it could be a contributing factor to prevent lipid damage, that is essential to improve organoleptic characteristics and to control stress during the processes of conservation and commercialization.

TEMPERATURE

Increases in surface seawater temperatures (2-4.5 °C) are probable to occur during the next decades (IPCC, 2007). Most physiological processes are temperature-dependent on various organisms; then, the increase of environmental temperature may lead to metabolic activation, which combined with an increase in O_2 consumption, initiates oxidative stress (Allakhverdiev et al., 2008; Halliwell & Gutteridge, 1989).

Matozzo et al. (2013) studied the effects of temperature changes (22 and 28°C), at 34 psu and 8.1 pH, in gills and digestive glands of *Chamelea gallina* and *M. galloprovincialis*. In the clams, only the thiobarbituric acid reactive substances in gills, and the CAT in the digestive gland, were affected. However, no modifications were observed in these parameters evaluated in the tissues of the mussel. In *M. galloprovincialis* respiratory tissues, hyperthermia (30°C) induced a p38 mitogen-activated protein kinase (p38-MAPK) activation in a rapid (maximum at 30 min), sustained (over 120 min) and considerable way; whereas the effect of hypothermia was not so intense (Kefaloyianni et al., 2005). The moderate response of p38-MAPK to hypothermia could be attributed to the fact that these organisms are routinely subjected to hypothermic stress and have consequently developed multiple adaptive responses in order to preserve their function under analogous conditions (Sheehan & Power, 1999; Lesser & Kruse, 2004).

An & Choi (2010) also evaluated physiological alterations at 10, 20 and 30°C in the ark clam *Scapharca broughtonii*. Water temperature changes significantly increased antioxidant SOD and CAT mRNA expression and activity in the digestive glands and gills in a time-

dependent manner at 30°C compared to the other temperatures. H_2O_2 concentrations, LPO, aspartate aminotransferase and alanine aminotransferase levels also increased significantly in a time-dependent manner in the high-temperature treatments, while lysozyme activity decreased. Therefore, in an early-stage exposure to high temperatures, oxidative stress was regulated by antioxidant enzymes, but continued thermal stress resulted in an increase the oxidative stress and damage due to the diminished protection.

Green-lipped mussels *Perna viridis* were observed to increased activities of antioxidants such as SOD, CAT, GPX, GR and GST both in gills and digestive glands under long-term exposures to 32 compared to 26°C, suggesting the activation of physiological mechanism to scavenge the ROS produced during heat stress. Also decreased values of GSH on long exposures to temperature stress indicate utilization of this antioxidant, either to scavenge ROS or to act in combination with other enzymes (Verlecar et al., 2007).

The results on different bivalves exposed to temperature variations suggested that they alter the oxidative metabolism by modulating antioxidant enzyme activities. Even more, as the frequency of extreme thermal events increases, intertidal animals may activate their cellular defense and repair mechanisms; otherwise, they may accumulate higher concentrations of cellular damage due to heat stress (Jimenez et al., 2016).

SALINITY

Salinity is a major controlling factor in coastal systems whose fast change, often associated to tidal oscillations, causes drastic alterations on aquatic communities by promoting a physiologically stressful environment. Hyposaline conditions may be produced by dilution factors such as rainy periods and fresh water inputs by rivers, snow or glacial melting. Meanwhile, hypersaline situations are due to salts concentration events such as marine water evaporation. Salinity is one of the main controlling factors of growth, causing great constrains on species productivity and diversity, and a variety of physiological responses (Regalla et al., 2007; Kirrolia et al., 2011; Verdelhos et al., 2015). Osmotic stress exerts considerable oxidative stress, associated with enhanced ROS generation, in organism living in the intertidal zone. Bivalve species are able to tolerate large salinity changes, even though they do not show osmo-regulatory abilities (Evans, 2009).

Freitas et al. (2017) exposed *M. galloprovincialis* to high (35 psu) and low (14 psu) salinities. Mussels presented lower metabolic activity (also related to lower energetic expenditure) at high salinity compared to low salinity organisms. This lower metabolic activity, probably related to valves closure, helped to mitigate the increase of lipid damage in this condition. At low salinity, despite the rise of CAT and SOD activities, lipid damage increased, probably as a result of ROS overproduction from higher electron transport system activity. Carbonic anhydrase slightly augmented at stressful salinity conditions, a mechanism of homeostasis maintenance. Matozzo et al. (2013) also studied the effects of salinity changes in gills and digestive glands of *M. galloprovincialis* and *C. gallina*. They observed that 28, 34 and 40 psu conditions significantly influenced the oxidative parameters (CAT, SOD, GST, thiobarbituric acid reactive substances) of the bivalves; although the variation pattern varied depending on the species and tissues analyzed. Even more, Hamer et al. (2008) previously investigated the exposure to reduced seawater salinities and its effect on DNA integrity, p38-

MAPK activation, metallothionein induction and O_2 consumption rate on the gills of the mussel *M. galloprovincialis*. O_2 consumption rate was dependent to salt concentrations and increased considerably to about 51 and 65% in 28 and 18 psu, respectively, compared to control mussels (37 psu). DNA integrity was negatively impacted by hyposalinity. Reduced salinities (18.5 and 11 psu) resulted in significantly higher p38-MAPK phosphorylation, whereas at 28 psu it was significantly decreased compared to control. The concentration of metallothioneins in mussels' gills was reduced at 28 and 18.5 psu, while it was significantly higher at 11 psu.

In two estuarine commercial clam species *Cerastoderma edule* and *S. plana*, biomarker responses on the foot around their optimal salinity value of 20 psu (from 10 to 35 psu) were studied (Gonçalves et al., 2017). They found a decrease on SOD enzymatic activities in both species, but the GST was reduced in *S. plana* and increased in *C. edule*. Similarly, Tankoua et al. (2011) also demonstrated the influence of salinity in some classes of biomarkers (GST, acetylcholynsterase, lactate dehy-drogenase and digestive enzymes) determined in *S. plana*. When unsaturated fatty acids were analyzed in these two bivalve species, their diversity and quantity presented the highest values at the extreme salinity treatments compared to control organisms.

In the ark shell *S. broughtonii* the response to hyposalinity and hypersalinity was studied in digestive glands, gills and hemolymph (An & Choi, 2010). The authors hypothesized that salinity changes increase oxidative stresses and SOD and CAT provided the antioxidant defense and protection. SOD was the first-line of defense against O_2^-, since SOD mRNA expression and activity increased when H_2O_2 concentrations in the hemolymph were high. As a second-line of defense, CAT mRNA expression and activity increased to reduce H_2O_2 concentrations. They also found that the lysozyme activity decreased in the hemolymph throughout the experiment, suggesting that salinity changes negatively affected immunity and reduced lysozyme activity. The observed increases in aspartate aminotransferase and alanine aminotransferase levels indicated that tissue damage also occurred facing the salinity changes.

The present findings demonstrated that bivalve's oxidative status and metabolic capacity were negatively affected by salinity changes, with alterations that may lead to physiological impairments namely on mussels reproductive output, growth performance and resistance to disease, with ecological and economic implications (Freitas el al., 2017).

OXYGEN PARTIAL PRESSURE (pO_2)

Marine coastal environments can become hypoxic or anoxic, changing completely the geochemistry of the areas. The rocky intertidal zone is a highly variable environment that ranges from fully aquatic to fully terrestrial conditions over the tidal cycle (Jimenez et al., 2016). Even more, low pO_2 is a frequently condition of many estuaries, swamps, and tidal flats, which receive high amounts of organic matter from coastal runoff (Abele et al., 2017). Conditions may worsen during warm summers when benthic biological and chemical O_2 demands increase. Gills of bivalve filter feeders serve as a particle filter and as gas exchanger at the same time; meanwhile the mantle is also involved in gas and ionic exchange (Henry and Saintsing, 1983). They might therefore be prone to suffer both elevated ROS levels and oxidative damage related to continuous contact between these tissues and modifications in the

pO_2. To cope with these situations, the bivalve shell enables its occupant to behaviorally adjust the internal environment with respect to dissolved gases, nutrients and the composition of bacterial communities in the surroundings of the soft tissues (Abele & Philipp, 2012).

Rivera-Ingraham et al. (2013) studied the effect of hypoxia and anoxia exposures in gills of *M. edulis*. Exposure to declining pO_2 caused an increase of gill metabolic rate between 21 and 10 kPa, a pO_2 range in which whole animal respiration is reported to be oxyregulating. Exposure of the animals to severe anoxia caused an onset of anaerobiosis (succinate accumulation). Concentrations of ROS decreased strongly during anoxic exposure of the mussels and increased upon reoxygenation. This ROS burst induced LPO in the mantle, but neither was protein carbonyl levels increased, nor did the tissue SOD and CAT activities and GSH concentration change. Further, analysis of apoptosis markers (activity of caspases 3/7) indicated no induction of cell death in the gills. The authors conclude that these hypoxia tolerant intertidal mussels did not suffer major oxidative stress in gill under these experimental conditions. *M. edulis* from high shore (naturally experienced 6 h air exposure, twice a day) and low shore (naturally experienced 2-2.5 h air exposure, few days a month) was also studied in gills and digestive glands collected after 2 h of air exposure. The authors found that the antioxidant enzymatic activities (CAT, SOD, GST, GPX, GR) were higher in mussels living in the upper intertidal zone, suggesting an ability to adjust their antioxidant defenses in response to the stressful situation induced by living in this part of the habitat (Letendre et al., 2009).

Mussels *P. perna* were exposed to air for 24 h showing a clear increase in the levels of LPO in the digestive gland and gills, while oxidative DNA damage increased only in the gills. In mussels re-submersed for a period of 3 h after the 24 h of air exposure these values returned to pre-aerial exposure levels. On the other hand, in a group of mussels exposed to air for 18 h and then to 1 h of re-submersion, SOD, CAT, GPX, GR, glucose-6-phosphate dehydrogenase, and total GSH were not modified. Only a 52% increase in the GST activity was observed in the digestive gland, which remained elevated after 1 h of re-submersion (Almeida et al., 2005).

Several studies have demonstrated that bivalves from tidal shores maintain respiratory independence against variable and diminishing ambient pO_2 by inducing ventilation and circulation of mantle-cavity water in order to maintain a sufficiently oxygenated state of their tissues (Bayne, 1971; Taylor & Brand, 1975; Brand & Morris, 1984; Strahl et al., 2011; Abele & Philipp, 2012; Rivera-Ingraham et al., 2013). However, physiological normal conditions, in the absence of water, require a complex and finely tuned set of mechanisms working in close coordination (França et al., 2007). In this context, enzymatic antioxidant defences suggest having the ability to acclimate during the hypoxic state (Chandel et al., 1998; Chandel & Schumacker, 2000) or following a hypoxic episode (Boveris & Chance, 1973; Loschen et al., 1973; Duranteau et al., 1998), where ROS are released from ubisemiquinone during tissue reoxygenation. Therefore, the location of organisms on the shore should be taken into account in sampling for ecotoxicological studies.

BIOTOXINS

Temperature changes and nutrient pollution in coastal areas contributes to the advance and maintenance of harmful planktonic toxins; however, physical, biological, and other

chemical factors may promote harmful responses (Sellner et al., 2003). An enhancement of human activity in coastal areas has increased eutrophication processes which resulted augmentation in frequency, intensity and geographical distribution of harmful algal blooms (HAB) during the last several decades (Derwent et al., 1998; Ibelings et al., 2007; Aguiar et al., 2011). These blooms can produce an extensive range of phycotoxins from a variety of microalgal species (Glibert et al., 2005). Aquatic animal species, such as bivalves, can accumulate bloom forming algae and their toxins by filtering water containing them, while less amounts are directly absorbed in a dissolved manner (Burmester et al., 2011). Responses of some mussel species to algal toxins have been previously described (Pflugmacher et al., 1998; Contardo-Jara et al., 2008; Burmester et al., 2011; Vareli et al., 2012; Kwok et al., 2012). The capacity to bioaccumulate and respond to toxins differs in a species-specific manner among bivalves and the harmful microalgae on which they may feed (Cembella et al., 1993; Nagai et al., 1996; Smolowitz & Shumway, 1997; Shumway, 2006; Hégaret et al., 2007 a,b). These relationships suggest that bivalves are more than simple transference vectors of phycotoxins to humans or other feeders at higher trophic levels. In fact, it has been reported that some types of marine toxins can reduce growth and reproduction rates of bivalves and could be lethal for some species (Shumway et al., 2006; Samson et al., 2008).

Digestive glands from *M. edulis platensis* were isolated from specimens collected in the Argentinean Sea during summer, winter and spring (in the presence of harmful planktonic toxins) (González & Puntarulo, 2016). It was shown that SOD and GST activities decreased in winter and spring, compared to samples collected in summer. During the spring season, the bivalves were facing oxidative stress, as indicated by the increases measured on hydrophilic (ascorbyl radical/ascorbate) and lipophilic (lipid radical/α-tocopherol) cellular media indexes of redox balance and on 2′,7′ dichlorofluorescein diacetate oxidation rate. Meanwhile, under physiological conditions, these biomarkers seem to be adequately controlled to keep steady state concentrations of damaging reactive species far from dangerous levels.

Qiu et al. (2013) studied the effect of a paralytic shellfish toxin (PST)-producing dinoflagellate, *Alexandrium tamarense,* on the muscle and digestive glands of the scallops *Patinopecten yessoensis* and the mussels *M. galloprovincialis*. Their results showed that ROS was quickly generated and then disappeared in the tissues of scallop and mussel in the PST-accumulating and depurating periods, and that a different response of antioxidant enzymes to ROS occurred in the two different bivalves. Only GPx enzyme was induced to remove ROS in the scallop, but SOD, CAT, and GPx enzymes were stimulated to avoid oxidative damage in the mussel. *M. galloprovincialis* was also sampled to study the possible impairments of physiological parameters under naturally exposed to algal yessotoxins (YTX) levels (Buratti et al., 2011). Clear correlations were established between the increase of homo-YTX (and concomitant decrease of YTX) and the increase of cAMP levels, thus providing further insights into the mechanisms of action of these compounds. Similarly, direct correlations were established between the increase of homo-YTX, cAMP levels and multixenobiotic resistance gene products expression at submaximal algal concentrations. On the contrary, at the maximum levels of algal accumulation, multixenobiotic resistance gene expressions were greatly reduced. A significant reduction of lysosome membrane stability was also observed in paralytic shellfish poisoning- or lipophilic toxin-positive mouse bioassay mussels, reinforcing the hypothesis that lysosomes are relevant targets of YTXs. Considering the significance of this biomarker as predictive of detrimental effects at the organism level (Viarengo et al., 2007; Moore et al., 2008), this result suggests the development of a stress syndrome in

mussels due to algal toxin accumulation. This apparent reduction of health status was concomitant with the inhibition of the multixenobiotic resistance-related gene expression in the mussels, therefore with a possible reduction in their defense system. These results corroborate previous reports indicating alterations of mussel physiological functions after exposure to algal toxins and suggest some molecular targets.

Hemolymph of the bivalve *Mytilus chilensis* was analyzed after the injection of an extract of the marine microalgae *Alexandrium catenella* which produces saxitoxin (STX) (Núñez-Acuña et al., 2013). The authors evaluated the expression of 13 candidate genes associated with cellular stress and immune response. Analysis by qPCR showed higher gene transcription levels in *M. chilensis* injected with STX than in control mussels. High levels of differential gene expression were observed for SOD, CAT, ferritin and heat-shock protein genes. To a lesser extent, ependymin, fibrinogen-like, galectin and mytilin B genes were also significantly more expressed in hemocytes of mussels injected with STX that in control mussels. These results provided insights into how marine toxins could modulate the innate immune system of marine invertebrates.

Besides deep investigations regarding contaminated seafood toxicity to humans with an association to economic losses, many studies were also undertaken to examine the influence of algal toxins on bivalves themselves since they lead to significant ecological damage (Geraci et al., 1989; Shumway, 1990; Bourdelais et al., 2002). Biotoxins may affect mussel physiological pathways, including hemocyte functionality, lysosome membrane stability, antioxidant enzymes activities, gene expression and cell signaling.

CONCLUSION

The revision on the biomarkers responses in bivalves affected by environmental stressors associated to the global climate change undertaken in this chapter indicates a high modulation capability of biochemical responses of the organisms. In this fluctuating global context, organisms will be chronically exposed to elevated temperature, a decreased pH, changes in salinity, modifications during seasons, pO_2 and biotoxins presence. The energy costs associated with the found stress responses in this chapter may alter organism energy budget and translate it into negative effects on fitness. We verified biomarkers related to the commented environmental stressful changes in ten mussel species (*M. coruscus, M. galloprovincialis, M. chilensis, M. edulis chilensis, M. edulis, M. edulis platensis, A. atra atra, P. viridis, P. perna, M. mondiolus*), five species of clams (*S. plana, D. cuneatus, C. gallina, S. broughtonii, C. edule*), one scallop (*P. yessoensis*) and two oysters (*C. virginica, C. gigas*). The variations in environmental factors caused by climate change generate a situation of oxidative damage and biochemical changes in bivalves as a direct or indirect consequence.

According to the data tested and the biomarkers evaluated form all the mentioned authors we found that some of them seemed to respond in a better way than others depending on the environmental conditions faced by the bivalves (Figure 2). When analyzing the acidification factor, it was revealed that GSH related enzymes and lipid damage were augmented. The antioxidant enzymes studied over different seasons showed a high variability effect according to the species and their habitat latitude, so they do not seem the best indicator of stress. Other

biomarkers should be tested instead, like hydrophilic and lipophilic indexes (González & Puntarulo, 2016). However, when these molluscs were exposed to increments in temperature, many antioxidant enzymes were activated. Salinity changes generated DNA and lipid damage with antioxidant enzymes modifications. Even more, various signal transduction pathways are involved in the regulation of the environmental stress response, apoptosis and facilitation of the repairmen of damaged proteins and other cellular components (Woessmann et al., 1999). In particular, p38-MAPK has been characterized as the principal stress-kinase responsive to fluctuations in ambient osmolality and temperature (Zhang & Cohen, 1996; Kultz & Burg, 1998; Gon et al., 1998). The effect of the diminished pO_2 also evidenced an increase in the DNA and lipid damage even though the increment observed in the activity of the antioxidant enzymes. Good biomarkers associated to biotoxins were ROS tests and oxidative stress indexes. Genes codifying for antioxidants enzymes and their activities were also affected but in different ways according to the studied toxin and bivalve species.

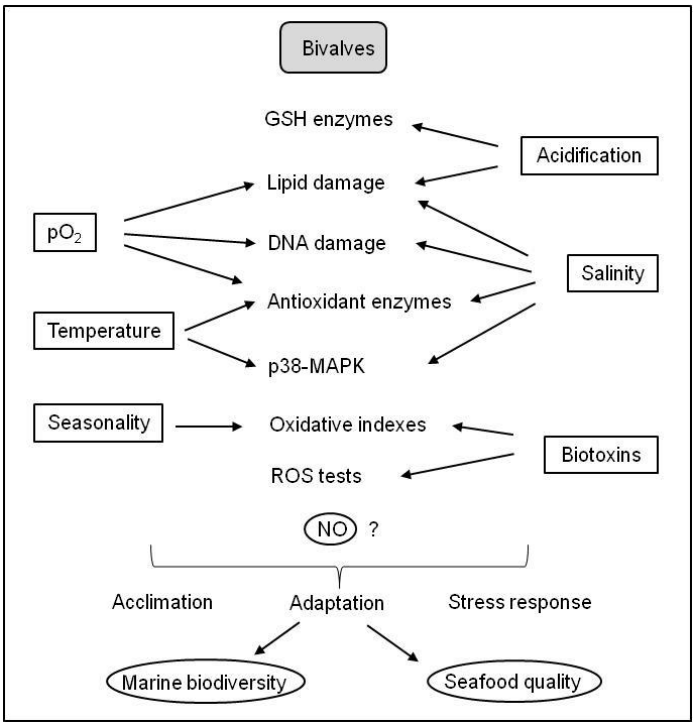

Figure 2. Biomarkers used to evaluate the effect and consequences of climate change on bivalves over the last few years.

Even more, nitric oxide (NO) has been shown to act as intracellular or transcellular signal and as cytotoxic host defense compound (Moncada et al., 1991; Knowles & Moncada, 1994; Moncada & Higgs, 1995). ROS and reactive nitrogen species cellular pathways are related through the reaction of O_2^- with NO to generate peroxynitrite ($ONOO^-$). $ONOO^-$ is a nitrating agent and a potent oxidant able to modify proteins, lipids and nucleic acids (Lu et al., 2014). González & Puntarulo (2016) compared NO content in digestive glands from *M. edulis platensis* isolated during three seasons. Spring samples showed a 2.7-fold NO increased compared to summer; however, this enhancement was significantly lower than the

value reached during winter. Thus, seasonality seems to be one factor responsible for altering NO content in this species. Tomanek et al. (2011) hypothesized that oysters exposed to elevated pCO_2 can cause oxidative and nitrosative stress by the ROS production reacting with $ONOO^-$. Future studies may include NO measurements as a possible biomarker for climate change stressors (Figure 2).

This chapter expands our understanding of multifactorial effects on the marine ecosystem in the specificity of investigated biomarkers and biotests, providing insight into the acclimation, adaptive and stress response processes of bivalves. Finally, such ecological and biogeochemical changes in the oceans could have profound consequences for marine biodiversity and seafood quality with deep implications for fishery industries in the upcoming decades (Figure 2) (Doney et al., 2009; Mora et al., 2013; Jin et al., 2015).

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Chapter 123

THE EFFECTS OF ASCORBIC ACID ON LIPID OXIDATION DURING THE PROCESSING OF *MYTILUS EDULIS CHILENSIS* IN THE BEAGLE CHANNEL (TIERRA DEL FUEGO)

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ABSTRACT

The peculiarity of membrane lipids with high polyunsaturated fatty acids content in mollusks, suggests a special pattern for the development of lipid oxidation, decreased organoleptic characteristics and loss of product value for sale. In the Puerto Almanza area, Tierra del Fuego, mussel extraction is carried out for fresh commercialization in local cities. The antioxidants application in different marine resources at any stage of processing has been effective for the oxidative control. Our objective was to evaluate the level of lipid oxidation (tiobarbituric acid reactive species content, TBARS) and non-enzymatic antioxidants content in mussel (*Mytilus edulis chilensis*) for commercialization, after the treatment with ascorbic acid (AH^-). Mussels extracted from offshore batch culture, were exposed to different treatments: T1: dry control, T2: control in seawater without antioxidant, T3: exposed to AH- (10 mM) and T4: exposed to AH- (5 mM). Subsamples of each treatment were taken at 0, 6 and 24h, for analysis. A TBARS increase of 36% and ascorbyl radical content ($A^{\bullet}$) were observed during the first 24h on T1. The AH^- incorporated by the mussels showed an antioxidant activity avoiding lipids oxidation during the first stage of processing (24h of exposure) comparing T1 and T4. These results showed the generation of oxidative stress in mussels during dry condition.

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The AH^- content in frozen mussels decreased significantly after 24h of exposure to the antioxidant in T1 and T4, probably due to consumption. In addition, both $A^\bullet$ as the $A^\bullet/AH^-$ index, increased significantly comparing T1, T3 and T4 treatments. This indicates that the manipulation conditions, transfer and cold storage generated an oxidative stress situation.

Overall, our results indicated that the maintenance in water for 24h and that the addition of AH^- (5 mM) after the mussel extraction, are beneficial during the transfer period and avoid the stress generated by manipulation of *M. edulis chilensis*. Higher concentrations of the AH^- could produce an effect contrary (pro-oxidant) in 24h. Further, cold storage (-20 ºC) for 5 days, regardless of the addition of antioxidants, does not prevent the oxidative lipid damage nor improve product quality.

Keywords: antioxidant, ascorbic acid, *Mytilus edulis chilensis*, oxidative stress

INTRODUCTION

Placed at the southern extreme of South America (54°52'50,01" S, 67°45'21,82" W), the Beagle Channel is a drowned glacial valley that connects the Pacific and Atlantic oceans trough the islands of Tierra del Fuego. Due to its particular location, it is considered a key environment for the study of interactions between the sub-Antarctic and the Antarctic regions (Pérez-Barros et al., 2004). Navarino and Isla Grande de Tierra del Fuego act as barriers to give this part of the Beagle channel the character of a semi-enclosed water body. Low surface salinity (2.5-2.7 PSU) from intense local inputs of great amounts of glacial meltwater (Klöser, 1996) can produce a strong stratification, which will remain as long as intense winds are absent (Villafañe et al., 2001). Ushuaia city, the biggest settlement on the channel with about 70000 inhabitants, exerts an increasing anthropic pressure by the way of discharges of urban and industrial effluents to its waters (Torres et al., 2009).

ROS generate damage to macromolecules through the reaction of these species with proteins, DNA and lipids constituting the biological systems (Elstner, 1982; Halliwell and Gutteridge, 1984). Proteins and peptides are composed of amino acids that are the object of attack both free radicals and other reactive molecules (Dalle-Donne et al., 2003). Studies on oxidative damage to amino acids have provided sufficient information about changes in the macrostructure of proteins. Lipid oxidation is the oxidative deterioration suffered by polyunsaturated fatty acids (PUFA) (Girotti, 1985). This process occurs through a mechanism that involves free radicals and causes cytotoxic effects that include membrane structural alterations, decreased fluidity, increased permeability of cellular components, and inactivation of enzymes and membrane transporters (Girotti, 1985; Halliwell, 1999).

The non-enzymatic antioxidant defenses are low molecular weight biomolecules that exert their activity through different mechanisms. These antioxidants can be liposoluble, so they are related to the protection of membranes (tocopherols, carotenoids, ubiquinols), as well as water-soluble (glutathione, ascorbic acid), phenolic compounds (flavonoids) and complex molecules. Ascorbate (AH^-) is located in all subcellular compartments (Smirnoff, 2000), being able to intervene in spontaneous reactions with free radicals generated in a biological system (Buettner and Jurkiewicz, 1993). It´s an excellent antioxidant and participates in both enzymatic and non-enzymatic reactions. Direct reactions not catalyzed enzymatically include the role of AH^- as a trap for hydroxyl, $^1O_2$ and O_2^- (Foyer et al., 1994). Additionally, it can

protect biological membranes through the regeneration of oxidized α-Tocopherol (α-T), being probably it´s most important function (Packer et al., 1979; Buettner and Jurkiewicz, 1996). However, the AH^- in high concentrations and in presence of transition metals, can act as a pro-oxidant agent. The $A^{\bullet}/AH^-$ ratio can be used as an indicator of the balance between damage caused by free radicals and antioxidant protection (Galleanoet al., 2002).

Considering the Beagle Channel characteristics explained above, an incipient mussel aquaculture (*Mytilus edulis chilensis*) has developed in the area in the last years, representing an important commercial resource for local populations. Shelf-life extension can be achieved by various preservation methods, i.e., refrigeration, freezing, salting, brining (wet salting), icing, smoking, glazing, drying, frying, modified atmosphere packaging, supplementation with antioxidant (Gandotra et al., 2012). Mussels are an excellent source of long-chain polyunsaturated acids. The particularity of the membrane lipids of marine organisms, with a high content of polyunsaturated fatty acids (PUFA), suggests a special pattern for the lipid oxidation and consequent reduction of energy as a source for human consumption. This oxidation causes rancid flavors and odors, which make it unacceptable from the consumer's point of view. The development of lipid oxidation can occur during the stage of collection, processing and cold storage for subsequent sale and consumption, depending on variables such as: period and temperature of storage, natural antioxidants, presence of physical or chemical treatments and packaging (Fernández-Reiriz et al., 1995). For this reason, in some of the production stages of this sea food, the food industry incorporates natural or synthetic antioxidants (Cuppett, 2001; Pokorný, 2007). The supplementation with antioxidant is a food preservation method used to extend the shelf life and to retain the quality of various foods.

The main goal of the present study, was to evaluate the levels of lipid peroxidation and content of non-enzymatic antioxidants in *Mytilus edulis chilensis*. The research was developed with organisms extracted from commercial cultivation, being developed in Almanza, Tierra del Fuego. The determinations were made in the different stages of the usual processing of the organisms destined for commercialization, after treatment with ascorbic acid.

MATERIALS AND METHODS

Experimental Design

To determine the effects of oxidative damage and antioxidants responses in mussels, 420 individuals of commercial size were harvested from mussel farmers in Bahía Brown, Almanza, Tierra del Fuego. The mussels were obtained manually from the floating rafts, and were immediately transported to the processing plant, where the animals were submitted to different treatments. The organisms were divided into 4 groups of 100 individuals each and were conditioned in tanks with filtered sea water and controlled temperature and salinity during 24h. The following treatments were then applied: T1: dry control, T2: control in seawater without antioxidant, T3: exposed to AH^- (10 mM) and T4: exposed to AH^- (5 mM). Sub-samples of 10 mussels were taken at 0, 6 and 24h in order to determine lipid damage and antioxidant content for each treatment. Additionally, sub-samples were taken and preserved in -20°C for 5 days. The mussels were processed until biochemical’s determinations. For

determination on mussel's tissue, 10 g of sample was homogenized in 20 ml of distillated water and pH was measured using a pH probe (Woyewodaet al., 1986).

Physico-Chemical Determination in the Sampling Area

The parameters were evaluated at 1m depth in the water column. The following were measured: pH, temperature, dissolved oxygen, and salinity, using a Horiba U-10 multiparameter probe. The accuracies of the readings were 0.01 for the pH and 0.1 for the temperature, dissolved oxygen and salinity. With the same probe used for *in situ* measurements, it was determined the temperature and pH in the containers corresponding to each experimental treatment.

TBARS Content

The homogenates were treated with 30% (w/v) trichloroacetic acid and 50 mM potassium phosphate buffer (pH 7.0). After centrifugation, the content of TBARS was determined in the supernatant spectrophotometrically at λ = 535 nm, using TBARS standards that were created by the acid hydrolysis of 1, 1, 3, 3 tetraethoxypropane, according to Malanga *et al*., (2004).

Lipid Soluble Antioxidants Content

The α-T content mussels was quantified by reverse-phase HPLC with electrochemical detection using a Bioanalytical Systems LC-4C amperometric detector with a glassy carbon working electrode at an applied oxidation potential of 0.6V (Desai, 1984). Samples were extracted with 1 mL of ethanol and 4 mL of hexane. After centrifugation at 600 ×g for 10 min, the hexane phase was removed and evaporated to dryness under N_2. Extracts were dissolved in methanol/ethanol (1:1) and injected for HPLC analysis (Desai, 1984). D,L-α-tocopherol (Sigma) was used as standards.

Ascorbyl Radical Content (A•)

A Bruker ECS 106 spectrometer was used for A• measurements. Homogenates from mussels were prepared in pure dimethylsulfoxide (DMSO) (1:3) and the spectra were scanned at room temperature under the following conditions: 50 kHz field modulation, microwave power 20 mW, modulation amplitude 1 G, time constant 655 ms, receiver gain 1 105, microwave frequency 9.81GHz, and scan rate 0.18G/s (Giulivi and Cadenas, 1993). Quantification was performed according to Kotake et al., (1996). The amount of spin adduct was calibrated using an aqueous solution of TEMPO, introduced into the same cell used for spin trapping. EPR spectra of spin adduct solution and TEMPO solution were recorded at the same spectrometer settings and the first-derivative EPR spectra were double integrated by a computer attached to the EPR spectrometer to obtain the area intensity, and then the concentration of spin adduct was calculated using the ratio of these areas.

Ascorbate Content (AH^-)

The content of AH^- was measured by reverse-phase HPLC. The samples were homogenized in metaphosphoric acid (10%, w/v) according to Kutnink et al., (1987). The content of ascorbate in the cell homogenates was quantified by reverse-phase HPLC with electrochemical detection using a LC-4C amperometric detector with a carbon working electrode at an applied oxidation potential of 0.6 V. A Supelcosil LC-18 column was

stabilized with metaphosphoric acid (0.8%, w/v) and a freshly prepared solution of ascorbic acid in metaphosphoric acid (10%, w/v) (1 µg/ml) was used as standard.

Considering that a AH^- concentration was variable between the different treatments, the $A^{\bullet}/AH^-$ ratio was calculated in order to show more precisely the non-generation of an oxidative stress situation after supplementation with ascorbic acid.

Statistical Analyses

Data in the text and tables are expressed as mean ±S.E.M. of five independent experiments, with two replicates in each experiment. Statistical tests (Mann-Whitney) were carried out using GraphPadInStat, version 3.01.version 5.0 (Abacus Concepts, Inc., Berkeley).

RESULTS

The physico-chemical parameters measured on the Beagle Channel (Almanza) in the sampling area can be seen in Table 1. They are within the average values for the area during the time of year in which the sampling was done (Hernando et al., 2007).

The seawater pH value in the treatment containers were close to neutrality and no significant variations were observed over time in the treatment without addition of antioxidants. However, in the containers supplemented with ascorbic acid, significant decreased values were observed after 6h independently of concentrations ($P< 0.05$, Table 2). Regarding to seawater temperature, the values in the treatment containers were close to the Beagle Channel values (Table 1) and no significant variations were observed over time or treatments ($P > 0.05$, Table 2).

Table 1. Physico-chemical parameters in the Beagle Channel for sampling area

pH	8.36 ± 0.05
Conductivity (mS/cm)	43.3 ± 0.9
Dissolved oxygen (mg/l)	12.0 ± 0.8
Temperature (°C)	9.5 ± 0.4
Salinity (PSU)	27.2 ± 3.2

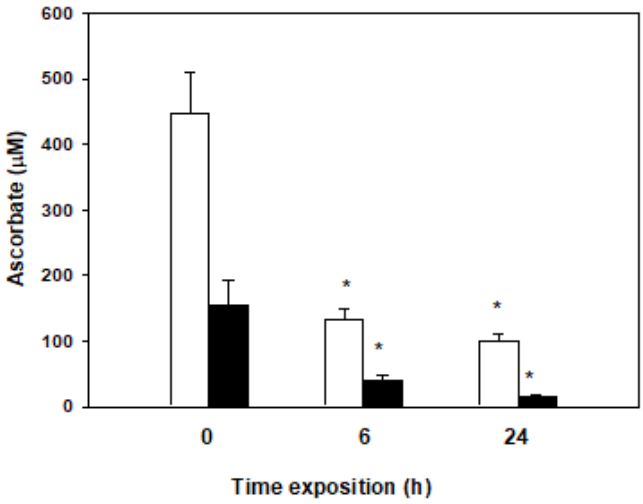
Table 2. Seawater pH and temperature for different treatments with *Mitylus edulis chilensis*

	Time (h)	T1	T2	T3	T4
pH	0	nd	7.65 ± 0.05	3.35 ± 0.02*	3.81 ± 0.01*
	6	nd	7.81 ± 0.02	3.87 ± 0.03*	4.01 ± 0.02*
	24	nd	7.68 ± 0.02	3.72 ± 0.03*	3.93 ± 0.02*
Temperature	0	9.4 ± 0.2	9.3 ± 0.2	9.4 ± 0.3	9.3 ± 0.2
(°C)	6	9.8 ± 0.1	9.6 ± 0.2	9.4 ± 0.1	9.8 ± 0.4
	24	9.8 ± 0.2	9.6 ± 0.2	9.5 ± 0.3	9.7 ± 0.5

T1: dry control, T2: control in seawater without antioxidant, T3: exposed to AH- (10 mM) and T4: exposed to AH- (5 mM).

***** Significantly different from the value of T2 at any experimental time in the same maintenance conditions ($P< 0,05$).

The AH^- content in seawater before to the incorporation of the mussels in the different treatments were: 0.071 ± 0.005 µM for T2 (control in seawater without antioxidants); 446 ± 65 µM in T3 (seawater with 10 mM ascorbic acid) and 154 ± 38 µM in the T4 (seawater with 5 mM ascorbic acid), but decreased significantly ($P < 0.05$) in the containers that were supplemented with ascorbic acid after 6 and 24h (Figure 1).



* Significantly different from the ascorbate value at time 0 under the same culture conditions ($P < 0.05$).

Figur 1. Ascorbic acid content in seawater for different treatments. (□) T3: high AH^- concentration (10 mM); (■) T4: low AH^- concentration (5mM).

After exposing the mussels to ascorbic acid for 24h at T = 10.2°C, no significant differences were observed in the internal pH, except an increase of 5.5 to 6.2 from 0 to 6h of exposure, in mussels from T3. Non-significant differences were observed in the pH values of all the mussels treated during 5 days of cold storage (5.8 ± 0.3). The TBARS content for the mussels during the first stage of processing, exposed or not to ascorbic acid during 6h, did not show significant differences comparing with time 0 ($P > 0.05$). However, after 24h, a significant decrease ($P < 0.05$) was observed in mussels exposed to seawater supplemented with the highest concentration of ascorbic acid (10 mM). On the other hand, there was a significant increase ($P < 0.05$) of the TBARS content in mussels kept dry for 24 h compared to the value at time 0, immediately after being collected (Table 3).

The AH^- content increased significantly ($P< 0.05$) in the mussels from the containers supplemented with ascorbic acid, independently of the concentration for 6 and 24h (Table 3). A typical EPR spectrum for the $A^\bullet$ in the presence or absence of mussel tissue is shown in Figure 2. No significant differences were observed on it for any of the treatments ($P> 0.05$, Table 3). The $A^\bullet$ content and the ratio $A^\bullet/AH^-$ were determined in order to evaluate if the supplementation with ascorbic acid generated an oxidative stress situation “per se”. A non-significant increase ($P> 0.05$) was observed in the ratio $A^\bullet/AH^-$ at 6h in T1. However, for the same exposure time, a significant decrease was observed after supplementation with 10 mM of ascorbate (Table 3).

Table 3. Ascorbate content, ascorbyl radical, ratio $A^{\bullet}/AH^{-}$, TBARS and α-T for *Mytilus edulis chilensis* samples exposed to different treatments during the first stage of processing

	Time (h)	T1	T2	T3	T4
Ascorbate	0	2.1 ± 0.4	2.1 ± 0.4	2.1 ± 0.4	2.1 ± 0.4
(nmol/mg FW)	6	4 ± 2	3 ± 1	25 ± 9*,**	8 ± 3*,**
	24	4 ± 1	2.6 ± 0.7	9 ± 2*,**	5 ± 1*
$AH^{\bullet}$	0	2.0 ± 0,7	2.0 ± 0,7	2.0 ± 0,7	2.0 ± 0,7
(pmol/mg FW)	6	6 ± 2	4 ± 2	5 ± 4	7 ± 5
	24	3 ± 1	3 ± 1	8 ± 4	3 ± 1
$A^{\bullet}/AH- 10^{-3}$	0	0.95 ± 0.06	0.95 ± 0.06	0.95 ± 0.06	0.95 ± 0.06
	6	1.5 ± 0,8	1.3 ± 0.4	0.23 ± 0.08*	0,88 ± 0.04
	24	0.75 ± 0,03	1.5 ± 0.6	0.91 ± 0.04	0.69 ± 0.07
TBARS	0	2.5 ± 0.4	2.5 ± 0.4	2.5 ± 0.4	2.5 ± 0.4
(nmol/mg FW)	6	2.2 ± 0.7	2.6 ± 0.1	3.1 ± 0.1	2.3 ± 0.1
	24	3.4 ± 0.1*	2.6 ± 0.4	1.7 ± 0.1*,**	2.2 ± 0.6
α-T	0	4.1 ± 0.9	4.1 ± 0.9	4.1 ± 0.9	4.1 ± 0.9
(pmol/g FW)	6	4.8 ± 0.8	2.0 ± 0.4	3.9 ± 0.3	2.9 ± 0.4
	24	1.8 ± 0.9*	1.3 ± 0.5*	1.6 ± 0.5*	2.5 ± 0.3*

T1: dry control, T2: control in seawater without antioxidant, T3: exposed to AH^{-} (10 mM) and T4: exposed to AH^{-} (5 mM).

* Significantly different from the ascorbate value at time 0 under the same culture conditions (P< 0.05).

**Significantly different from the ascorbate value at 6 and 24 h in samples without seawater exposure (P< 0,05).

*Significantly different from the $A^{\bullet}/AH^{-}$ values at time 0 under the same culture conditions (P< 0,05).

*Significantly different from α-T values at time 0 under the same culture conditions (P< 0,05).

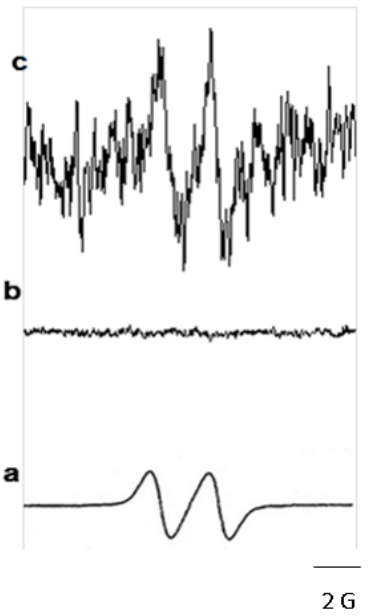


Figure 2. Characteristic spectrum of $A^{\bullet}$ by EPR. (a) simulated spectrum using the following parameters: g = 2.00518 and a_H = 1.8 G, corresponding to $A^{\bullet}$ (Jurkiewicz and Buettner, 1994). (b) Spectrum of the reaction medium (DMSO) in the absence of the mussel homogenate. (c) Spectrum of $A^{\bullet}$ in the presence of the mussel homogenate.

In the second stage of processing, after 5 days of storage at -20°C, the TBARS mussels content in presence or absence of ascorbic acid, showed no statistically significant differences (P> 0.05, Table 4). However, after 5 days of storage, the TBARS values obtained for all the treatments were significantly higher (100 times high) than those obtained in the first stage of processing (Table 3). The AH^- content decreased significantly (46.7%, P< 0.05), in mussels coming from the different groups in relation to the initial content (Table 4). Only a significant decrease in ascorbate content was observed after 24h of exposure to ascorbic acid for T1 and T4. Both the $A^\bullet$ content and the $A^\bullet/AH^-$ ratio (oxidative stress indices) increased significantly between T1 and T4. The α-T content showed a significant decrease after 6h of treatment for T2 and T3 (P <0.05, Table 4).

Table 4. Ascorbate content, ascorbyl radical, ratio $A^\bullet/AH^-$, TBARS and α-T for *Mytilus edulis chilensis* samples exposed to different treatments during the second stage of processing (5 days of storage at -5 °C)

	Time (h)	T1	T2	T3	T4
Ascorbate	0	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3
(nmol/mg FW)	6	0.5 ± 0.9	1.2 ± 0.4	0.7 ± 0.3	1.0 ± 0.3
	24	0.3 ± 0.1*	1.4 ± 0.5	1.1 ± 0.6	0.5 ± 0.1*
$AH^\bullet$	0	0.10 ±0.02	0.10 ±0.02	0.10 ±0.02	0.10 ±0.02
(pmol/mg FW)	6	1.7 ± 0.4	0.21 ±0.05	2.1 ±0.8	0.9 ± 0.2
	24	0.7 ± 0.1	0.19 ±0.03	nd ±	3.0 ±0.8
$A^\bullet/AH^- 10^{-3}$	0	0.20 ± 0.03	0.20 ± 0.03	0.20 ± 0.03	0.20 ± 0.03
	6	3.2± 0.4	2.1 ± 0.7	4 ± 1	2.2 ± 0.9
	24	2.0 ± 0.7	1.2 ± 0.8	nd	7 ± 3
TBARS	0	22 ± 4	22 ± 4	22 ± 4	22 ± 4
(nmol/mg FW)	6	20 ± 2	22 ± 5	20 ± 2	15 ± 3
	24	22,4 ± 0,1	26 ± 5	27 ± 3	22 ± 2
α-T	0	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
(pmol/g FW)	6	1.8 ± 0.8	0.5 ± 0.2**	0.7 ± 0.1**	1.5 ± 0.4
	24	3 ± 1	1.2 ± 0.4	1.2 ± 0.5	1.1 ± 0.4

T1: dry control, T2: control in seawater without antioxidant, T3: exposed to AH^- (10 mM) and T4: exposed to AH^- (5 mM).

* Significantly different from the ascorbate value at time 0 under the same culture conditions (P< 0.05).

**Significantly different from α-T values in samples without seawater exposure during 6h (P< 0,05). nd: no determined.

DISCUSSION

Molluscs removed from their natural marine habitat are under constant stress due to changes in the environment, such as temperature and anaerobiosis. Various methods and techniques are used to measure stress in live mussels (Khan et al., 2006). The mussel species that inhabits naturally the Beagle Channel is used in the mussel farming, being *Mitylus edulis chilensis* very abundant in the area (Calvoet al.,1998). This species has been previously characterized from physiological and biochemical analyses (Giarratano, 2010). The antioxidants concentrations values in mussels of T1 (controls at time 0) match those observed by Giarratano and Amin (2010); Duarte et al., (2011); Giarratanoet al., (2010), in mussels

transplanted for ecotoxicology studies. The organisms that are in better physiological responses conditions will respond better to the stress generated by the activities of handling, processing and storage (Gonzalézet al., 2017).

The adverse effects on mollusks due to oxidative stress should be minimized to reach commercialization standards. Many studies have shown an increase in the mortality rate and loss of product quality due to damage to the internal tissues of mollusks (Harding et al., 2004). All fresh foods of marine origin (fish, mollusks) are susceptible to deterioration caused by both microbial activity and chemical reactions. Lipid damage occurs easily and limits the organoleptic properties during the storage of such products (Chaijan et al., 2006). This deterioration produces free fatty acids that under oxidation conditions produce low molecular weight compounds responsible for rancidity, loss of aroma and flavor. The mechanisms of lipid oxidation reactions can be characterized by three steps: initiation, propagation and termination reactions. This phenomenon can be modified by intrinsic and extrinsic factors, such as: fatty acid composition, concentration of pro-oxidants-antioxidants, metals, enzymes, pH, temperature, ionic strength and oxygen consumption. Lipid oxidation can be initiated and accelerated by several mechanisms, including ROS. The lipid oxidation products can interact with thiobarbituric acid and develop a pink colored compound (TBARS), establishing a method to measure lipid oxidation and is commonly used in mollusks to measure it´s quality (Jardine *e*t al., 2002, Khan et al., 2006). Several studies had shown that the values of TBARS increase during the cooking and storage of different products: turkey, chicken, mackerel, sardines, steaks, pork (Cuppett, 2001, Khan et al., 2006). According with these previous studies, our results showed a 36% increase in TBARS content during the first 24h of processing in T1 (control).

The balance between pro and endogenous antioxidants as well as exposure to different environmental variables, determine the susceptibility to oxidative damage in marine organisms (Gonzaléz *et* al., 2015). In this study we shift the balance (damage/protection) to antioxidants, through physical treatment with different concentrations of ascorbic acid. This technique has been used efficiently to control lipid oxidation during cold storage of fish for 6 months (Erickson 1997). In other studies, the opposite effect has been seen, which would indicate that many variables such as concentration, application technique, storage period, among others, play an important role in the efficiency of the use of ascorbic acid as an antioxidant in sea products. In our study, the ascorbic acid incorporated by *Mytilus edulis chilensis* showed an antioxidant activity to control lipid oxidation during the first stage of processing (24h exposure) between the T1 and those exposed to ascorbic acid (5 mM) (T3). However, the ascorbic acid is highly susceptible to oxidation at high pH levels (Jacobsen *et al*., 2001). This could explain partially the considerable decrease in antioxidant activity after 5 days of storage. On the other hand, this decrease in activity could eventually indicate a pro-oxidant action of ascorbic acid on the lipids of mussels during storage.

The $A^{\bullet}$ content can be considered as a final product of radical transformations in biological systems of antioxidant defense and alsoused as an early and global indicator of oxidative stress. Some fundamental aspects that allow the use of $A^{\bullet}$ as an index of oxidative stress, are the following: (a) $A^{\bullet}$ has a relatively low reduction potential that allows ascorbic acid to react as an antioxidant with virtually any radical that is generated in a biological system (Buettner and Jurkiewicz 1993); (b) $A^{\bullet}$ is produced in very early stages of oxidative stress, while the concentration of ascorbic acid only decreases if regeneration is not enough.

Previous research has shown that when variables such as pH, concentration of catalytic metals and ascorbic acid levels are controlled, the use of $A^{\bullet}$ is useful as an index of oxidative stress (Malanga et al., 2012). However, since the generation of $A^{\bullet}$ depends on the oxidation of AH^-, and the reaction of AH^- with free radicals, the use of the concentration of $A^{\bullet}$ by itself, as an index of oxidative stress, would not be adequate in systems where the content of ascorbic acid varies in relation to the oxidative condition. For this reason, it is more adequate to use the index $A^{\bullet}/AH^-$, as a possible tool to evaluate a situation of oxidative stress in different biological systems (Stegmann and Schuler, 1993; Kozak et al., 1997; Galleano et al., 2002; Gonzalez et al., 2008; Lattuca et al., 2009; Malanga et al., 2004; 2012;2016). In our study we determined only a non significant increase in mussels exposed to 6h in dry conditions (T1) in coincidence with an increased $A^{\bullet}$. These results could indicate the generation of a minor oxidative stress in the mussels extracted in Almanza, which are kept dry during their transfer to the Processing Plant located at 700 kilometers. However, this situation was not observed in those organisms that were kept in seawater or incubated with ascorbic acid during the same period.

A decrease in the α-T content was also observed coincident with that observed in other studies in fish and mussels (Brannan and Ericsson 1996; Tseng et al., 2005; Khan et al., 2006). In addition, such decrease was also observed in other non-enzymatic antioxidants, such as glutathione (data not shown) and could be related to the presence of free Fe or other metals that after a prolonged storage time, lead to cell death with changes in lipid oxidation. Previous work has shown that re-immersion for 12, 24 and 48h prior to the processing activities, reduces the stress response increasing the quality of the product and prolonging it´s commercial life (Harding et al., 2004). The data obtained in this work show that the maintenance in water for 24h is beneficial for the transfer of the mussels and that not only the use of the antioxidant aggregate would be beneficial in the prevention of the stress generated by the manipulation of the mussel *Mytilus edulis chilensis*.

The TBARS content in mussels during the second stage of processing, in the presence or absence of ascorbic acid, did not show significant differences ($P > 0.05$). However, after 5 days of storage, the values observed for all treatments were significantly higher than those obtained in the first stage of processing, probably as a consequence of freezing. These results would indicate that the different forms of ascorbic acid could eventually have a pro-oxidant action on the lipids of mussels during storage. Similar results were observed by Khan et al., (2006) for *Mytilus edulis* in crops. On the other hand, the content of ascorbic acid in mussels decreased significantly after 24h of exposure to ascorbic acid in dry exposure (T1) and for the lowest concentration used (T4), probably due to consumption by organisms. Ascorbic acid can react with peroxyl radicals to give hydroperoxides and $A^{\bullet}$, which can be evaluated. This has been observed in *in vivo* and *in vitro* studies (Zhang and Omaye 2000). In the conditions of this work, it was observed that both the content of $A^{\bullet}$ and the ratio $A^{\bullet}/AH^-$ increased significantly when the mussels were maintained in the absence of seawater or when were exposed to the ascorbic acids in both concentrations, indicating the generation of minor oxidative stress.

As was previously shown, there were a decrease in α-T in mussels exposed to seawater without ascorbic aggregate and in those exposed to 10 mM of the antioxidant after 6h of treatment and 5 days of cold storage. For *in vitro* studies, Yamamoto (2007) observed that vitamin E is oxidized under prolonged storage conditions. These results allow us to ensure

that cold storage (-20°C) for 5 days, regardless of the treatment, does not improve the quality of the product.

Overall, the results of this study, as well as previous data in other aquatic organisms (Perez 2009, Vicetti et al., 2005), have shown that not always the increase in the concentration of a single antioxidant is the best option for the improvement of the product quality. Currently, the combined use of antioxidants with a synergistic effect is recommended. On the other hand, these results are not enough to accept or reject this type of treatment and must be completed with other studies such as: the evaluation of oxidative damage to proteins; the evaluation of other enzymatic and non-enzymatic antioxidants, as well as the quantification and identification of lipids present in mussels at different seasonal moments.

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Chapter 124

A SEA OF PROBLEMS: IMPACTS OF PLASTIC POLLUTION ON OCEANS AND WILDLIFE*

Tuesday, October 29, 2019
U.S. House of Representatives
Subcommittee on Water, Oceans, and Wildlife
Committee on Natural Resources,
Washington, DC

The Subcommittee met, pursuant to notice, at 2:27 p.m., in room 1324, Longworth House Office Building, Hon. Alan S. Lowenthal [Member of the Subcommittee] presiding.

Present: Representatives Lowenthal, Sablan, Van Drew, Case, Cox, Neguse, Cunningham; McClintock, Lamborn, and Graves.

Also present: Representative Haaland.

Dr. LOWENTHAL. The Subcommittee on Water, Oceans and Wildlife will come to order.

I may look like Congressman Huffman, but I am not. [Laughter.]

Dr. LOWENTHAL. I know it is a shock. I am Congressman Lowenthal. Congressman Huffman is back in Sonoma, dealing with the wildfires that are there, and never was able to get back here to Washington. We are all hoping that the fires subside, that many people are safe, and that Mr. Huffman returns soon.

With that, the Subcommittee on Water, Oceans, and Wildlife will come to order.

The Subcommittee is meeting today to hear testimony on "A Sea of Problems: the Impacts of Plastic Pollution on Oceans and Wildlife."

Under Committee Rule 4(f), any oral opening statements at hearings are limited to the Chairman, the Ranking Minority Member, the Vice Chair, and the Vice Ranking Member. This will allow us to hear from our witnesses sooner, and help Members keep to their schedules.

* This is an edited, reformatted and augmented version of Oversight Hearing, before the Subcommittee on Water, Oceans, and Wildlife, of the Committee on Natural Resources, U.S. House of Representatives, One Hundred Sixteenth Congress, First Session, Serial No. 116–27, dated October 29, 2019.

Therefore, I ask unanimous consent that all other Members' opening statements be made part of the hearing record if they are submitted to the Subcommittee Clerk by 5 p.m. today, or the close of the hearing, whichever comes first.

Hearing no objections, so ordered.

STATEMENT OF THE HON. S. ALAN LOWENTHAL, REPRESENTATIVE IN CONGRESS FROM THE STATE OF CALIFORNIA

Dr. LOWENTHAL. I am going to open up now, and I want to welcome all the witnesses. We are here today to discuss a pressing environmental issue, and that is plastic pollution.

Certainly, single-use plastics have made life easier, but these materials come at a much higher cost than many would like to admit. Plastics last for centuries in the natural environment, and are found nearly everywhere on our planet.

Last year, I witnessed the impact of plastic pollution on wildlife in Antarctica, one of the few places on earth that has been relatively untouched by human activity, but certainly not untouched by the scourge of plastics.

Personally, I have been involved in trying to tackle the growing plastic crisis for over 20 years, working with my constituent and friend, Captain Charles Moore, who created the scientific research organization Algalita, and who did some of the early research on the plastic garbage gyre.

There is an estimated 8 million metric tons of plastic that enter the oceans each year at a rate of about one garbage truck per minute, threatening biodiversity and accumulating in the seafood that we eat and in the water that we drink. Plastics have even been found in water samples right here in the Capitol Visitors Center.

Plastics are also making climate change worse. The global life cycle emissions from one year's plastic production throughout the United States are about the same as 462 coal-fired power plants per year, and that number is rising.

Plastic production is an environmental justice issue, also. Petrochemical factories and incineration facilities are often located in low-income communities, where local health impacts and air quality impacts are quite significant, but frequently are ignored.

Finally, in this Subcommittee, we need to look at solutions to deal with, for example, ghost fishing gear, fishing gear that has been lost at sea but continues to catch fish, marine mammals, turtles, birds, and corals.

It is clear that we need to reduce plastic pollution. Higher recycling commitments, and bans and taxes on single-use plastic items can be part of the solution, but we must expand our tools to address this growing environmental and public health problem.

In this Committee, we switched to reusable pitchers and glasses for water, rather than the disposable plastic water bottles we see so often around the Capitol. But not every switch is as easy, and not everyone has the option.

The financial burden of cleaning up pollution should not be solely on the taxpayers. It is imperative that the companies that manufacture and sell these products take ownership of their environmental impacts. Congress needs to step up, too.

It is for this reason that I have been working on comprehensive legislation with Senator Udall. Our legislation seeks to create a more circular approach by putting in place an

extended producer responsibility program, implementing recycling content standards, as well as phasing out certain single-use-only items that have more sustainable alternatives.

I am excited to announce that we should have a discussion draft of this legislation quite soon, which we will disseminate publicly, and I encourage all of you to let me know your thoughts and comments after its release.

Some Federal agencies are also doing their part. NOAA's Marine Debris Program recently funded 14 new projects addressing aspects of this problem. However, the $2.7 million provided to these projects doesn't even come close to addressing the scale of the ocean plastic problem.

The bottom line is this: We need to do more, we need to look at a broader range of solutions that are going to prevent wildlife from being strangled, and to keep microplastics from ending up on our plate.

With that, I look forward to hearing more from our witnesses about their ideas.

[The prepared statement of Dr. Lowenthal follows:]

PREPARED STATEMENT OF THE HON. ALAN S. LOWENTHAL, REPRESENTATIVE IN CONGRESS FROM THE STATE OF CALIFORNIA

Today we're here to discuss a pressing environmental issue: plastic pollution.

Certainly, single-use plastics have made life easier. But these materials come at a much higher cost than many would like to admit. Plastics last for centuries in the natural environment and are found nearly everywhere on our planet. Last year I witnessed the impacts of plastic pollution on wildlife in Antarctica, one of the few places on earth that has been relatively untouched by human activity.

Personally, I have been involved in trying to tackle the growing plastic crisis for over 20 years, working with my constituent and friend, Captain Charles Moore, who created the scientific research organization Algalita and who did the early research on the pacific garbage gyre.

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Plastics are also making climate change worse. The global lifecycle emissions from one year's plastic production are about the same as 462 coal fired power plants per year – and that number is rising.

Plastic production is an environmental justice issue too. Petrochemical factories and incineration facilities are often located in low-income communities, where local health impacts and air quality impacts are significant but often ignored.

Finally, in this subcommittee, we need to look at solutions to deal with ghost fishing gear—fishing gear that's been lost at sea but continues to catch fish, marine mammals, turtles, birds, and corals.

It's clear that we need to reduce plastic pollution. Higher recycling commitments and bans and taxes on single-use plastic items can be part of the solution, but we must expand our tools to address this growing environmental and public health problem.

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I am excited to announce that we should have a discussion draft of the legislation very soon, which we will disseminate publicly, and I encourage all of you to let me know your thoughts and comments after it is released.

Some federal agencies are also doing their part - NOAA's Marine Debris Program recently funded 14 new projects addressing aspects of this problem. However, the $2.7 million provided to these projects doesn't even come close to addressing the scale of the ocean plastic problem.

The bottom line is this: we need to do more and we need to look at a broader range of solutions to prevent wildlife from being strangled and to keep microplastics from ending up on our plate.

With that, I look forward to hearing more from our witnesses about their ideas, and I will now invite the Ranking Member to share his remarks.

* * *

Dr. LOWENTHAL. I will now invite the Ranking Member to share his remarks.

STATEMENT OF THE HON. TOM MCCLINTOCK, REPRESENTATIVE IN CONGRESS FROM THE STATE OF CALIFORNIA

Mr. MCCLINTOCK. Thank you, Mr. Chairman. The Subcommittee meets today to hear testimony on plastics and their impact on our oceans.

From the tenor of the written testimony, it appears the Majority is blaming American consumers for the plastic waste that reaches our oceans, and is proposing to place restrictions on them that will dramatically reduce the convenience and higher quality of life that plastics have contributed to our modern society, while increasing costs dramatically.

Blaming America first seems to be a recurring theme, but the facts paint a very different picture. A 2017 study published in the *Environmental Science & Technology* magazine found that between 88–95 percent of all the plastic debris that enters our oceans comes from 10 rivers, none of which is anywhere close to the United States—8 of those rivers are in Asia, and 2 are in Africa.

According to a 2015 study, the top 20 marine plastic polluters produced as much as 10.76 million metric tons of waterborne plastic debris. The United States generated just 0.11 million

metric tons, barely 1 percent. Indeed, the entire United States contributed less waterborne plastic pollution than North Korea.

So, who does the Majority blame for this? American consumers. But, as Jeane Kirkpatrick once observed, they always blame America first.

According to the EPA, Americans have increased plastic recycling from 20,000 tons in 1980 to 3.1 million tons in 2015. That is a 155-fold increase. American consumers go to great lengths to responsibly dispose of plastic waste, and the numbers show that. American consumers are heroes, not villains, in this fight against plastic pollution of our oceans. We should be celebrating them, not punishing them.

Yet, that is just what draconian restrictions on plastic use would do, starting with the 1.7 million families who depend on plastics manufacturing to put food on the table, roofs over their heads, and taxes into our government coffers. The single largest state employing them remains my home state of California, where 80,000 Californians are directly employed in the plastics industry.

The misplaced object of the left's ire appears to be single-use plastic containers, the toothpaste tube, the shampoo bottle, the plastic bag. They criticize them as wasteful, since the plastic is used once and discarded, and yet takes between 50 and 1,000 years to decay. Well, if they are properly disposed of—and Americans do—I have to ask, what exactly is that problem?

The most common single-use packaging of the ancient world, once we had progressed from animal skins and gourds, was the amphora, usually a ceramic. A massive hill called Mount Testaccio in Rome is composed of discarded amphora, which have not degraded in nearly 2,000 years. Yet, the world is not worse for it, and the Romans were infinitely better off for it. Which begs the question: If we are going to ban single-use plastic containers, exactly what will replace them?

How about your toothpaste? Before plastics, toothpaste came in collapsible metal tubes. Do the opponents of plastics find this a more environmentally-friendly container? The toothpaste tube was invented to protect consumers from the unhygienic practice of getting toothpaste in glass jars and dipping your toothbrush into them. Shall we return to glass jars? Before that, toothpaste came in powdered form in cardboard boxes and wax paper, which required mixing a batch every time you wanted to brush your teeth.

Plastics have largely replaced aluminum as the best container to protect food against food spoilage. Before aluminum, it was tin. It takes 4 pounds of bauxite, usually by strip mining, and 7½ kilowatts of electricity to make 1 pound of aluminum. Do the plastic critics really think an environmentally-friendly alternative is to return to the era of metal containers?

Before metal containers, glass was commonly used. Glass takes roughly 1 million years to decompose, 1,000 times longer than the longest estimate for plastic decomposition. I suppose we could go back to cardboard and paper, but I remember the campaign a decade ago to ban paper bags as wasteful and environmentally offensive, so we dutifully replaced them with plastic bags, which have now attracted the ire of the environmental left.

Single-use plastics, properly disposed of, mean greater convenience and lower prices for American consumers, and a much smaller environmental footprint than all of the different packaging materials that they have replaced.

So, I am very interested in hearing today why Americans, who have an exemplary record of responsible plastic disposal and recycling, are to blame for the excesses of other people in

other countries, and why those same Americans should now be punished with higher prices, less convenience, and a lower standard of living.

And, finally, I would like to know what are the plastics critics proposing as an alternative to plastic containers that they haven't already rejected over the years.

I yield back.

[The prepared statement of Mr. McClintock follows:]

PREPARED STATEMENT OF THE HON. TOM MCCLINTOCK, RANKING MEMBER, SUBCOMMITTEE ON WATER, OCEANS, AND WILDLIFE

The sub-committee meets today to hear testimony on plastics and their impact on our oceans. From the tenor of the written testimony, it appears that the majority is blaming American consumers for the plastic waste that reaches our oceans and is proposing to place restrictions on them that will dramatically reduce the convenience and higher quality of life that plastics have contributed to our modern society.

Blaming America first seems to be a recurring theme, but the facts paint a very different picture. A 2017 study published in the Environmental Science and Technology Magazine found that between 88-95 percent of all the plastic debris that enters our oceans comes from ten rivers – none of which is anywhere close to the United States: eight of those rivers are in Asia and the other two are in Africa.

According to a 2015 study, the top 20 marine plastic polluters produced as much as 10.76 million metric tons of waterborne plastic debris. The United States generated just 0.11 million metric tons – or barely one percent. Indeed, the entire United States contributed less waterborne plastic pollution than North Korea.

Who does the majority blame for this? American consumers. But as Jeanne Kirkpatrick once observed, they always blame America first.

According to the EPA, Americans have increased plastic recycling from 20,000 tons in 1980 to 3.1 million tons in 2015. American consumers go to great lengths to responsibly dispose of plastic waste – and the numbers show that.

American consumers are heroes – not villains – in the fight against plastics pollution of our oceans. We should be celebrating them and not punishing them!

Yet that is just what Draconian restrictions on plastic use would do, starting with the 1.7 million families who depend on plastics manufacturing to put food on the table, roofs over their heads and taxes into our coffers. The single largest state employing them remains my home state of California, where 80,000 Californians are directly employed in the plastics industry.

The misplaced object of the left's ire appears to be single-use plastic containers: the toothpaste tube, the shampoo bottle, the plastic bag. They criticize them as wasteful, since the plastic is used once and discarded and yet take between 50 and 1,000 years to decay.

If they are properly disposed of – and Americans do that better than just about any other people on this planet – I have to ask, what exactly is the problem? The most common single-use packaging of the ancient world – once we had progressed from animal skins and gourds – was the amphora, usually a ceramic. A massive hill called Mt. Testaccio in Rome is

composed of discarded amphorae, which have not degraded in nearly 2,000 years. Yet the world isn't the worse for it – and the Romans were infinitely better off for it.

Which begs the question, if we are going to ban single use plastic containers, exactly what will replace them? How about your toothpaste? Before plastics, toothpaste came in collapsible metal tubes. Do the opponents of plastics find this a more environmentally friendly container? The toothpaste tube was invented to protect consumers from the unhygienic practice of getting toothpaste in glass jars and dipping your toothbrush into them. Shall we return to glass jars? Before that, toothpaste came in powder form in cardboard boxes and wax paper, which required mixing a batch every time you brushed your teeth.

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Single use plastics – properly disposed of – mean greater convenience and lower prices for American consumers, and a much smaller environmental footprint than all the different packaging materials that they replaced.

So I'm very interested in hearing why Americans – with an exemplary record of responsible plastic disposal and recycling – are to blame for the excesses of other people in other countries; and why those same Americans should now be punished with higher prices, less convenience and a lower standard of living. And finally, I would like to know what the plastics critics are proposing as an alternative to plastic containers, that they haven't already rejected over the years.

* * *

Dr. LOWENTHAL. I am going to ask unanimous consent that the gentleperson from New Mexico, Representative Haaland, be allowed to sit on the dais and participate in today's proceedings.

Without objection, that is ordered.

Now I am going to introduce our witnesses.

Our first witness is Mr. Ted Danson. You may know him better as Michael on "The Good Place," or Sam on "Cheers." But Mr. Danson is also the Vice Chair of the Board of Directors at Oceana, where he has been closely involved since its inception.

Our next witness will be Mr. Juan Parras, who is the Founder and Executive Director at the Texas Environmental Justice Advocacy Service, or TEJAS.

Following him we will hear from Dr. Jenna Jambeck, Professor of Environmental Engineering at the University of Georgia, and the lead author of a groundbreaking study on plastic.

And, finally, our last witness will be Tony Radoszewski, who is the President and CEO of the Plastics Industry Association.

Let me remind all the witnesses that, under our Committee Rules, they must limit their oral statements to 5 minutes, but that their entire statement will appear in the hearing record.

When you begin, the lights on the witness table will turn green. After 4 minutes, the yellow light will come on. Your time will have expired when the red light comes on, and I will ask you to please complete your statement.

I will also allow the entire panel to testify before questioning witnesses.

The Chair now recognizes Mr. Danson to testify.

Welcome to our Committee.

STATEMENT OF TED DANSON, ACTOR, ADVOCATE, AND BOARD MEMBER, OCEANA, LOS ANGELES, CALIFORNIA

Mr. DANSON. I would like to thank the Chair and Ranking Member, and members of the Committee for the opportunity to testify on plastic pollution.

I am the Vice Chair of Oceana's Board of Directors. Oceana is the largest international advocacy organization dedicated solely to ocean conservation. I have been working on ocean issues for more than 30 years. In the late 1980s, I co-founded the American Oceans Campaign, which then joined with Oceana in 2002. I am here to testify today about the growing problem of plastic pollution that is threatening our oceans.

Almost from the moment we wake up, to the time we go to bed, we are faced with throwaway plastic. We face it when we brush our teeth with a toothbrush made of plastic, and squeeze toothpaste out of a plastic tube, and when we wash our hair with shampoo and conditioner from plastic bottles. The rest of our daily routines might include one or several coffees in cups with plastic lids, lunch in plastic take-out containers with plastic utensils, and grocery shopping, where single-use plastic is unavoidable. There isn't a place on earth untouched by the pollution from all this plastic.

The list of marine animals affected by plastic pollution grows. Plastic has been consumed by an estimated 90 percent of seabird species, and eaten by every species of sea turtle. Even our corals are threatened.

In addition to polluting the marine environment, plastic poses a risk to human health. We are now seeing plastic in our water, our food, soil, air, and bodies. Plastic particles have been found in everything from honey and beer to salt and tea.

Plastic is also affecting our climate. If plastic was a country, it would be the planet's fifth largest emitter of greenhouse gases. With plastic production rates anticipated to increase, so will plastic's effects on the climate and oceans.

The most important thing to remember about plastic is that it lasts for centuries. This is what makes single-use plastics so profoundly flawed. They are created from a material made to last forever, but are designed to be used once and thrown away.

Simply improving recycling rates will not solve the plastic crisis. Of all the plastic waste ever generated, only 9 percent has been recycled. That means the vast majority was sent to a landfill, incinerated, or ended up polluting our natural environment, including our oceans. Recycling is like trying to mop up water from an overflowing bathtub, while the faucet is still running. We need to turn off the faucet and reduce the production of plastic.

Companies need to significantly reduce the amount of single-use plastic they are putting onto the market, and offer consumers plastic-free choices for their products. Unfortunately, companies aren't doing enough, and that is why we need your help.

Policies governing the production and use of single-use plastic are effective, and these policies are becoming more common around the world and across this country. The European Union, Peru, Chile, and Canada have all announced or are implementing policies to reduce plastic pollution. U.S. cities, counties, and states have taken the initiative, passing policies to reduce single-use plastics. But ultimately, comprehensive U.S. Federal action is needed.

This Committee should use its authority to tackle the problem. I applaud you for stopping the use of plastic water bottles in Committee hearings.

The National Park Service had a policy to encourage national parks to stop selling water in plastic bottles. Unfortunately, the policy has been reversed. The Committee should make our national parks, wildlife refuges, marine sanctuaries, and other Federal lands and waters into single-use plastic-free zones.

I urge Congress to pass Federal legislation that stops plastic pollution at the source, that significantly reduces the production of this everlasting pollutant, that holds corporations responsible for this global crisis, and enables states and cities to continue to lead the way on solutions.

Don't fall for the false promise of recycling. And please don't stoop to incineration. We must stop the runaway increase in plastic production and reduce the amount of plastic that companies are making and foisting on us, because it will last for centuries. We have no more time to waste. Thank you.

[The prepared statement of Mr. Danson follows:]

PREPARED STATEMENT OF TED DANSON, VICE CHAIR, OCEANA BOARD OF DIRECTORS, LOS ANGELES, CALIFORNIA

Good afternoon. Thank you, Chairman Huffman, Ranking Member McClintock and members of the committee, for the opportunity to testify today on plastic pollution's effects on our oceans. My name is Ted Danson, and I am the vice chair of Oceana's board of directors. Oceana is the largest international advocacy organization dedicated solely to ocean conservation. We work in North, South and Central America, Asia and Europe to advocate for science-based policies that will restore the ocean's abundance and biodiversity.

I've been working on ocean issues for more than 30 years. My interest in the oceans started when one day, I decided to take my daughters — who were 4 and 8 years old at the time — to go swimming at the beach in Southern California. We were ready to go and running toward the water, but were stopped by a sign that said, "no swimming, ocean polluted."

My girls couldn't believe it, and neither could I. The ocean was closed. They asked me, "Why, why can't we go swimming — in this beautiful ocean?" So, in the late 1980s, I co-founded the American Oceans Campaign to clean up beaches and the ocean. And for 15 years, we worked to protect the oceans from oil drilling and other threats.

To expand the capacity of the American Oceans Campaign, we decided to join with Oceana in 2002. Oceana has protected more than 4.5 million square miles of ocean and won

over 200 victories to stop overfishing, habitat destruction, pollution and the killing of threatened species. I am here today to testify about the growing problem of plastic pollution that is threatening our oceans.

Almost from the moment we wake up to the time we go to bed, we are faced with throwaway plastic. We face it when we brush our teeth with a toothbrush made of plastic and squeeze toothpaste out of a plastic tube, and when we wash our hair with shampoo and conditioner from plastic bottles. The rest of our daily routines might include one or several coffees in cups with plastic lids, lunch in plastic takeout containers with plastic utensils, and grocery shopping, where single-use plastic is unavoidable.

If you tried to avoid the plastic typically encounter in a day, you'd hit countless obstacles. There was an article in *The New York Times* earlier this year about people who managed to maintain generally plastic-free lifestyles — their days involved using homemade shampoo, toothpaste and more. This effort is extraordinarily admirable, but not many could manage it.[1] Millions of consumers should not have to restructure their daily routines to avoid plastic when the country's leading producers of food, personal care products and other everyday staples could start using sustainable alternatives to single-use plastic, stopping the problem at the source.

Plastic hasn't been around for as long as you might imagine, considering the level of plastic pollution we're seeing in the environment. It wasn't being used for consumer goods like beverage bottles until the 1940s. By the 1950s, we had entered the era of "throwaway living" — meaning our current culture of relying on single-use, disposable materials to make our lives more efficient and convenient. Plastic was convenient for producers too — it was a cheap, durable and lightweight material. This trend's environmental impact was evident within just a few years. Disposable items were suddenly cluttering roadsides around the country.

Fast forward to today, and we're seeing plastic floating on the surface of the sea, washing up on the world's most remote coastlines, melting out of Arctic sea ice and sitting at the deepest point of the ocean floor.[2] There isn't a place on earth untouched by plastic pollution. In fact, it's now cemented in our fossil record. For the first time, researchers have documented plastic building up exponentially in the sediments off the coast of Santa Barbara, California, that precisely mirrors the massive expansion in global plastic production from 1945 to the present decade.[3]

We are leaving behind a permanent legacy of plastic pollution for future generations.

The list of marine animals affected by plastic pollution is continually growing. Plastic has been consumed by an estimated 90% of seabird species and eaten by every species of sea turtle.[4] Some organisms, such as corals, appear even more attracted to plastic than food.[5]

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[1] Kurutz, S (2019) Life Without Plastic Is Possible. It's Just Very Hard. *The New York Times*. Available: https://www.nytimes.com/2019/02/16/style/plastic-free-living.html. Accessed Oct 23, 2019.

[2] Lavers JL and Bond JL (2017) Exceptional and rapid accumulation of anthropogenic debris on one of the world's most remote and pristine islands. *PNAS* 114: 6052-6055. doi: 10.1073/pnas.1619818114; Peeken I, Primpke S, Beyer B, *et al.* (2018) Arctic sea ice is an important temporal sink and means of transport for microplastic. *Nature Communications* 9. doi: 10.1038/s41467018-03825-5; Chiba S, Saito H, Fletcher R, *et al.* (2018) Human footprint in the abyss: 30 year records of deep-sea plastic debris. *Marine Policy* 96: 204-212. doi: 10.1016/j.marpol.2018.03.022.

[3] Brandon JA, Jones W and Ohman MD (2019) Multidecadal increase in plastic particles in coastal ocean sediments. Science Advances 5. doi: 10.1126/sciadv.aax0587.

[4] Wilcox C, van Sebille E and Hardesty BD (2015) Threat of plastic pollution to seabirds is global, pervasive and increasing. *PNAS* 112: 11899-11904. doi: 10.1073/pnas.1502108112; Kuhn S, Bravo Rebolledo EL and van

What's worse, studies have shown when corals come into direct contact with plastic debris, their likelihood of disease increases from 4% to a staggering 89%.[6] At least 17% of the species observed to be affected by marine debris are listed as threatened or near threatened with extinction by the International Union for the Conservation of Nature, indicating that marine plastic debris may be contributing to the possibility of these species' extinction.[7]

One study estimated that up to 51 trillion microplastic particles were present in the ocean in 2014. This number is only expected to increase as plastic continues to pour into our oceans and breaks up into smaller pieces.[8]

In addition to polluting the marine environment, plastic poses a risk to human health. We're now seeing plastic in our water, our food, our soil, our air and our bodies.[9] Plastic particles have been found in everything from our water and beer to honey, salt and tea.[10] The particles also make their way into the seafood we eat.[11] Scientists are still studying the potential impacts the plastic particles themselves are having on our health.

Plastic is also affecting our climate. If plastic was a country, it would be the planet's fifth-largest emitter of greenhouse gases.[12] Studies have shown that plastic contributes to climate change by using fossil fuels and emitting greenhouse gases throughout its life cycle, from production and transportation to waste management. Plastic at the ocean's surface and on land continually releases methane and other greenhouse gases throughout its existence, and these emissions increase as plastic breaks apart in sunlight.[13] With plastic production rates anticipated to increase, so will plastic's effects on our climate.

Franeker JA (2015) Deleterious Effects of Litter on Marine Life. In: *Marine Anthropogenic Litter*. Cham: Spinger International Publishing.

[5] Rotjan RD, Sharp KH, Gauthier AE, *et al.* (2019) Patterns, dynamics and consequences of microplastic ingestion by the temperate coral, *Astrangia poculata*. *The Royal Society*. doi: 10.1098/rspb.2019.0726.

[6] Lamb JB, Willis BL, Fiorenza EA, *et al.* (2018). Plastic waste associated with disease on coral reefs. *Science* 26: 460-462. doi: 10.1126/science.aar3320.

[7] Gall SC and Thompson RC (2015) The impact of debris on marine life. *Marine Pollution Bulletin* 92: 170–179. doi: 10.1016/j.marpolbul.2014.12.041.

[8] van Sebille, E., Wilcox, C., Lebreton, L., et al. (2015) A global inventory of small floating plastic debris. Environmental Research Letters.

[9] -- (2019) Plastic and Health: The Hidden Costs of a Plastic Planet. Center for International Environmental Law. 84p.; Boots B, Russell CW and Green DS (2019) Effects of microplastic in soil ecosystems: above and below ground. *Environmental Science and Technology*. doi: 10.1021/acs.est.9b03304; Schwabl P, KöppelS, Königshofer P, *et al.* (2019) Detection of various microplastics in human stool: a prospective case series. *Annals of Internal Medicine*. doi: 10.7326/M19-0618; Dris R, Gasperi J, Mirande C, *et al.* (2017) A first overview of textile fibers, including microplastics, in indoor and outdoor environments. *Environmental Pollution* 221: 453–458. doi: 10.1016/j.envpol.2016.12.013.

[10] Kosuth M, Mason SA and Wattenberg EV (2018) Anthropogenic contamination of tap water, beer, and sea salt. *PLoS ONE* 13. doi: 10.1371.journal.pone.0194970; Hernandez LM, Xu EG, Larsson HCE, *et al.* (2019) Plastic teabags release billions of microparticles and nanoplastics into tea. *Environmental Science and Technology*. doi: 10.1021/acs.est.9b02540.

[11] Li J, Green C, Reynolds A, Shi H and Rotchell JM (2018) Microplastics in mussels sampled from coastal waters and supermarkets in the United Kingdom. Environmental Pollution 241: 35–44. doi: 10.1016/j.envpol.2018.05.038; Rochman CM, Tahir A, Williams SL, et al. (2015) Anthropogenic debris in seafood: Plastic debris and fibers from textiles in fish and bivalves sold for human consumption. Scientific Reports 5 doi: 10.1038/srep14340.

[12] Zheng J and Suh S (2019) Strategies to reduce the global carbon footprint of plastics. Nature Climate Change 9: 374–378. doi: 10.1038/s41558-019-0459-z; -- CO2 Emissions | Global Carbon Atlas. Available: http://www.globalcarbonatlas.org/en/CO2-emissions. Accessed Oct 9, 2019a.

[13] -- (2019) Plastic and Climate: The Hidden Costs of a Plastic Planet. Center for International Environmental Law.

Perhaps the single most important thing to remember about plastic is that it lasts for centuries.[14] Most of the plastic you've used in your lifetime still exists on the planet in some form or another. This is what makes single-use plastics so profoundly flawed. Single-use plastics are created from a material made to last forever, but are designed to be used once and thrown away. Sometimes single-use plastics are only used for a few moments before polluting the earth for years to come.

Half of all the plastic ever made in our planet's history was produced in the past 15 years.[15] Plastic production is expected to quadruple between 2014 and 2050, rising 40% in just the next decade.[16] Waste-management options don't have a chance at keeping up. Take recycling, for instance. Now that companies are seeing their names on the bottles floating in the ocean and polluting our beaches, plastic producers frequently tout their commitments to improving recycling rates and their investments in waste-management systems. They proclaim recycling as the panacea to our plastic problem.

But of all the plastic waste ever generated as of 2015, only 9% has been recycled. That means the vast majority, 91%, either was sent to a landfill, was incinerated or ended up polluting our natural environment — including our oceans.[17] Simply improving recycling rates will not solve this crisis.

In fact, not everything that goes into the recycling bin actually gets recycled. Some is disposed of in landfills or lost in the recycling process. Some is turned into lower-value products, known as "downcycling." And some is exported to developing nations with less robust waste management systems. This means the plastic we thought was being recycled often ends up in a landfill or in the ocean on the other side of the globe.[18] The United States is no exception. In 2015, plastic recycling rates in the U.S. were only 9%.[19] The U.S. and other developed countries have been adding to the problem by shipping some of our plastic waste to countries in Asia because it's cheaper than dealing with it at home.[20]

The truth is, recycling can't solve the ever-growing plastic crisis. Recycling is like trying to mop up water from an overflowing bathtub while the faucet is still running. We need to turn off the faucet and reduce the production of single-use plastic. Companies that have created this problem need to change the way they do business. They must do more than recycle. We need them to significantly reduce the amount of single-use plastic they are putting onto the market and offer consumers plastic-free choices for their products.

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[14] -- (2018) A Guide to Plastic in the Ocean. *NOAA's National Ocean Service.* Available: https://oceanservice.noaa.gov/hazards/marinedebris/plastics-in-the-ocean.html. Accessed June 6, 2019.

[15] Geyer R, Jambeck JR and Law KL (2017) Production, use, and fate of all plastics ever made. *Science Advances* 3. doi: 10.1126.sciadv.1700782.

[16] UNEP and GRID-Arendal (2016) Marine Litter Vital Graphics. Nairobi: United Nations Environment Programme and Arendal: GRID-Arendal.; -- (2019) Plastic and Climate: The Hidden Costs of a Plastic Planet. Center for International Environmental Law. 108p.

[17] Geyer R, Jambeck JR and Law KL (2017) Production, use, and fate of all plastics ever made. *Science Advances* 3. doi: 10.1126.sciadv.1700782.

[18] Brooks AL, Wang S and Jambeck JR (2018) The Chinese import ban and its impact on global plastic waste trade. *Science Advances* 4. doi: 10.1126/sciadv.aat0131.

[19] -- (2018) Advancing Sustainable Materials Management: 2015 Tables and Figures. Environmental Protection Agency.

[20] -- (2019) Global Exports of Plastic Scrap by Country and Year (in metric tons). *Institute of Scrap Recycling Industries, Inc.* Available: https://www.isri.org/docs/default-source/commodities/international-scrap-trade-database/plastic-ex-comtrade-2019-28mar 2019.pdf?sfvrsn=6. Accessed Sept 30, 2019.

Unfortunately, those companies aren't doing enough, and that's why we need your help. It's up to our national, state and local governments to require companies to reduce single-use plastic. Policies governing the production and use of single-use plastic are the most effective way to stem the flow of it into our oceans, and these policies are becoming more common around the world.[21]

The European Union, Peru, Chile and Canada have all announced or are implementing policies to reduce plastic pollution. The United States should create a national policy that comprehensively addresses the plastics crisis threatening our future. U.S. cities, towns, counties and states have recognized the urgency of the issue and taken the initiative on their own, passing policies to reduce single-use plastics. Effective policies include bans, taxes, deposit return systems and extended producer responsibility.

Here are a few examples:

In 2018, the European Union announced a phaseout of single-use plastics by 2021. The Single-Use Plastics Directive bans single-use plastic products, including plates, cutlery, polystyrene food and beverage containers, and other items that are estimated to represent 85% of single-use plastic found on beaches in the EU.[22]

Earlier this year, Santa Monica, California prohibited food and beverage sellers from offering disposable food ware, including plates, cups, bowls, trays and utensils, made predominantly with plastic. The city has already banned expanded polystyrene products.[23]

In 2019, Vermont passed a law that includes a ban on single-use plastic bags, a ban on expanded polystyrene food service products, a minimum 10-cent tax on recyclable paper bags, a ban on single-use plastic stirrers, and a policy making straws available by request-only in food service establishments.[24]

On the federal level, this committee should use its authority to tackle the plastic pollution problem. I applaud you for stopping the use of plastic water bottles in committee hearings and votes, the rest of Congress should take this same step. There's no need to wait. In 2011, the National Park Service implemented a policy to encourage national parks to stop selling water in plastic bottles.

Unfortunately, the policy has been reversed. The committee should make our national parks, national wildlife refuges, marine sanctuaries and other federal lands and waters into single-use plastic free zones, stopping the sale of single-use plastics including plastic beverage bottles throughout the Department of Interior system.

Local and state policies move us in the right direction, and ultimately comprehensive U.S. federal action is needed — and soon. I urge you, our policy-makers tasked with protecting our country's natural resources, to pass federal legislation that stops plastic pollution at the source, significantly reduces the production of this everlasting pollutant, holds corporations responsible for this global crisis and enables states and cities to continue to lead the way on solutions. Don't fall for the false promise of recycling and don't stoop to incineration. We must stop the runaway increase in plastic production and reduce the amount

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[21] UNEP (2018) Combating marine plastic litter and microplastics: An assessment of the effectiveness of relevant international, regional and subregional governance strategies and approaches - summary for policy makers. Available: https://papersmart.unon.org/resolution/uploads/unep_aheg_2018_1_inf_3_summary_policy_makers.pdf. Accessed Jul 31, 2019.

[22] Directive 2019/904 of the European Parliament and of the Council of 5 June 2019 On the Reduction of the Impact of Certain Plastic Products on the Environment, 2019 O.J. (L 155) 1–19 (EU).

[23] SANTA MONICA, CAL., MUN. CODE ch. 5.44 (2019).

[24] VT. STAT. ANN. tit. 10 §§ 6691–6700 (effective July 1, 2020).

of plastic companies are making and foisting on us, because it will last for centuries. We have no more time to waste.

Local and state policies move us in the right direction, and ultimately comprehensive U.S. Federal action is needed—and soon. I urge you, our policy makers tasked with protecting our country's natural resources, to pass Federal legislation that stops plastic pollution at the source, significantly reduces the production of this everlasting pollutant, holds corporations responsible for this global crisis and enables states and cities to continue to lead the way on solutions. Don't fall for the false promise of recycling and don't stoop to incineration, we must stop the runaway increase in plastic production and reduce the amount of plastic companies are making and foisting on us, because it will last for centuries. We have no more time to waste.

QUESTIONS SUBMITTED FOR THE RECORD BY REP. VELÁZQUEZ TO TED DANSON

Question from Rep. Nydia M. Velázquez:

In my district, the Brooklyn Bridge Park Conservancy reported that over 75% of the waste discovered in their cleanup project was single-use plastics — particularly straws and plastic bottles. As you mentioned in your testimony, recycling alone will not address the worsening plastic crisis. We need timely action from both consumers and producers. Throughout your time working on this front, what corporations or industries have been the most unresponsive to advocates' request to start using sustainable alternatives to single-use plastic?

Answer: Solving the plastic pollution crisis will require efforts from all companies and industries producing unnecessary single-use plastic, but some industries have played a larger role in the problem than others. The 2018 International Coastal Cleanup found that the most commonly collected plastic items included plastic grocery bags, plastic straws, plastic stirrers, plastic lids, plastic takeout containers, foam takeout containers, plastic beverage bottles and plastic bottle caps. Plastic bottles were among the top three most common plastic items found in Break Free from Plastic's global cleanup this past September.

The responsibility for curbing the amount of plastic beverage bottles and plastic bottle caps ending up in our waterways should fall on the companies producing these products, but unfortunately, we're not seeing significant progress. If you go to your average supermarket or lunch counter wanting a beverage or a salad, you'll often find your only choices have a plastic package. Four decades of the industry knowing about the plastic pollution problem hasn't changed that. Beverage companies continue to tout their recycling commitments as a solution to the problem rather than switching to more sustainable packaging. Some of these companies even make vague promises to reduce their use of virgin plastic that lack quantifiable goals, making it impossible for us to hold them accountable.

Similarly, it is no surprise that plastic bags, straws, stirrers, lids and takeout containers are ending up in our oceans when they're so readily available at retailers and restaurants. These are single-use items that these companies could choose to avoid, but we haven't seen enough take the initiative to stop using these items or swap them out for less harmful

alternatives. Policies like plastic bag, straw and polystyrene bans that have passed in cities, counties and states around the country are effective in driving widespread change around these items.

Companies have the power to greatly reduce the amount of plastic flowing into our oceans by quitting their reliance on plastic packaging and giving consumers plastic-free choices. We need to demand that change now and implement policies that support it.

* * *

Dr. LOWENTHAL. Thank you, Mr. Danson.

The Chair now recognizes Mr. Parras to testify for 5 minutes. Welcome to the Committee.

STATEMENT OF JUAN PARRAS, FOUNDER, EXECUTIVE DIRECTOR, TEXAS ENVIRONMENTAL JUSTICE ADVOCACY SERVICES (TEJAS), HOUSTON, TEXAS

Mr. PARRAS. I, too, thank you, Chairman Lowenthal and Ranking Member McClintock. I am Juan Parras with Texas Environmental Justice Advocacy Services (TEJAS). TEJAS has been working on environmental justice issues along the Houston Ship Channel for over 16 years. We work at the intersection of human rights and social justice issues.

We call Houston home and share that home with the largest petrochemical complex in the Nation, the second-largest in the world. It is also the largest city with no zoning, meaning that refineries and petrochemical plants, storage tanks, and other industries and infrastructures can be built on the fence line of communities bordering them.

Ninety-nine percent of plastic is derived from fossil fuels. Of those plastics produced, they are derived from either fracked gas or oil. The explosion of natural gas products has led to an ever increasing demand for natural gas liquid, rich in the chemicals that serve as the building block of plastic production.

Naphtha is a product of oil refining. It is another key element of plastic production. Only five companies account for over half of global naphtha sales: British BP, Chevron, ExxonMobil, Shell, and China National Petroleum Corporation. Four of the five have refining capacities along our coast within an hour of our front-door communities.

We are already exposed to a dangerous mix of toxic pollutants, both authorized and unauthorized, released by many different industrial sources located along the Houston Ship Channel. Over the last several years, that petrochemical complex has been expanding. Post-Hurricane Harvey, we began tracking the emissions and realized that the expansions seen in our communities were related to a rapidly, ever-growing market in plastic. Ethane crackers, terminals, and logistics plants all centered around one thing, the production of plastics.

We understood that these expansions were focused on ethylene crackers and LNG facilities. However, we now understand the major economic pivot oil and gas is undergoing, shifting from traditional production into new forms of petroleum utilization.

However, as they expanded, so too did the instability of these petrochemical plants, and we have seen an increase of chemical disasters in the Houston Ship Channel. In the most

recent fire, 37 people were injured, some with first-degree burns. Workers were initially evacuated, but later required to re-enter the plant as the fire was still burning.

To compound the problems, the Commission's Baytown air quality monitors had malfunctioned during the event, and thus deprived community members of invaluable air quality data to protect their health.

While those fires blazed, community members were wholly unaware of the fire or proper shelter-in-place. ExxonMobil has a 10-year investment of $20 billion in their expansion projects for the Gulf of Texas.

Recent disasters: the ExxonMobil fire on March 16, 2019; the ITC Fire on March 17, 2019, where over 8 cities were held hostage under a chemical plume 47 miles long and 17 miles wide; the ExxonMobil Olefins fire on July 31, 2019, where 37 workers were injured; and on September 20, 2019, where nine chemical barges collided after Tropical Storm Imelda damaged evacuation routes.

A recent report for the Center for International Environmental Law found that if trends in the oil consumption continue as expected, the consumption of oil by the entire plastic sector will account for 20 percent of the total consumption by 2050. A recent study uncovered two-thirds of the 90 plastic-related facilities in the Houston region violated air pollution control laws over the last 5 years, and were subject to environmental enforcements. But many more exceeded their permits and were not penalized.

State records show these compounding emissions result in cumulative impacts on neighboring communities, including an increased risk for developing cancer and other health conditions. Plastic poses a distinct risk to public health, from wellhead to waste. From our dinner table to the depths of our oceans, every part of the chain that creates plastic harms us.

Plastic is being produced near vulnerable communities, predominantly people of color, poor people, indigenous, and immigrant people who have to pay the price in shortening the life span of our children and elderlies.

And I see that I am out of time, but I will submit the entire document. Thank you.

[The prepared statement of Mr. Parras follows:]

PREPARED STATEMENT OF JUAN PARRAS, EXECUTIVE DIRECTOR, TEXAS ENVIRONMENTAL JUSTICE ADVOCACY SERVICES (T.E.J.A.S.)

T.E.J.A.S has been working on environmental justice issues along the Houston Ship Channel for over 16 years, we work at the intersection of human rights and social justice issues. We call Houston home and share that home with the largest petrochemical complex in the nation, second-largest in the world. It is also the largest city with no zoning. This means you can put parks, homes, and preschools next petrochemical facilities, refineries, storage tanks and other industry infrastructure, in fact you can find living examples in our community of Manchester and throughout the Gulf Coast. 99% of plastic is derived from fossil fuels. Of those plastics produced they will derive from either fracked gas or oil. The explosion of natural gas production has led to ever increasing demand for natural gas liquid, rich in the chemicals that serve as the building blocks of plastic production. Naphtha, a product of oil refining is another key of production. Only five companies account for over half of global

naphtha sales: BP, Chevron, ExxonMobil, Shell and China National Petroleum Corporation. Four of five have refining capacity along our coast within an hour of our front door.[25]

We are already exposed to a dangerous mix of toxic pollutants, both authorized and unauthorized, released by many different industrial sources located along the Houston Ship Channel. Over the last several years that petrochemical complex has been expanding. Post hurricane Harvey we began tracking emissions and came to understand that the expansions hitting our communities were related to a rapidly, and ever-growing, market in plastic. Ethane crackers, terminals, and logistics plants all centered around one thing: the production of plastic. We understood that these expansions focused on ethylene crackers and LNG but now we began to understand the major economic pivot oil and gas is undergoing, shifting from traditional production into new forms of petroleum utilization. However, as they grew, so too did the instability of these petrochemical plants and with it has come an increase in chemical disasters.

In the most recent fire, 37 people were injured, some with first-degree burns. Workers were initially evacuated but later required to reenter the plant as the fire was still burning. To compound the problem, the Commission's Baytown air quality monitors malfunctioned during the event and thus deprived community members of invaluable air quality data to protect their health. While those fires blazed community members were wholly unaware of the fire or proper shelter-in-place procedures. ExxonMobil has a 10 year investment of $20 billion in their Grow the Gulf Project.

Recently Disasters:

- ExxonMobil Fire March 16, 2019.
- The ITC fire, March 17, 2019 over 8 cities held hostage under a chemical plume 47 miles long 17 miles wide.[26]
- ExxonMobil Olefins Fire, July 31, 2019, 37 workers were injured.[27]
- September 20, 2019, 9 chemical barges collide after Tropical Storm Imelda damaging evacuation routes.[28]

In a recent report the Center for International Environmental Law found that, "If trends in oil consumption continue as expected, the consumption of oil by the entire plastics sector will account for 20% of the total consumption by 2050."

A recent study by uncovered "two-thirds of the 90 plastics-related facilities in the Houston region violated air pollution control laws over the last five years and were subject to enforcement actions. But many more exceeded their permits and were not penalized, state

[25] Center for International Environmental Law, *Fueling Plastics: Fossils, Plastic & Petrochemical Feedstocks* (September, 2019), available electronically https://www.ciel.org/wp-content/uploads/2017/09/Fueling-Plastics-Fossils-Plastics-Petrochemical-Feedstocks.pdf.

[26] https://www.click2houston.com/news/how-it-happened-a-timeline-of-the-deer-park-chemical-fire.

[27] https://www.houstonchronicle.com/news/houston-texas/houston/article/ExxonMobil-s-Baytown-fire-the-latest-in-a-14270558.php#photo-18007536.

[28] https://www.khou.com/article/traffic/i-10-east-freeway-shut-down-after-barges-break-loose-hit-bridge/285-d522e91e-1a54-4b2d-9269- fe44d75f6c81.

records show."[29] These compounding emissions result in cumulative impacts for neighboring communities, including an increased risk for developing cancer and other health conditions.

The production of plastic releases toxics like 1,3, butadiene, benzene, ethane, styrene, toluene. In the short term they look like: headaches, fatigue, weakness, memory loss, nausea, nose bleeds, unconsciousness. In the long term: asthma, anemia, central nervous system damage, childhood leukemia and other cancers, kidney and liver damage, sterility, and even death.[30] The effect is even more severe on children, seniors and the already sick.

Plastic poses a distinct risk to public health from wellhead to waste. From our dinner table to the depths of our oceans. Every part of the chain that creates plastic harms us. Plastic had to be produced near vulnerable communities that used fossil fuel that were extracted next to PEOPLE. BLACK, BROWN, POOR, INDIGENOUS, IMMIGRANT and so many others had to pay the price in shortening the lives of our children's health. The devastating extraction of from our land that shakes our earth. The production of plastic treats us as disposable, as a byproduct that can be ignored. OUR LIVES CANNOT AND WILL NOT BE SACRFICED FOR CONVIENCE.

The American Chemistry Council predicts industry will invest in $204 billion by 2030 on 334 new and expanded facilities in the US alone.[31]

We know our community is not alone in this struggle. The Gulf Coast is known for housing some of the most sophisticated refining capacity in the world. This should not come at the detriment of use at the fenceline.

For us on the fenceline, this is not an exercise in paper pushing or number crunching: not addressing this issue with the necessary enforcement disproportionately harms people of color. There is no amount of money that can make up health impacts from additional emissions and also fugitive emissions associated with additional units or points of emission.

It is vital that community voices be heard at the decision-making table, these are the daily decisions that can drastically alter the outcomes for generations to come. Legislation and policies that safeguard our already overburdened is necessary for our survival. You don't have to lose a child, mother or friend to understand our fight for life.

Juan Parras,

Executive Director

Texas Environmental Justice Advocacy Services.

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The following documents were submitted as supplements to Mr. Parras' testimony. These documents are part of the hearing record and are being retained in the Committee's official files:

[29] Environmental Integrity Project, *Growth of Houston-Are Plastics Industry Threatens Air Quality and Public Safety* (September 5, 2019), available electronically at https://www.environmentalintegrity.org/wp-content/uploads/2019/09/Plastics-Pollution-on-the-Rise-report-final.pdf.

[30] https://www.ciel.org/wp-content/uploads/2019/02/Plastic-and-Health-The-Hidden-Costs-of-a-Plastic-Planet-February-2019.pdf.

[31] https://www.americanchemistry.com/Policy/Energy/Shale-Gas/.

- Report, Plastics Pollutions on the Rise: Growth of Houston-Area Plastics Industry Threatens Air Quality and Public Safety, Environmental Integrity Project, September 5, 2019
- Brief on Plastic in the Gulf Coast—Buildout Hazards to Human Health and Microplastics
- Fueling Plastic Series—Center for International Environmental Law
  - Fueling Plastics: Fossils, Plastics, & Petrochemical Feedstocks
  - Fueling Plastics: How Fracked Gas, Cheap Oil, and Unburnable Coal are Driving the Plastics Boom
  - Fueling Plastics: Plastic Industry Awareness of the Ocean Plastics Problem
  - Fueling Plastics: Untested Assumptions and Unanswered Questions in the Plastics Boom
- Plastics & Climate: The Hidden Costs of a Plastic Planet—Center for International Environmental Law
- Plastic & Health: The Hidden Costs of a Plastic Planet
- Videos
  - The Story of Plastic Teaser
  - How Plastic Production Pollutes Small Towns
  - Manchester—Tejas

QUESTIONS SUBMITTED FOR THE RECORD TO MR. JUAN PARRAS

Mr. Parras did not submit responses to the Committee by the appropriate deadline for inclusion in the printed record.

Question Submitted by Rep. Lowenthal

Question 1. Mr. Parras, the environmental justice component and how frontline communities are affected by the health impacts of plastic refining and production facilities are too often overlooked. Can you please describe for the Committee the relationship between the plastic life cycle and environmental justice?

1a. The industry is looking to increase production over the next decade, if the projections for increasing plastic production come true, what will the impacts of new facilities be on communities of color and other overburdened communities?

1b. How can Congress better support EJ communities in their fight against the plastic industry?

1c. Given the relationship between greenhouse gas emissions and plastic production, how much of an impact would phasing out single-use plastics have on curbing greenhouse gas emissions?

Question Submitted by Rep. Velázquez

Question 1. Can you describe the relationship between the plastic life cycle and environmental justice? What institutional systems are in place that have allowed these impacts to occur?

Question Submitted by Rep. Cox

Question 1. Mr. Parras, while the research is still out on microplastics' human health impacts, it sounds like the communities that TEJAS works with have some experience with that question. Recently, I introduced a bill to help prevent asthma in rural communities. Is asthma a potential concern with plastic production and incineration?

* * *

Dr. LOWENTHAL. Thank you, Mr. Parras.

The Chair now recognizes Dr. Jambeck to testify for 5 minutes. Welcome to the Committee, Dr. Jambeck.

STATEMENT OF DR. JENNA JAMBECK, PROFESSOR OF ENVIRONMENTAL ENGINEERING, UNIVERSITY OF GEORGIA, ATHENS, GEORGIA

Dr. JAMBECK. Thank you, Chairman Lowenthal, Ranking Member McClintock, and the rest of the Subcommittee. I am honored to be here to testify at this hearing.

My name is Jenna Jambeck, I am a professor of Environmental Engineering at the University of Georgia and a National Geographic Fellow. I have been conducting research on solid waste for over 23 years, with related projects on marine debris itself for 18, especially projects regarding location and spatial analysis, quantification and characterization, and global plastic waste management.

I have also witnessed and sampled plastic in the ocean, sailing across the Atlantic in 2014. I have co-developed the Mobile Litter Logging App—Marine Debris Tracker, which was funded by the NOAA Marine Debris Program in 2011, where over 2 million items have been logged by people all over the world.

I have previously testified to the Senate on this issue, to the Subcommittee on Fisheries, Water, and Wildlife.

I am also a participant in the International Informational Speakers Program with the U.S. State Department. This has brought me to 13 different countries and economies around the world to engage with governments, academics, NGOs, and citizens on this issue.

I have submitted a longer written document, but my testimony today is my opinion based upon my background and experience conducting research on marine debris plastic and waste.

When I testified previously to Congress in 2016, I spoke to educate and raise awareness of this issue based upon my research. But we now know we have a major problem with

plastic ending up in our environment and in the ocean. The science on this issue has increased rapidly just in the past 4 years.

We now know we have produced 8.3 billion metric tons of plastic as of 2017. And since about 40 percent of this is used for packaging and single-use items, it means that 6.3 billion of that had become waste by 2015.

So, what have we done with that waste? How did we manage it? We have recycled about 9 percent of that cumulatively, those vary locally. But, on average, globally recycled only about 9 percent. Another 12 percent had been incinerated. That means 79 percent has ended up either in a landfill or in the open environment.

As a result of weathering and exposure to sunlight, plastic that is in the environment doesn't biodegrade. It simply fragments into smaller and smaller pieces, and with an unknown fate, I would say, of the smallest particles that we can't even measure yet.

You heard the number in our *Science* paper in 2015. We estimated the global quantity of plastic entering the oceans at 8 million metric tons in 2010, and that is equal to about a dump truck of plastic entering every minute. So, although there have been actions taken globally to stop the business-as-usual projection of this input doubling by 2025, plastic production use and population growth are all driving factors that have resulted in an increase of plastic used and in our waste streams.

We can all agree we want to keep plastic out of the ocean in the first place. There is a tremendous opportunity for continued bipartisan support and action on this issue. In the intervention framework I developed in 2016, we start all the way upstream with reducing waste generation, especially in places with high per-person waste generation rates, like here in the USA. Our waste generation rate is two to six times that of many countries around the world, especially still economically developing countries. And this reduction can be obtained through a combination of individual choice, policies, and industry-led changes.

For when we do need packaging, there needs to be a more distinct connection between design, material choice, and end-of-life management of materials. Currently, the waste management system has to deal with whatever comes their way. This is one contributing factor to the historical practice of exporting nearly 50 percent of our plastic recycling to other countries, primarily those of lower income, which contributes to the environmental pollution in their country, as well.

Engagement of all stakeholders across all points of this issue, from production, to use, to management, is critical to make sure all voices are heard. So, one reminder I always have to give: there are people behind all the numbers I gave you. We need to collectively come up with creative, socially and culturally appropriate solutions, because we are all here today presenting to you. I am optimistic we can do that, and I will continue to work hard on science to inform policy. But everyone has an important role to play.

In my last points, I want to encourage you to try two experiments.

First, for the next 24 hours, take note of everything that you touch that is plastic. From this you will see how widely used and useful the material is, but it also makes you reflect upon where and when are the right times and places to use this material.

Second, go outside on a scavenger hunt for litter—you won't likely have to go far—and look at each item you find as a message for you, the figurative or sometimes literal message in a bottle. Ask yourself three questions: one, what is it; two, how did it get here; and three, what are we going to do about it?

Community-based data collection and citizen science within a framework and structure can contribute to critical data needed to inform circular materials management in communities. And I believe questions like these can empower citizens, NGOs, corporations, and policy makers like you to take the most relevant, impactful action for their country, state, or community.

Thank you.

[The prepared statement of Dr. Jambeck follows:]

PREPARED STATEMENT OF JENNA R. JAMBECK, PHD, PROFESSOR OF ENVIRONMENTAL ENGINEERING, COLLEGE OF ENGINEERING, UNIVERSITY OF GEORGIA, ATHENS, GEORGIA; NATIONAL GEOGRAPHIC FELLOW

Key Points

Based upon my testimony, the top five recommendations for how Congress can best support research, cleanup, or prevention efforts to combat marine debris are:

1) Funding the current agencies and initiatives, as well as new research through other agencies to provide science to further determine human health impacts (e.g., micro and nanoplastics) and mitigate this issue through the entire value chain of plastic (e.g., fate and transport of plastic in the environment, new materials and product design), which can provide economic innovation and growth, and also inform policy. Community-based data collection and citizen science with proper frameworks and structure can contribute to critical data needed to inform circular materials management in communities on the front lines of waste management.
2) To support prevention domestically, Congress could support legislation to reduce waste generation to reduce leakage of especially plastic packaging (and those items found in typical beach cleanups), like deposit-return schemes which show a 40% reduction in beverage containers where in place in the USA, as well as, for example, product stewardship/extended responsibility initiatives to increase the collection and value of waste.
3) To support prevention internationally, we can continue to provide funding through USAID and other bilateral initiatives, which I have seen give NGOs the opportunity to catalyze action, improve infrastructure and the economy, in countries like Vietnam, Philippines and Indonesia. We also need to make sure our exports are not negatively impacting other countries and support development in other countries so they may participate in trade using standards such as the OECD. We can also determine if our trade-agreements can influence other countries improvement of environmental standards, including solid waste management.
4) Show support for global initiatives to assist with the reduction of plastic entering the ocean and improvement of waste management infrastructure development around the world (e.g., with world development banks, NGOs and industry), along with technology and knowledge transfer to other countries on solid waste management

through, for example, the U.S. State Department, US EPA and NOAA. The newest USAID CCBO funding is one aspect of this process.

5) Derelict fishing gear is one of the most dangerous types of debris in the environment. Supporting the development of a program (through an agency) for fisherman to drop off gear that is broken or that they find could help this program (providing collection and disposal in areas where DFG has an impact). NOAA Marine Debris Program "Fishing for Energy" or similar could continue and/or expand.

Introduction

I would like to thank Chairman Huffman, and the rest of the Water, Oceans and Wildlife Subcommittee for the opportunity to testify at this hearing to examine plastic's impact on the ocean. It is an honor and privilege to be with you today. My name is Jenna Jambeck and I am a Professor of Environmental Engineering at the University of Georgia and a National Geographic Fellow. I have been conducting research on solid waste issues for 23 years with related projects on marine debris since 2001, especially projects regarding location and spatial analysis of debris, debris quantification and characterization, global plastic waste mismanagement and technology/mobile device usage (mapping, etc.). I have also sampled open ocean plastic sailing across the Atlantic and co-developed the mobile app, Marine Debris Tracker, funded by the NOAA Marine Debris Program. I have presented at three Capitol Hill staffer briefings, a Global Ocean Commission meeting, the 2015 Our Ocean Conference, a 2015 G7 workshop, and at the White House Office of Science Technology and Policy (OSTP). I also serve as the U.S. representative on an Advisory Panel for the United Nations Environment Program Global Partnership on Marine Litter. I testified on May 17, 2016 to the Senate Subcommittee on Fisheries, Water and Wildlife on this topic. I have been in the International Informational Speakers Program with the US State Department since 2017 and have been to thirteen different countries/economies working on the issue of marine debris and plastic waste in public environmental diplomacy (Chile, Philippines, Indonesia, Japan, South Africa, Vietnam, Jordan, Israel, South Korea, India, Bulgaria, Taiwan, and China). My testimony today is my opinion, based upon my background and experience in studying marine debris and plastic pollution.

Context

I think it is important to provide context and introduction similar to when I gave testimony to the US Senate in 2016, the US regulatory history is always relevant. I grew up in the 1970s outside a small town (fewer than 3,000 people) in Minnesota. Like many people at the time, we managed our trash by taking it to the landfill and putting it in ourselves. I always found it fascinating to see what people throw away – and I have seen bowling balls to bologna in landfills. In graduate school, my fascination turned into a passion for studying solid waste management as an environmental engineer. Environmental engineers can also design urban drinking water and wastewater facilities, but to me, solid waste management felt like it most closely involved people. Unlike the small effort required to turn off a faucet or flush a toilet (even a sensor can do this with no human effort), we all have to decide daily

what to consume, what materials to use, what *is* and *is not* "solid waste" in our own home, and then whether to give away, discard, compost or recycle unwanted materials. The human component of solid waste management, and the direct interaction with people, is an aspect of my work that continues to be essential to my work.

In 1976 Congress passed the Resource Conservation and Recovery Act (RCRA) that required the U.S. EPA (typically through the states) to regulate solid and hazardous waste.[32] "Open dumping" was prohibited and replaced by engineered and regulated landfills, composting and recovery systems.[33] RCRA also specifically called for research to inform solutions, including demonstrations and special studies on measures to reduce the generation of waste, waste collection practices, and economic incentives to promote recycling and waste reduction (among other things).[34] Because of RCRA, we had outstanding progress in solid waste management, just in my lifetime. When I heard about our trash ending up in the ocean in 2001, I knew we must be contributing to it from the land, and started down the path of my current research. In this testimony, I am going to illustrate the direct connection between the solid waste (trash) we produce on land and the plastic found in our ocean, recalling that the human component goes hand in hand with local, state, regional, national and international initiatives to address this problem.

Introduction

Marine debris has been recognized as a contamination issue for more than 50 years[35] but the laser-focus on plastic has occurred just in the past 5-7 years. Plastic completely changed our world after it's expanded use in World War II, and global annual plastic production has increased from 1.7 million metric tons/yr in 1950 to 360 million metric tons/yr (not including polyester fibers) in 2019.[36] Along with a steep increase in production, we have seen a resulting increase in plastic in the waste stream from 0.4% in 1960 to 12.7% in 2012 (by mass) in the U.S. All traditional plastics do not biodegrade, but only fragment into smaller, ultimately microscopic or nanoscopic, pieces. A cumulative 8.3 billion metric tons of plastic has been produced since 1950.[37] Since approximately 40% of plastic is used for packaging and single use items, this means that 6.4 billion metric tons has become waste by 2015 (Figure 1). Globally, on average, we have recycled only about 9% of plastic, with 12% recycled and 79% ending up in our landfills or in the environment. With cumulative quantities projected to reach 34 billion metric tons of production and 12 billion metric tons of waste, the management of plastic in the waste stream is only continuing to grow.

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[32] Resource Conservation and Recovery Act (RCRA) - Public Law 94-580, October 21, 1976, (42 U.S.C. 6901-6992; 90 Stat. 2795), as amended by P.L. 95-609 (92 Stat. 3081), P.L. 96-463 (94 Stat. 2055), P.L. 96-482 (94 Stat. 2334), P.L. 98-616 (98 Stat. 3224), P.L. 99-339 (100 Stat. 654), P.L. 99-499 (100 Stat. 1696), P.L. 100-556 (102 Stat. 2779).

[33] Code of Federal Regulations (CFR) Title 40, Parts 239 – 282.

[34] https://www.epa.gov/aboutepa/new-law-control-hazardous-wastes-end-open-dumping-promote-conservation-resources.

[35] Ryan, P. (2015). A Brief History of Marine Litter Research, in Marine Anthropogenic Litter, Bergmann et al. (eds.), Springer, New York, NY.

[36] Plastics Europe, https://www.plasticseurope.org/application/files/9715/7129/9584/FINAL_web_version_Plastics_the_facts2019_1 4102019.pdf.

[37] Geyer, R., Jambeck, J.R., Lavender Law, K. (2017). Production, use, and fate of all plastics ever made, Science Advances, 19 Jul 2017, Vol. 3, no. 7.

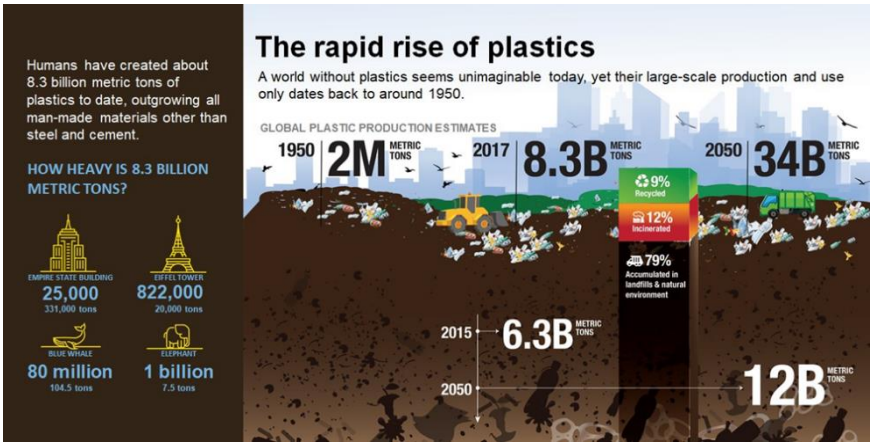

Figure 1. Global materials flow of plastic.

Polymers that make up the plastics that we commonly encounter are listed in Table 1. But plastics also contain additives to alter color, texture, shape, form, antimicrobial surfaces, make it flame retardant, and for other properties.[38] The wide variety of available additives results in thousands of different plastic material compounds for particular purposes, creating a diverse array of plastic materials that end up in our trash, which can make recovery and recycling challenging. In the USA, the per person waste generation rate ranges from 4.48 to 6 lbs/person/day (2 to 2.7 kg/person/day), depending on the reference examined.[39] This is 2-6 times the waste generation rates of many countries around the world.[40] The recycling percentage for all plastic in the USA is the same as the global average, with only about 9% of plastic recycled, although rates for individual polymers vary (Table 1).[41]

Table 1. Common polymers, uses and density related to seawater

Polymer	Recycling Number	Sink or Float in Seawater	Common Use(s)	USA Recycle Rate
Polyethylene Terephthalate (PET)	1	Sink	Individual beverage bottles, textiles	18.4%
High Density Polyethylene (HDPE)	2	Float	Gallon jugs, some personal care product and detergent bottles	10.3%
Polyvinyl Chloride (PVC)	3	Sink	Piping, siding (construction)	Negligible
Low Density Polyethylene	4	Float	Retail bags, thin film plastic	6.2%
Polypropylene	5	Float	Bottle caps, yogurt containers, toys	0.9%
Polystyrene	6	Sink (expanded floats)	Foamed/expanded PS in packaging	1.3%
Others	7	Nylon sinks	Fishing nets (nylon), carpet	22.6%

[38] Additives have been mixed into plastic compounds since they have been in the consumer market: Deanin, R.D. (1975). Additives in plastics, *Environmental Health Perspectives*, 11: 35-39.

[39] https://www.epa.gov/facts-and-figures-about-materials-waste-and-recycling/national-overview-facts-and-figures-materials#Generation; https://erefdn.org/national-waste-generation-recovery-and-disposal-assessment/.

[40] http://datatopics.worldbank.org/what-a-waste/.

[41] https://www.epa.gov/sites/production/files/2018-07/documents/smm_2015_tables_and_figures_07252018_fnl_508_0.pdf.

Since plastic "degrades" through fragmentation, the result is microplastic (smaller than 5 mm in size) in the environment. Secondary microplastics are formed by the fragmentation of larger items. Primary microplastics are manufactured in these small sizes. Some sources of primary microplastic are resin pellets and microbeads. Resin pellet loss has been addressed by the industry though their Operation CleanSweep program,[42] and recent federal legislation banned microbeads in personal care products as of 2018.[43] Secondary microplastics are found on our coastlines, in our sediments, and floating in the ocean aggregating in the five oceanic gyres. Using the largest available ocean microplastics dataset, a recent study estimated that 15 to 51 trillion particles, with a mass of 93 to 236 thousand metric tons, are floating on the sea surface globally; this is equivalent to only about 1% of the estimated input of plastic waste to the ocean from land in a single year.[44] Where the remaining plastic debris is in the ocean remains a major unanswered question. The majority of field sampling to date captures only particles larger than approximately one-third of a millimeter in size, but increasing numbers of reports of synthetic fibers (from clothing and woven ropes, for example) in freshwater and marine environments, and even in air, make microfibers now a major concern.[45] And, while many people think of marine debris as being only in the ocean environment, the Great Lakes are governed by NOAA's Marine Debris Program, and are known to be contaminated with plastic (REF) and not to be overlooked are inland riverine inputs of which there are two global estimates for, but could make up 5% to 50% (likely around 25%) of the global inputs of plastic into the ocean.[46]

In the last decade, scientific research into marine debris, and especially plastic, has increased. In 2011, a scientific working group was convened at the National Center for Ecological Analysis and Synthesis (NCEAS). I was honored to be a part of this working group that spent three and a half years synthesizing data to describe the scale and impact of trash in ocean ecosystems. At least nine scientific articles have been produced from this group describing information to date,[47] advancing the science. The NCEAS work, along with other recent scientific work, has brought attention to the issue of plastic in the oceans further validating action at the global scale by the G7, G20, United Nations, and multinational global funding entities like the World Bank, the Global Environment Fund (GEF). In 2018, the Save Our Seas Act was passed with unanimous bipartisan support. And Save Our Seas 2.0 is in the legislative process now.

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[42] https://opcleansweep.org/.

[43] https://www.congress.gov/114/plaws/publ114/PLAW-114publ114.pdf.

[44] van Sebille E, Wilcox C, Lebreton L, Maximenko N, Hardesty B D, van Franeker J A, Eriksen M, Siegel D, Galgani F and Law K L 2015 A global inventory of small floating plastic debris, *Environmental Research Letters*, 10 124006.

[45] Woodall, L. C., Gwinnett, C., Packer, M., Thompson, R.C., Robinson, L.F., Paterson, G.L. (2015). Using a forensic science approach to minimize environmental contamination and to identify microfibers in marine sediments. *Marine Pollution Bulletin*, 95(1), 40-46; Watts, A.J.R., Urbina, M.A., Corr, S., Lewis, C., Galloway, T.S. (2015). Ingestion of Plastic Microfibers by the Crab Carcinus maenas and Its Effect on Food Consumption and Energy Balance, *Environmental Science & Technology*, 49 (24), 14597-14604.

[46] Lebreton, L. C. M. et al. River plastic emissions to the world's oceans. *Nat. Commun.* 8, 15611 (2017); Schmidt, C., Krauth, T., Wagner, S. *Environ. Sci. Technol.*, 2017, 51, 21, 12246-12253; Lechner, A., Keckeis, H., Lumesberger- Loisl, F., Zens, B., Krusch, R., Tritthart, M., Glas, M., Schludermann, E. (2014). The Danube so colourful: A potpourri of plastic litter outnumbers fish larvae in Europe's second largest river, *Environmental Pollution*, 188, 177-181.

[47] I reference some of them in this document, but the full list is available online here: https://www.nceas.ucsb.edu/projects/12645#.

Similar to RCRA in the 1970's, sound science should be used when determining policies and solutions. Today, we have sufficient evidence to guide action to reduce inputs of plastic into the ocean. In parallel, new scientific information should be created to help us better understand the sources, sinks and impacts of plastic in our oceans.

Impacts from Plastic Marine Debris

I will cover impacts briefly here, with further detail able to be obtained in my previous testimony to the Senate.[48] In 1966, two U.S. Fish and Wildlife Service employees, Karl W. Kenyon and Eugene Kridler, were among the first scientists to document plastic and wildlife interactions when they discovered plastic was consumed by seabird (Albatross) chicks that had died in the Hawaiian Islands National Wildlife Refuge.[49] Since that time, many individuals of a multitude of different species of wildlife have been found to be impacted by plastic. Like in the Albatross chicks in 1966, ingestion of and entanglement are the most commonly reported interactions. A comprehensive critical review of the literature on marine debris impacts was led by Dr. Chelsea Rochman in the NCEAS group. Of the 296 perceived threats of debris to wildlife that were tested, 83% were demonstrated (proven), and 82% of those were from plastic. There is evidence of impacts to individual animals and to assemblages of organisms suggesting decision-makers should take action in order to avoid risk of "irreversible harm."[50]

Lost fishing equipment (e.g., nets and traps) can "ghost fish," or drift while continuing to catch fish and kill wildlife. This can have an impact on the fishing and shellfish industry. One study in Puget Sound alone analyzed 870 recovered "lost" gillnets and found 31,278 invertebrates (76 species), 1036 fishes (22 species), 514 birds (16 species), and 23 mammals (4 species); 56% of invertebrates, 93% of fish, and 100% of birds and mammals were dead when recovered.[51] When experts were asked which marine debris item poses the greatest risk to marine life, fishing-related gear ranked first, followed by balloons and plastic bags.[52]

Marine debris can present physical hazards to shipping, boating, fishing and industrial systems by blocking navigation, fouling boat propellers, clogging water intakes or blocking pumping systems. Coastal tourism is also affected by marine debris and other litter. In the 1980s, when medical waste was found on some beaches, communities lost millions of dollars from a decline in tourism and increased costs for beach cleanup maintenance.[53] A 2014 study by the NOAA Marine Debris Program in Orange County, CA found that 1) residents are concerned about marine debris, and it significantly influences their decisions to go to the

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[48] https://www.epw.senate.gov/public/_cache/files/8/0/8074ded1-5986-4a9b-b033-2eb69e66993f/B775115948AB5A3C80BDDB5B0287E8B3.jambeck-testimony.pdf.

[49] Kenyon, K. W., & Kridler, E. (1969). Laysan Albatrosses swallow indigestible matter. Auk, 86, 339–343, also referenced in Ryan, P. (2015). A Brief History of Marine Litter Research, in Marine Anthropogenic Litter, Bergmann et al. (eds.), Springer, New York, NY.

[50] Rochman, C.M., Browne, M.A., Underwood. A.J., van Franeker, J.A., Thompson, R.C., Amaral-Zettler, L.A. (2016). *Ecology*, 97(2), 302-312.

[51] Good, T.P., June, J.A., Etnier, M.A., Broadhurst, G., (2010). Derelict fishing nets in Puget Sound and the Northwest Straits: Patterns and threats to marine fauna, *Marine Pollution Bulletin*, 60(1), 39-50.

[52] Wilcox, C., Mallos, N., Leonard, G.H., Rodriguez, A., Hardesty, B.D. (2016). Using expert elicitation to estimate the impacts of plastic pollution on marine wildlife, *Marine Policy*, 65 (2016), 107-114.

[53] NRC (National Research Council) Committee on Shipborne Wastes, Clean Ships, Clean Ports, Clean Oceans, National Academy Press, Washington D.C., 1995.

beach, 2) No marine debris on the beach and good water quality are the two most important beach characteristics to them, and 3) Avoiding littered beaches costs Orange County residents millions of dollars each year. If the debris were reduced by just 25%, it would save residents roughly $32 million dollars in reduced travel to other beaches.[54] UNEP estimates the financial damage of plastics to marine ecosystems globally is $13 billion each year.[55] A recent study outlined that there are negative impacts to almost all marine ecosystem services, negative impacts to human wellbeing (fisheries, heritage and recreation) at a cost of $3300 to $33,000 per metric ton of marine plastic per year, equaling $264 billion per year at the mid-input estimate.[56]

Plastic also hosts an entire microbial community termed the "plastisphere."[57] Plastic can transport non-native species and provide habitat for microbes that might not otherwise thrive, but we don't yet know the full extent of this microbiome on ocean microbiology or the broader ocean ecosystem. Plastics in the ocean are associated with chemicals. This includes organic compounds like flame retardants, pesticides, and polychlorinated biphenyls (PCBs) that accumulate on the plastic from surrounding water. It also includes the additive ingredients of the plastic that can leach into the surrounding environment. Thus, plastic can transport these compounds around the world and be another potential source of contaminants to wildlife.[58] Some of the additives to plastic have come under question for toxicity,[59] but we don't yet know the full impact they have on aquatic systems.[60] Still, there has been evidence of the transfer of chemicals from plastic to fish in the lab, causing liver toxicity and impacting functions of the endocrine system and to other organisms in the field.[61] Plastic particles and fibers have also been found in the stomachs of fish, and in shellfish sold for human consumption.[62]

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[54] Chris Leggett, Nora Scherer, Mark Curry and Ryan Bailey, Assessing the Economic Benefits of Reductions in Marine Debris: A Pilot Study of Beach Recreation in Orange County, California, Industrial Economics, Inc., for the NOAA Marine Debris Program, 2014.

[55] Raynaud, J. (2014). Valuing Plastic: The Business Case for Measuring, Managing and Disclosing Plastic Use in the Consumer Goods Industry, UNEP, Plastic Disclosure Project, Trucost.

[56] Beaumont, N., Aanesen, M., Austen, M., Börger, T., Clark, J., Cole, M., Hooper, T., Lindeque, P., Pascoe, C., Wyles, K., Global ecological, social and economic impacts of marine plastic, Marine Pollution Bulletin, Vol 142, 2019, Pages 189-195.

[57] A recent summary article that references multiple scientific references on this: Samoray, C. (2016). Ocean's plastics offer a floating fortress to a mess of microbes, Science News Magazine, February 9, 2016; Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., (2013). Life in the "Plastisphere": Microbial Communities on Plastic Marine Debris, *Environmental Science & Technology*, 47 (13), 7137-7146.

[58] Same as note 6. Plus, a good overview is Rochman, C. (2015). The Complex Mixture, Fate, and Toxicty of Chemicals Associated with Plastic Debris in the Marine Environment, in Marine Anthropogenic Litter, Bergmann et al. (eds.), Springer, New York, NY.

[59] For example, antimicrobial – Yueh, M. and Tukey, R.H. (2016). Triclosan: A Widespread Environmental Toxicant with Many Biological Effects, *Annual Review of Pharmacology and Toxicology*, 56: 251-272; flame retardants – Agency for Toxic Substances and Disease Registry, Toxic Substances Portal - Public Health Statement for Polybrominated Diphenyl Ethers (PBDEs), September 2004 (accessed May 11, 2016) http://www.atsdr.cdc.gov/phs/phs.asp?id=899&tid=94.

[60] Teuten, E. L., Saquing, J. M., Knappe, D. R. U., Barlaz, M. A., Jonsson, S., Björn, A., … Takada, H.. (2009). Transport and Release of Chemicals from Plastics to the Environment and to Wildlife. *Philosophical Transactions: Biological Sciences*, 364(1526), 2027–2045.

[61] Rochman, C.M., Hoh, E., Kurobe, T., The, S.J., (2013). Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress, *Scientific Reports* 3, No. 3263; Rochman, C.M., Kurobe, T., Flores, I., The, S.J., (2014). *Science of the Total Environment*, Vol. 493, 656-661; Jang, M., Shim, W.J., Han, G.M., Rani, M., Song, Y.K., and Hong, S. H. Styrofoam Debris as a Source of Hazardous Additives for Marine Organisms, *Environmental Science & Technology*, Article ASAP, DOI: 10.1021/acs.est.5b05485.

[62] Rochman CM, Tahir A, Williams SL, et al. Anthropogenic debris in seafood: Plastic debris and fibers from textiles in fish and bivalves sold for human consumption. Scientific Reports. 2015;5:14340.

Input into the Ocean from Mismanaged Plastic Waste

In the NCEAS group, as we started compiling information about sources and inputs of plastic into the ocean, we quickly concluded that mismanaged solid waste (trash) made up a large portion of the input. Other inputs include, but are not limited to, commercial fishing gear, shipping, recreational boating and fishing, and catastrophic events. Our first objective was to quantify mismanaged waste from land. To make the estimate of plastics entering the ocean from waste management, we developed a comprehensive framework (Figure 1).

Our methods for this estimate were to look at per person waste generation rates in 2010 from 192 countries with a coastline in the world. Because people's activities nearest the coast are responsible for most of the plastic going into the water, we limited our analysis to a 50km strip of the coastline. From there, we looked at what percent of that waste is plastic, and what percentage of that is mismanaged waste (which means litter or when waste is not captured and dumped on the land). From there we had three scenarios of input into the ocean: low, mid and high.

The results were that in 2010, we estimate that 275 million metric tons (MMT) of plastic waste was generated in 192 countries. Of that, 99.5 MMT of this waste was generated within 50km of the coastline, and 31.9 MMT was mismanaged. We then estimated that between 4.8 and 12.7 MMT (a mid-scenario of 8 MMT) reached the oceans[63] (Figure 2). This annual input of plastic is equal to 5 grocery-size bags filled with plastic going into the ocean along every foot of coastline in the world.

Figure 2. Plastic waste inputs from land into the ocean in 2010.

[63] Jambeck, J. R., Andrady, A., Geyer, R., Narayan, R., Perryman, M., Siegler, T., Wilcox, C., Lavender Law, K., (2015). Plastic waste inputs from land into the ocean, Science, 347, p. 768-771.

The U.S. is one high income country on the list, and while our waste management systems are well-designed and very effective, and the only mismanaged waste is from litter, we have a large coastal population and a large waste generation rate. If we look to the future, and assume a business as usual projection with growing populations, increasing plastic consumption and increased waste generation, but no increase in capture of waste, by 2025, the 8 million metric tons doubles – with a cumulative input by 2025 of 155 million metric tons.

Import-Export of Plastic Waste

While recycling and the circular economy have been touted as potential solutions to this issue, one can see from the recycling percentages given in the introduction, we have a long way to go for recycling to be significant. Approximately half of the plastic waste intended for recycling has been exported to hundreds of countries around the world (Figure 3).

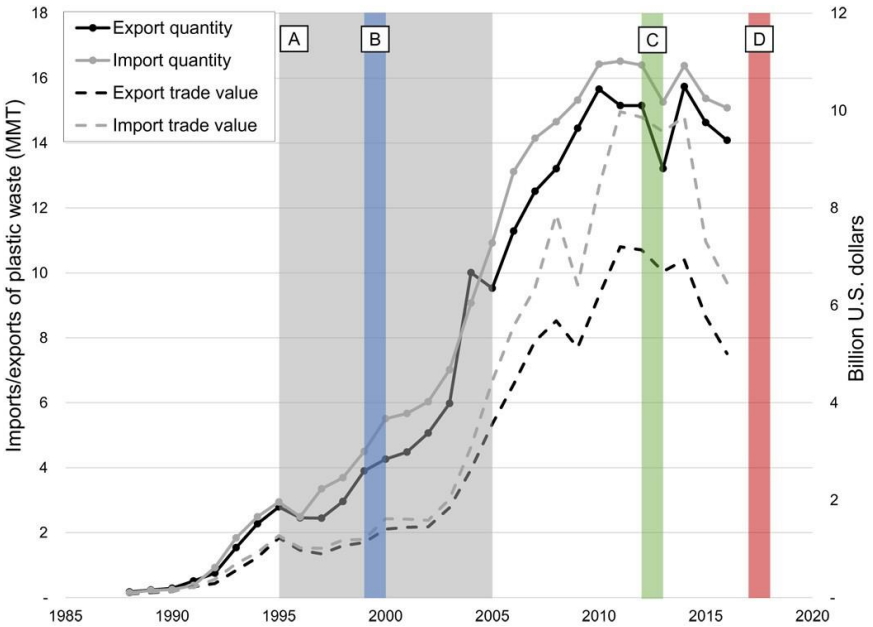

Figure 3. Trade of plastic waste in mass and trade value (UN Comtrade data).

Before their import restrictions (resulting really in a ban) in 2017, China had imported a cumulative 45% of plastic waste since 1992.[64] Compiled commodity trade data by Amy Brooks in my research group illustrated that higher-income countries in the Organization for Economic Cooperation (OECD) have been exporting plastic waste (70% in 2016) to lower-income countries in the East Asia and Pacific for decades. An estimated 111 million metric tons of plastic waste is displaced with the new Chinese policy by 2030 begging the question of where this plastic goes now and will continue to go – and causing one of the biggest

[64] Brooks, A., Wang, S. Jambeck, J. (2018). The Chinese import ban and its impact on global plastic waste trade, Science Advances, 20 Jun 2018: Vol. 4, no. 6, DOI: 10.1126/sciadv.aat0131.

economic disruptions to recycling ever to happen in the USA. With 89% of historical exports consist of polymer groups often used in single-use plastic food packaging (polyethylene, polypropylene, and polyethylene terephthalate), bold global ideas and actions for reducing quantities of nonrecyclable materials, redesigning products, and funding domestic plastic waste management are needed. The USA and others who have exported to countries that lack waste management systems are responsible for some of this mismanagement. In China alone, this added another 11% of plastic mass to their waste stream to manage in 2015. Rethinking trade agreements and the balance of resources to be able to participate in trade for countries (like small island sates) that need to, is important. This is also a large global economic system that involves the livelihood of millions of people around the world. Improving their conditions and protecting the environment should be paramount. New amendments to the Basel Convention have put requirements on exporting countries to at least notify and get consent for shipments.[65] The USA could help lead efforts to both improve and develop domestic infrastructure while participating in responsible global trade of recycled materials.

It's a Global Issue

Once plastic is in our oceans, it becomes a global issue and poses great logistical and economic challenges to get it out. In addition, the plastic is not always visible (although we find it everywhere we look, we have only quantified a fraction in our ocean compared with what is going in), so understanding potential risk to our ecosystems requires two things: 1) understanding the impact and 2) understanding the exposure. Our recent estimate of plastic entering the oceans informs the second part – exposure, just how much plastic is going into the ocean? But it also makes us ask – where is all the plastic going? While we know action will help "turn off the faucet" of plastic input (see potential interventions, below), there are still gaps in the sources, distribution, fate and impacts of plastics in the ocean that need more research if we want to continue to move forward in addressing this issue based upon science.[66]

Interventions and Mitigation Strategies

I developed the framework below for my 2016 testimony[67] and would like to submit it again with some ideas, further explanation and answers to some of the questions posed by the senators in this hearing. This framework provides intervention points (1 through 5) and then a list of potential (but not all encompassing) interventions that may occur at the various points. In general, this represents a hierarchy of interventions. However, the most "bang for your buck" interventions will depend on the needs of the specific geography addressing the issue, however, in many cases, all geographies have points along the entire framework that will help

[65] http://www.basel.int/Implementation/Plasticwastes/Overview/tabid/6068/Default.aspx.

[66] A good recent review of why it is important to move forward with science –based solutions is provided in Rochman, C. (2016). Strategies for reducing ocean plastic debris should be diverse and guided by science, *Environmental Research Letters*, 11 014006.

[67] https://www.epw.senate.gov/public/_cache/files/8/0/8074ded1-5986-4a9b-b033-2eb69e66993f/B775115948AB5A3C80BDDB5B0287E8B3.jambeck-testimony.pdf.

reduce debris and plastic going into the ocean. Some interventions can be immediate and some will take more time. The framework starts on the left with the most "upstream" interventions and ends with a last chance to capture the material before it enters the ocean. In many cases the interventions offer the opportunity for economic innovation and growth. The USA could be a leader in several of these categories of interventions.

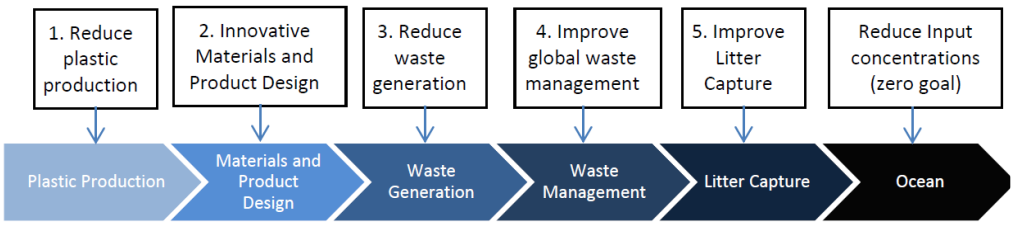

Figure 4. Intervention and Mitigation Strategies along some Points in the Plastic Value Chain I'll now discuss some potential intervention points identified in Figure 2 in a bit more detail.

1. Reducing Plastic Production

Plastic production is one of the "book ends" of the plastic value chain. Other than a few of the past 65 years, global plastic production has increased annually, and is anticipated to continue to do so into the near future. Although it comes from fossil fuels for the most part, and is produced from monomers that come from the processing of oil and natural gas, these monomers (e.g., ethylene and propylene) are used to make many different compounds, not just polymers. As long as other common chemicals are made, it is likely that polymers will continue to be made as well. And, as economies around the world continue to develop, packaged goods become more prevalent. Unless the industry changes its own course, this stage is mostly influenced if levers in other stages are pushed (e.g., demand is decreased for other reasons along the value chain). Reduction in demand comes primarily from the points given below.

1. Consumers demanding less packaging or no packaging (some markets)
    a. Not everyone has access to clean water, for example, so can't always make the choice of a reusable bottle, but these choices taken collectively where possible do make a difference
2. Local initiatives (e.g., bans, taxes)
    a. These are often very local-specific, but are also becoming more common
    b. Mass of items removed may be relatively small, but numbers of items are also important – there is more than one way to measure debris (e.g., mass, count, etc.)
3. Voluntary industry actions
    a. Industry has become more engaged on this issue – I wonder if they will volunteer some changes to help in the future as well?
    b. The reality is that all signs point to further growth in waste generation, as well as plastic use, especially where economic development is occurring or predicted to occur in the future.

2. Innovative Materials and Product Design

New materials development and product design take time to advance, so these activities need to be happening now – and they are, but even more time and resource investment is needed. Overall, I think Green Engineering principles,[68] if followed during material development and product design, would help to avoid many of the externalities of plastic that we are dealing with currently. In addition, circular economy concepts, emerging all over the world now, will be important to also apply to plastic materials. Both of these guiding principles promote non-toxic materials, ultimately with the capability of biodegrading and/or being recycled. Materials and products made with more homogenous compounds would make recycling more efficient and effective. Materials and products can be designed to retain their value, for collection, recovery and recycling. Several of these concepts are outlined in Ellen MacArthur Foundation's report on the "The New Plastic Economy: Rethinking the future of plastics," which focuses specifically on packaging.[69] The University of Georgia has combed environmental engineering and polymer chemistry in a successful and rapidly expanding New Materials Institute with centers on biodegradable polymers and circular materials management to develop and test materials to reduce the flow of plastic into the ocean. NMI has become part of a National Science Foundation (NSF) Industry–University Cooperative Research Centers (IUCRC) that has over 30 corporate partners interested in more sustainable and biodegradable polymer products. These industry-research groups participate in pre-competitive research and development as new materials need to scale to be economical for all to use. There is no doubt that developing alternative materials without the unintended consequences of traditional plastics will spark innovation and economic growth in the USA where truly biodegradable polymer production facilities (e.g., Polyhydroxyalkanoates (PHA)), like the ones in Georgia owned by Danimer Scientific and RWDC are creating jobs. There are many current corporate commitments to change materials, use more recycled materials, and be more circular with materials – many of these commitments have been made at the Our Ocean meeting that just occurred for the sixth time in Oslo Oct 23-24. $652 million was committed by governments, corporations and NGOs to reduce ocean pollution, including plastics. Commitments to move to redesign were made by Unilever and PepsiCo, for example, moving to reduction in virgin plastic use and increases in recycled content.[70] Specific points are given for redesign and material substitution below:

1. Sustainable packaging associations (pre-competitive collaborations)
    a. E.g., UGA's New Materials Institute IUCRC, Sustainable packaging coalition, Green-Blue: These pre-competitive environments could help develop alternatives, standardize packaging and help packaging retain value so that it is easier to recycle and less leakage will occur if it has value.
2. Truly biodegradable alternatives (e.g., PHA)
    a. PHA is expanding in the market in the USA and is creating economic value (new facility opening in Kentucky – several open in Georgia already). While it may biodegrade if littered in the environment, it should still be managed in the solid

[68] http://www.acs.org/content/acs/en/greenchemistry/what-is-green-chemistry/principles/12-principles-of-green-engineering.html.

[69] https://www.ellenmacarthurfoundation.org/publications/the-new-plastics-economy-rethinking-the-future-of-plastics.

[70] https://ourocean2019.no/wp-content/uploads/2019/10/20191025-Commitments-1616.pdf.

waste system, and be thoughtful about where used (in currently non-recyclable items, for example). But it has the possibility of being home-composted as well. The USA is currently a leader in the development of this material.

   b. An important distinction should be made with polylactic acid (PLA), a popular corn-based polymer is bio-based and industrial compostable (avoids using fossil fuels as feedstock), but it will not biodegrade in home composting or in the ocean. It will not biodegrade if littered on land. It has to reach a high temperature (reached in industrial composting) to be able to biodegrade.

3. Packaging with more value (e.g., single, homogenous materials, design for recycling/end-of-life)
   a. This can be helped by collaborations between industry, brands and waste managers/experts
4. Design out problematic items/materials (e.g., caps/lids)
   a. Similar to how aluminum can "pop-top" opening was changed to a tab that stayed on (so the pull tabs did not get littered), we can innovate design for items that leak into the environment (if data is collected – see intervention point 5, last chance capture).

3. Reduce Waste Generation

In places like the U.S., where we already have high per person waste generation rates, we can examine methods of waste reduction. For example, some of us have the luxury of being able to make choices about single use items we use daily. The majority of us have access to clean drinking water infrastructure so we can use a reusable water bottle, reusable coffee mug, bring a reusable bag to the grocery store, and say "no" to straws (or get reusable ones). These seem like small and mundane things, but what our research on plastic input showed is that since population density is such a big driver of these inputs, just one small choice, taken collectively, can make a big difference. There is a bit of a "chicken and egg" scenario here though, consumers can make choices, but they also need availability and access to those choices. For example, it might be hard to not buy bottled water if you don't have access to a drinking fountain or water filling station. But this is also where policies regarding specific items of concern can provide motivation. Waste reduction can also occur from participation in new collaborative and sharing economies. These new paradigms are emerging and technology and social media are helping to move them forward. People are choosing to own less and "share" more. It started with car and bike shares, but has expanded to tools, and even clothing. As people become more aware of the issue of plastic in our environment, they are demanding companies reduce waste themselves, and help provide the right choices and infrastructure for people to reduce their own waste generation.

Specific points on waste reduction below – and asking the question, *can we decouple waste generation from economic growth?* I get very excited to see what my students and young innovators will create in this category daily.

1) Using reusable items (e.g., bottles, mugs, bags, etc.) and if this is challenging for citizens, I ask them to think about why and what change is needed so it is possible at the government or corporate level? Then advocate for that change.
2) Sharing, Collaborative Economy concepts

   a. Bike shares, car shares, tool shares, clothing rental, etc. these all reduce the need to purchase and create waste (facilitated by technology), but still meet people's needs and can still create revenue for the companies providing the services.
   b. How can these concepts be related to packaging? (see 2.b)
3) Decouple waste generation with economic growth (facilitated by technology)
   a. Reuse programs (using mobile phones, which many people have globally, especially where rapid economic growth is occurring)
   b. RFID, mobile phones, smart-labels, etc. (e.g., RFID water refill stations exist for both Coca-Cola and PepsiCo products, but are not yet widely distributed yet)

4. Improve Waste Management Globally

Improving waste management globally could go a long way to keeping a large mass of plastic out of the ocean (realizing mass is not the only meaningful metric for plastic – volume, count, shape, or impact to wildlife are other metrics). For example, in our *Science* paper the top 20 countries' mismanaged plastic waste encompassed 83% of the total input in 2010. But with a combined strategy, in which total waste management is achieved in the 10 top-ranked countries and plastic waste generation is capped, a 77% reduction could be realized by 2025. That sounds simple. We know how to design waste management systems, but in light of the context I gave at the beginning, waste management is much more than just a design challenge, it also has deep social and cultural dimensions. So we need to work together at a combination of local and global initiatives, and we need global participation from various stakeholders along the entire value chain of plastic (see following section on Circularity Assessment Protocol). Per person waste generation is coupled with economic development and, in many cases, the waste stream has fairly quickly changed characteristics to include more plastic. There are still many people in both the U.S. and globally that are unaware of the consequences of plastic in our aquatic environment.

Globally, innovation and creativity is needed in this space and people are heeding the call. Large, global NGOs are partnering with local groups in areas of concern to try to implement culturally appropriate mitigation strategies. Infrastructure is being integrated into existing informal waste management sectors in the hopes of continuing and improving people's livelihoods. U.S.-based groups can help in efforts for this global problem by connecting with groups who are trying to address these issues in their own countries, and there is a lot of work to be done. Some concepts that can be drivers in this area: zero waste (reduce disposal or destruction of waste to as close to zero as possible) and product stewardship/extended producer responsibility (waste management responsibility is shared or is the entire responsibility of product manufacturers). Plastic reuse and recycling can grow if the right economic structure is in place to motivate the collection of plastic waste and its reprocessing. Many local groups in global communities need some added support to elevate and expand what they are already doing to bring it to scale. Policies like deposit-return schemes reduce the quantity of plastic that reaches the environment. In US states that have these schemes, a 40% reduction of beverage containers is observed.[71]

Solid waste collection can be a hyper-local activity and can look different in each country, city and even neighborhood. Plastic has made it a more complicated and created a

[71] Schuyler, Q., Hardesty, B.D., Lawson, TJ., Opie, K., Wilcox, C., Economic incentives reduce plastic inputs to the ocean, Marine Policy, Volume 96, 2018, Pages 250-255.

rapid change in the waste stream that we were ill prepared for. It creates a waste stream that is more varied and dynamic than we have ever experienced before. It has proved to be quite a challenge for waste operators and municipalities to manage. I have developed a "Five C" approach for this intervention point.

1) Collect: May be traditional, on-demand, or decentralized waste collection
   a. Collection innovation is needed – revers logistics may play a role
2) Capture: Material Recovery Facilities, waste depots, waste banks, community centers (e.g., "punto limpio" in Chile)
3) Contain: Recycling or engineered disposal
4) Context and 5. Culture – these can "make or break" the success of a potential intervention. The local community and stakeholders absolutely need to be engaged and involved from the start through the end of any project and not just led through it, but their local and indigenous knowledge is critical.

5. Litter Capture

Litter capture and collection is the last point to keep materials from entering the ocean. It is reserved for mostly the litter that occurs from inadvertent littering, lack of awareness and behavior issues. After outreach and education to prevent litter in the first place, there are street sweeping, municipal litter clean-up programs and stormwater catchment systems, all which will only be conducted in their respective jurisdictions. An innovative example of a final catchment device is the Baltimore Water Wheel.[72] Operated off of mechanical and solar energy in Baltimore Harbor, "Mr. Trash Wheel" has booms that skim the surface of the harbor and direct the floating trash to the conveyer system that removes it from the water and places it into a dumpster to be managed properly.

Non-governmental organization and volunteer cleanups to remove litter have been occurring for years. These events certainly help to keep litter from entering the ocean, and they are also a source of data. The Ocean Conservancy's International Coastal Cleanup is now in its 33rd year and it not only helped to remove 0ver 10,500 metric tons of debris from beaches in 2018, but it has spread awareness and education as well. For the first time in 2017 and also in 2018, the top ten items found on beaches for the ICC were all plastic. In 2011, my colleague Dr. Kyle Johnsen and I co-developed a mobile app called Marine Debris Tracker at the University of Georgia funded by the NOAA Marine Debris Program. The Marine Debris Tracker mobile app and citizen science program allow for the collection of global standardized data at a scale, speed, and efficiency that wasn't previously possible.[73] It also spreads awareness and education about this issue wherever it is used. Individuals all over the world have helped to clean-up or document over 2 million items – by simply hitting a few buttons on their mobile phone to tell us what they found. User metrics provide a ranking and our largest group user is the Georgia Sea Turtle Center protecting and caring for Sea Turtles on Jekyll Island, GA and one of our largest individual user is in Omaha, NE (not far from the Missouri River) where he has collected over 87,000 pieces of litter alone, over the past 7.5 years. We, along with our app users, have fostered an online community through social

[72] http://baltimorewaterfront.com/healthy-harbor/water-wheel/.

[73] Jambeck, J.R., Johnsen, K. (2015). Marine Debris Tracker: Citizen-based Litter and Marine Debris Data Collection and Mapping, *Computing in Science and Engineering*, 17(4), 20-26; http://www.marinedebris.engr.uga.edu/.

networks – everyone is supportive of each other's efforts and individuals know that they are a part of a large global effort. There is now enough (opportunistic) data in the database to start to examine characteristics and trends based upon the spatial and temporal data provided by our extremely dedicated users. Data is critical to informing upstream solutions and can really empower communities and decision-makers to be able to take actions driven by data. Last-chance cleanup points are summarized below.

1. Engineered, mechanical systems
    a. Mr. Trash Wheel or other engineered devices
2. Manual (by hand)
    a. Cleanups (e.g., ICC by Ocean Conservancy)
    b. Use of ocean-bound plastic can catalyze the development of infrastructure since the material now has value – often a much higher value than it did previously (e.g., Parley, Dell, NextWave plastics)
3. Data to feed back to Interventions 1 through 4 in the Framework
    a. E.g., Marine Debris Tracker developed by UGA (or other apps) to collect data
    b. Could make upstream choices/changes based upon what is leaking into the environment

Community-Based Data Collection and Assessment

Communities are the at the front lines of this issue. They are where solid waste is managed and many decisions and development of waste management systems are made. They also experience the direct impacts of plastic pollution in their local environment. It is important we work with communities in the decision-making process to be able to come together on realistic and viable solutions. After I began traveling for the US State Department for the International Informational Speakers Program in 2017 (that has now brought me to 13 countries), I often find myself in the same situation over and over again. Speaking with governments and communities about this issue, they would say to me, "Well now that we know more about this issue, what can we do?" and I would pause (since I had not often been there very long typically), and tell them that they have the local and indigenous knowledge for solutions to this issue – they know their own context and culture. But I could also look around and take note of what I saw to contribute data for them to use… from what stores and cafes were selling in packing, from waste and recycle bins I saw, to litter on the ground. I also thought more about the concept of the circular economy – being touted as a solution to this issue, what does it really mean at the community level? How does a community move closer, or even see where they are at, related to the circular economy? In addition the community systems are an inherently complex, sociotechnical system, which is difficult to define with traditional metrics. There was a need for a methodology and a framework that provides a baseline understanding, illustrates the impacts of changes in the system, and facilitates useful knowledge exchange between cities, while allowing for flexible adaptation to local knowledge and expertise.

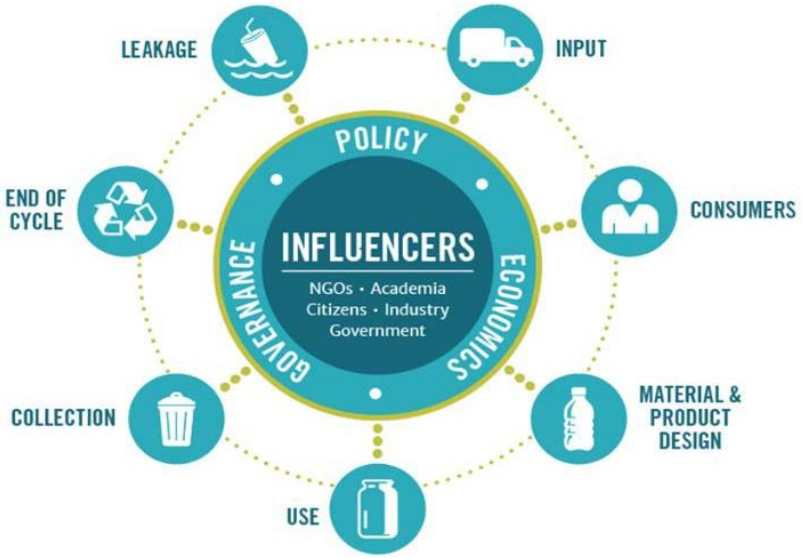

Figure 5. The Hub and Spoke model of the Circularity Assessment Protocol (CAP).

This is the context for how our Circularity Assessment Protocol (CAP) was developed in our Center for Circular Materials Management (the only center of its kind in the USA), in our New Materials Institute at the University of Georgia. Conducted in collaboration with a community and eventually by the community itself, the CAP characterizes seven community components: 1) inputs, 2) consumers, 3) product design, 4) use, 5) collection, 6) end-of-cycle management (e.g., waste management), and 7) plastic leakage into the environment. Various influencing factors drive this system including governance, economics, policy and legislation (e.g., bans, taxes, extended producer responsibility). Furthermore, multiple stakeholders exist at every level of the CAP influencing the complex system and these include citizens, government, industry, NGOs and academia. While the hub and spoke model illustrates the CAP (Figure 4), it is a complex system with components inherently interconnected to each other and to life-cycle impacts beyond each component.

While the CAP is a framework approach to addressing marine litter originating from land-based sources, it can also include data collection for marine or water-based sources through parallel research questions, and the quantity of leakage from this sector can be characterized during litter assessments (e.g., if fishing gear is an issue, it is typically evident on litter surveys on land as well). The framework supports points of intervention and actions, including guidance on effective impact (in terms of environmental and economic) to improve circularity. The CAP can help to inform a community by giving them a baseline assessment to work from and direct potential actions to take to improve the areas that most need it, and to answer specific questions they have about their own community. The CAP can inform and support the government to define policies and good practices related to solid waste management and infrastructure, including facilitating an understanding of solid waste and plastic management through a social lens. This can provide an understanding of people's actions (both local and transient) which will inform policy and interventions.

The CAP is being used for projects funded by the World Bank, National Geographic, the Asia-Pacific Economic Cooperation (APEC) through the Ocean Conservancy, and USAID. Projects are completed or active in the Seychelles, Philippines (metro Manila), Chile, India,

Bangladesh and at least two places in the USA, one small island community and a large metropolitan coastal city. A scaled-down version of CAP is being conducted in 30 small island and coastal community stopovers around the world with eXXpedition, and further development of the CAP for communities to conduct the process themselves within the framework is underway.

The U.S. Can Be a Global Leader in Addressing This Issue

Once plastic enters the ocean, it quickly becomes a global problem. The United Nations Environment Program has been addressing this issue through the Global Partnership for Marine Litter, with resolutions anticipated out of a meeting later this month. There is also the discussion of a global agreement with the potential for flexibility of countries to be able to reach reduction goals as they see fit. But the U.S. should be a leader in addressing this global issue, and it has in some ways. The U.S. Department of State has worked on this issue through the G7, G20 and Our Oceans conference. The NOAA Marine Debris Program started in 2006 with the Marine Debris Reduction Act (reauthorized in the Save Our Seas Act) and is one of the few agencies to provide grant assistance to community groups and research. The U.S. EPA has a Trash Free Waters Program that has expanded recently in bringing in partners and pilot sites around the U.S. NOAA and the U.S. EPA (chair and vice chair, respectively) lead the Interagency Marine Debris Coordinating Committee (IMDCC), a multi-agency body responsible for streamlining the federal government's efforts to address marine debris. Representatives meet to coordinate a comprehensive program of marine debris activities and make recommendations for research priorities, monitoring techniques, educational programs, and regulatory action. The IMDCC participants are the U.S. Army Corps of Engineers, U.S. Navy, U.S. Coast Guard, U.S. Fish and Wildlife Service, Bureau of Safety and Environmental Enforcement, Department of Justice, Environmental and Natural Resources Division, Department of State, Office of Marine Conservation, and the Marine Mammal Commission. Another group that has worked on U.S.-based marine debris issues is the National Fish and Wildlife Foundation. While U.S. scientists, universities, and research groups are at the forefront of the science of marine debris, there have only been a few research grants funded through the National Science Foundation and NOAA. Even while a multitude of domestic agencies and research groups have been working on this issue, resources are limited for addressing this issue and meeting our goals in being global leaders. Multi-agency cooperative programs could further advance the science of plastic contamination and pollution while also providing future economic benefits through startup companies and whole new industries. Community-based and citizen science programs to collect badly needed data, like our CAP using the Marine Debris Tracker mobile app can be used in the USA, as well as around the world.

Summary

Based upon my testimony, the top five recommendations for how Congress can best support research, cleanup, or prevention efforts to combat marine debris are:

1) Funding the current agencies and initiatives, as well as new research through other agencies to provide science to further determine human health impacts (e.g., micro and nanoplastics) and mitigate this issue through the entire value chain of plastic (e.g., fate and transport of plastic in the environment, new materials and product design), which can provide economic innovation and growth, and also inform policy. Community-based data collection and citizen science with proper frameworks and structure can contribute to critical data needed to inform circular materials management in communities on the front lines of waste management.
2) To support prevention domestically, Congress could support legislation to reduce waste generation to reduce leakage of especially plastic packaging (and those items found in typical beach cleanups), like deposit-return schemes which show a 40% reduction in beverage containers where in place in the USA, as well as, for example, product stewardship/extended responsibility initiatives to increase the collection and value of waste.
3) To support prevention internationally, we can continue to provide funding through USAID and other bilateral initiatives, which I have seen give NGOs the opportunity to catalyze action, improve infrastructure and the economy, in countries like Vietnam, Philippines and Indonesia. We also need to make sure our exports are not negatively impacting other countries and support development in other countries so they may participate in trade using standards such as the OECD. We can also determine if our trade-agreements can influence other countries improvement of environmental standards, including solid waste management.
4) Show support for global initiatives to assist with the reduction of plastic entering the ocean and improvement of waste management infrastructure development around the world (e.g., with world development banks, NGOs and industry), along with technology and knowledge transfer to other countries on solid waste management through, for example, the U.S. State Department, US EPA and NOAA. The newest USAID CCBO funding is one aspect of this process.
5) Derelict fishing gear is one of the most dangerous types of debris in the environment. Supporting the development of a program (through an agency) for fisherman to drop off gear that is broken or that they find could help this program (providing collection and disposal in areas where DFG has an impact). NOAA Marine Debris Program "Fishing for Energy" or similar could continue and/or expand.

As environmental engineers, we manage all solid waste that comes our way. But by connecting our activities on land with what ends up in our oceans, and through that awareness, realizing that we should be thinking about end-of-life in materials development and product design stages, we can shift the paradigm of "waste" to materials management. Also, the worldwide interest on this topic has put the spotlight on global solid waste management infrastructure needs, and so we need to collectively come up with creative, socially and culturally-appropriate mitigation strategies. Collectively, we hold the key to this problem. By changing the way we think about waste, reducing at source, designing products for their end-of-life management, valuing secondary materials, collecting, capturing and containing our waste, we can open up new jobs and opportunities for economic innovation, and in addition, improve the living conditions and health for millions of people around the world while protecting our oceans.

Previous questions from Congress:

1. *What are some of the most promising innovations?*

In my opinion some of the most interesting and promising innovations are the ones that decouple waste generation from economic growth. How can we meet people's needs and increase livelihood without creating more waste to manage? Sharing and collaborative economy concepts, RFID cups, using technology to connect people and facilitate sharing and reuse programs all lead to potential interventions. Reduce waste generation in the first place. See page X, above.

2. *What is role of PLA and other bio-based plastics?*

I think there is a role for material and product innovation and bio-based and biodegradable (truly) polymers will be a part of the solution. However, these materials are being produced at relatively low quantities right now, so they are not going to be a big market share for some time. And thought needs to go into what they replace as well as life-cycle tradeoffs. And an understanding of situational biodegradability is critical. See page X, above.

3. *Fisherman incentives*

I think incentives for fisherman to collect or bring back gear would be a way to get some of the most deadly gear out of the ocean and marine environment. I think also supplying a place for fisherman to put used gear is important (e.g., dumpster or recycle bin at the port). Tracking and transparency of nets – and really all plastic (as much as feasible) could help keep the material out of the ocean because we would have a better inventory of it.

4. *What are some of the root causes?*

Responsibility – while not particularly popular in the USA, product stewardship is an important concept to discuss here. From an engineering standpoint, when a company wants to build a development/civil engineering project, there often is a partnership with the community. One example, I live near an above ground storage tank farm, and trucks come and go from it regularly. There were likely road improvements needed to be able to build the tank farm and the company who constructed it may have contributed to that infrastructure since they were building at this site. In some ways, this can be analogous to selling products in a country or location that does not have infrastructure to manage the waste created from those products. I don't think companies knew the issues this would bring. And I think they want to help based upon new awareness, but we are certainly playing "catchup" with the issue now. Besides policies in other countries, some companies are doing this individually, but many still don't know how to help with infrastructure. I think that facilitating this in some way could be significant – maybe it will all be individual public-private partnerships, but some thought could go into how to facilitate companies engaging in shared responsibility. Ultimately it will take shared actions by industry, municipalities, and citizens to make significant positive change on this issue.

As often said, there is no one solution to this issue, but an integrated approach is needed to reduce and eliminate plastic entering and impacting our ocean.

QUESTIONS SUBMITTED FOR THE RECORD TO JENNA R. JAMBECK, PHD PROFESSOR OF ENVIRONMENTAL ENGINEERING, COLLEGE OF ENGINEERING, UNIVERSITY OF GEORGIA

Questions from Rep. Alan Lowenthal

1. Why is it important to work with local communities to identify their sources of plastic pollution and possible solutions?

It is important to work with local communities because plastic inputs and waste are created and managed at the community level, i.e., our communities are on the front lines. So understanding their needs, context and situation is important. Even if a federal policy is enacted, the communities will be impacted. Disposal and recycling are commonly different from community to community. Community engagement, including co-creation, or at least buy-in, on potential solutions is critical to implementation and participation. While local solutions can scale to make them larger and more impactful, exploring what communities need can inform federal legislation.

As referenced in my written testimony, one example is the Circularity Assessment Protocol (CAP), developed in the Center for Circular Materials Management (the only center of its kind in the USA), in the New Materials Institute at the University of Georgia. Conducted in collaboration with a community and eventually by the community itself, the CAP characterizes seven community components: 1) inputs, 2) consumers, 3) product design, 4) use, 5) collection, 6) end-of-cycle management (e.g., waste management), and 7) plastic leakage into the environment. Various influencing factors drive this system including governance, economics, policy and legislation. Furthermore, multiple stakeholders exist at every level of the CAP influencing the complex system and these include citizens, government, industry, NGOs and academia. While a simple hub and spoke model illustrates the CAP, and data collection is rapid and easy to collect through a collaborative effort by the community members and researchers, it is a complex system with components inherently interconnected to each other.

One of the largest benefits to CAP is that it can help to inform and empower a community by giving them a starting assessment to work from and direct potential actions to take to improve the areas that most need it, and to answer specific questions they have about their own community. The CAP can inform and support the government to define policies and good practices related to solid waste management and infrastructure, including facilitating an understanding of solid waste and plastic management through both and technical *and* social lens. This can provide an understanding of people's actions (both local and transient) which will inform policy and interventions.

Other community-based work that I have participated in is the National Geographic Sea to Source Expedition along the Ganges River in India. This expedition focuses on plastic pollution in three key areas: land, water and people. On land, we collect data about the input and use of plastic in communities, how waste is collected and managed, and characterize the movement and type of plastic in the environment. The water team studies plastic pollution in the air, water, sediment and species in and around the river. The socioeconomic team surveys local communities along the expedition route to better understand awareness and perceptions

of plastic pollution, household plastic waste management and local solutions for addressing this issue. During the expeditions, we engage the local community, and work with stakeholders to empower then to find context-sensitive solutions that can help drive a long-term positive change. This kind of interdisciplinary and community-based work, incorporating easy-to follow citizen science methods and cutting-edge technology can be a spark for continued change on this issue. Similar kind of work could be conducted in major river waterways in the USA as well. Previous data on the USA is only an estimated model based upon reported solid waste infrastructure. And, as one of the largest waste generators in the world, we really don't know (except for a few exceptions where collection takes place, like Mr. Trash Wheel[74]) what plastic leaks into and from our waterways in our own backyard.

2. There was a lot of discussion on the societal relevance of plastic as it is. What innovations and alternatives are available or coming very soon?

I think the USA was sold short by the hearing discussion that there was no alternatives and no other material to use besides traditional plastic. E.g., we have solved the "what to do without plastic to hold toothpaste problem" and there are solid toothpaste "chews" in several different brands available packaged without plastic, including one very successful women-owned and operated US-based company called Bite.[75] The USA in many ways is, and can continue to expand, in leading the world on innovative materials and alternatives to traditional plastic. Already polylactic acid (PLA) exists and a large amount of R&D has been conducted in the USA on it. While it does not avoid all unintended consequences of traditional plastic, it does avoid using fossil fuels as a feedstock and serves as an example to the economic growth and development of a new material that serves the needs of traditional plastics but is different from it in some ways. As stated in the testimony though, an important distinction should be made with PLA, as it will not biodegrade in home composting or in the ocean. It will not biodegrade if littered on land. It has to reach a high temperature (reached in industrial composting) to be able to biodegrade.

Also included in my testimony is an entire section on Innovation summarized here:

Overall, I think Green Engineering principles,[76] if followed during material development and product design, would help to avoid many of the externalities of plastic that we are dealing with currently. In addition, circular economy concepts, emerging all over the world now, will be important to also apply to plastic materials. Both of these guiding principles promote non-toxic materials, ultimately with the capability of biodegrading and/or being recycled. Materials and products made with more homogenous compounds would make recycling more efficient and effective. Materials and products can be designed to retain their value, for collection, recovery and recycling. The University of Georgia has combined environmental engineering and polymer chemistry in a successful and rapidly expanding New Materials Institute with centers on biodegradable polymers and circular materials management to develop and test materials to reduce the flow of plastic into the ocean. NMI has become part of a National Science Foundation (NSF) Industry–University Cooperative Research Centers (IUCRC) that has over 30 corporate partners interested in more sustainable

[74] https://www.mrtrashwheel.com/.

[75] https://bitetoothpastebits.com/.

[76] http://www.acs.org/content/acs/en/greenchemistry/what-is-green-chemistry/principles/12-principles-of-green-engineering.html.

and biodegradable polymer products. These industry-research groups participate in pre-competitive research and development as new materials need to scale to be economical for all to use. There is no doubt that developing alternative materials without the unintended consequences of traditional plastics will spark innovation and economic growth in the USA where truly biodegradable polymer production facilities (e.g., Polyhydroxyalkanoates (PHA)), like the ones in Georgia owned by Danimer Scientific and RWDC are creating jobs (see more in the answer below to question 3). Specific points for redesign and material substitution are:

A. Sustainable packaging associations (pre-competitive collaborations)
    a. E.g., UGA's New Materials Institute IUCRC, Sustainable packaging coalition, Green-Blue: These pre-competitive environments could help develop alternatives, standardize packaging and help packaging retain value so that it is easier to recycle and less leakage will occur if it has value.
B. Truly biodegradable alternatives (e.g., PHA)
    a. PHA is expanding in the market in the USA and is creating economic value (new facility opening in Kentucky – several open in Georgia already). While it may biodegrade if littered in the environment, it should still be managed in the solid waste system and be thoughtful about where used (in currently non-recyclable items, for example). But it has the possibility of being home-composted as well. The USA is currently a leader in the development of this material.
    b. Danimer Scientific in collaboration with Frito-Lay is working on PHA packaging as well, so a major brand is making this shift too, scaling this to more USA-based economic growth.
C. Packaging with more value (e.g., single, homogenous materials, design for recycling/end-of-life)
    a. This can be helped by collaborations between industry, brands and waste managers/experts
D. Design out problematic items/materials (e.g., caps/lids)
    a. Similar to how aluminum can "pop-top" opening was changed to a tab that stayed on (so the pull tabs did not get littered), we can innovate design for items that leak into the environment (if data is collected at last chance capture).

3. Is there a positive economic impact from the development of alternatives to traditional plastic?

Yes, while there is an economic component to traditional plastics to the economy and jobs, the alternatives can create similar output and work opportunities (see some in the answer to question 2, above). And the USA can be at the forefront of this change.

One specific example is a company called RWDC that works closely with the New Materials Institute at the University of Georgia. RWDC has just purchased a property in Athens, GA for their first production facility. They have already hired approximately 40 people and will bring 100 jobs to Athens-Clarke County, Georgia (one of Georgia's 91 persistently poor counties) in the next year, and an estimated 210 jobs after 5 years. There is another site in Monroe, GA, where another 86 jobs will be created within the next two years. And this is just one company growing as quickly as it can in the USA.

4. What are some of the benefits and trade-offs from switching away from traditional plastics?

There is no doubt that plastic has changed our society and culture. It has brought us many things we rely on every day – this was the point of my 24-hour experiment. But, do we really need it for all those things? Some things yes, medicine, electronics, many what we call "durable goods" – but the single-use plastic, the packaging, and what ends up in the environment (the second and other critical part to the experiment I presented!) – how much of that needs to be plastic? We are not going to get rid of all plastic, but I think we need to be more thoughtful about where, when, and how we use it.

Here are some examples of trade-offs that we might consider while thinking about plastic. Certainly plastic has brought light-weight benefits to food packaging, transport and allows food to be stored in sanitary ways, protecting the embodied energy that went into that food. Many times the carbon footprint of that food is large. Something to ponder, where do we draw the lines in these analyses? Why does our food have such a high carbon footprint/embodied energy? Should all food be distributed through the current model if it requires plastic packaging? I encourage people to think "out of the packaging container" and outline all the ways we can change the delivery of products and design of packaging. But, the best thing, environmentally-speaking, is to not produce any waste in the first place, so that lends itself to reusable items. However, for when packaging is needed, what then, should it be made out of? Life-cycle assessments (LCA) were referred to in the hearing and I have conducted LCAs on various waste management scenarios myself.[77] More upstream, product LCAs can inform packaging choices, so we can compare carbon footprint, energy use, water consumption, etc. of two products, for example a plastic v. a reusable bag. While the energy input or carbon footprint for production, for example, may be more for the reusable bag, the fact that you do not have to manage waste after it's end-of-life is an energy and carbon off-set. While the plastic bag is light, it will have to be transported to a recycling or disposal facility and then managed there. In a carbon balance scenario, plastic does not release carbon at end of life, because as far as we know it does not practically degrade, so while it is not a benefit that it remains forever in a landfill, it does not release carbon while there. In addition, plastic bags have been known to jam up recycling systems at material recovery facilities (MRFs) and blow from landfills, making containment a challenge (and requiring human effort and machines to manage at landfills). These two situations do not fit into an LCA in a straight-forward way. And a last major limitation of this kind of LCA is that there is no way to include a littered plastic bag ending up in the ocean and a turtle eating it and dying. Animals killed from plastic litter does not fit into any LCA. So there are trade-offs that are a challenge to compare, and we need a better way to look at the systems holistically, even beyond our typical LCA. At a minimum, we need to be able to acknowledge, and talk through some of these tradeoffs, in a systematic way.

[77] Jambeck, J., Weitz, K., Townsend, T., Solo-Gabriele, H., (2007). CCA-treated Wood Disposed in Landfills and Life- cycle Trade-Offs With Waste-to-Energy and MSW Landfill Disposal in the U.S., Waste Management, Volume 27, Issue 8, 2007, Pages S21-S28. http://www.sciencedirect.com/science/article/pii/S0956053X07000773 Thorneloe, S., Weitz, K., Jambeck, J., (2007). Application of the U.S. Decision Support Tool for Materials and Waste Management, Waste Management, 27 (2007) 1006–1020. http://www.sciencedirect.com/science/article/pii/S0956053X0700058X.

Questions from Rep. Nydia M. Velazquez

1. In your testimony you highlight corporate commitments made at the Our Ocean meeting in Oslo, can you describe what steps exactly are in motion to help reduce plastic pollution in the environment? Is it enough?

The Our Ocean Commitments are available here: https://ourocean2019.no/wp-content/uploads/2019/10/20191025-Commitments-1616.pdf.

For the first time that I can recall a company, Unilever, committed to an absolute reduction of plastic use. They are finding alternative ways of delivering products, as PepsiCo announced purchasing Soda Stream an alternative delivery mechanism for carbonated beverages as well. Other companies and governments made commitments too (and my mentioning those two companies by no means is an endorsement in any way). But no, these commitments are still not enough for a couple reasons. First, the corporations have the capacity to go further with these commitments and make them more impactful, but the commitments continue to get stronger each year, so they do indicate movement in the right direction. Another reason it is not enough is that I think multiple entities need to be involved to create a larger positive impact. No one "group" (e.g., industry, government, NGO) can do this alone. For example, corporations designing and using packaging need to speak with the waste management companies and these two systems, the input and the management, should be better integrated. I still see a lot of issues related to design and management that could be addressed by these two end-of-the-spectrum entities working together. For example if product stewardship or extended producer responsibility is considered, the impacts to the waste management companies – and their input – needs to be considered and heard. For all groups working on, and involved in, this issue – if each group makes some compromises, the shift each entity needs to make can be smaller in order to meet in the middle, yet still creating a truly impactful way forward. I recommend a US-based summit where the relevant stakeholders can gather together to actively negotiate how new federal policies could be endorsed in order to better protect the environment for all.

Questions Submitted by Rep. Cox

Question 1. A recent study found 16 microplastic fibers in a single half-liter sample of water taken from the Capitol Visitor's Center. How did the microplastics get into the Capitol Visitor's Center drinking water or anybody's drinking water for that matter?

Answer. I would have to see this study's methods to be able to comment on this specific result, but microfibers and microplastics have been found in freshwater, tap drinking water, groundwater and wastewater in published studies.[78] This same research was a review of these published studies, and they found that methods are still widely conducted and not standardized, and in order to really find out the risk to human health from exposure, these methods need to be standardized to high levels. So to properly answer your question, there

[78] Albert A. Koelmans, Nur Hazimah Mohamed Nor, Enya Hermsen, et al. Microplastics in freshwaters and drinking water: Critical review and assessment of data quality, Water Research, Volume 155, 2019, Pages 410–422.

needs to be more research conducted based upon common research methods and standards. This would be a good role for the U.S. EPA to play in the USA, to direct the methods and standards for comparative purposes.

At this point without more data, we can only guess at the sources of the fibers and particles. We know fibers are generated from washing clothes and unless otherwise captured,[79] these go out with our wastewater to either septic or treatment plants (when treated). In cases where not treated, they would be directly discharged to the environment. Although we know that typically over 90 percent of the fibers can be removed from the wastewater treatment facility,[80] it means they end up in the sludge that settles out and then is either managed at a landfill or in some cases, applied to the land where run-off could reintroduce them to the environment again. We also know that fibers are transported by air, so atmospheric deposition (mostly regional, near-range likely) could be a transport into our freshwaters.[81] So, it can end up in our source water from point source (wastewater), run-off and from the air. And, although drinking water is treated and many particles are removed, it is possible that some could remain. There has not been an investigation into the drinking water distribution system and its contribution, if any, to microplastic in water, but it is doubtful for microfibers as far as I am aware. If water is stored in an open glass in a room, microfibers will very likely fall into it—they are in the air all around us. Identifying them as a polymer with FTIR or Raman, for example, is very important so that we correctly identify if they are plastic or not.

Question 2. What do we know about the human health impacts of ingesting microplastics?

Answer. We really don't know at this point—there are likely studies underway on this topic, but the potential impacts are not easy to study and if some of the plastics are at the nanoscale level, they are not able to be analyzed or identified at this point with current analytical capability. We know we are exposed through beverages we consume (including water) and some of the food we eat (e.g., salt), but we don't yet know the impact to humans. I also recommend referring to Dr. Chelsea Rochman's recent testimony to the House on this issue.[82]

Question 3. Oftentimes we turn to alternatives to address environmental challenges like plastic pollution. In the case of climate change, we might use renewable power instead of coal. In the transportation sector, we see people switching to electric vehicles. However, there are always bumps in the road when we make these transitions, and it's our job here in Congress to smooth those out. Take the idea of adopting alternatives to plastic as an example. Explain to the Committee why we have not seen a more rapid transition to biodegradable plastics or plastic alternatives.

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[79] Hayley K. McIlwraith, Jack Lin, Lisa M. Erdle, et al. Capturing microfibers—marketed technologies reduce microfiber emissions from washing machines, Marine Pollution Bulletin, Volume 139, 2019, Pages 40–45.

[80] JingSun, Xiaohu Dai, Qilin Wang, Mark C.M. van Loosdrecht, et al. Microplastics in waste- water treatment plants: Detection, occurrence and removal, Volume 152, 1 April 2019, Pages 21–37.

[81] Steve Allen, Deonie Allen, Vernon R. Phoenix, et al. Atmospheric transport and deposition of microplastics in a remote mountain catchment, Nature Geoscience volume 12, pages 339–344 (2019).

[82] https:// docs.house.gov / meetings / AP / AP06 / 20190919 / 109934 / HHRG-116-AP06-WState- RochmanC-20190919.pdf.

Answer. I think the biggest reason here is cost. Traditional plastics are so inexpensive. There are alternatives developed and companies are working hard to scale them (see my answer to Rep. Lowenthal's Question 2, above). But the cost makes it challenging until they are able to scale. The development and manufacturing of alternative materials will have economic growth and provide job opportunities in the USA (also see my answers in Rep. Lowenthal's Question 3, above), so like your other examples for climate change, we can see transitions to different businesses and job growth, while making some of these changes. Policies that level the playing field for other materials and products would be helpful.

Question 4. What are some of the actions that Congress could take to allow for increased adoption of more recyclable and environmentally friendly alternatives to plastic?

Answer. As mentioned above, policies to level the playing field in the cost of materials for use can help here. These could include a tax or fee on certain kids of traditional resins, bans, and required design and procurement standards. Again, I think that these kinds of actions should take into account the impact on all relevant stakeholders to be able to move forward with a balance in terms of compromise. In some cases, end-of-life policies have an upstream impact, e.g., depending on how a product stewardship policy is written, it can impact design of products and materials chosen as well. The example from Norway that I often talk about it is the Extended Producer Responsibility (EPR) law in Norway influenced upstream design and recyclability of products. By requiring a certain percent of PET to be recycled, a company formed to help make this happen and in order to reach the needed recycling rates in the most efficient way, the design of PET bottles were changed so that they could be recycled bottle-to-bottle by Infinitum.[83]

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Dr. LOWENTHAL. Thank you, Dr. Jambeck.
The Chair now recognizes Tony Radoszewski to testify.
Welcome to the Committee, Mr. Radoszewski.

STATEMENT OF TONY RADOSZEWSKI, PRESIDENT AND CEO, PLASTICS INDUSTRY ASSOCIATION, WASHINGTON, DC

Mr. RADOSZEWSKI. Good afternoon, Chairman Lowenthal, Ranking Member McClintock, and members of the Subcommittee. Thank you for having me here today. My name is Tony Radoszewski, and I am the President and CEO of the Plastics Industry Association. We call ourselves PLASTICS for short, and we use that term proudly.

Plastics were first developed by John Wesley Hyatt in the 19th century as a synthetic replacement for billiard balls. Yes, that is right, billiard balls. Ivory was expensive, and the process of collecting it was gruesome and inhumane. So, Hyatt tinkered around in his lab and developed the material that could behave like ivory, but at a fraction of the cost, and at a fraction of the environmental impact. That has been the story of plastics from their genesis to

[83] https://infinitum.no/english/about-us.

today. It is a material that meets or exceeds the performance of other materials, and does so at a fraction of the cost, and with lower environmental impact.

Since they were first developed, plastics have grown to make hospitals safer, surgeries less invasive, patient care more sterile, safer, effective, and affordable. In a century-and-a-half since they were invented, plastics have also made cars, trucks, and planes more efficient, more affordable, more environmentally friendly, and, ultimately, safer.

Plastic pipe brings fresh water to people and takes wastewater away for treatment in the most economical and environmentally sustainable way. Plastics have also made food last longer, improving health and safety to millions across the world.

The plastics industry employs 993,000 people in the United States. The state with the largest number of plastics employees is California, where 79,700 men and women are directly employed by our industry. I can say with confidence that none of them got into this business in order to pollute our oceans and waterways. I can also say with confidence that many of them entered the industry with a passion to improve the safety and quality of a lot of people. That our products end up where they shouldn't upsets me. And I am sure every one of those nearly 1 million people who work in this industry feel the same way. But it is a fact. It is also a fact that a staggering 8 million tons of plastic ends up in the world's oceans each year, 90 percent of which originates from 10 rivers in Southeast Asia and Africa. The remaining 10 percent comes from elsewhere around the world. That is a great deal of value being wasted when these products end up in lakes, rivers, and, ultimately, oceans.

Our industry agrees with everyone in this room that there is a plastic waste problem. The urgency of the situation cries out for a solution more thoughtful than simply saying "no" to a material that lowers greenhouse gas emissions, is more efficient to produce than other materials like metal, paper, and glass, and has delivered numerous benefits to society as a whole.

Study after study, including one conducted recently by the California Water Board, has shown that banning of plastic products simply drives consumers to other less sustainable materials. Bans have a very minor impact on litter, if they have any impact at all.

Plastics are used in such a diverse array of applications because they are the best option when all considerations are evaluated. In a free market society, consumers decide which products provide the best value and performance. In so many applications the chief characteristics of plastics—that is, their lower weight, durability, flexibility, and versatility—constantly make them superior to other competing materials.

Plastic bags became popular due to concerns about how many trees were being cut down to make paper bags. Plastic bottles are lighter and don't break as easily as glass ones, reducing product loss and shipping costs. When they are disposed of properly, these plastic products have a smaller environmental footprint than identical products made of other materials.

Rather than trying to deny the value of plastics, we need to head in the opposite direction, and aim to preserve and enhance their value so that they are worth too much to waste. This can happen by investing in recycling and waste management infrastructure.

We continue to support legislation that would provide grants to the Environmental Protection Agency, to state and local entities to improve recycling infrastructure, which is what we need to close the loop on these issues.

We have also supported the Save Our Seas 2.0 Act, which aims to improve efforts to combat marine debris, and is currently seeing action in various Senate committees, with companion legislation having been introduced here in the House.

The industry itself has stepped up to this challenge by innovating, like it always has, developing new chemistries, investing in new recycling and collection technologies, developing ways to convert plastic waste into energy, and creating the supply to meet the demand for recycled plastic content. Still, we need the support of Federal, state, and local authorities to ensure that no American has to wonder if the bottle they toss into the blue bin will end up being recycled, or if it will end up as landfill fodder.

Perhaps I should sum up our industry's position with a recent quote from Japan's Prime Minister, Shinzo Abe: "We shouldn't treat plastic as an enemy, nor ostracize those who use it. What is needed is appropriate management of trash, and to search for solutions through innovation."

On a personal note, I love this industry. I have worked for it for nearly 40 years. I sincerely believe that plastics are among humankind's greatest innovations, and that they have delivered an enormous benefit to public health and commerce all over the world. We need to learn how to live with these materials, because I can assure you we would never want to have to live without them.

Thank you, and I look forward to your questions.

[The prepared statement of Mr. Radoszewski follows:]

PREPARED STATEMENT OF TONY RADOSZEWSKI, PRESIDENT AND CEO, PLASTICS INDUSTRY ASSOCIATION

Good afternoon Mr. Chairman, the ranking member and members of the subcommittee. Thank you for having me here to speak today.

My name is Tony Radoszewski and I am the president and CEO of the Plastics Industry Association. We call ourselves PLASTICS for short, and we use that term proudly.

Founded in 1937, we're the only association that supports the entire plastics supply chain, and we have a track record of fostering collaboration between each segment of the industry.

We believe in working to make our members and the industry more globally competitive. We believe in advancing sustainability and being a good steward of resources. We believe in promoting plastics manufacturing as a viable career option.

We provide education to the industry and to the public about plastics. We support technology-driven innovation to solve problems. We work to change the public's perceptions about plastics and show how they impact our lives for the better. We understand what's important to our members' business and we advocate on their behalf to enact sustainable policies and create sustainable business growth for the industry.

Our councils, committees and events such as our signature global tradeshow NPE®, bring the boldest and brightest innovators, influencers and new technologies together to create connections, expand business growth and showcase our industry.

We're dedicated to helping our members shape the future and make a positive impact every day.

Plastics themselves were first developed by John Wesley Hyatt in the 19th century as a synthetic replacement for ivory in billiard balls.

Ivory was expensive and the process of collecting it was gruesome and inhumane. So, Hyatt tinkered around in his lab and developed a material that could behave like ivory but at a fraction of the cost and a fraction of the environmental impact.

That's the been the story of plastics from their genesis to today; it's a material that does what other materials can't and does so at a fraction of the cost and a fraction of the environmental impact of other materials.

Since they were first developed, plastics have grown to make hospitals safer, surgeries less invasive, patient care more sterile and effective and affordable— they do things in the medical realm that could scarcely have been dreamt of by the original innovators and creators of this material: stents, prostheses, bandages, replacement hips, shoulder sockets, knees, antimicrobial surfaces, dissolvable sutures, syringes, pill bottles, contact lenses and on and on and on.

In the century and a half since they were invented, plastics have also made cars, trucks and planes more efficient, more affordable, more environmentally friendly and safer.

In the United States and around the world, plastic pipe brings fresh water to people and takes wastewater away for treatment in the most economical and environmentally sustainable way. In developing countries, this one aspect has significantly improved the health and viability of millions of people.

And a similar story takes place in food packaging. Plastics make food last longer and enabled it to travel farther to help feed those most desperately in need of assistance. Again, peoples' quality of life, especially in developing countries, is dramatically improved due to the use of plastics.

Why would anyone want to ban such a material?

The plastics industry employs 993,000 people in the U.S. The state with the largest number of plastics employees is California, where 79,700 men and women are directly employed by our industry. I can say with confidence that none of them got into this business in order to pollute our oceans and waterways.

That our products end up where they shouldn't upsets me and every one of those nearly one million people who not only rely on this industry to make a living, but innovate with passion.

But it is a fact. It is a fact that a staggering eight million tons of plastics ends up in the world's oceans each year—90% of which originates from 10 rivers in southeast Asia and Africa. The remaining 10% comes from elsewhere around the world. There's a great deal of value being flushed down the drain when these products end up in lakes, rivers and ultimately oceans.

Our industry agrees that there is a plastic waste problem. But the urgency of the situation cries out for a solution more thoughtful than simply saying no to a material that lowers greenhouse gas emissions, is more efficient to produce than other materials like metal, paper and glass, and has delivered numerous benefits to society as a whole.

Study after study—including one conducted recently by the California water board—has shown that banning a plastic product simply drives consumers to other less sustainable materials. Bans have a very minor impact on litter, if they have any impact at all.

Take plastic bags, for instance.

Plastic bags make up extremely small percentages of the waste and litter streams, which is why banning them doesn't have much of an impact. According to the EPA, they make up 0.3% of municipal solid waste and they typically make up less than 1% of litter (branded plastic retail bags made up 0.8% of litter in New Jersey, for example).

Alternatives to plastic bags are also often worse for the environment. Paper, woven polypropylene, and cotton/canvas bags all have a higher carbon footprint than traditional plastic bags. The UK, Denmark, and Quebec governments all did studies on this and came to a similar conclusion—plastic bags are the best environmental option at the checkout counter.

California's plastic bag ban led to an increase in carbon emissions due to increased paper bag usage as well as skyrocketing trash bag sales, which use more plastic (see NPR article and the study). Overall, if you ban plastic bags, you will see fewer of them around. But consumers will switch to options that have a much higher carbon footprint, and litter and waste won't be meaningfully changed for the better.

This is true for bags but also for product bans in general. As an example, McDonalds in the United Kingdom and Ireland banned plastics straws and replaced them with paper ones. The company recently was forced to admit that the new paper straws weren't recyclable. Many consumers also don't like paper straws either. As mentioned before, banning a product drives consumers to use other less sustainable and less functional options while having a negative economic impact on the industry and its workers.

Plastics and plastic products exist for a reason.

They're used in such a diverse array of applications because they are the best option when all considerations are evaluated. In a free market society like we enjoy here in the U.S., the marketplace is driven by consumer demand, which determines which products provide the best value and performance. In so many applications, the chief characteristics of plastics—that is, their lower weight, durability, flexibility and versatility—constantly make them superior to other competing materials.

Even products that we encounter here in the U.S. in our day-to-day lives solve problems. Plastic bags became popular due to concerns about how many trees we were cutting down to make paper bags. Plastic bottles are lighter and don't break as easily as glass ones, reducing product loss and shipping costs. When they're disposed of properly, these plastic products have a smaller environmental footprint than identical products made of other materials.

Rather than trying to deny the value of plastics, we need to head in the opposite direction and aim to preserve and enhance their value so that they're worth too much to waste. This can happen by investing in recycling and waste management infrastructure.

We continue to support legislation that would provide grants through the Environmental Protection Agency to state and local entities to improve recycling infrastructure—which is what we need to close the loop on these issues.

This could be as simple as an education program on recycling in a particular community to the provision of new optical sorting equipment within existing Materials Recovery Facilities (MRFs). Simply put, we need to improve the collection of materials as one way of keeping it from becoming waste in a landfill, or litter in the ocean or along the side of the road. We believe having a reliable, steady supply of recovered material will encourage companies to use more recycled content.

Making it easier for consumers to recycle is a major factor in keeping our products out of the water and other environments where they do not belong. We would certainly support efforts to raise awareness on the impact of littering and better waste management practices.

But this should not be the only tool deployed to address this challenge. The industry supports voluntary, industry-led or public-private initiatives designed to increase the recovery of plastic materials that meet the standards of Sustainable Materials Management (SMM) analysis. Such initiatives could include programs aimed at increasing the use of post-consumer recycled material or bioplastics, as long as the industry has been involved in the creation of such initiatives, and they can be supported by economic analysis, adequate supply and transition time and remain consistent with other regulatory requirements pertaining to the manufacture and use of the product, such as food packaging safety rules.

Additionally, any potential language that imposes a fee on containers or packaging should apply to all materials—not just plastic—as all materials are found in the waste stream.

PLASTICS advocates for the use of SMM as a guiding policy principle—one that considers the entire ecosystem of the product and prioritizes the use of materials and processes that consider total energy and resource inputs throughout the entire lifecycle of a product and minimizes associated waste. SMM's holistic approach achieves this goal by using metrics like greenhouse gas emissions, water usage and transportation efficiencies for different materials, and comparing their advantages while meeting economic, social and environmental requirements. With that in mind we would caution against any product ban that does not consider the implications of what would replace that product. In many cases, what is broadly considered a "single-use" plastic product is the more environmentally sound choice when considering the manufacturing process, shipping and recyclability over the life of the product. Shortsighted bans would only create more problems without proper, detailed analysis.

We've also supported the Save Our Seas 2.0 Act which aims to improve efforts to combat marine debris and is currently seeing action in various Senate committees with companion legislation having been introduced here in the House.

Save Our Seas 2.0 is an important, bipartisan step forward to address the critical issue of marine waste and its impact on the environment. The legislation will build upon the progress the industry is making to address marine debris across the world. New proposals like the Marine Debris Response Trust Fund, as well as more research to understand the root causes of this global issue and federal support for improving water and waste management infrastructure are all critical to any effort to comprehensively address the threat marine debris poses to our oceans and waterways.

The industry itself has stepped up to the plastic waste challenge by innovating like it always has—developing new chemistries, investing in new recycling and collection technologies, developing ways to convert plastic waste into energy and creating the supply to meet the demand for recycled plastic content.

In addition to finding new ways to increase the effectiveness of traditional recycling—typically a curbside pickup program or local drop off—the industry has explored advanced recycling through the use of new additives like compatibilizers that help incompatible resins chemically bond, and property enhancers that improve the strength, quality and ultimately value of recycled materials.

The industry is also building on processes like chemical recycling, pyrolysis and gasification. Each of these processes are used to turn plastic polymers back into individual monomers—allowing materials to be reused in a variety of ways. In these processes, the chemical building blocks that make up the recycled plastic are recovered. The fundamental

building blocks can in some cases be repolymerized endlessly, giving them the qualities of brand-new, or virgin, resin.

The transformation can occur through a variety of processes, all of which avoid combustion, or burning, of plastics.

Chemical recycling is any process by which a polymer is chemically reduced to its original monomer form so that it can eventually be processed (re-polymerized) and remade into new plastic materials that go on to be new plastic products.

Chemical recycling helps us overcome the limits of traditional recycling. It also helps manufacturers continue to push the boundaries of how, and where, recycled plastics can be used. Chemical recycling has long been used for nylons, and the industry is working to make it possible for other resin types.

Pyrolysis, sometimes called "plastics to fuel," turns non-recycled plastics from municipal solid waste (garbage) into a synthetic crude oil that can be refined into diesel fuel, gasoline, heating oil or waxes. Using pyrolysis to convert non-recycled plastics into ultra-low sulfur diesel (ULSD) fuel reduces greenhouse gas emissions by 14% and water consumption by 58%, and it saves up to 96% in traditional energy use as opposed to ULSD from conventional crude oil.

Gasification turns non-recycled materials from municipal solid waste (garbage) into a synthesis gas, or "syngas," which can be used for electric power generation or converted into fuel or chemical feedstocks, such as ethanol and methanol, some of which can also be used to make new plastics that go into consumer products.

Numerous companies are already engaged in these processes across the country.

Agilyx, an alternative energy company, recycles polystyrene (which most people know as Styrofoam™) into high-value petrochemicals. Agilyx's polystyrene recycling process creates like-new materials while generating fewer greenhouse gases than manufacturing does.

Shaw Industries Group uses chemical recycling for nylon and polyester fiber in carpets. The company has invested more than $20 million to convert products that were once seen as waste into valuable resources. They reclaimed and recycled more than 800 million pounds of carpet from 2006 to 2015.

Resinate Materials Group collects chemicals from plastic materials and works to promote the practical and economical value of chemically recycled plastics. The company has found several high-value applications for the chemicals harvested from recycled medical plastics. It uses certain types of recycled packaging to create coatings, adhesives and sealants.

Patagonia, an outdoor clothing brand, chemically recycles non-wearable Capilene® polyester and fleece products. Today, the brand features a collection of products made completely from recycled materials. Patagonia's chemical recycling process uses 76% less energy than the process used to make new polyester.

Beyond that, the industry continues to expand its energy recovery capacity, which enables companies to convert post-use, non-recyclable plastics into a range of useful products such as fuels and electricity. Unfortunately, there are still some items that we can't recycle at this time and these items are typically sent to landfills.

Energy recovery technologies are changing that. They complement recycling to add a new dimension to the solid waste management toolkit.

It all starts with waste. Municipal solid waste is an underutilized resource of energy that can boost energy security, reduce landfill waste and lower greenhouse gas emissions. Energy recovery is a powerful process that has the potential to change the way we fuel the world. If

all the non-recycled plastics in municipal solid waste were converted to oil instead of landfilled, these plastics could power up to nine million cars per year.

When it comes to traditional recycling, companies are making big investments and commitments to collect more material and find new uses for it.

For instance, here are a few recent examples of companies investing in expanding recycling:

- GDB International is making "sizeable investments" in New Jersey and Ohio to pelletize plastics that were previously being sent to China.
- PureCycle Technologies is building $120 million polypropylene recycling facility in Ohio.
- East Terra built a new facility in Indiana.
- Merlin Plastics in British Columbia and Peninsula Plastics in California have made significant investments in mixed plastics recycling for the west coast.
- Azek invested in 100-million-pounds per year processing line for PE films in Illinois.
- Green Tech Solution plans to invest $75 million in a new plastics and metals recycling facility in Blacksburg, South Carolina.
- The Carton Council invested in artificial intelligence and robotics to help MRFs sort recycled materials more efficiently in Colorado, Minnesota and Florida.

That's just an example list. Additionally, we are seeing major shifts in the behaviors of plastics material suppliers who are forming strategic relationships with recyclers and brands. Again, some examples:

- Indorama entered a joint venture with Loop Industries for PET monomers from chemical recycling.
- Americas Styrenics has an off-take agreement with Agilyx for styrene monomer from chemical recycling. This joint venture is now call Regenyx and is moving quickly to commercial scale operations.
- LyondellBasell entered an agreement with Suez to jointly own QCP. This joint venture leverages the two partners' strengths and provides a platform for growth.
- Pepsi signed a multi-year supply agreement with Loop Industries.
- BP has an off-take agreement for oil produced by RES Polyflow from their pyrolysis system.
- A partnership was announced between the ReVital Polymers startup Pyrowave, and global plastics producer INEOS Styrolution to recycled polystyrene packaging.

The plastics industry is changing the ecology of how plastics are made and the supply chains that create them.

Brand Owners are also making unprecedented commitments to using recycled content. Those growing commitments are being tracked in the Sustainable Packaging Coalition's Goals database.

There is not currently sufficient quantity and quality of material in the market today to meet the 2025 goals that have been set by big name companies. New investments will help meet that demand, but we must find a way to grow the supply of material available to feed the

growing domestic recycling market— namely by implementing legislation that helps accomplish this goal.

The U.S. plastics recycling industry is undoubtedly in a period of transition, but it is certainly not dead. As a result of some of the challenges facing this sector, the U.S. domestic processing capability and capacity are growing more and more robust and able to handle more. The industry believes we must focus on how to improve our collection and recovery systems to expand recovery opportunities for more plastic products—while also creating new supplies of recycled plastics to feed domestic investments.

As an organization, PLASTICS has taken a leading role in promoting the aforementioned investments, commitments and technologies and exploring other ways to combat marine debris and deliver solutions to the end-users of our products.

PLASTICS leads the Pacific Northwest Secondary Sorting Demonstration project—a 60-day recycling demonstration that involves installing a portable secondary sorting system where selected materials from four regional MRFs will be further sorted. This innovation will help create six additional streams of recyclables which will reduce waste going to landfills or adversely affecting our environment.

Our Transportation and Industrial Plastics (TIP) Committee participates in the End-of-Life Vehicle (ELV) Recycling Project. Launched in 2015, the ELV project aims to demonstrate the viability of collection and recycling of auto plastics from ELVs and build a basic recovery model, beginning with thermoplastic polyolefin (TPO), which can be eventually expanded upon to include a broader range of resins and parts. To date, a variety of testing has been conducted on TPO recovered from bumpers and initial evaluation suggests there could be strong demand for the recycled TPO if the right end markets are identified. Through collaboration with various other association and member companies, PLASTICS works to prove out those end markets, creating new opportunity for auto recyclers to generate revenue.

PLASTICS' Flexible Film and Bag Division launched the New End Market Opportunities (NEMO) for Film Project in 2017, which aims to develop a reliable source of materials for companies that can use recycled plastic bags, wraps and films in their products.

We're also a part of the Materials Recovery for the Future (MRFF) project which aims to make it easier for MRFs around the country to empower their communities with the ability to recycle flexible packaging—again, bags and wraps but also punches and other packages—in their normal recycling stream and curbside.

PLASTICS also offers a number of tools and resources to companies in the industry that they can use to make their own operations more sustainable:

We help educate companies on how they can turn their waste into valuable resources, or eliminate waste altogether using the tools offered through PLASTICS' Zero Net Waste program. Through this program manufacturers learn how to maximize diversion—achieving in some cases 90% recycling rates and even 100% recovery rates—engage employees in environmental efforts and avoid landfill costs and generate revenue by recycling.

Since the 1980s, PLASTICS and the American Chemistry Council (ACC) have jointly operated Operation Clean Sweep (OCS), an international stewardship program designed to prevent resin pellet, flake and powder loss and help keep this material out of the marine environment.

More recently we've hosted a series of presentations for the industry focused on advancing sustainability, specifically on subjects like energy reduction through the Better

Plants Program, zero net waste, sustainability 101 for new professionals, water reduction, benchmarking, transportation efficiency and calculating economic impacts.

Despite all these efforts, we still need the support of federal, state and local authorities and new legislative solutions to ensure that no American has to wonder if the water bottle they toss in the blue bin will end up being recycled or if it will end up as landfill fodder.

Perhaps I could sum up our industry's position with a recent quote from Japan's Prime Minister Shinzo Abe: "We shouldn't treat plastic as an enemy, nor ostracize those who use it. What's needed is appropriate management of trash and to search for solutions through innovation."

Plastics are among humankind's greatest innovations and they've delivered an enormous benefit to public health and commerce all over the world. We need to learn how to live with these materials, because I can assure you, we would never want to have to live without them.

Thank you.

QUESTIONS SUBMITTED FOR THE RECORD BY REP. MCCLINTOCK TO MR. TONY RADOSZEWSKI, PLASTICS INDUSTRY ASSOCIATION

Question 1. During the hearing the topic of green house gas emission, as it relates to plastic products, was brought up on several occasions. Can plastics play a role in reducing green house gas emissions?

Answer. Thank you for the follow-up question regarding plastics' impact on greenhouse gas emissions. To put it simply, plastics reduce greenhouse gasses when compared to currently available alternative materials. As I mentioned the day of the hearing, plastics would be replaced with less sustainable options if bans on plastics were implemented. Life cycle analyses continuously show how plastics is the better choice to reduce greenhouse gas. Whether that is by light-weighting vehicles which increases fuel mileage and decreases emissions, or the fact that paper, woven polypropylene and cotton/canvas bags all have a higher carbon footprint than traditional plastic bags. I could go on, but I will let the science speak for itself. I've included several studies that illustrate what I am referencing. It cannot be overstated: plastic as a material improves the overall picture as it relates to greenhouse gasses when looking at the full life cycle of a product.

Plastics' lighter weight minimizes their environmental footprint by decreasing production of waste, energy use and carbon emissions through the full life cycle of the product. Beyond energy savings and water conservation, plastics help preserve the shelf-life of food, thereby preventing food waste, a huge problem worldwide. According to the EPA, most uneaten food decays in landfills, where it accounts for 34 percent of U.S. methane emissions (methane is a powerful greenhouse gas that is 21 times more harmful to the environment than CO2.[84])

Many people think glass bottles are "greener" than plastic. But glass bottles require 46 percent more greenhouse gases and 55 percent more energy to produce than plastic bottles do.[85]

The American Chemistry Council (ACC) released several studies showing the positive impact plastics can have versus alternatives. In particular, a Franklin Associates studies, "Life

[84] https://www.scientificamerican.com/article/earth-talk-waste-land/.
[85] https://posterng.netkey.at/esr/viewing/index.php?module=viewing poster&doi=10.1594/ ecr2015/C-2599.

Cycle Impacts of Plastic Packaging Compared to Substitutes in the United States and Canada" from April 2018[86] and "Life Cycle Inventory of Packaging Options for Shipment of Retail Mail-Order Soft Goods" from April 2004, pgs. ES15–17.[87]

Additionally, a study by Trucost estimates that substitution of plastic components with alternative materials in passenger vehicles sold in North America in 2015 would lead to an increase in lifetime fuel demand for those vehicles of over 336 million liters (89 million gallons) of gasoline and diesel, and at an environmental cost of $2.3 billion. This equates to an environmental cost increase of $169 per gasoline or diesel passenger car sold in North America in 2015. As another example, improved skin-type plastic packaging for sirloin steak can cut food waste by almost half compared to conventional plastic packaging (34 percent waste to 18 percent waste) with environmental savings of $606 per metric ton of beef sirloin sold. This equates to environmental savings of over $2.2 million for every additional 1 percent of sirloin steak sold in improved packaging in the USA. This case study illustrates the significant environmental net benefits that plastic food packaging can deliver where it helps to avoid the waste of resource intensive food products.[88]

On a national level, to substitute the 14.4 million metric tonnes of plastic packaging in the six packaging categories analyzed in one study, more than 64 million metric tonnes of other types of packaging would be required. The substitute packaging would require 80 percent more cumulative energy demand and result in 130 percent more global warming potential impacts, expressed as CO2 equivalents, compared to the equivalent plastic packaging.[89]

A study by Denkstatt which looked at the impact of plastic packaging on life cycle energy consumption and greenhouse gas emissions in Europe showed that substituting plastic packaging with other materials would on average increase the respective packaging mass by a factor 3.6. The study also showed life cycle energy demand would increase by a factor 2.2 or by 1,240 million GJ per year, which is equivalent of 27 Mt of crude oil in 106 VLCC tankers or comparable to 20 million heated homes.

Additionally, greenhouse gas emissions would increase by a factor 2.7 or by 61 million tonnes of CO2-equivalents per year, comparable to 21 million cars on the road or equivalent to the CO2-emissions of Denmark.[90]

It is our conclusion that plastic is the best overall material to use for a variety of reasons and these studies show over and over again sustainability is a success story of our material.

Thank you again for the opportunity to testify and for your follow-up question.

* * *

Dr. LOWENTHAL. Thank you for your testimony, Mr. Radoszewski.

I am going to remind the members of the Committee that Rule 3(d) imposes a 5-minute limit on questions. And now, the Chairman is going to recognize Members for any questions they may wish to ask members of the panel or the witnesses.

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[86] https://plastics.americanchemistry.com/Reports-and-Publications/LCA-of-Plastic-Packaging- Compared-to-Substitutes.pdf.

[87] https://www.oregon.gov/deq/FilterDocs/LifeCycleInventory.pdf.

[88] https://plastics.americanchemistry.com/Plastics-and-Sustainability.pdf.

[89] https://plastics.americanchemistry.com/Education-Resources/Publications/Impact-of-Plastics- Packaging.pdf.

[90] https://denkstatt.eu/download/1994/.

I am going to recognize myself for 5 minutes of questions. My first question goes to Dr. Jambeck.

I want to follow up on something you have said, but also something that the Ranking Member spoke about in his introduction, where he said there is no real problem here in the United States, the real amount of plastics in the ocean really come from other countries, Asian countries and African countries.

So, Dr. Jambeck, in your work, how much of the waste is entering the oceans from China, Vietnam, and other southeastern Asia countries. Can you tell us, is this the real picture of the origins of the waste?

Can you tell us more about the full impact of the United States' role in contributing to oceans debris and plastic waste?

And has that been partially hidden by our reliance on exporting our waste primarily to Asia?

Can you respond to that? That is really how it was framed.

Dr. JAMBECK. Yes. That is a big question.

But, certainly, when we first did those calculations of the global impact of plastic into the ocean, we couldn't take into account that import-export aspect. So, what we did see were these influencing factors—really rapidly developing economies, where infrastructure to manage the waste that comes with the increased waste generation, that comes with economic growth, that infrastructure was lagging behind.

The areas that have been referred to here, so many of those rapidly developing economies, is where we saw the most leakage. But as I mentioned in my testimony, our per-person waste generation rate is two to six times that within the United States. And if we look at leakage as a percentage of what we generate, the reason the United States is the only high-income country within the top 20 countries within that original paper is because of our waste generation rates.

So, in terms of a contribution to the global plastic quantity of waste, the 6.4 million that I mentioned—or billion, excuse me—we are a major contributor.

So, what has become an issue that started in the 1990s, in terms of our single stream recycling, to make it easier we can put everything in one bin. That meant our commodities, as well as the WTO encouraging global trade, and China needing material for manufacturing, becoming the manufacturing hub of the world, that set up this rapid increase in exporting of our recycled materials. And for me, we looked at recycled plastic. Over half of that had been going to China until they stopped that in the end of 2017, which caused a cascade impact on our recycle industry within the United States itself. So, that has been a major problem, because we were relying on lower-income countries to manage that material, in many cases with China having trouble managing their own, and then us exporting on top of that. That contributes to pollution in those countries, as well.

So, it is very interconnected and complex, but I hope that clarifies some.

Dr. LOWENTHAL. Thank you. I want to talk a little, raise some questions about—we know about the waste and plastics, and how much going into the ocean. But the question is how does this impact species?

The first question is, we have just seen the IPBES—I hope that I pronounced it right—report that was released earlier this year that included plastic pollution as a threat to marine biodiversity. So, it is seen as a threat.

My first question is, Mr. Danson, do you know if plastic is affecting species that are in danger of extinction?

We are trying to understand not only how it gets into the ocean, but what some of the impacts are.

Mr. DANSON. Some of the impacts. Turtles, every species of turtle, is either on the endangered species list or close to it. And every species of turtle has ingested plastic. Plastic doesn't go away completely. It just breaks down into smaller and smaller pieces, so a turtle or a sea mammal or another fish may ingest that plastic. They think that they are full, because their stomachs are full of plastic, so they stop eating and they starve to death.

Albatross end up dipping into what they think is some sort of something they like to eat in the water, but it is plastic. Then they feed it to their child, their little bird, and the bird dies for the same reason; they starve to death.

So, yes, it is having an impact on whales, on many species.

Dr. LOWENTHAL. Thank you. I think my time has been up, so I am going to yield. I will now call upon Representative Graves, who looked very good sitting on the Democratic side there for a while, and we welcome him back.

[Laughter.]

Mr. GRAVES. Thank you, Mr. Chairman. I have recruited a number of your Members to come to our side.

Dr. LOWENTHAL. Are you coming to my district this weekend? Mr. GRAVES. I look forward to it. And I want to make note—Mr.

Lowenthal and I, I was arguing with him awhile back and I said, you need to come see the people that I represent, the communities that I represent, so you understand why I say the things that I do, and why I vote the way that I do. And to his credit, he came down and spent 3 days in Louisiana. And I put him on a boat, an airboat. We put you on an airplane or a helicopter, maybe, took him all over the place, made him eat crawfish, all sorts of things—plastic-free crawfish.

So, I do want to thank you, and I am looking forward to going over to your part of the country——

Dr. LOWENTHAL. It is going to be great.

Mr. GRAVES [continuing]. To see if we can talk some wisdom into those people.

[Laughter.]

Dr. LOWENTHAL. But now you have to ask questions. Now you have 5 minutes. Thank you.

Mr. GRAVES. No, seriously, thank you very much. Dr. LOWENTHAL. Thank you.

Mr. GRAVES. I appreciate the friendship and I am looking forward to the opportunity to meet with some of your constituents. Thank you all very much for being here. And I want to be clear that I very much appreciate all of your efforts to remove plastic from the waste stream, a goal that I very much share. I represent part of the coast of Louisiana, and one of the top commercial and recreational fishing destinations and producers in the United States. And not just fishing for fun, but for subsistence, and a really important part of our culture, community economy in south Louisiana.

Look, we can talk end game for a minute, but I am curious. There is a huge part of the waste stream that exists right now. You have plastic in the oceans. You have plastic that is somewhere in the recycle chain, as we know, with what China has done.

What do we do right now, just putting the long-term aside, looking at the incredible waste streams that are in the ocean—and I am well aware and supportive of some of the legislation that we have pushed out of the House to deal with that. But what do we do with the current waste stream of plastic?

The current waste stream that is supposed to be recycled, but with a China ban has created some problems with where it goes, what do we do with the plastic that is in the ocean?

If you were king for the day, could make any decision, what would you do?

Mr. Danson, I would like to ask all of your opinions.

Mr. DANSON. I would reduce single-use plastic. It is designed to live forever, and yet you use it once and throw it away. You take the easy things like that, that aren't really necessary——

Mr. GRAVES. Can I just clarify my question, though?

My point, though, is you have plastic that has already been singularly used, and so it is already somewhere in the waste stream. Whether it is in our oceans, it is somewhere in the shipping or somewhere, where it is going to be recycled, but it is somewhere in the waste stream already. How do we handle that waste stream? Mr. DANSON. I am not sure. If it is in the ocean, I am not sure you can. It is like oil. Once it is in the water column, you are not going to get it out. You may be able to scoop some of the obvious bigger pieces out. You can do beach cleanup, and all of that. But really, compared to the amount of plastic that is about to be produced in the next 20, 30 years, it is going to be scaled up. You just can't compete with the amount of plastic production by recycling and picking up on the beaches.

Mr. GRAVES. Thank you. Mr. Parras?

Mr. PARRAS. What I see in our neighborhoods and communities all over the country is that plastic, it is actually made to be disposable, it seems like. It is affordable because it is plastic, so what happens is that people just don't consider it as trash, or as valuable, so they get rid of it. And until we start actually either charging more for the production of plastic so that we can have major cleanups, that may help.

Mr. GRAVES. Thank you. Dr. Jambeck—and I just want to reemphasize I am talking about the existing load that is there. I am interested to hear—our last witness talked a little bit about some of the technologies moving forward, but please.

Dr. JAMBECK. Sure, so quickly, what is already in existence, probably the easiest thing to grab are nets, something that your area is well familiar with, and they are one of the materials, typically nylon, very valuable, and could be recycled.

The problem with what already exists is the diversity of plastic that is there, the challenges with recycling that. Most of it is getting landfilled here in the United States, so that is not the best thing. We wish that more of it could be recycled.

Mr. GRAVES. Thank you.

Mr. RADOSZEWSKI. Thank you. Today, our industry, the four value components of our value chain, from the resin manufacturers, the machinery manufacturers, processors, and end users are all actively engaged in recycling and re-use of these products, ranging from sorting to the plastics that are most predominately used in recycling, PET and high-density polyethylene, and then also developing technologies that can sort out the other materials and develop enough of a waste stream so that they can be used in applications.

The other technologies that are being used right now are chemical recycling, in which we can take the products back to their basic form, re-polymerize it, and use it again in food

contact packaging, where before, if it is recycled, we can't use it in food packaging. So, these are technologies that we are actively involved in right now.

Mr. GRAVES. Thank you. I yield back.

Dr. LOWENTHAL. Thank you. I now recognize Representative Case for 5 minutes.

Mr. CASE. Thank you, Chair. The Ranking Member asked two, I think, good questions.

The first is that he asked what exactly is the problem, and the second question that he asked was why should Americans take the blame for the excesses of the rest of the world. Those are two good questions in this debate.

As to the first question, I will give a couple of examples from my perspective. In the state of Hawaii, we have the largest marine monument in our country, Papaha¯ naumokua¯ kea National Marine Monument. And there we get somewhere around 52 metric tons of marine debris, almost all ghost fishing gear, every year. Every year.

Now, why is that a problem? Well, it wrecks coral reefs, which are endangered around the world, and it degrades into smaller parcels, which then are ingested by our marine life. We have 1,400 Hawaiian monk seals left in the entire world, and declining. They get entrapped in this debris and die. That species is highly endangered. We have invasive species from elsewhere in the world hitching a ride on ghost fishing gear to Hawaii, the endangered species capital of the world, where we cannot take that kind of external impact.

We have in Hawaii—I went, on the first World Reef Day on June 1 of this year, to the north shore of Oahu to a beach in Kahuku, where I tried to clean up a coastline with Sustainable Coastlines Hawaii, one of many grassroots organizations across our country trying to do something about it on a micro level. A beach that I used to walk on that was pretty white is now all different colors: green, yellow, red. Very small particles of plastics not degraded, but down into the level of ingestion at the very lowest levels of marine life. Now, that is what the problem is.

As to the Ranking Member's second question, why should we take the hit when the rest of the world isn't doing anything about it. I think that is a really legitimate question, because it reminds me greatly of the debate over climate change, where, essentially, the same question is posed: Why should we reduce our emissions when the rest of the world is not doing that?

And that leads us to international agreements, as what I can see as being one of the only ways to get at this problem from an international perspective.

So, Mr. Danson, does Oceana partner with international organizations toward an international solution to plastics in the ocean, given that it does put us at a disadvantage for us to unilaterally curb our plastics use from several perspectives, and yet we need to do it. Cities and counties and states throughout the country are doing that. The City of Honolulu is doing it right now. Are you partnering with the rest of the world to try to find those international agreements?

Mr. DANSON. Yes, I believe we are. I think there are literally thousands, or at least 1,000 groups around the world working on plastics. This is a united effort. I can get you more specifics when I talk to the staff of Oceana.

I mean, we haven't talked about climate change and greenhouse gases, but plastic is such a huge part of that story. I don't see how we cannot address our plastic, our greenhouse gas emissions—and if we don't do that, how we expect the rest of the world to follow along.

So, yes, sorry, that is my——

Mr. CASE. OK. Thank you very much.

Mr. Radoszewski, you stated in your testimony that you and your industry are supportive of Save Our Seas 2.0, which is a bipartisan, bicameral bill introduced in both the House and the Senate, and that calls for much greater studies, some incentives at the Federal level, but it also calls for pursuing international agreements that would curb plastic use, especially single-use plastic use around the world.

And your testimony sounded to be inconsistent with that position, that part of Save Our Seas 2.0. Are you supportive of pursuing international agreements whereby the entire world would agree to a reduction in plastic use and a reduction of dumping of plastics into the oceans?

Mr. RADOSZEWSKI. I would say we are involved and eagerly working with international organizations to find solutions to the problems that exist today. We are engaged with—whether it is the British Plastics Union, the Canadian Plastics Industry Association, the New Zealand Plastic Association, working in consortium with them to define those abilities to minimize the waste in the ocean, and in the land, as well.

Mr. CASE. OK. That doesn't sound like what I am talking about. It sounds like you are working with the rest of the plastics industry around the world to manage it going into the oceans, but not necessarily reducing it.

Mr. RADOSZEWSKI. Reducing or reusing or recycling, there are a lot of different options that we are looking at. And in the Save Our Seas 2.0, there are many parts of it that we do like and other parts that we would still like to negotiate with.

Mr. CASE. OK, thank you.

Dr. LOWENTHAL. Thank you. I now recognize the Ranking Member for 5 minutes of questions.

Mr. McClintock.

Mr. MCCLINTOCK. Thank you, Mr. Chairman.

First, I don't think Mr. Case was listening very carefully to what I said. I was referring specifically to properly-disposed-of plastics, plastics put in landfills, incinerated or recycled, none of which gets into the ocean.

And we know that America accounts for less than 1 percent of plastic marine pollution.

So, even if we went to the extreme of banning all plastics in the United States, in addition to having a devastating effect on the economy, it would at best affect just 1 percent of plastic pollution in our oceans.

But Mr. Radoszewski, Dr. Jambeck asked a very intriguing question. Think about how much plastic you touch every day. Isn't that an indication of how useful plastic has become in our daily lives?

Mr. RADOSZEWSKI. Absolutely. If you look at what plastics have replaced in the past, whether it is glass, paper, steel, aluminum, the reason why there is so much plastic is it is the best choice in terms of many of the packaging applications that it finds itself——

Mr. MCCLINTOCK. Isn't her question also a warning of how our quality of life would decline if the left is successful in restricting or banning it?

Mr. RADOSZEWSKI. Well, I would think a lot of things that we have taken for granted today would be gone, and the accessibility to those foodstuffs that give us a higher quality of life, not only to Americans on the East and West Coast, but in the middle of the country, and poorer areas, as well. The availability to get foods to different parts of the world because of lower transportation costs, and the food stays safer and healthier and fresher are all reasons why the quality of life, not only in the United States but across world, has increased.

Mr. MCCLINTOCK. I think Dr. Jambeck's question also begs a correlated question. Let's think about everything that we touch every single day. Everything is either mined or it is grown, is it not?

Mr. RADOSZEWSKI. I would think that would be right.

Mr. MCCLINTOCK. I don't know of a single exception to that. And that then opens a new question, and that is, what is the alternative to plastics? I used the example of a toothpaste tube. What would be the alternative to that?

Mr. RADOSZEWSKI. Well, I think, even in your original testimony, you mentioned what it used to be. And, as far as we know, the only thing we could do is go back to what it was. And that would mean glass bottles. That would mean lead, I think, was what was once used in toothpaste tubes, because of the softness of it.

So, if you go backward, you are talking about materials that have a higher carbon footprint, take more energy to produce, usually weigh more. The transportation costs also increase, so you have that aspect, as well.

Mr. MCCLINTOCK. So, at this juncture in our technology and science and advancement of our civilization, plastics are the most environmentally friendly alternative that we have, if we are to engage in the commerce that makes our civilization possible, is it not?

Mr. RADOSZEWSKI. I think that is very right. In fact, again, I go back to the point of—let's look at food packaging. The ability to get a bratwurst at any place in the country at any time because it is wrapped in plastic and has a foam board packet, which is made of styrene, makes it accessible to everybody. Your meat stuffs, your sausage containers for your breakfast patties, all those are packaged in plastics because they get product to the shelf economically, safely, and fresh.

Mr. MCCLINTOCK. I am curious, Mr. Danson. How are we going to get our toothpaste, for example? How do you propose that we package our toothpaste in the future? You want to ban plastic containers? You want to go back to metal tubes or glass jars?

Mr. DANSON. You know, I don't really know the answer to that.

Mr. MCCLINTOCK. Well, that is the problem, isn't it? I have not heard a single alternative offered by the critics of plastics. And I think it has become very clear that plastics we have found to be a far better solution, economically and environmentally, to the materials that we have used in the past.

Mr. Radoszewski, tell me how a ban on single-use plastics would impact the overall economy.

Mr. RADOSZEWSKI. I think it would be detrimental to it. It could have an effect of putting people out of work. I don't think there is a quick response to supply the demand that the marketplace has created for these products. So, you would have a shortage of goods. You would have an economic decline because of lack of innovation of materials that we are seeing in the plastics industry. There is a whole host of things that would be affected immediately with some of these immediate bans——

Mr. MCCLINTOCK. And what would happen to consumer prices?

Mr. RADOSZEWSKI. They would go up. I mean it is a simple example of supply and demand. If the demand is not satisfied by the supply, the price goes up.

Mr. MCCLINTOCK. Well, our automobiles, for example, instead of using plastic materials, would go back to using metal materials. I mean, I am just looking at these nameplates right here. They are plastic. In a previous day, they were brass, much more expensive and much harder on the environment to mine. Is that correct?

Mr. RADOSZEWSKI. It is. And, in fact, if you look at the CAFE standards, one of the reasons the automobile industry has been able to meet those standards over the last couple of decades is because of the incorporation of higher-performing plastics that do the same performance as metal——

Mr. MCCLINTOCK. So, once again, it is blame America first, let's harm the American consumer, even though the American consumer is responsibly disposing plastic products, and without any alternative. That, to me, sounds almost childlike.

I yield back.

Dr. LOWENTHAL. Thank you, Ranking Member. I now call upon Representative Cunningham for 5 minutes of questions.

Mr. CUNNINGHAM. Thank you, Mr. Chairman. And thank you for holding this hearing today on an issue that is near and dear to my heart, and also our constituents in the 1st District of South Carolina, which stretches from Charleston all the way down to Hilton Head.

This issue is certainly on the minds of South Carolinians, many of whom dedicate their free time to support local beach cleanups in an effort to preserve our beautiful, God-given natural resources. And I am proud to represent so many of these conservation leaders. The local Surfrider Foundation chapter in my Congressional District hosts beach cleanups almost every single weekend.

And we also have Andrew Wunderley of the Charleston Waterkeeper, who has made it his livelihood to protect and restore the quality of Charleston's waterways, while fighting for the right to swimmable, drinkable, fishable water.

And, today, I actually came up here from Charleston with some of the plastic treasures that were recently found on our shoreline over the weekend from the Goose Creek Reservoir, which is the source of the Goose Creek water supply. So, let's see what we have here today— and this was just found this weekend.

It looks like we have a used piece of Styrofoam here. We have a plastic water bottle; a single-use straw; a single-use plastic bag, and this actually looks like it has been kind of shredded or nibbled on, more than likely ingested by some type of marine life, this is what is left of it right now; some other straw, a shredded straw— we have all seen the pictures of sea turtles ingesting these and the damage that causes; a glass jar; and it looks like a potato chip bag, plastic.

And this isn't abnormal, unfortunately. This has become kind of the norm of what washes up on our shore lines or into our water-ways every single weekend, and a lot of people in this room are aware of it.

In fact, earlier this year NOAA published a report on the economic impacts of marine debris. And without objection, I would like to enter this report for the record.

Not surprisingly, this report found that getting rid of debris from our beaches can have a significant positive impact on the tourism economy. That is kind of a no-brainer.

Mr. Danson, every year the Ocean Conservancy's International Coastal Cleanup Report shows the most frequently found items on the beach. In 2017, data showed, for the first time, that the top 10 most commonly found items were all made of plastic. And that trend continued in 2018.

So, Mr. Danson, is what you saw here today, is this typical of the items typically found in beach cleanups, in your experience?

And how do these discoveries help shape policy?

Mr. DANSON. Well, they are all single-use plastics, which is something we would like to reduce. They are all very convenient and easy for us to use in our everyday life, but create incredible problems, everything from greenhouse gases to sea animals dying from ingesting it. That is our disposable lifestyle, of which I am part of. It is very hard to deal with that every day.

But people are coming up with solutions. There is a toothpaste called Bite that now comes in a little jar that is a powder, and you add water. That creates jobs and money and taxes. So, there are alternatives that we need to find.

It has been incredibly useful, and now it has become incredibly dangerous. And I think that is the argument, not that the left or the right has any monopoly on being smart about things. It is this is a problem for all of us, and we all need to find ways to do it. And I do believe we are capable of that.

Mr. CUNNINGHAM. I appreciate it, Mr. Danson, and I appreciate you all being here today.

Unfortunately, my time is coming to a close. I know there has been some discussion here today as far as where the United States is, as far as the polluting and cleanup and everything. But I think we should all agree that the United States of America is a leader, and we should lead on this issue. And no matter where we fall in the list of polluters, we should be leading by example and being more responsible, being more of like a Sam, instead of the Norm, if you will.

[Laughter.]

Mr. CUNNINGHAM. But just being out in the front on this, and recognizing that this is not sustainable, and we have to do every single thing in our power to make that come to an end.

So, I appreciate the work you all are doing, I appreciate the time here today. And, with that, I would yield back.

Dr. LOWENTHAL. Thank you, Mr. Cunningham. And I now recognize Congressman Sablan for 5 minutes of questions.

Mr. SABLAN. Thank you very much, Mr. Chairman, for holding today's hearing.

In my first few months here in Congress, in my first year, I had this naïve thought. If there was a possibility for some committee members to get on an airplane and fly over this garbage patch that is in the Pacific—now it has a new name, actually—it is the Great Pacific Garbage Patch, and it is located just a little north of Hawaii, and right next to a place called Micronesia.

I come from the Northern Marianas, which is a part of Micronesia—called Micronesia because it is a lot of small islands together. And you take all of those islands together, all of them, and put them together, it is hardly a large part of this garbage patch.

We have in the Northern Marianas, islands that are conservation islands—and unless you are a scientist with a permit, you can't get on these islands. But there have been scientists who have gotten permits and gotten on and found, to their dismay, that they had to collect bags and bags and bags of garbage, plastic garbage.

I don't mean any disrespect to all of you, thank you.

Mr. Danson, sir, thank you very much for so many wonderful hours of great entertainment. I enjoyed your show, "Cheers."

I also noticed, among the four witnesses on the table, sir—Mr. Rado——

Mr. RADOSZEWSKI. It is OK. Call me Tony. How about that?

Mr. SABLAN. OK, Tony. Among all the four witnesses, you are the only one with a plastic bottle of water.

Mr. RADOSZEWSKI. Right.

Mr. SABLAN. Yes. I mean you really are for your product. [Laughter.]

Mr. RADOSZEWSKI. Sir, if you would like me to comment on that, I——

Mr. SABLAN. No, I am not asking you for a comment, it was just an observation, sir. You didn't have to bring that, because there are glasses of water in front of you.

But, you see, these Micronesian Islands, yes, we probably contributed to some of this debris. But we are not responsible for that debris, and that thing is floating and growing. And it is one day going to cover Micronesia. Micronesia is—the area is the size of the 48 contiguous states.

So, what do we do about that?

Dr. Jambeck, how much effort and resource would you think it would take to clean up this garbage patch?

Dr. JAMBECK. What is floating out there is only about 3 percent of what we think is going in every year. So, it is not a large amount. But you are absolutely right in that what is floating often ends up on islands like yours that sort of interrupt those currents. To be honest, the best way to sort of get that out is if it is ending up on land, and then cleaning that land, like they do in Hawaii. There are folks who are trying to design systems to collect out in the Great Pacific Garbage Patch. But there are a lot of resources that go into that, and that is similar to the analogy of mopping up your bathroom floor while the tap is on.

Mr. SABLAN. OK. Just imagine what it would be like for Hawaii if that garbage gets any closer and just keeps going on land, because tourism is their major industry.

I don't have an answer to the problem. I really don't. I do have a serious concern, because I eat a lot of fish, reef-caught fish, and tuna caught by trawling, and everything.

I agree that these things get into the fish, so it gets into what I eat, probably, most likelihood. But I don't know. I don't have an answer. I am not as smart as the four of you sitting at the witness table, but we do need to act on, get something going, and try to find a way to resolve this, and maybe find an alternative to plastic that is not going to hurt people's jobs, you know?

There has to be something. We are a much better Nation than we think we are, than we give ourselves credit for.

My time is up. Thank you, Mr. Chairman.

Dr. LOWENTHAL. Thank you, Mr. Sablan. Next, the Chair recognizes Mr. Neguse for 5 minutes of questions.

Mr. NEGUSE. Thank you, Mr. Chair, and thank you for hosting this important hearing.

The topic of plastic in our waters and oceans cannot be more pressing. A study conducted by the U.S. Department of the Interior and the U.S. Geological Survey, aptly and alarmingly called "It's Raining Plastic," was published in May, and found that plastic was found in 90 percent of rainfall samples in Denver and in Boulder, Colorado, which happens to be—Boulder, in particular—the area that I represent in Congress, amongst many others.

An earlier study found that people are swallowing an average of 5 grams of plastic every week, about the weight of a credit card. For my constituents, who are suffering from this reality every day, ultimately, for the people across this Nation and the world who are doing the same, it is imperative that we address this issue. It just so happens, Mr. Chair, quite fittingly—literally, 1 week ago, or a week-and-a-half ago, on October 16, 2019, a constituent

of mine—her name is Annie, she is a sophomore at Fort Collins Polaris Expeditionary Learning School in my district—wrote to me about this very issue, about the issue of microplastics in our world's oceans and water systems at large.

And in her letter she said, "I am such a small part of this world, but I want to do everything I can to fix this problem." I am certainly inspired by her commitment to fixing this problem, and am heartened by the Chairman's decision to host this important hearing, and my fellow Committee members in their attempt to address this issue collectively, and, of course, to the witnesses who have joined us, and to their testimony.

I will confess I had a number of competing scheduling commitments, from both a hearing perspective as well as meetings, but I was watching the testimony and some of the exchanges on the television in our office. And there was one exchange in particular that was a bit interesting to me, and I had noticed that Mr. Danson, you didn't have an opportunity to really respond to the question that was being posed by the gentleman from California, Mr. McClintock.

So, I would like to go back to the point that he made about toothpaste. In 1984, how old were you, Mr. Danson?

[Laughter.]

Mr. NEGUSE. I mean, if you are comfortable sharing it, of course. I don't want to——

Mr. DANSON. Tough question. I was born in 1947. Would you do the math for me?

[Laughter.]

Mr. NEGUSE. I am a lawyer, not a mathematician, unfortunately. But I believe that that would put you at, what 34——

Mr. DANSON. Sounds right.

Mr. NEGUSE. Forty-three. I think that is right.

Dr. LOWENTHAL. No, 47.

Mr. NEGUSE. In 1984, when you were 43, what kind of——

Mr. DANSON. Thirty-three.

Dr. LOWENTHAL. Thirty-three.

Mr. DANSON. Go ahead. I am old. Go on. [Laughter.]

Mr. NEGUSE. That is all right. I don't want to get stuck on your age, Mr. Danson. What kind of car were you driving back in the 1980s?

Mr. DANSON. The 1980s? Mr. NEGUSE. Yes.

Mr. DANSON. A Ford Explorer for a while.

Mr. NEGUSE. A Ford. And I take it it probably wasn't an electric car, right?

Mr. DANSON. No. But I did have the first EV–1.

Mr. NEGUSE. All right. And I suspect you might have been renting back then, or you owned a home. Did your home have solar panels back then?

Mr. DANSON. No, it did not.

Mr. NEGUSE. No. And my point is this, the reason why I ask. I was born in 1984. I am 35 today. I have a daughter who is 14 months old. And I think a lot about the world that she will inherit. And much of the work that we do here in this Committee and in this Congress is about fighting to make sure that the world she inherits is a better one than we did.

The transformative changes that have happened just in the last 35 years since I was born have been dramatic, right? And you have chosen, amongst many other citizens in our country—and, of course, several of the panelists here—to try to make a difference, to adopt strategies in your own life and the way in which you conduct yourself to be environmentally

conscious and, of course, taking advantage of the technological capabilities that have also changed.

So, this notion that we can't adapt, that removing microplastics—suddenly we all will be amiss—with the realities of trying to replace the plastic tube that carries toothpaste, to me is a false choice. Fundamentally, we all collectively are going to have to adopt strategies that enable us to move into a future in which microplastics are not polluting our planet and in the communities that we are all so lucky to call home. That, to me, is what this hearing should collectively be about.

So, to the extent, Mr. Danson, that you would care to respond further, I know you did talk a little bit about some of the alternatives to toothpaste containers, and toothpaste brushes that are non-plastic options, but if you care to also illuminate further, or expound further on that—

—

Mr. DANSON. Just briefly, I do know that people will invent new things, and create more jobs, and not create stuff that is worse for the climate.

But just in general, if you are talking about your children, then you are talking about climate change. You just are. And you are talking about greenhouse gases. And if you are talking about greenhouse gases, and we are in the middle of a Committee about ocean plastic, you have to acknowledge that the plastic is coming from petroleum and chemicals, and that life span, from the time of production to it lying on a beach, is the equivalent, all of the plastic, as the fifth-largest emitter of greenhouse gases.

So, if you want to take care of your children, you have to start addressing these incredibly inconvenient things that we have all gotten used to, and enjoy. But they are no longer good for us, and they are going to land on our children and our grandchildren in a huge way.

Mr. NEGUSE. Thank you. I yield the balance of my time, and apologize to Mr. Danson for revealing his age.

[Laughter.]

Mr. NEGUSE. And with that, I yield back.

Dr. LOWENTHAL. The gentleman yields back. How old are you?

Mr. NEGUSE. Thirty-five.

Dr. LOWENTHAL. All right. [Laughter.]

Dr. LOWENTHAL. Thank you. I would like to thank the witnesses for their valuable testimony and the Members for their questions. I found this very interesting.

The members of the Committee may wish to have some additional questions for the witnesses, and we are going to ask you to respond to these in writing.

Under Committee Rule 3(o), members of the Committee must submit witness questions within 3 business days following the hearing, and the hearing record will be held open for 10 business days for their responses.

Just before I end, I want to introduce into the record a journal article from Volume 9 of the journal *Nature Climate Change* of 2019, which was a study that showed that the global life cycle greenhouse gas emissions from conventional plastics which were produced in 2015 were 1.8 billion metric tons of carbon dioxide equivalent. This is approximately the annual emissions, as I pointed out in my introduction, of 462 coal-fired power plants. That is what we are just talking about in terms of CO2 emissions. I want to get that formally into the record.

Mr. MCCLINTOCK. A point of order, Mr. Chairman.

Dr. LOWENTHAL. Yes?

Mr. MCCLINTOCK. I am wondering whose time are you speaking on? Because we are out of questions. If we are, I am prepared to engage——

Dr. LOWENTHAL. No, I am just introducing something into the record.

Mr. MCCLINTOCK. And there is no objection.

Dr. LOWENTHAL. Thank you.

If there is no further business, without objection, this Committee stands adjourned.

[Whereupon, at 3:43 p.m., the Subcommittee was adjourned.]

* * *

November 12, 2019

Chairman Jared Huffman
United States Congress
1527 Longworth House Office Building
Washington, DC 20515
Ranking Member Tom McClintock
United States Congress
2312 Rayburn House Office Building
Washington, DC 20515

RE: Written Testimony for October 29, 2019 Hearing from Kathleen Pitre, Chief Sustainability Officer at Ball Corporation

Chairman Huffman, Ranking Member McClintock, and members of the Committee:

I write on behalf of Ball Corporation, the world's largest aluminum beverage and aerosol can maker, to provide testimony for your October 29, 2019 hearing, "*A Sea of Problems: Impacts of Plastic Pollution on Oceans and Wildlife.*"

Founded in 1880, Ball Corporation is the leading manufacturer of innovative sustainable packaging solutions to customers around the globe. In addition to our packaging solutions business, Ball also provides sensors and satellites to the federal government through our subsidiary Ball Aerospace. We employ 18,300 people in 90 manufacturing locations with operations in North and Central America, South America, Europe, Asia, the Middle East and Africa.

Sustainability is one of our top priorities at Ball and is supported by global policies, quantitative targets, and tools for performance monitoring. We strive to put the right people, processes and partners in place to help us create long-term shared value for our company and our stakeholders.

Ball is very concerned about the increasing amount of plastic that pollutes our waterways and oceans and believe fundamental change is required. If we want to mitigate, and even reverse, the plastic pollution crisis, government as well as business need to take action.

Sustainable Packaging

Packaging is critical to delivering consumer products safely, conveniently and in good condition, and it preserves and protects beverage, aerosol, and other products as they move through supply chains.

Relative to glass, plastic and other substrates, aluminum cans exemplify a circular economy. They are infinitely recyclable, easily collected and sorted, and are by far the package with the highest economic value in the recycling stream. Currently, the US recycles 50% of its aluminum cans, Europe recycles 75% and Brazil recycles 97%, given the right policy, aluminum is capable of getting close to a 100% recycling rate in the U.S, too.

By contrast, plastic is the least recycled substrate with only an estimated 5% of plastics being effectively recycled around the world, while 40% are deposited in landfills and another third end up in ecosystems such as our world's oceans and rivers. It is estimated that 8 million tons of plastic leaks into the ocean every year. This is the equivalent of dumping the contents of one garbage truck into the ocean every minute, and it's only getting worse. If nothing changes, the rate of pollution is expected to increase at the equivalent of two garbage truck's worth per minute by 2030 and four per minute by 2050. By then, there could be more plastic in the ocean than fish.[91]

Government Response

The "take, make, waste" paradigm has created products that are extremely cheap to produce yet very expensive to be recycled. For many products, technical capabilities are insufficient to fully close the material loop and significant losses persist in material quality and quantity from established recycling processes. Simply put, these materials cannot be kept in the material loop; they are hard to recycle adequately and will eventually become trash. It is just cheaper to continue using the primary materials instead of recycled ones.

Since these products do not reflect the true cost of the environmental degradation they cause, both consumers and manufacturers have no incentive to change their habits, further exacerbating the plastic crisis. Communities everywhere are struggling to take in the sheer amount of plastic waste that enters the waste stream, and now that countries like China no longer accept our trash, this waste is increasingly landfilled or, worse yet, finds its way into our oceans and waterways. We need proactive government leaders to step in and instigate change.

In the absence of federal legislation, states across the nation have taken the initiative and begun to introduce measures to mitigate the growing environmental and human health crisis. These bills offer a range of solutions to plastic pollution, including recycling content minimums, extended producer responsibility programs, taxes, and even outright bans. While some of these might not be politically feasible at the federal level now, Congress has the responsibility to, at the very least, provide these states with the guidance, expertise and resources necessary to inform sensible policy.

[91] Ball Sustainability Report, p. 5.

Possible Solutions

A circular economy strives to create favorable conditions for economically viable recycling, rather than developing lowest cost products that are not recycled and require expensive recycling technologies to be recovered. Instead of trying to develop end-of-the-pipe solutions, businesses must invest in creating truly circular products with the end goal of preventing waste in the first place.

While there are a variety of policy tools Congress could use catalyze change, we believe there are three areas where legislators can start:

- *Define Recyclability*: Currently, there is no single yardstick for measuring "recycling" and, as a result, businesses have inappropriately twisted this word to give the façade that their products are recyclable. Recyclability claims suggest that it is a great achievement when a product is recyclable. The truth is that recyclability is only an achievement when a product can be consistently and infinitely recycled, and the material can be kept in the loop without loss in material quality. Congress should clearly define "recycling" so that it reflects the goals of circularity rather than another marketing ploy.
- *Promote Smart Packaging Design*: Many products today are made of multiple materials and consist of different pieces that vastly complicate sorting and processing. Congress should incentivize industry and business to use homogenous products - those made of only one material or designed for easy disassembly - that do not require complex processing in order to be reused or recycled.
- *Offer Technical Assistance*: As states like California, Massachusetts, Washington and many others begin considering legislation regarding plastic, the federal government and its agencies, at the very least, should act as a resource for technical assistance. Many of these bills propose sweeping changes to the states' waste management industries specifically, and to the economy more broadly. The federal government should be a resource for every state, no matter their policy preferences.

Establishing the right policy is a key component to achieve a truly circular economy. Opening an honest discussion about how we design and recycle packaging materials is the place to start. We cannot recycle our way out of this problem. Rather, we need to develop packaging solutions with circularity in mind from the beginning. This is the only way we will begin to stem the tide of plastic in our oceans.

We commend you for scheduling this hearing and thank you for your work on this important national matter.

SUBMISSION FOR THE RECORD BY REP. LOWENTHAL

GOVERNMENT OF THE DISTRICT OF COLUMBIA
Department of Energy and Environment

NOV 13 2019

Representative Jared Huffman
Chairman
Subcommittee on Water, Oceans, and Wildlife
Natural Resources Committee
1324 Longworth House Office Building
Washington, DC 20515

Representative Tom McClintock
Ranking Member
Subcommittee on Water, Oceans, and Wildlife
Natural Resources Committee
1329 Longworth House Office Building
Washington, DC 20515

Subject: Written Testimony for recent U.S. House of Representatives hearing entitled *A Sea of Problems: Impacts of Plastic Pollution* on *Oceans and Wildlife*

Dear Rep. Huffman and Rep. McClintock:

Thank you for your interest in addressing the impacts of plastic pollution on the world's oceans. Plastic pollution is one of the great environmental challenges of our time and will not be solved without robust participation from federal, state, and local governments. We welcome this opportunity to provide written testimony and stand ready to work with you on further efforts to address this issue.

The District has been a leader in the nation for over a decade on addressing trash, particularly plastic materials, in our local waterways. According to a 2015 article published in the journal, *Science*, researchers estimate over 8 million metric tons of trash enter the oceans annually from land-based sources.[92] In a heavily urbanized area like the District, a large portion of that trash comes from litter being conveyed via storm sewer and combined sewer systems to estuaries like the Potomac and Anacostia Rivers. In response to this problem, the District, in collaboration with U.S. Environmental Protection Agency (EPA) and upstream jurisdictions, established a total maximum daily load (TMDL) for trash in the Anacostia River. As a result, the District is compelled by EPA and our own priorities to reduce trash in the Anacostia River as part of our efforts to make the Anacostia fishable and swimmable once more. We believe addressing trash, including plastic pollution, starts upstream in our streams, rivers, and estuaries. Trash, especially plastic, affects our local waterways as much as it does the oceans.

We are very proud of our accomplishments over the past decade and would like to share details of some of our efforts in hopes that this work can, and will be, emulated elsewhere throughout the nation. Three important components have helped us to effectively manage

[92] Jambeck, J.R., R. Geyer, C. Wilcox, T.R. Siegler, M. Perryman, A. Andrady, R. Narayan, & K.L. Law. 2015. Plastic waste inputs from land into the ocean. Science 347: 768-771.

trash in our waterways: regional partnerships, using sound science to inform policy, and taking multi pronged, innovative approaches.

Regional Partners/Zips

The issue of trash impacting the Potomac River watershed began over a decade ago thanks to a regional collaboration facilitated primarily by the Alice Ferguson Foundation of Accoceek, MD. Since 2005, the Alice Ferguson Foundation has held an annual Potomac Watershed Trash Summit in the DC metropolitan area. This summit brings federal, state, and local government agencies, elected officials, and non-governmental organizations together to discuss efforts to reduce trash in the watershed. This would not be possible without grant funding from the NOAA Marine Debris Program, which has been an invaluable partner in regional efforts to address this issue.

Example of trash condition s in Watts Branch, a tributary lo the upper Anacostia River in Washington, DC.

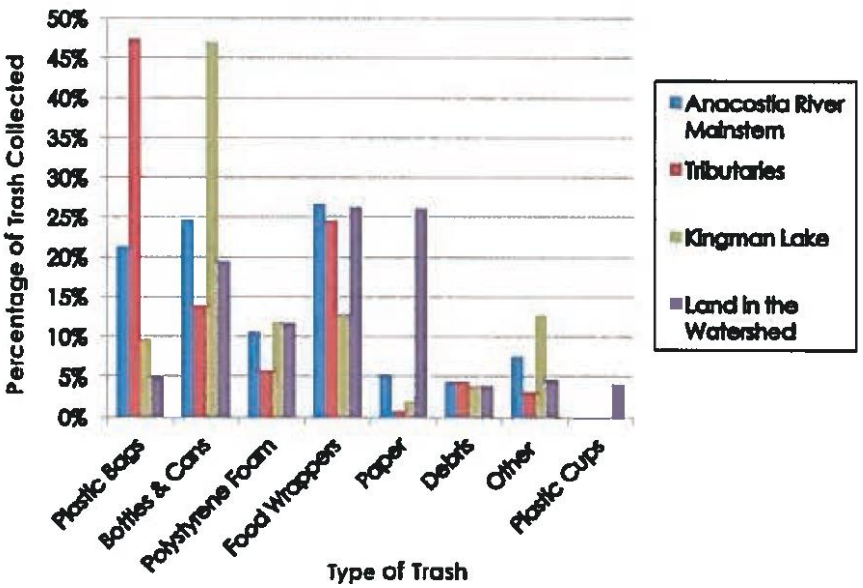
Over the years, participants have implemented key efforts discussed at this summit including a regional anti-littering campaign, innovative local policies for reducing trash, and regional collaborations to establish the TMDL for trash for the Anacostia, one of the most urbanized tributaries to the Potomac River. The summit brought together leaders from the District, the state of Maryland, local Maryland counties, EPA, and local advocates to reach common ground on establishing the TMDL. Without this regional partnership it is very unlikely this effort would have been successful. As we will highlight more specifically, several new innovative approaches for trash reduction have been implemented since that time.

In 2016, the District, Montgomery County, and Prince George's County reaffirmed their commitment to making the Potomac and Anacostia River free of trash by signing the

Anacostia River Accord. The Accord was signed by the District's Mayor Muriel Bowser, as well as Montgomery County Executive Isiah Leggett, and Prince George's County Executive Rushern Baker. In addition, the Anacostia Watershed Restoration Partnership, housed at the Metropolitan Washington Council of Governments, has convened a trash working group to develop consistent methods for tracking and reporting trash reductions across all three jurisdictions. This is further evidence of the importance of regional partnerships at combatting the issue of trash in our waterways.

Using Sound Science to Inform Policy

One of the first things the District did to address the problem of trash in the Anacostia River was conduct a two-year comprehensive study of trash conditions. The District Department of Energy and Environment (DOEE) funded the Anacostia Watershed Society of Bladensburg, MD to conduct surveys of litter along the river and its many tributaries, and monitor trash loads from storm sewer outfalls. The study provided two important pieces of information: (1) data on the most common types of trash in the Anacostia River and its watershed and (2) data on total weight of trash entering the Anacostia River on an annual basis. The first dataset informed District policies targeted to address specific types of trash most common in our waterways such as single-use plastic bags and expanded polystyrene foam products. The graph below shows the most common types of trash found out of 44 different categories sampled along DC shorelines in the Anacostia River, its tributaries, Kingman Lake (a semi-enclosed lake in the Anacostia River), and land in the Anacostia River watershed. The District utilized the second dataset on trash loads to develop the trash TMDL and identify and strategically address hotspots in the watershed which are conveying above average amounts of trash to the river.



Graph displaying most common types or trash found by count in 2008 in the Anacostia River, its tributaries, Kingman Lake, and land in the Anacostia watershed.

Since 2016, DOEE has been working with the Metropolitan Washington Council of Governments to sample trash along rivers and streams throughout the District. We provide this data annually to U.S. EPA Region III. This work builds upon a larger monitoring dataset the Council of Governments has been collecting in the Maryland portion of the Anacostia River watershed since the early part of this decade, making it one of the most robust datasets for trash for a waterway anywhere in the nation. Having this data is imperative to making future strategic decisions for implementation and informing development of new policies.

Our local partners have also been monitoring for microplastics in local waterways. Water samples collected in the Anacostia River by Anacostia Riverkeeper in 2019 contained microplastic concentrations as high as 696 microplastic particles per liter. To provide some perspective, samples taken in another highly urbanized river, the River Thames in London, England, revealed concentrations as high as 84.1 microplastic particles per liter.[93] A recent report from the Chesapeake Bay Program Scientific and Technical Advisory Committee revealed that microplastics are ubiquitous throughout the Chesapeake Bay and its watershed (which includes the District); however more research needs to be done on the potential ecological effects of microplastics. The report recommends conducting an ecological risk assessment looking at the effects on multiple species of importance to the bay and its watershed. A copy of the report can be found at: https:// pub-data.diver.orr.noaa.2ov/marine-debris/midatlantic/FINAL.STAC%20Re port Microplastics.pdf.

Multi-Pronged, Innovative Approaches

As with most environmental challenges, there is no "silver bullet" for eliminating the harm trash poses to our waterways and wildlife. The District has devised a plan that utilizes innovative policies, trash capture technologies, education, and outreach.

As mentioned previously, our monitoring efforts helped us to determine the most common types of trash found in our waterbodies. The District utilized monitoring data described previously to justify the need to reduce three of the most common types of trash found during sampling: single-use plastic bags, expanded polystyrene foam products (commonly referred to as Styrofoam™), and other food service ware.

In 2009, the District enacted the Anacostia River Clean Up and Protection Act (also known as the Bag Law) to create a five-cent fee on single-use plastic bags. Starting January 1, 2010, consumers in the District pay the fee at the time of purchase in a restaurant, grocery, liquor, or convenience store. DOEE employs an inspection team to ensure businesses are in compliance with the law. Revenue, fines, and other contributions generated by the law goes into the Anacostia River Clean Up and Protection Fund to pay for projects like trash capture devices, stream restoration, stormwater management projects, education, outreach, and administrative costs. In Fiscal Year 2019, the Department found that 77% of businesses inspected were in compliance with the law. More information on the District's Bag Law, including annual revenue and expenditure reports, are available at https;://doee.dc.gov/bugs.

[93] For more information, please go to the Anacoslia Riverkeeper story map, *Single-Use River: Microplastics in the Anacostia River*, at https://www.arcgis.com/apps/Cascade/index.html?appid=0ff0d351069a477c915570513f01d082.

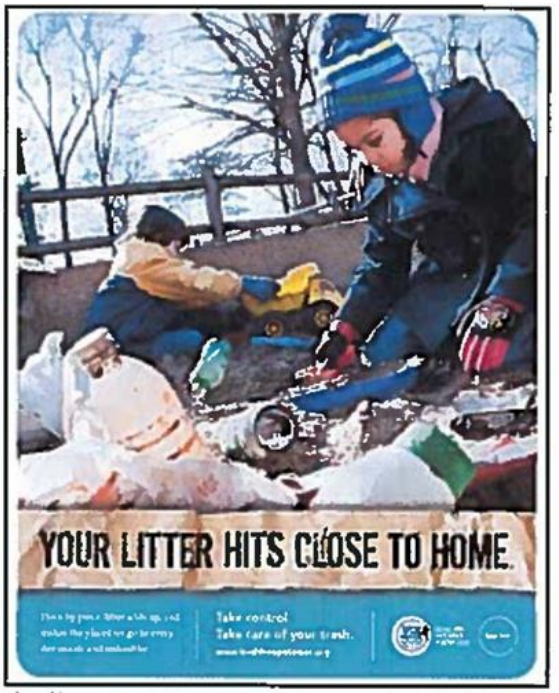
In 2014, the District enacted the Sustainable DC Omnibus Amendment Act, which includes restrictions on food service ware packaged and intended for consumption without further preparation. Specifically, a ban on food service ware made of Styrofoam™ (foam) took effect January 1, 2016, and the law requires food service ware to be made of recyclable or compostable materials starting January 1, 2017. Plastic straws were banned as part of the recyclable and compostable requirements in October of 2018. As with the Bag Law, DOEE inspects businesses to make sure they are in full compliance with the law. In fiscal year 2019, DOEE found that 97% of District businesses inspected were no longer using foam, and compliance rates with the new plastic straw and stirrer ban were rapidly increasing. Further information on the District's food service ware requirements is available at https://doee.dc.gov/foodseryiceware.

The District has also implemented innovative structural controls for capturing trash. Since 2009, the District has installed nine trash traps in the Anacostia River watershed. These traps have varied from proprietary products to custom devices designed by local non-profits. The pictures below display examples of the devices. These devices have primarily been funded by the Anacostia River Clean Up and Protection Fund and other local funding sources through grants to local nonprofits to design. install, and maintain these devices. The nonprofits also collect data on trash collected in the traps to further inform policy and management. Since installation of the first device in 2009, these devices together have helped capture and remove over 60,000 lbs of trash and debris from the Anacostia River and its tributaries.

Photo of a Bandalong™ liner trap designed and installed by Stormwater Systems of Cleveland, GA, along a tributary to the Anacostia River. The District has funded the installation and maintenance of nine structural controls like this one from funding sources such as the Anacostia River Clean Up and Protection Fund generated from the five cent fee on single -use plastic retail bags.

Lastly, the District has led many education and outreach activities over the years to change behavior. In 2010, DOEE funded the Alice Ferguson Foundation (AFF) to conduct a study of littering behavior that guided the development of an anti-littering campaign

throughout the District. The campaign's central message, *"Your Litter Hits Close to Home,"* was based on AFF's social marketing research that found people were most impacted by the effects litter has on their personal space, health, and well-being. Below is an example graphic from the anti littering campaign. Other local governments in the Potomac River watershed have adopted these campaign materials.



Example poster from the DOEE and Alice Ferguson Foundation Anti-Littering Campaign.

In closing, I want to again thank you and the Subcommittee on Water, Oceans, and Wildlife for your interest in this important subject. As the nation's capital, the District has an important role to play in restoring urban waterways. We have set the stage for reducing trash in our rivers and streams using multi-faceted, innovative approaches, but we are not done. We are truly alarmed by the potential impact of microplastics on our local waterways. Additional research is needed looking at the broader ecological effects of microplastics, including effects on living resources important to recreation and commerce.

I would encourage the subcommittee to peruse reports on our monitoring efforts. If you are interested in receiving copies, or have any other questions regarding our efforts to reduce trash in our waterways, please contact Matt Robinson of the DOEE Watershed Protection Division at matthew.robinson@dc.gov or (202) 442-3204.

Sincerely,
Tommy Wells
Director

SUBMISSION FOR THE RECORD BY REP. LOWENTHAL, STRATEGIES TO REDUCE THE GLOBAL CARBON FOOTPRINT OF PLASTICS

Jiajia Zheng and Sangwon Suh*

Over the past four decades, global plastics production has quadrupled [1]. If this trend were to continue, the GHG emissions from plastics would reach 15% of the global carbon budget by 2050 [2]. Strategies to mitigate the life-cycle GHG emissions of plastics, however, have not been evaluated on a global scale. Here, we compile a dataset covering ten conventional and five bio-based plastics and their life-cycle GHG emissions under various mitigation strategies. Our results show that the global life-cycle GHG emissions of conventional plastics were 1.7 Gt of CO2-equivalent (CO2e) in 2015, which would grow to 6.5 GtCO2e by 2050 under the current trajectory. However, aggressive application of renewable energy, recycling and demand-management strategies, in concert, has the potential to keep 2050 emissions comparable to 2015 levels. In addition, replacing fossil fuel feedstock with biomass can further reduce emissions and achieve an absolute reduction from the current level. Our study demonstrates the need for integrating energy, materials, recycling and demand-management strategies to curb growing life-cycle GHG emissions from plastics.

Global production of plastics grew from 2 Mt to 380 Mt between 1950 and 2015, at a compound annual growth rate of 8.4% (ref. [1]). Globally, 58% of plastic waste was discarded or landfilled, and only 18% was recycled in 2015 [1]. It is estimated that 4.8–12.7 Mt of plastic waste generated by coastal countries entered the ocean in 20103. Growing alongside the volume of global production and consumption of plastics are the diverse concerns on their impacts on the ecosystem and human health [4–7]. However, relatively little attention has been paid to their contributions to climate change. Although the chemical industry as a whole is responsible for about 15% of global anthropogenic GHG emissions [8], the magnitude of global life-cycle GHG emissions from plastics has yet to be quantified.

Various strategies to reduce GHG emissions from plastics have been discussed in the literature, such as replacing fossil fuel-based plastics with bio-based plastics [9–11]. Bio-based plastics generally show lower life-cycle GHG emissions than their fossil fuel-based counterparts12. It is estimated that substituting 65.8% of the world's conventional plastics with bio-based plastics would avoid 241–316 MtCO2-equivalent (CO2e) yr–1 (ref. [13]). Both biodegradable and non-biodegradable forms of bio-based plastics are available on the market [14]. Bio-based non-biodegradable polymers such as bio-polyethylene (bio-PE) and bio-polyethylene terephthalate (bio-PET), also referred to as 'drop-in' polymers, offer virtually identical properties to their fossil fuel-based counterparts. However, bio-based biodegradable polymers, such as polylactic acid (PLA), polyhydroxyalkanoates (PHAs) and thermoplastic starch (TPS), display different mechanical and chemical properties [12]. Strategies to promote bio-based plastics have been initiated by the European Commission and countries such as Japan, Korea and Thailand [15, 16]. In 2017, the total global production of bio-based plastics reached 2.05 Mt, and is projected to grow by 20% over the next five years [17].

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Low-carbon energy is another strategy to reduce the life-cycle GHG emissions of plastics. Under a 100%-renewable-energy scenario, the GHG emissions from US plastics production could be reduced by 50–75% (ref. [18]). Another strategy to reduce the GHG emissions from plastics is recycling, which reduces, in part, carbonintensive virgin polymer production [19] while preventing GHG emissions from some end-of-life (EoL) processes such as incineration [20].

However, the literature so far has focused on a subset of plastic types, mitigation options or geographical locations in isolation [18, 21]. Here, we develop a dataset that covers GHG emissions from resin production, conversion and EoL processes for ten fossil fuel-based and five bio-based plastics. We then integrate the dataset with projections of global plastics demand and GHG mitigation strategies. We evaluate the following mitigation strategies and their combinations:

1) Bio-based plastics. Fossil fuel-based plastics are gradually substituted by bio-based plastics until they are completely phased out by 2050. Although bio-based plastics can be derived from a variety of feedstocks, here we model corn and sugarcane given their dominance in the current market [11].
2) Renewable energy. The energy mix of the plastics supply chain is gradually decarbonized and reaches 100% renewables (that is, wind power and biogas) by 2050. Emissions under the current energy mix are modelled for comparison.
3) Recycling. Recycling rates of EoL plastics gradually increase and reach 100% by 2050. For comparison, we also model the emissions under a projected EoL management mix scenario and a 100% incineration/composting scenario.
4) Reducing growth in demand. The current annual growth rate of global plastics demand (4%) is reduced to 2%.

We examine these strategies as illustrative scenarios, rather than as realistic projections of future trajectories, with the purpose of envisioning their potentials for GHG mitigation. We acknowledge that achieving 100% recycling or renewable energy may be neither practical nor economically feasible in reality. Details on these scenarios can be found in Supplementary Table 1.

Our analysis shows that conventional (fossil fuel-based) plastics produced in 2015 emitted 1.8 GtCO2e over their life cycle, excluding any carbon credits from recycling (Figure 1). The amount corresponds to 3.8% of the 47 GtCO2e emitted globally that year [22]. The resin-production stage generated the majority of emissions (61%), followed by the conversion stage (30%). Of all plastic types, polyester, polyamide and acrylic (PP&A) fibres had the highest GHG emissions in both stages. The polyolefin family (polypropylene, PP; low-density/linear low-density polyethylene, L/LLDPE; and high-density polyethylene, HDPE), which accounts for nearly 50% of the world's plastics consumption, was also a significant contributor. GHG emissions from bio-based plastics are not considered for 2015 given their negligible market share (<1%).

The EoL stage accounted for 9% of total life-cycle emissions, excluding the carbon credits from recycling. Incineration was the dominant source of GHG emissions among EoL processes. Landfill generated the least GHG emissions, although the process handles the largest share of plastic waste (58%). The recycling process itself generated 49 MtCO2e. However, if the displacement of carbon-intensive virgin polymer production by recyclates is

considered, the GHG emissions of recycling would go down to negative 67 MtCO2e, and the total emissions from the EoL stage would be reduced from 161 MtCO2e to 45 MtCO2e. In this case, the total global life-cycle GHG emissions of plastics become 1.7 GtCO2e, or 3.5% of the global annual GHG emissions in 2015.

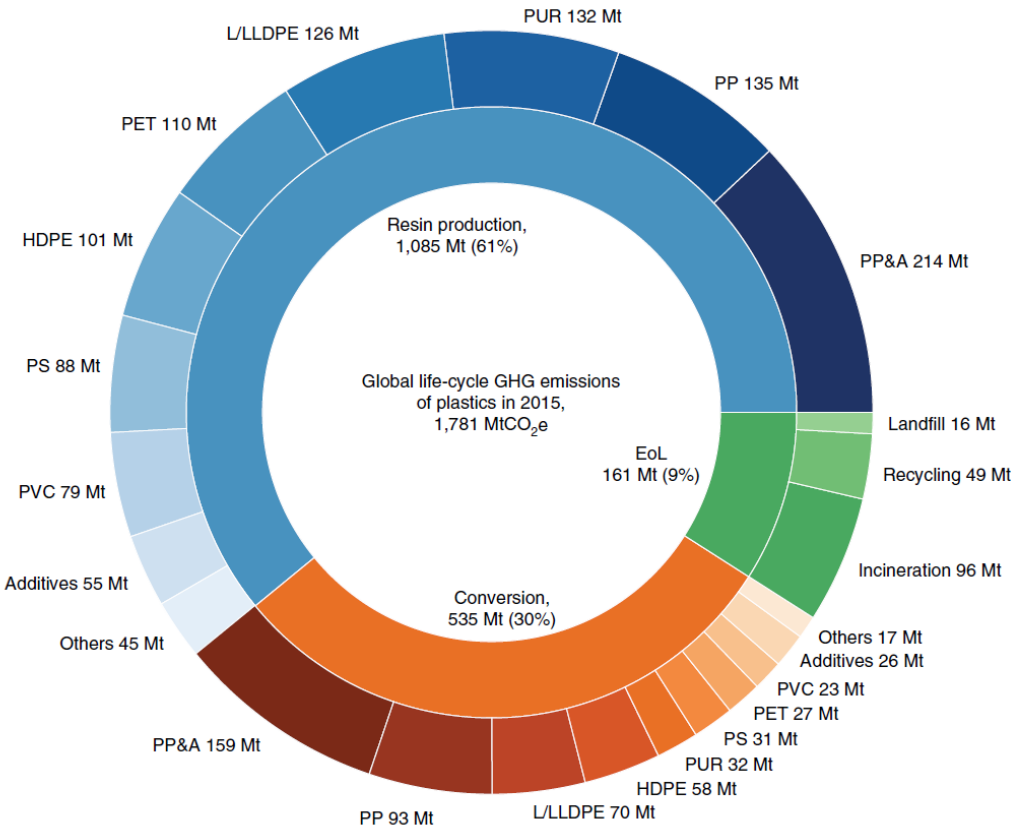

Figure 1. Global life-cycle GHG emissions of conventional plastics in 2015 by life-cycle stage and plastic type. Carbon credits generated by recycling are not included. Blue, orange and green represent the resin-production, conversion and EoL-management stages, respectively. The emissions from each stage are broken down by plastic type or EoL-treatment method, indicated with different shades of the corresponding colour. PUR, polyurethane.

Under the current trajectory, the global life-cycle GHG emissions from plastics are poised to grow rapidly (Figure 2a). The global economy produced 407 Mt of plastics in 2015, with an average annual growth rate of 4% between 2010 and 2015 [1]. Following this trend, annual plastics production is expected to grow to 1,606 Mt by 2050, and the life-cycle GHG emissions are expected to grow from 1.7 GtCO2e in 2015 to 6.5 GtCO2e in 2050, using the projected EoL-management mix change1, and maintaining the current energy mix (the baseline is the blue solid line in Figure 2a). If all plastic waste is incinerated by 2050, total annual emissions will reach 8.0 GtCO2e (a 22% increase from the baseline). Recycling all plastic waste, however, would reduce the emissions to 4.9 GtCO2e by 2050 (a 25% reduction from the baseline).

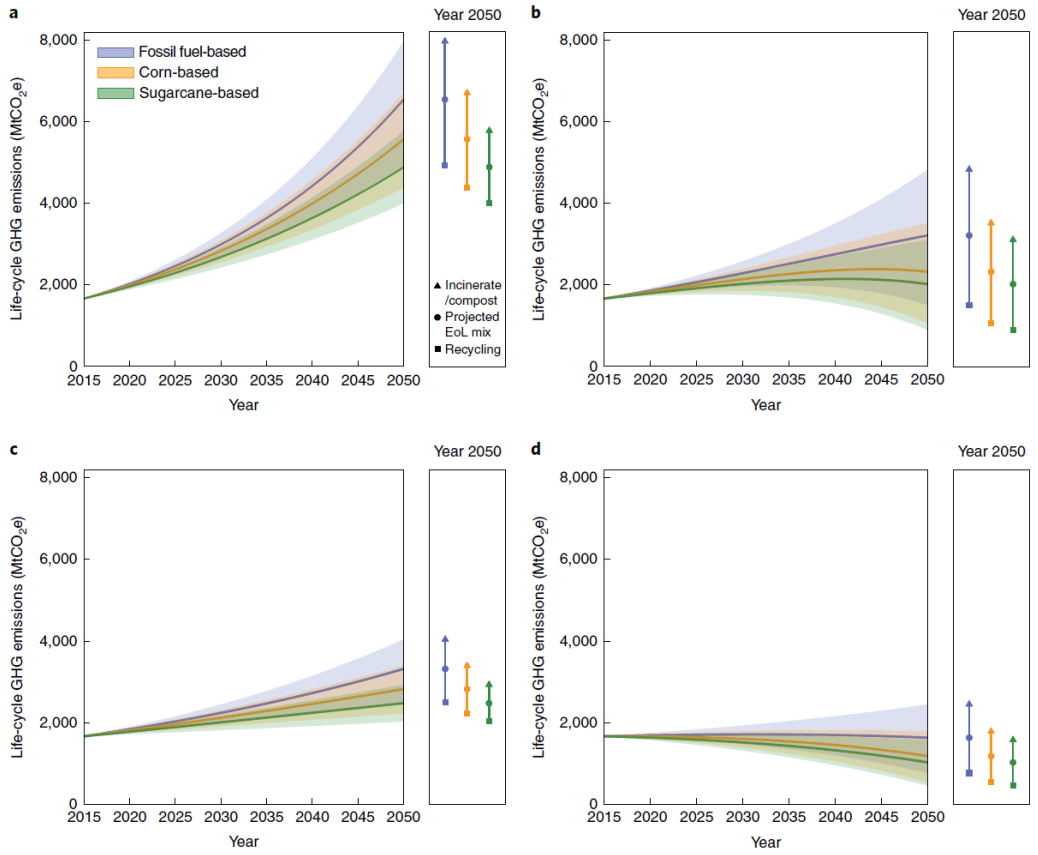

Figure 2. Global life-cycle GHG emissions of plastics under scenarios of different feedstock sources, energy mixes, EoL management strategies and growth in plastics demand for 2015–2050. a, Plastics demand grows at 4% yr−1 under the current energy mix. b, Plastics demand grows at 4% yr−1, and the energy mix decarbonizes by 2050. c, Plastics demand grows at 2% yr−1 under the current energy mix. d, Plastics demand grows at 2% yr−1, and the energy mix decarbonizes by 2050. Solid lines represent the projected EoL-management mix (Supplementary Table 10); whereas shaded areas represent ranges due to EoL options. The bars on the right side of each panel represent ranges due to different EoL options in 2050.

With a plastics demand growth rate of 4% yr−1, it has been estimated that a complete replacement of fossil fuel-based plastics with corn-based plastics would reduce global life-cycle GHG emissions of plastics to 5.6 GtCO2e by 2050 under the current energy mix and the projected EoL mix, which is 1.0 GtCO2e (or 15%) less than the baseline (Figure 2a). If all EoL drop-in plastics are incinerated and all EoL biodegradable plastics are composted, global life-cycle GHG emissions of corn-based plastics would increase to 6.7 GtCO2e. Recycling all EoL bio-based plastics, however, would reduce the emissions to 4.4 GtCO2e. Sugarcane-based plastics can further reduce global life-cycle GHG emissions of plastics to 4.9 GtCO2e, which is 1.7 GtCO2e (or 25%) less than the baseline, with a range between 5.8 GtCO2e (100% incineration/composting) and 4.0 GtCO2e (100% recycling). A 100% recycling scenario for fossil fuel-based plastics in our model results in similar, or even lower, emissions compared to bio-based plastics with the projected EoL mix (Figure 2a,b, sidebars). This

implies that the recycling of conventional plastics may be as beneficial as using renewable feedstock.

An energy decarbonization scenario shows substantial potential to reduce GHG emissions (Figure 2b,d). On average, switching to 100% renewable energy would reduce life-cycle GHG emissions from plastics by 62% in 2050, assuming 4% yr−1 growth in demand. Even if fossil fuel sources (petroleum, natural gas and coal) serve as the sole feedstock for future plastics production, using 100% renewable energy can achieve 51% reduction (projected EoL mix) compared to the baseline, although the absolute total emissions would double the 2015 level by 2050. However, recycling all EoL plastics under 100% renewable energy allows 77%, 84% and 86% reductions in life-cycle GHG emissions from fossil fuel-, corn- and sugarcane-based plastics, respectively. This result shows that absolute reduction of emissions can only be achieved by combining aggressive deployment of renewable energy and extensive recycling of plastics.

Reducing plastics demand growth rate from 4% to 2% yr−1 reduces emissions by 56% (under the current energy mix) to 81% (under low-carbon energy) relative to the baseline in 2050 (Figure 2c,d). Using 100% renewable energy keeps the emissions virtually constant at the 2015 level for fossil fuel-based plastics with projected EoL mix, and replacing them with bio-based ones brings the emission levels down further. Among all the scenarios tested, the global life-cycle GHG emissions of plastics were the lowest under the 100% sugarcane-based plastics with 100% renewable energy combined with 100% recycling and reduced demand growth, which achieved 0.5 GtCO2e yr–1, or 93% reduction from the baseline. This demonstrates that a drastic reduction in global life-cycle GHG emissions of plastics would be possible in a technical sense, but it would require implementing all of the four strategies examined at an unprecedented scale and pace.

Figure 3 shows the breakdown of GHG emissions by life-cycle stage, for each kilogram of plastics derived from different feedstock types. The total life cycle GHG emissions for fossil fuel-based, corn-based and sugarcane-based plastics are on average 4.1, 3.5 and 3.0 kgCO2e per kg plastic in 2050, respectively, under the current energy mix (Figure 3a). Under a 100%-renewable-energy scenario, however, the average life-cycle emissions will be reduced to 2.0, 1.4 and 1.3 kgCO2e per kg plastic, respectively (Figure 3b). Plastics derived from renewable feedstock (assuming projected EoL mix) generate lower GHG emissions over the whole life cycle compared to their fossil fuel-based counterparts regardless of the energy system used.

The resin-production and conversion stages are major contributors to the life-cycle GHG emissions of all feedstock types under the current energy mix (Figure 3a). However, under the 100% renewable-energy scenario, incineration becomes the largest contributor to the total emissions for bio-based plastics (Figure 3b). Under the 100%-renewable-energy scenario, recycling generates fewer carbon credits, as the low GHG emissions of renewable energy undercut the carbon benefits of avoiding virgin polymer production.

In summary, our results show that none of the four strategies— namely bio-based plastics, renewable energy, recycling and demand management—can achieve sufficient GHG mitigation for absolute reduction below the current level on their own; only when implemented in concert can these strategies achieve the much-needed absolute reduction. Among them, decarbonization of the energy system—which is an economically more favourable option for GHG mitigation compared to the use of bio-based plastics [18]—shows the greatest potential. Even if fossil fuel feedstock is used as the sole source for plastics

production, a 100%-renewable-energy scenario will reduce the average life-cycle GHG emissions by half from the baseline emissions. If combined with extensive recycling or demand management, decarbonization of energy can maintain the current level of GHG emissions until 2050. Reducing GHG emissions even further to achieve absolute reduction from the current level requires large-scale adoption of bio-based plastics in addition to implementing all of the other three strategies examined.

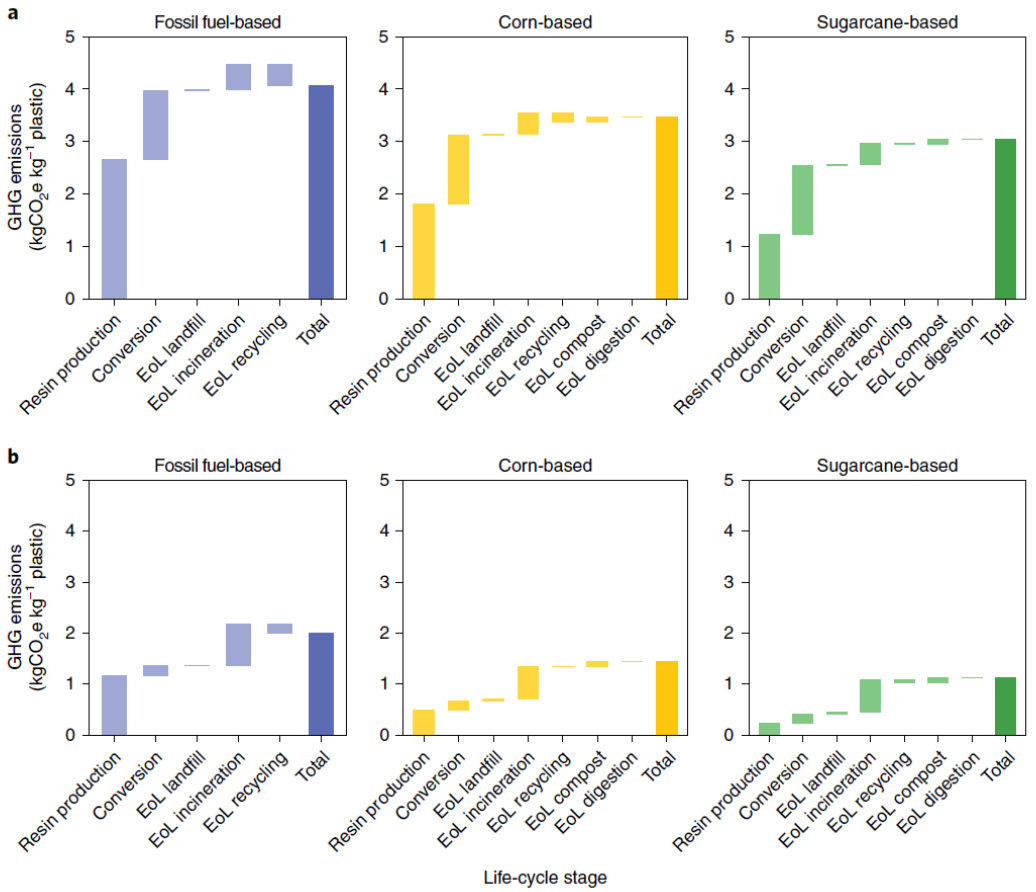

Figure 3. GHG-emissions breakdown by life-cycle stage of plastics derived from different feedstock types under two energy-mix scenarios in 2050. a, GHG emissions under the current energy-mix scenario in 2050. b, GHG emissions under a 100%-renewable-energy scenario in 2050. Emissions results are based on the scenario with a 4% yr–1 growth rate for plastics demand and the projected EoL-management mix (Supplementary Table 10). Carbon credits generated by recycling are considered.

Going forward, we see both opportunities and challenges in reducing the life-cycle GHG emissions of plastics. The current global average plastics recycling rate of 18% (ref. 1) certainly presents substantial room for further improvement. The low price of fossil fuel-based plastics, however, is a key barrier to dramatically increasing recycling rates. Together with technological innovations in plastics recycling, fiscal policies, such as carbon pricing and incentivising recycling infrastructure expansion, should be considered to overcome such barriers [23, 24].

Replacing fossil fuel-based plastics with bio-based plastics is shown to play an important role in GHG mitigation. Nevertheless, our results show that the emissions of bio-based plastics are highly dependent on the EoL-management method chosen. Composting or incinerating bio-based plastic waste, for example, showed similar or even higher GHG emissions than the scenario in which 100% fossil fuel-based plastics were used under the projected EoL mix in 2050. Moreover, EoL management of bio-based—especially biodegradable—plastics requires systematic changes such as separate collection and recycling infrastructure, since inclusion of biodegradable plastics in the mix of conventional plastic waste can affect the quality of the recyclates [25]. Furthermore, composting of biodegradable plastics in home composting conditions or natural environments is much less effective than in industrial composting facilities [14]. Finally, the land-use implications of a large-scale shift to bio-based plastics require further research. In 2017, land use for bioplastics was reported to be 0.82 million hectares (or 0.016% of global land area), which would increase to 0.021% in 2022 under the projected market growth [17]. A complete shift of the plastics production of approximately 250 million tonnes to bio-based plastics would require as much as 5% of all arable land [26], which, depending on where they take place, may undermine the carbon benefits of bio-based plastics. The use of lignocellulosic or waste biomass as feedstock, and growing material crops in fallow lands, would alleviate the pressure of cropland expansion and associated GHG emissions from land-use change.

Our study shows that an aggressive implementation of multi-layered strategies would be needed in order to curb the GHG emissions from plastics. GHG-mitigation strategies are often implemented within energy, materials, waste-reduction and management policies in isolation. Our results indicate that absolute reduction in life-cycle GHG emissions of plastics requires a combination of the decarbonization of energy infrastructure, improvement of recycling capability, adoption of bio-based plastics and demand management.

Online Content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41558-019-0459-z.

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Author Contributions

J.Z. performed the research and analysed the data. S.S. conceived the idea and designed the study. Both authors wrote the manuscript.

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The authors declare no competing interests.

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[LIST OF DOCUMENTS SUBMITTED FOR THE RECORD RETAINED IN THE COMMITTEE'S OFFICIAL FILES]

Submission for the Record by Rep. Cunningham

— Contracted Report to NOAA 2019, The Effects of Marine Debris on Beach Recreation and Regional Economies in Four Coastal Communities: A Regional Pilot Study

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Chapter 125

CLEANING UP THE OCEANS: HOW TO REDUCE THE IMPACT OF MAN-MADE TRASH ON THE ENVIRONMENT, WILDLIFE, AND HUMAN HEALTH*

THURSDAY, SEPTEMBER 26, 2018
U.S. SENATE,
COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS,
Washington, DC.

The committee met, pursuant to notice, at 10:05 a.m. in room 406, Dirksen Senate Office Building, Hon. John Barrasso (chairman of the committee) presiding.

Present: Senators Barrasso, Carper, Inhofe, Boozman, Wicker, Fischer, Rounds, Ernst, Sullivan, Cardin, Whitehouse, Gillibrand, Booker, and Van Hollen.

OPENING STATEMENT OF HON. JOHN BARRASSO, U.S. SENATOR FROM THE STATE OF WYOMING

Senator BARRASSO. I call this hearing to order.

Today the Committee will examine the issues of man-made trash that is polluting the oceans, also known as marine debris.

While marine debris can range from metals, glass, rubber, paper, and textiles, the vast majority of marine debris is plastic.

Plastic plays an incredibly important role in our lives. As a doctor, I have seen firsthand how plastic can be indispensable in the field of medicine and healthcare. Similarly, plastic is crucial in virtually every aspect of modern society and economy, including the field of environmental protection. This doesn't mean, of course, that plastics should end up in our rivers, in our lakes, in our streams, and in our oceans.

* This is an edited, reformatted and augmented version of Hearing before the Committee on Environment and Public Works, United States Senate, One Hundred Fifteenth Congress, Second Session, Publication No. S. HRG. 115–385, dated September 26, 2018.

It is estimated that around 8 million metric tons of plastic waste ends up in the world's oceans each year. While some of this plastic is dumped directly into the ocean, like discarded fishing nets, most of the plastic flows from rivers.

Experts believe that about 90 percent of all plastic pollution flows into the oceans from just ten rivers, eight of which are in Asia. It is estimated that Asia contributes to about 80 percent of all ocean plastic. Specifically, China, Indonesia, the Philippines, Thailand, and Vietnam are responsible for more plastic pollution than the rest of the world combined.

By now, many Americans have heard of the Great Pacific Garbage Patch. This is an area in the Pacific Ocean where currents have concentrated plastic and other man-made trash. It now stands as the world's largest concentration of marine debris.

Similar debris concentrations exist elsewhere throughout the world. In fact, plastic has been found in almost all corners of the ocean.

Plastic takes at least 450 years to degrade completely; sometimes much longer than that. In the meantime, the debris will continue to entangle and kill marine wildlife, as well as threaten human health. If little is done to stem the accumulation of plastic in the ocean, experts believe that by 2050 there will be more plastic in the ocean than fish, as measured by weight.

Earlier this summer, National Geographic dedicated its June issue to this problem. It is a remarkable cover where it looks like it is an iceberg, but actually it is an upside-down plastic bag. And I don't know is responsible for this, but Senator Whitehouse and I both had this on our desks in our offices and pointed to this as he and I were talking about another issue.

I think it is a remarkable effort by National Geographic to display in picture form something that we know is a major problem affecting——

Senator WHITEHOUSE.

[Comment made off microphone.]

[Laughter.]

Senator BARRASSO. I defer to my colleague from Rhode Island.

Senator WHITEHOUSE. Isn't this bipartisanship?

[Laughter.]

Senator WHITEHOUSE. Here is a visual demonstration.

Senator BARRASSO. Well, it says, "Some people see ocean plastic as a looming catastrophe worth mentioning in the same breath as climate change." I have it from the plastic standpoint. I am glad, Jonathan, that you, Dr. Baillie, National Geographic's Executive Vice President and Chief Scientist, are able to join us here today.

Environmentalists aren't alone in recognizing this problem. Plastic manufacturers also acknowledge we need to address this problem.

Earlier this summer, another one of our witnesses today, Cal Dooley, announced that he would extend his tenure atop the American Chemistry Council, as he said, to "fight the spread of mismanaged plastic waste and help lay the foundation for a sustained global industry effort to address it." Thank you.

Likewise, Coca-Cola, which is also represented here today and one of the world's biggest producers of plastic bottles, has taken steps to confront the problem. In January this year, Coca-Cola announced that it would "help collect and recycle a bottle or can for everyone it sells by 2030."

Today the Committee will want to hear what more can be and should be done. Specifically, we want to know what private industry, what local and State governments, what

the Federal Government, and what international institutions should help do to address the crisis.

I would like to point out that today's hearing follows one that Senator Sullivan held as Chairman of the Subcommittee on Fisheries, Water, and Wildlife in May 2016. It also follows the efforts, as well, as those of Senator Whitehouse to pass the Save Our Seas Act, which I understand is under consideration as we speak.

I want to thank them for their leadership on this issue.

I am going to give each of you an opportunity to say what you would like, a few words, about that after I turn to Ranking Member Carper. This issue is very important to the Ranking Member, to whom I now turn for his opening comments.

OPENING STATEMENT OF HON. THOMAS R. CARPER, U.S. SENATOR FROM THE STATE OF DELAWARE

Senator CARPER. Thanks so much, Mr. Chairman. Thank you very much for holding this hearing.

I really want to say to Sheldon and to Dan, thank you for your wonderful leadership. Thank you for demonstrating how we can work across the aisle on really important issues. I think we are going to make some real progress, including today. So, thank you both.

This is actually a timely issue for us in Delaware. Every year for 31 years we have done something called the Annual Delaware Coastal Cleanup. We start just north of Ocean City, Maryland all the way up to Pennsylvania, about 100 miles, and we pick up trash, all kinds of trash. This is a photograph that was taken at Fox Point State Park, not too far from actually Martha and I live in Wilmington.

We had a lot of kids who showed up. There were scouts and there were people who were just there for a good time and, frankly, for a good cause, and we netted about four tons of trash going up and down the Delaware coast on Saturday.

You might not believe the types of items we cleanup. No, you probably would believe it. From tennis balls, plastic water caps, lip balm containers, a metal baseball bat, and a whole lot more. Four tons is a massive amount of marine debris, and that is just from one cleanup weekend along Delaware's 100-mile coast.

Those numbers pale in comparison to the amount of trash in our oceans. As the Chairman has already mentioned, the infamous Great Pacific Garbage Patch, the largest mass of marine debris floating in the ocean, is over 300 times the size of Delaware and nearly the size of Alaska.

If you were able to lift Alaska up off the face of the earth and put one end of it on the U.S.-Canadian border, the other end would stretch all the way to Mexico. Imagine that. There is a massive marine debris floating in the ocean that is that big.

As we all know, all this debris has serious impacts on our water quality, on our wildlife, on our food chain, and while the extent of its impacts are not fully known, we know that hundreds of species interact with plastics. Plastic consumption can harm wildlife and all states of life. Recent research suggests it can also decrease reproduction rates. We also know that tiny plastic particles called microplastics may be present in our drinking water and in the food that all of us consume.

Cleanup efforts like the Delaware Coastal Cleanup provide hands-on opportunities for citizens of all ages to learn about this global problem and to contribute to the solution. Not just talk about it, not just worry about it, but to do something about it. However, most environmental experts agree that stopping debris from ending up in our waters in the first place is more of an urgent priority.

We thank all of our witnesses for coming today. We are going to hear about potential solutions from our esteemed panel this morning.

As Co-Chair and Co-Founder of the Bipartisan Senate Recycling Caucus, along with my Republican partner, Senator John Boozman, I want to mention recycling is one such solution.

Delaware is a little State. It doesn't have a whole lot of space for landfills, so we had to get serious not too long ago about recycling. As Governor, I signed two executive orders to improve and promote recycling. The first established a citizens' workgroup on recycling to evaluate recycling in our State. The second established a goal of a 30 percent diversion rate for recyclables from Delaware's solid waste stream.

Delaware's recycling activities continue to grow with the implementation of the universal recycling law in 2010, which eventually led to curbside recycling collection for all single-family households and commercial businesses. These practices work for both our environment and for our economy.

I am proud of our State's work, but while Delaware has made some strides, good strides, other States struggle. I will just say it is a mixed bag. I think we were late to the game. Other States were a little bit ahead of us. But we are making great strides now. Some other States and communities, frankly, aren't doing their share; they are not doing enough. Maybe we can inspire them.

In many places it is cheaper to dispose of recyclable materials in landfills. These items can then make their way into our waters, unfortunately. This problem worsened when China announced earlier this year that it would no longer accept plastic waste from other countries to convert into new plastic-containing products.

Why is that a big deal? Because China was previously taking 30 percent of U.S. plastic waste for recycling. We, as a Nation, will need to invest in better waste management and recycling infrastructure to address challenges like this. We also need to find creative ways to finance these investments. Further, we may want to consider proposals to incentivize the use of recyclable plastics for manufacturing purposes.

All that said, the Federal Government cannot undertake this effort alone. In the last several years, corporations and industry partners have stepped up and really led the way. Good for you.

To our witnesses today testifying on behalf of these partners, we are truly grateful for your work and for the commitments that you have already made to recycling and help prevent debris from entering our oceans.

Agreeing on solutions and figuring out how to implement them will not be easy, but I am encouraged by the strong bipartisan support and leadership of our two colleagues from Alaska and from Rhode Island. With their continued resolve and with the help of the rest of us, I believe we can put our heads together, put our hands together and make a real difference on this issue, and I look forward to doing so.

Thank you one and all.

Senator BARRASSO. Thank you, Senator Carper.

Senator Sullivan, anything you would like to say?

OPENING STATEMENT OF HON. DAN SULLIVAN, U.S. SENATOR FROM THE STATE OF ALASKA

Senator SULLIVAN. Thank you, Mr. Chairman and Ranking Member Carper, for holding this very important hearing today. This is important for the Country, for the world, for Rhode Island, for New Jersey. It is certainly important for my State, Alaska, which has more coastline than the rest of the lower 48 States combined.

The prevalence of marine debris on our shores is a chronic issue. As noted, this marine debris results from a number of man-made sources, including derelict fishing gear, poor solid waste management practices, major storm events, and everyday litter.

But, as the Chairman mentioned, this is a preventable issue. Of the plastics that enter the oceans from land, more than half comes from just five developing countries. In Asia, ten river systems, eight in Asia and two in Africa, contribute almost 90 percent of land-based ocean plastics. To me, this presents a huge opportunity to curb this issue at its source globally.

I want to emphasize what has already been stated, but for the media covering this hearing, hold your breath. This is a fiercely bipartisan issue.

[Laughter.]

Senator SULLIVAN. It does happen here. Matter of fact, it happens quite a lot. Senator Whitehouse, I am going to talk about all the great work he has done. Senator Booker also has been a huge champion of this.

To just give you a little sense of the work that has been done, this past year, Senator Whitehouse and I have engaged early and often with the EPA, the Commerce Department, the U.S. Trade Representative's Office, the State Department, the American Delegation to the G7, other countries in the G7, countries in the G20; and what has resulted is a growing strong commitment to pursue marine debris prevention goals through future international trade agreements and development aid agreements.

This is an important step forward, actionable step to impact curbing this man-made plight on our oceans that we all agree is a big problem.

Last Congress, Senator Whitehouse and I, in this Committee, as Chair and Ranking Member of the Wildlife Subcommittee, held a hearing on the issue of marine debris. Much of which came out of that hearing is now in our Save Our Seas Act, the SOS Act, which I am happy to report we think is going to be hot-lined and passed today, we hope, in the Senate.

It has already passed once. The House liked it so much they added a bunch of other elements to it, and we are going to try to repass it again here in the Senate today.

This bill would serve to strengthen the Federal response capabilities to marine debris disasters, combat land-based marine debris resources, and encourage interagency coordination in stemming the tide of ocean plastics and, importantly, encourage the Trump Administration to pursue international agreements with regard to this challenge. And I think, talking to the senior members of the Administration, they are already there, so we are hopeful this is going to become law soon.

Senator Whitehouse and I are also talking about an SOS 2.0 bill, and I know Senator Booker is interested in that as well. It is my hope that this hearing will help provide ideas and momentum for the goals on what we think would be a good followup bill.

Finally, again in the spirit of bipartisanship, last night I had the honor of presenting the International Conservation Caucus Foundation, the ICCF, Teddy Roosevelt International

Conservation Award to Senator Whitehouse at an annual gala event. Although to make sure it stays a little partisan, I was glad to note that this was an award named after one of America's great Republican presidents.

Thank you again, Mr. Chairman, for holding his hearing and giving me an opportunity to speak on this issue. We look forward to a very good, robust discussion today.

Senator BARRASSO. Thank you, Senator Sullivan. Congratulations, Senator Whitehouse. The floor is yours.

OPENING STATEMENT OF HON. SHELDON WHITEHOUSE, U.S. SENATOR FROM THE STATE OF RHODE ISLAND

Senator WHITEHOUSE. Thank you, Chairman Barrasso. I will note that Senator Sullivan, my friend and colleague, was quite restrained last night in his comparisons of the relative size of his State and mine, which I thought was a kindness certainly appreciated by the Senator from Rhode Island.

Let me first thank you, Chairman, for holding this hearing. You and the Ranking Member have been very great to work with. I appreciate your focus on this. It is, I think, a really productive opportunity for us and I am grateful to you.

I want to also thank you and the former Chairman, Senator Inhofe, sitting beside you, because both of you have been able to overcome the disability of living in landlocked States in order to take a very positive interest in the marine debris problem.

I want to particularly thank Senator Inhofe, who became an original co-sponsor of the SOS bill that Dan and I worked on. I appreciate his support and leadership for it. Senator Inhofe is a powerful legislator, and when he puts his shoulder behind something, it tends to happen, so I give him a lot of credit for his support for our Save Our Seas bill.

Senator Sullivan has been an incredible partner in all of this, and I want to pay a lot of respect to him for his work. We wouldn't have even had the original hearing had Senator Sullivan not been able to successfully negotiate with the Commerce Committee, particularly the Fisheries Subcommittee of the Commerce Committee, to allow this to go forward, because there are turf issues involved.

Fortunately, the chairman of the Fisheries Subcommittee of the Commerce Committee is also Dan Sullivan, so he was able to have that conversation with himself and reach an agreement to go forward in the Environment and Public Works Committee and have that hearing. It is from that hearing that the interest of Senator Inhofe and others was provoked, and from that hearing that the SOS bill went forward.

We do expect that it will pass the Senate by unanimous consent again today, with some of the additions that our friends who see a bill moving want to take an opportunity to add things to. That has been what has slowed it down. It has not been a lack of enthusiasm for the underlying bill; it has been other people saying, wow, something good is happening, let's see if I can get my thing on it.

So it has been a very, very positive experience and Dan's leadership has been phenomenal not only legislatively, but also with pushing really hard on the Administration to make this a policy priority in the Administration. He has been harassing the trade

representative, the White House, the Department of Commerce. He has been very, very energized, and I appreciate that very much.

I also want to express my appreciation to our former colleague in Congress, Cal Dooley, who is here for the American Chemistry Council, and I would like to put into the record the press release that the American Chemistry Council put out when it announced the extension of Mr. Dooley's tenure.

The reason I want to put it into the record is that in one small page it has four separate mentions of how important the American Chemistry Council thinks solving the marine debris problem is and very strong personal statements of commitment and determination by Mr. Dooley, so I think that puts us in a very good opening position.

Senator BARRASSO. Without objection, submitted. [The referenced information follows:]

DOOLEY TO REMAIN AT ACC THROUGH THE END OF 2019

WASHINGTON (June 11, 2018) – The American Chemistry Council (ACC) today announced that Cal Dooley has agreed to delay his retirement and extend his tenure as President and CEO through 2019. In April, Dooley announced his intention to retire at the end of 2018. The decision to delay retirement comes after ACC's Annual Meeting which took place June 4th through June 6th in Colorado Springs, CO, where the board of directors agreed that the chemicals and plastics industry must take a global leadership role to reduce and ultimately eliminate plastic waste.

"Cal's leadership at ACC has been essential to the industry's success in recent years. As ACC members embark on an effort to reduce and eliminate plastic waste in the years to come, the ACC officers felt strongly that Cal's experience and leadership were essential to aligning the global industry around a coordinated strategy," said Bob Patel, ACC Chairman and Chief Executive Officer of Lyondell Basell. "With a little arm twisting and agreement from his gracious wife Linda, we were able to convince Cal to stay on to lead the development of this critical effort."

"The global chemicals and plastics industry has an imperative to fight the spread of mismanaged plastic waste that is increasingly littering our rivers, oceans and landscapes. While plastic products provide countless health, safety, lifestyle and sustainability benefits, those benefits cannot be fully realized unless we take swift and aggressive actions to make the most of all resources and leverage technology to dramatically increase rates of reuse, recycling and recovery of all plastic products," said Dooley. "Ending plastic waste is an issue of personal, as well as professional interest, and I am excited to help lay the foundation for a sustained, global industry effort to address it."

Korn Ferry International was retained to conduct the search for Dooley's replacement when his retirement was announced in April 2018. With Dooley's announcement today, Korn Ferry's efforts have been suspended, but they will resume their search in mid-2019.

* * *

Senator WHITEHOUSE. We clearly need to do things to clean up our oceans and to clean up the rivers; there are a few of them that are flowing this into our oceans. We have a map here of some of the places around the world which are the top 10 sources, as you will see. They focus on the Pacific, which is one of the reasons that Senator Sullivan has been so strong on this and Senator Murkowski has pledged to work on this through our Oceans Caucus as well.

Senator Carper showed the beach cleanup in Delaware. We do beach cleanups in Rhode Island as well. We do our beach cleanups with trash bags. Senator Sullivan and Senator Murkowski have beach cleanups in Alaska where they have to use front-end loaders, dumpsters, barges because they are on a very, very burdened Pacific coast.

So, it is a few countries and it is a few rivers that are the main sources, and we can do a lot to try to clean that up through trade policy treaties and simple public shaming and friendly persuasion.

We also need to work, and this is where the American Chemistry Council will come in so strongly, on trying to find ways to actually have plastic biodegrade in the oceans. It breaks down into smaller and smaller bits, but it doesn't actually biodegrade into natural elements. It can do that often in a landfill because the composting heat will help it break down, but in the ocean that doesn't happen; and we need to do research in order to find products that will allow that to happen without undercutting the fundamental value of plastic, which is that it lasts a bit.

We need to worry about entanglements and try to help our fishermen cleanup the oceans as they are out there. We see too much marine life dying from ghost fishing gear that still sweeps the ocean and kills, but with no gain to anyone because nobody ever recovers it.

Finally, I think we need to understand the consequences for human health of plastic at the micro level beginning to get into the human diet in a way that the human species has never experienced before through our long history. We have eaten a lot of things through our long history, but it has all been stuff that fundamentally came back to certain natural elements. To have microscopic plastic now in our diet is something new that we need to undertake health research into.

So, I appreciate this going forward and I thank very much my friend, Senator Sullivan, for what a superb leader and partner he has been on this, and I look forward to working with him productively on SOS 2.0, along with all who are present here today. Thank you.

Senator BARRASSO. Thank you very much, Senator Whitehouse, for your leadership.

Thank you, Senator Sullivan.

We now will hear from our witnesses. Today we are joined by four: Dr. Jonathan Baillie, Executive Vice President and Chief Scientist of the National Geographic Society; Hon. Cal Dooley, President and Chief Executive Officer of the American Chemistry Council; Mr. Bruce Karas, who is Vice President of Environment & Sustainability at Coca-Cola North America; and Dr. Kara Lavender Law, who is Research Professor of Oceanography at the Sea Education Association.

I want to remind the witnesses your full written testimony will be made part of the official hearing record today. Please keep your statements to 5 minutes so we will have plenty of time for questions.

I look forward to hearing the testimony and I would like to begin with Dr. Baillie.

STATEMENT OF JONATHAN BAILLIE, EXECUTIVE VICE PRESIDENT AND CHIEF SCIENTIST, NATIONAL GEOGRAPHIC SOCIETY

Mr. BAILLIE. Good morning. Thank you, Chairman Barrasso, Ranking Member Carper, and the distinguished members of the Committee. I would like to thank you for holding this timely hearing on cleaning up the world's oceans. I would also like to congratulate Senator Whitehouse on his award last night. Congratulations.

The Committee's leadership on this global crisis is critical, and I am grateful to have the opportunity to share my expertise as a representative of National Geographic.

I am going to talk about the scale of the crisis; then I am going to discuss the implications for wildlife, for people, and for the economy; and then I am going to close discussing what National Geographic is doing and what we can do better as a Nation.

The use of plastics is rapidly increasing throughout the world and is now a major threat to the environment, to marine species, human health, and the economy. As you can see on this map, over here and over here, the problem of plastics is global, is visible, and it is harmful. But it is also solvable.

Today there are 9.2 billion tons of plastics in this world, and annually we are producing about 500 million tons of plastics, 40 percent of which is just used once and then discarded. There is estimated to be about 150 million tons of plastics just floating around our oceans and our marine environment, and no one knows really how long it takes for these plastics to biodegrade. Of course, it depends on the particular plastics, but estimates range between 450 years to never.

This leaves our world with an ever-increasing amount of plastic waste. It is a problem we can no longer ignore.

Research indicates that hundreds of marine species consume plastic or become entangled in it. The animals confuse plastic bags or small plastic fragments for food, and it is absolutely devastating to see a sea bird fly in and feed it chick plastic waste unknowingly.

Species face entanglement in plastic packaging such as six-pack rings, as well as ghost nets, fishing nets that have been cut loose or are simply lost.

And we know that plastics have already entered the food chain. Microplastics have been found in 114 aquatic species, more than half of which we actually consume. Organic pollutions also fasten on to these plastic particles. And then there are nanoplastics.

Now, this is concerning, as the full implications are unknown. We, however, do know that plastics are linked to everything from weight gain to brain development impairment in humans.

Now, ocean plastic waste is also a threat to our economy. The ocean supports over 28 million American jobs. One in six U.S. jobs is marine-related. And coastal areas account for 85 percent of the U.S. tourism revenue.

I could give you many more statistics, but it is clear that unchecked plastic pollution poses a major threat to this important component of the U.S. economy.

Now, National Geographic is stepping up. We are using our combined power of our cutting-edge science and exploration and our storytelling to draw attention to this critical issue and to help people understand all over the world what they can do.

In May 2018, we launched Planet or Plastic?, which has already been referred to. This is a multiyear initiative that is focusing on the plastic crisis and how we can stop single-use

plastics entering the oceans. We also give out many awards to explorers around the world, many of which are working on this particular issue, explorers like Heather Koldewey, who is working in the Philippines to help remove these ghost nets from the oceans and have them turned into carpet tiles. It is innovations such as this that we find very encouraging.

Can we please play the film? [Video played.]

Mr. BAILLIE. That is just one of our amazing explorers. Heather said there is hope, but not without major change, and that change has to start right here, right now, in the United States.

We are one of the most developed Nations in the world, and we have to ask ourselves why are we sending over half our plastic recyclables overseas instead of developing our own robust recycling capability? Why do we continue to use multilayer plastics like disposable coffee cups that can't be recycled? And why are we creating recycling standards that reduce confusion and address the fact that 91 percent of recyclable plastic is not being recycled?

Now, while federalism and regulations make addressing this issue challenging, we must shift how our Nation manages plastic design and recapture, a task that only the U.S. Federal Government is able to take on. To support this, National Geographic would like to offer to convene a summit in Washington, DC. To bring together policymakers, to bring together industry leaders, and to bring together other stakeholders to have a critical discussion about how the U.S. can take a leadership position in this space.

Now, imagine a future where we don't address this solvable issue. Imagine a future with billions of tons of plastics floating around the oceans, the impacts on species, the impacts on people and the economy. This is unthinkable. It is time for us to address this head-on. It is time for bold decisions and bold action. And it is time for the United States to take a leadership position to demonstrate best practice and to continue to drive innovation.

Thank you.

[The prepared statement of Mr. Baillie follows:]

WRITTEN TESTIMONY OF DR. JONATHAN BAILLIE, EXECUTIVE VICE PRESIDENT AND CHIEF SCIENTIST NATIONAL GEOGRAPHIC SOCIETY, BEFORE THE UNITED STATES SENATE COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS "CLEANING UP THE OCEANS: HOW TO REDUCE THE IMPACT OF MAN-MADE TRASH ON THE ENVIRONMENT, WILDLIFE, AND HUMAN HEALTH?," 26 SEPTEMBER 2018

Chairman Barrasso, Ranking Member Carper, and distinguished members of the Committee, I would like to thank you for holding this timely hearing about cleaning up our world's oceans. The Committee's leadership on this global crisis is critical, and I am grateful for the opportunity to share my expertise on behalf of National Geographic and to assist with your mission.

As my testimony will further demonstrate, understanding and addressing how man-made trash reaches and impacts our oceans is a top priority for National Geographic. Our world's oceans are deeply impacted by human activities, including overfishing, man-made pollutants, and beyond.

Due to its widespread and consistently increasing use, plastic is one of the top threats to the environment, marine wildlife, human health, and the economy. Whether it takes the form of abandoned fishing nets or litter making its way into our oceans, and whether the pieces are large or small, the world's unchecked use of plastics has sparked a global crisis.

Plastics: A Global Crisis

There are about 9.2 billion tons of plastic in the world today. And nearly 500 million tons of plastic continue to be produced annually.

About 40 percent — or roughly 180 million tons — of this annual total is only used once before being discarded. This leaves more than 6.9 billion tons, or over 75 percent of the plastic existing globally, as plastic waste.

Unlike most other consumer materials, no one knows how long it takes plastic to biodegrade completely, with estimates ranging from 450 years to never. This leaves our world with an ever increasing amount of plastic waste.[1]

An estimated 150 million tons of plastic are currently circulating in our marine environments.[2] And a 2015 study found that between 5.3 and 14 million additional tons of plastic enter the ocean each year.[3]

Due to plastic's relatively recent introduction, more research into its impact on our oceans is needed. However, some alarming consequences have already been identified.

Impact of Plastic Debris on Marine Wildlife[4]

There is a large body of evidence demonstrating that plastic debris is often deadly to marine wildlife. Unfortunately, the durability of plastic ensures that its presence in marine ecosystems is persistent at all levels, ranging from large marine mammals to the smallest krill.[5]

Research indicates that at least 700 marine animal species have consumed or become entangled in plastics. They face entanglement in plastics such as "ghost nets" — fishing nets that have been left or lost in the ocean by fishermen — or six pack rings. The animals confuse plastic bags or smaller plastic fragments with food. Plastics of all sizes are tied to everything from digestive track blockages to immediate death.[6]

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[1] Laura Parker, "Plastic: We made it. We depend on it. We're drowning in it." *National Geographic*, June 2018, 40-69.

[2] Oceans Caucus Foundation. "Marine Debris." Published March 15, 2015. http://www.ocfoundation.us/the-issues/marine-debris.

[3] Jenna Jambeck et al. "Plastic waste inputs from land into the ocean," *Science* 347, no. 6223 (2015): 768-771. http://science.sciencemag.org/content/347/6223/768.

[4] Natasha Daly, "A Toll on Wildlife," *National Geographic*, June 2018, 80-84.

[5] Amanda Dawson et al. "Turning microplastics into nanoplastics through digestive fragmentation by Antarctic krill," *Nature Communications* 9, Article number 1001 (2018). https://www.nature.com/articles/s41467-018-03465-9.

[6] Elizabeth Royte, "A Threat to Us?" *National Geographic*, June 2018, 84-87.

Photo by Brian Skerry, National Geographic.

Plastic debris not only exists in forms that we can see, such as bottles and plastic bags, but is also broken down into much smaller pieces by light exposure, ocean drift, and marine organism digestion. These microplastics — or pieces smaller than one-fifth of an inch — linger for centuries.[7] Scientists have already found microplastics in 114 aquatic species, more than half of which are consumed by humans.[8]

In addition, microplastics cause physiological issues in marine species. Pollutants that wash off the land and into the sea adhere to microplastics, causing problems ranging from organ damage to reproductive abnormalities in the marine organisms that consume them.[9]

We do not yet understand the full implications of plastic debris on marine wildlife. However, National Geographic and scientists around the world are working every day to better understand this issue.

Impact of Plastic Debris on Humans

Research has demonstrated that many of the fish and shellfish humans eat are consuming microplastics. It has also tied plastics to issues ranging from weight gain to brain development impairment.

We do not yet know at what quantity human consumption of microplastics will have adverse effects and how other factors such as food preparation affect microplastic toxicity.[10] However, we do know that microplastics have entered our food chain.

Scientists are expressing additional concern about the potential impact of nanoplastics on human health. Nanoplastics are less than 100 billionths of a meter in size and are created as microplastics continue to degrade. Their miniscule size makes them impossible for scientists to trace in the seafood humans consume. However, scientists worry that these tiny nanoplastics, which can penetrate cells and move into tissues and organs, pose a risk to humans.

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[7] Ibid.

[8] GESAMP, "Sources, fate and effects of microplastics in the marine environment: part two of a global assessment" (Kershaw, P.J., and Rochman, C.M., eds.). (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/ UNEP/ UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection). Rep. Stud. GESAMP No. 93 (2016): 220.

[9] Elizabeth Royte, "A Threat to Us?" *National Geographic*, June 2018, 84-87.

[10] Ibid.

Photo by Randy Olson, National Geographic.

Despite the scientific questions that remain at the micro- and nanoscales, the impact of marine plastic is evident at the macroscale. Oceans and coastal ecosystems provide an irreplaceable benefit to the U.S. economy. Our oceans support over 28 million American jobs. In fact, between the fishing, boating, tourism, recreation, and ocean transport industries combined, one in six U.S. jobs is marine-related. Additionally, U.S. consumers alone spend over $55 billion on fishery products, and coastal areas account for 85 percent of U.S. tourism revenue.[11]

Scientists are still in the early phase of studying how extensively the global plastic crisis has impacted and will continue to impact our world. However, they have already demonstrated that plastic pollution poses a tremendous threat to our oceans, the marine wildlife that inhabits them, and the U.S. economy.

My Background on the Issue

Prior to joining National Geographic in 2016, I spent 20 years at the Zoological Society of London (ZSL) working on projects focused on threatened species and their habitats in over 50 countries.

While at ZSL, I helped develop the Living Planet Index, Sampled Red List Index, and Wildlife Picture Index. I also worked with a network of 8,000 scientists to produce the first International Union for the Conservation of Nature (IUCN) Red List of Threatened Species using quantitative criteria to assess extinction risk. I then partnered with IUCN to produce the first list of the 100 most threatened animals, plants, and fungi and co-chaired the IUCN Regional Red List working group and the IUCN Special Survival Commission Pangolin Specialist Group.

I led the ZSL team that founded the EDGE of Existence program, which focuses on Evolutionarily Distinct and Globally Endangered (EDGE) species and supporting young scientists around the world working to protect animals facing extinction. While at ZSL, I also founded the Conservation Technology Unit and the Business and Biodiversity Programme.

[11] U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Ocean Service. "What does the ocean have to do with human health?" https://oceanservice.noaa.gov/facts/ocean-human-health.html.

I helped initiate United for Wildlife, a collaboration of seven of the most influential conservation organizations working to address illegal wildlife trade at scale. Under this collaboration, I led the development of the award-winning digital conservation leadership training platform, the Rhino Impact Investment, and the development of technology for nature.

It was also during this time that I co-led the first large-scale retail activism project to focus on ocean conservation: Project Ocean. The partnership between ZSL and Selfridges, a chain of high-end department stores in the United Kingdom, highlighted the unthinkable prospect of the world's major fisheries collapsing by 2050.

The retail platform provided the opportunity for conservationists from ZSL to reach a wider audience and have a global impact. Twenty-two non-governmental organizations (NGOs) dedicated to environmental and sustainable issues embraced the initiative, leading to public discussions between political figures at Selfridges on World Oceans Day 2011. Project Ocean culminated with a long-term joint project between Selfridges and ZSL: a 50-hectare, or 124-acre, marine protected area in the Philippines, aiming to safeguard fish species and their coral reef ecosystem.

The success of this initiative demonstrated the effect that public awareness initiatives can have on major global problems. Now, as National Geographic's Executive Vice President and Chief Scientist, I have access to an unparalleled media megaphone to address the issues facing our oceans today.

Planet or Plastic?

For 130 years, National Geographic has pushed the boundaries of exploration to further our understanding of the planet and empower us all to create a healthy and sustainable future for generations to come. We do this by investing in bold people and transformative ideas and sharing what we learn with audiences around the globe. Our ultimate goal is to achieve a planet in balance, one that provides for humanity and the untold millions of other species with which we live.

Today, our brand reaches millions of people of all ages in 172 countries every month. National Geographic publishes in 37 languages, our broadcast channels air in 43 languages, and our social media channels reach 436 million.

Through the power of our scientific research and storytelling, the integrity of our brand, and the reach of our global media platforms, we are uniquely positioned to engage with the public, to educate and raise awareness, and to build consciousness and help drive change.

In May of 2018, National Geographic launched *Planet or Plastic?*, a multiyear initiative aimed at raising awareness about the global plastic waste crisis and reducing the amount of single-use plastic that enters the world's oceans. The initiative was showcased in the June 2018 "Planet or Plastic?" issue of National Geographic magazine and across our digital platforms to educate our audiences about the crisis and to show them how to take accountability for this global emergency.

We're encouraging our audiences to pledge to take small, attainable steps, such as using reusable shopping bags, skipping straws, carrying a reusable bottle, and properly disposing of trash. We are demonstrating that when we work together, we can reduce single-use plastics and make a lasting impact on the global plastic crisis.[12]

[12] National Geographic. "Planet or Plastic?" https://www.nationalgeographic.com/environment/plasticpledge/.

June 2018 issue of National Geographic Magazine.

As of last month, our *Planet or Plastic?* education initiative had already generated over 938 million content views across our digital platforms. And our worldwide reach has prompted 47,000 people to take the pledge to reduce single-use plastics.

Investing in Change

National Geographic's commitment to addressing this issue goes beyond storytelling. It also includes grants to support hundreds of National Geographic Explorers. Through the power of our grants program, these bold individuals are changing the world by furthering the collective understanding of our planet and empowering the global community to generate solutions for a healthier and more sustainable future.

Stopping Plastic Pollution at Its Source[13]

Inland plastic can travel many miles to enter our world's oceans. A combination of direct dumping of waste into rivers and litter flowing from land to local waterways during

[13] National Geographic. "Plastics: Source to Sea." https://www.nationalgeographic.org/projects/ocean-plastics/.

rainstorms creates an unintentional plastic waste conveyor system. The waste from local waterways feeds into larger tributaries and rivers that ultimately transport the inland waste into our oceans. Via this mechanism, polluted rivers deliver a significant portion of the millions of tons of plastic that enter oceans annually.[14]

Through our grants program, we are partnering with scientists and innovators who are working diligently to find ways to keep plastics from ever reaching the ocean. To better understand how plastic waste flows to the ocean in the first place and to prevent it from ending up there, National Geographic Explorers will be journeying from the source of the plastic in the rivers to the ocean. Their goal: to understand the types and pathways of plastic in a river system and to provide science-based information that will engage citizens and help policymakers, businesses, and NGOs implement solutions to this growing crisis.

Improving Plastics Waste Management[15]

Approximately 80 percent of marine debris comes from land-based sources.[16] Improved solid waste management reduces the amount of plastics entering the oceans annually.

Award-winning National Geographic Explorer and environmental engineer Jenna Jambeck has researched marine debris since 2001 and has raised awareness via her research on plastic waste leakage into water systems. In her 2016 testimony before the Senate Committee on Environment and Public Works Subcommittee on Fisheries, Water, and Wildlife, Jambeck pointed to plastic pollution's economic and environmental impact as well as the ability to combat widespread plastic pollution via well-researched and culturally appropriate strategies.[17]

Today, Jambeck advances her work on finding scientifically supported and culturally relevant solutions with funding from National Geographic's grants program. She works in Vietnam — a country with an often-informal solid waste management system — to better understand and improve waste management, and to ultimately reduce the quantity of plastics entering water systems via local partnerships and education.

Cleaning Up Plastic Fishing Gear Pollution[18]

National Geographic Explorer Heather Koldewey works with ZSL to help communities clean up ocean plastic as part of the National Geographic Ocean Plastics Initiative. Through interdisciplinary research and conservation action at the interface between communities and the environment, Koldewey drives real change in the communities she touches.

Today, Koldewey works in the Philippines and beyond to combat ocean plastics, starting with ghost nets. Abandoned fishing gear comprises around 10 percent of all plastic trash in the oceans. More than 705,000 tons of fishing nets are lost yearly, according to the United

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[14] Laura Parker et al, "What Happens to the Plastic We Throw Out," *National Geographic*. https://www.nationalgeographic.com/magazine/2018/06/the-journey-of-plastic-around-the-globe/.

[15] National Geographic. "Grants Across the Globe: Working With The Independent Waste Collector Community In Vietnam To Reduce Leakage Of Plastic To The Ocean." https://www.nationalgeographic.org/grants/where-we-work/A1228097?filter=activity_status:All&q=Jenna%20Romness%20Jambeck.

[16] Oceans Caucus Foundation. "Marine Debris."

[17] *Marine Debris and Wildlife: Impacts, Sources and Solutions: Hearing before the U.S. Senate Committee on Environment and Public Works*, 114th Cong. (2016) (written testimony of Jenna R. Jambeck, Ph.D., Associate Professor of Environmental Engineering, College of Engineering, University of Georgia).

[18] Laura Parker, "These Communities Turn Discarded Fishing Nets Into Carpets," *National Geographic*, Published June 13, 2018. https://news.nationalgeographic.com/2018/06/heather-koldeway-explorer-nets-plastic-philippines- ocean-culture/?beta=true.

Nations,[19] and nearly half the weight of the Great Pacific Garbage Patch's surface debris is fishing gear.

Abandoned fishing gear is directly connected with a disproportionately large number of marine wildlife fatalities. World Animal Protection estimates that more than 100,000 large whales, sea lions, and seals are killed every year by fishing gear, in addition to an "inestimable" number of sea birds, turtles, and fish.

Koldewey saw this critical issue as an opportunity for partnership. By developing Net-Works, a collaboration between global carpet tile manufacturer Interface, Inc. and ZSL, she developed a successful community-based supply chain for retrieving and discarding fishing nets. Net-Works partners with coastal communities to collect discarded fishing nets, convert them into nylon yarn, and sell them to Interface to create carpet tiles. So far, the initiative has collected and recycled 208 tons of discarded fishing nets, removing these dangerous threats to marine wildlife from our oceans and empowering local communities with economic opportunity.[20]

Partnership for Change[21]

National Geographic is also partnering with Sky Ocean Ventures to create the largest global media campaign to reduce plastic litter in the ocean. We've committed $10 million to bring our scientific expertise, grants, and media reach to the partnership.

The partnership will make grants available to proposals that will measurably reduce plastic pollution before it reaches the ocean, including improved recycling, waste management, stakeholder engagement, and more. It will also convene a series of Innovation Challenges, uniting the best international minds around technologies designed to reduce plastic waste and its impact on oceans. Additionally, the partnership will create an event series engaging industry leaders, corporations, institutions and foundations around the issue of marine plastic pollution.

An Opportunity for U.S. Leadership

Our planet is at a crossroads. World plastic production is increasing exponentially. In 1950, 2.3 million tons were produced. In 1993, 162 million tons were produced. And in 2015, 448 million tons were produced.[22] As plastic production grows, so does the threat plastic waste poses to our oceans and the wildlife and economies that depend upon them.

However, as Laura Parker wrote in the June National Geographic magazine cover story, plastics have been a boon to humanity. They helped the Allies win World War II, "eased travel into space, and revolutionized medicine … In airbags, incubators, helmets, or simply

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[19] Eric Gilman et al, Food and Agriculture Organization of the United Nations, *Abandoned, lost or otherwise discarded gillnets and trammel nets: Methods to estimate ghost fishing mortality, and the status of regional monitoring and management*, 2016, FAO Fisheries and Aquaculture Technical Paper No. 600, Rome, Italy.

[20] Zoological Society of London. "Asia: Net-Works." https://www.zsl.org/conservation/regions/asia/net-works.

[21] National Geographic 2018. "Sky and National Geographic Work Together to Fight Ocean Plastic." http://press.nationalgeographic.com/2018/04/16/sky-and-national-geographic-work-together-to-fight-ocean-plastic/.

[22] National Geographic. "10 Shocking Facts About Plastic." https://www.nationalgeographic.com/environment/plastic-facts/.

by delivering clean drinking water to poor people in those now demonized disposable bottles, plastics save lives daily."[23]

Now, it's a matter of learning to balance the potential provided by plastic with the threat plastic waste poses to our world. We know that the ocean's currents ensure that marine plastic pollution will never be just one nation's problem. Any plastic waste that is introduced to the system will impact wildlife and economies around the globe.

This is why National Geographic launched the *Planet or Plastic?* initiative and why we are dedicated to encouraging consumers to end their reliance on the single-use plastics.

National Geographic is committed to continuing to raise international awareness through our media and education arms and to better understanding and addressing the issue through research and community partnership. We are working with corporations, NGOs, and other institutions around the world to eliminate single-use plastic and promote recyclability.

However, addressing this issue will require true global partnership. In this process, it's important to remember that our work must start with evaluating our own practices.

With *Planet or Plastic?,* we hope to do nothing less than transform consumer behavior — and we're starting at home. To kick off this multiyear initiative, National Geographic is conducting an internal audit of our headquarters in Washington, D.C., to assess how our own operations and supply chain use plastic. From the plastic wrappers in which our magazines were formerly distributed to the materials we use in our cafeteria, we're identifying and eliminating single-use plastics.

Now, it's time for the United States to do the same. The United States is one of the most developed nations in the world. So why aren't we addressing this issue head-on? Why are we relying on sending our plastic recyclables overseas instead of developing a robust recycling capability of our own? And why do we continue to use plastics that can't be recycled at all?

U.S. Plastic Waste

Today, an estimated 94 percent of Americans have access to recycling. However, our nation lacks standardization on which materials are considered recyclable and what community collection practices look like.[24] This contributes to this shocking metric: In the United States, 91 percent of recyclable plastic is not actually recycled.[25]

Additionally, more than half of plastic waste that is recycled in the United States has been exported to hundreds of countries around the world because our own recycling sector is under resourced. Historically, China has been responsible for recycling 45 percent the world's plastic and paper products.

However, this year, China stopped accepting recycling from abroad. This is related to the fact that many countries, including the United States, transitioned to single-stream recycling. Single-stream recycling was designed to streamline recycling and reduce consumer burden by allowing all recyclables — from paper to plastic to glass — to go into the same bin. However, this streamlining process means that much of the waste reaching China is too contaminated with non-recyclables to provide China with the profit margin needed to continue processing

[23] Susan Goldberg, "The Plastic Apocalypse," *National Geographic*, June 2018, 6.

[24] Drew Desilver, "Perceptions and realities of recycling vary widely from place to place," *Pew Research Center*, Published October 7, 2016. http://www.pewresearch.org/fact-tank/2016/10/07/perceptions-and-realities-of-recycling- vary-widely-from-place-to-place/.

[25] Faye Flam, "The Recycling Game Is Rigged Against You," *Bloomberg*, Published June 27, 2018. https://www.bloomberg.com/view/articles/2018-06-27/plastic-recycling-is-a-problem-consumers-can-t-solve.

the world's recyclables. As a result, estimates predict that 122 tons of potentially recyclable plastic will be diverted from Chinese recycling facilities, likely ending up in landfills.[26] However, the global shortage in recycling capabilities is only one piece of the issue.

Some plastics are too difficult to recycle in the first place. Due to their prevalence in our daily lives, multilayer plastics, which are used in products ranging from disposable coffee cups to snack packaging, are particularly problematic. Multilayer plastics incorporate materials such as paper or aluminum, tightly stacking thin layers of the materials alongside plastic and making the separation and processing of these materials nearly impossible. While material production advancements make these multilayer plastics cheap, readily available, and utilitarian, the non-recyclable nature of these single-use materials is a major contributor to the global plastic crisis.[27]

Unfortunately, this means that our nation's plastic waste footprint is an issue that consumers and individual organizations cannot solve alone. It requires a shift in how our nation as a whole manages plastic design and recapture, a task that only the U.S. federal government is able to take on.

Model Nation and Innovation

I understand that federalism and regulation concerns make addressing this issue challenging. However, we cannot stymie the global plastic crisis without U.S. federal government involvement.

The U.S. government can play a stronger role in addressing the global plastic crisis by convening conversations with industry leaders and encouraging them to present solutions and commit to targets. The United States is a global leader in innovation. Our government continuously invests in American ingenuity. Now, it must invest in solutions to the global plastic crisis through private sector and entrepreneurial innovation around better material design and recapture.

Only the federal government is positioned to implement the solutions developed by private sector leaders at a nationwide scale. But as part of our *Planet or Plastic?* initiative, National Geographic would like to offer our support to U.S. policymakers as they address this issue.

On behalf of National Geographic, I would like to offer to convene a summit in Washington, D.C., to bring together policymakers, industry leaders, and other essential stakeholders for a critical discussion about how the United States can lead in this space. The *Planet or Plastic?* Summit will ensure that the right voices are in the room as we look at the current state of plastic waste in the United States and, in turn, how to advance the American recycling industry, move away from non-recyclable and single-use plastics, and find better alternatives to plastic where possible. And equally importantly, National Geographic wants to bring our powerful storytelling to bear to amplify the results of this conversation and to raise awareness of what the United States is doing to address the global plastic crisis.

While the plastic crisis has no boundaries, the U.S. government must first take its own steps to protect the oceans and marine wildlife upon which one in six American jobs

[26] Amy Brooks, Shunli Wang, and Jenna Jambeck, "The Chinese import ban and its impact on global plastic waste trade," *Science Advances* Vol. 4, no. 6 (2018). http://advances.sciencemag.org/content/4/6/eaat0131.

[27] Lilly Sedaghat, "7 Things You Didn't Know About Plastic (and Recycling)," *National Geographic*, Published April 4, 2018. https://blog.nationalgeographic.org/2018/04/04/7-things-you-didnt-know-about-plastic-and-recycling/.

depend.[28] Perhaps the time has come to examine the feasibility of a federal regulatory approach that would provide minimum standards on plastic use and recycling. The approach could provide incentives and mandates — carrots and sticks — for states to adopt the right recycling protocols and provide national standards for plastic import, manufacturing, and use.

It's a bold solution, but this crisis requires one. It's time to stop the plastic pollution that got us into this mess in the first place — and to step up and be a true global leader on this issue.

Appendix

World Map Display of Plastic Waste in Oceans via National Geographic

"Drowning in Plastic," *National Geographic*, June 2018. 55-57. https://www.dropbox.com/sh/gdcr7416n8gpfxl/AAAHejRDyj9XCPnAuKvSBc3Za?dl=0&p review=00_MAP+plastics_grfx_map.jpg.

Recent Editorial Co-Authored by Jonathan Baillie

Jonathan Baillie and Ya-Ping Zhang, "Space for Nature," *Science* 361, no. 6407 (2018): 1051. http://science.sciencemag.org/content/361/6407/1051.

SENATE COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS, HEARING ENTITLED, "CLEANING UP THE OCEANS: HOW TO REDUCE THE IMPACT OF MAN-MADE TRASH ON THE ENVIRONMENT, WILDLIFE, AND HUMAN HEALTH?", SEPTEMBER 26, 2018

Questions for the Record for Dr. Jonathan Baillie
Executive Vice President and Chief Scientist
National Geographic Society

Chairman Barrasso

1. Experts seem to agree that stopping the flow of debris into the ocean is a bigger priority than cleaning up the debris already in the ocean. Is this an assessment that you agree with? If not, why not?

Yes, l agree. Studies have found that approximately 80 percent of marine debris comes from land-based sources. While stopping the flow of plastic debris from land into waterways and cleaning up the debris that already pollutes the world's oceans are important from an

[28] U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Ocean Service. "What does the ocean have to do with human health?" https://oceanservice.noaa.gov/facts/ocean-human-health.html.

environmental and economic standpoint, addressing the origins of plastic pollution would provide a major boost to combating this issue.

2. We know that five countries in Asia bear much of the responsibility for the increase in ocean plastic. That does not mean that we in the United States do not have a critical role to play. What additional steps can local and state governments as well as the federal government take to address this problem?

The U.S. government can play a stronger role in addressing the global plastic crisis by convening conversations with industry leaders and encouraging them to present solutions and commit to targets. The federal government has an advantage in that it maintains the capability to implement solutions developed by the private sector on a nationwide scale. I suggest that the federal government explore a federal regulatory approach that would provide minimum standards on plastic use and recycling. Local and state governments can also examine and address their waste management practices to stymie the paths by which plastics flow from land in their jurisdiction into waterways.

3. On January 24, 2018, the *Financial Times* published an article, by Clive Cookson, entitled, "The problem with plastic." It explained that:

> "While the personal-care industry is phasing out microbeads, concern is growing about another ubiquitous micropollutant: plastic fibres. Analysis shows these to be present in streams, rivers, lakes, and seas worldwide, as well as household drinking water. Their main source seems to be clothing and textiles made from synthetic fibres, which become detached in washing machines and are not filtered out by water-treatments plants."
>
> Do you agree with this assessment? If so, how do we begin to address this issue?

We know that microplastics such as plastic fibers have already entered our food chain and that pollutants adhering to these microplastics can cause issues like organ damage and reproductive abnormalities in the marine organisms that consume them. We also know that as microplastics degrade, they become nanoplastics, which have the ability to penetrate cells and move into tissues and organs. Per GESAMP, potential avenues of addressing the fiber issue could specifically involve enhanced washing machine filters or other new technologies that would limit the release of these microplastics during washing, such as laundry detergent additives and textile finishing treatments.[29]

In addition, there are companies that are looking into design-based solutions. For example, some fleece manufacturers are working to design materials that shed fewer fibers.

4. What regions of the world are the least studied when it comes to plastic pollution?

Plastic use and the problem of plastic waste have grown exponentially in a very short period of time. While there are regions of the world that may be less studied, the National

[29] http://ec.europa.eu/environment/marine/good-cnvironmcntal-status/descriptor-l0/pdf/GESAMP_microplastics%20full%20study.pdf.

Geographic Society is focusing initially on areas where plastic use is high and where high levels of plastic waste are seeping into the environment.

In fact, National Geographic is already taking steps to study plastic pollution in several of these areas by investing in the work of our scientists and explorers. Through our grants program, we partner with scientists and innovators who are working diligently to find ways to keep plastics from ever reaching the ocean.

For example, National Geographic Explorer Jenna Jam beck works in Vietnam, which has an often-informal solid waste management system, to better understand and improve waste management. Explorer Heather Koldewey works in the Philippines to help communities clean up ocean plastic as part of the National Geographic Ocean Plastics Initiative.

We have found that even highly remote areas like the uninhabited Henderson Island in the Pacific can play host to what is estimated to be the highest concentration of debris anywhere in the world. Further research could and should be conducted around the world on the plastic crisis. This is a global issue that affects the world's connected waterways and oceans.

Ranking Member Carper

5. Stopping the flow of marine debris into the ocean and mitigating its impacts are not problems that can be solved overnight. Long-term, thoughtful, and collaborative solutions are necessary in order to address the full scope of the issue. Solutions that have been discussed included: improving recycling; incentivizing the use of recycled materials in the global supply chain; developing more biodegradable alternatives; and changing manufacturing protocols. What steps can this Committee and the Congress take now to advance these potential solutions here in the United States?

The federal government should explore a federal regulatory approach that would provide minimum standards on recycling and the recyclability of plastic products. The approach could provide incentives and mandates-carrots and sticks for states to adopt the right recycling protocols and provide national standards for plastic import, manufacturing, and use. Only the federal government is able to implement change now on a nationwide scale.

6. Now that China has implemented its "Green Fence Policy," a ban on importing plastic waste, our market for these materials in the United States is flooded. China previously accepted 30 percent of our plastic waste. Local municipalities are now having even more trouble breaking even when collecting and recycling this waste. In your opinion, what are the best ways for the United States to address this new challenge?

Many countries, including the United States, use single-stream recycling. Single-stream recycling was designed to streamline recycling and reduce consumer burden by allowing all recyclables-from paper to plastic to glass-to go into the same bin. However. This streamlining process means that much of the waste reaching China is too contaminated with non-recyclables to provide China with the profit margin needed to continue processing the world's

recyclables. Many of the recycling facilities in the United States are facing the same issue now.

Our nation lacks standardization on which materials are considered recyclable and what community collection practices look like. This contributes to this shocking metric: In the United States, 91 percent of recyclable plastic is not actually recycled.

This can be addressed via a three-pronged approach:

1) *The United States must streamline its recycling standards and practices to reduce contamination.* I understand that federalism and regulation concerns make addressing this issue challenging. However, an effort on this scale is something that only the federal government-with the help of participatory stakeholders can manage. Our nation must explore a federal regulatory approach that would provide minimum standards on plastic use and recycling. The approach could provide incentives and mandates-carrots and sticks tor states to adopt the right recycling protocols and provide national standards tor plastic import, manufacturing, and use.
2) *We must move away from non-recyclable plastics that have no value to the recycling industry.* The U.S. government can play a stronger role in addressing the global plastic crisis by convening conversations with industry leaders and encouraging them to present solutions and commit to targets. The United States is a global leader in innovation. Our government continuously invests in American ingenuity. Now, it must invest in solutions to the global plastic crisis through private- sector and entrepreneurial innovation around better material design and recapture.
3) *We must educate consumers about what is recyclable and how it should be recycled.* Once recycling practices are standardized, the United States will need to engage in a nationwide education campaign to raise awareness, highlight recycling myths versus facts, and ensure consistency in our nation's consumer recycling practices. National Geographic is committed to using our media reach to aid in this education process.

Senator Markey

7. For the past 25 years, China has imported 45 percent of the world's plastic waste, over l00 million tons every year. From the United States, about 3,700 shipping containers of recycling went to China on a daily basis. That changed this past January, when China closed its borders to 24 categories of solid waste, leaving the United States and Europe without a market for much of our plastic trash. Municipal waste managers in the United States may now have to put recyclable plastic in landfills as falling prices and lack of demand for recyclables could also lead to recycling centers closing.

a. Does the closing of China's borders to many kinds of plastic waste force a fundamental shift in the U.S.'s recycling economy?

Yes, China's decision to stop accepting plastic recyclables from abroad requires a major shift in our nation's recycling economy. In addition to the three essential steps l outlined in question 6, we need to improve the design of plastics in the United States to ensure that they are recyclable in the first place.

One way to do this is by reducing our reliance on multilayer plastics, used in products ranging from disposable coffee cups to snack packaging, as they are particularly problematic. Multilayer plastics incorporate materials such as paper or aluminum, tightly stacking thin layers of the materials alongside plastic and making the separation and processing of these materials nearly impossible. While material production advancements make these multilayer plastics cheap, readily available, and utilitarian, the non-recyclable nature of these single-use materials is a major contributor to the global plastic crisis. We must find recyclable alternatives to multilayer plastics as well as better substitutes for single-use plastics wherever possible.

b. Will this increase the risk of more U.S. plastic ending up in the oceans in addition to landfills?

Yes, a global analysis of all plastics ever made showed that only 9 percent were recycled. The vast majority (79 percent) accumulated either in the environment or in landfills. Estimates predict that 122 tons of potentially recyclable plastic will be diverted from Chinese recycling facilities.[30] In all likelihood, this break in the global recycling system will lead to an additional influx of marine plastic pollution in our oceans.

Senator Merkley

8. In your testimony, you spoke about the need for U.S. leadership on marine debris. What are the most important policy steps the United States should be taking to address marine debris on an international scale?

Much of my background on this issue comes from my decades of experience as a scientist and from leading advocacy projects that convened a variety of business, conservation, and non-governmental organization (NGO) stakeholders in order to enact meaningful change on a large scale. Through this lens, and as I've outlined above, I believe the United States should explore methods for advancing the American recycling industry, moving away from single-use plastics, looking for plastic alternatives, where possible, and providing incentives and mandates for states to adopt the right recycling protocols and provide national standards for plastic import, manufacturing, and use.

[30] https://news.nationalgeographic.com/2017/07/piastic-produced-recycling-waste-ocean-trash-debris-environment/.

Senator Whitehouse

9. During your testimony, you offered to convene a summit of essential stakeholders in Washington, D.C., to discuss U.S. leadership in this area. Do you see a role for the federal government in this discussion? What other sectors and stakeholders will be involved?

The federal government has a vital role to play in this process as a convener of conversations with industry leaders in order to encourage them to present solutions and commit to targets to address the crisis. Members and senators, as well as their staff, on and beyond the committees of jurisdiction are important participants in the ongoing dialogue to resolve this issue. Industry leaders, NGOs, and other organizations are also key components of this process.

10. National Geographic magazine's recent marine debris issue punctuated the crisis with poignant photos and articles. What role do these stories and images play in encouraging change and support for marine debris solutions?

At National Geographic, we take our role in addressing this issue very seriously, whether through scientific research or storytelling. Our global reach across numerous media channels allows us to engage and educate audiences on issues affecting a planet we all share. By raising public consciousness and consumer awareness, National Geographic helps shine a light on global issues like plastic pollution. Increased public awareness leads to individuals making changes in their own lives and expanding those choices among their family, friends, and communities. Poignant storytelling and striking images are effective tools in illustrating issues that may otherwise go overlooked, and the global plastic crisis is a perfect example of that.

11. What misconceptions remain among the general public about marine debris?

A common misconception among the general public is that the biggest issue we face is the type of marine debris that we can see, such as bottles and plastic bags. Awareness is often low about the risk imposed by plastics breaking down into much smaller pieces by light exposure, ocean drift, and marine organism digestion. These microplastics-or pieces smaller than one-fifth of an inch-linger for centuries. Scientists have already found microplastics in 114 aquatic species, more than half of which are consumed by humans. In addition, microplastics cause physiological issues in marine species. Pollutants that wash off the land and into the sea adhere to microplastics, causing problems ranging from organ damage to reproductive abnormalities in the marine organisms that consume them.

Research has demonstrated that many of the fish and shellfish humans eat are consuming microplastics. It has also tied plastics to issues ranging from weight gain to brain development impairment. We do not yet know at what quantity human consumption of microplastics will have adverse effects and how other factors such as food preparation affect microplastic toxicity. However, we do know that microplastics have entered our food chain.

Scientists are expressing additional concern about the potential impact of nanoplastics on human health. Nanoplastics are less than 100 billionths of a meter in size and are created as microplastics continue to degrade. Their miniscule size makes them impossible for scientists

to trace in the seafood humans consume. However, scientists worry that these tiny nanoplastics, which can penetrate cells and move into tissues and organs, pose a risk to humans.

12. National Geographic launched its *Planet or Plastic?* campaign this summer. The initiative asks people to pledge to reduce their use of single-use plastics.

a. How many people have taken the pledge so far?

As of November I, 2018, our *Planet or Plastic?* education initiative had already generated more than 972 million content views across our digital platforms. And our worldwide reach has prompted more than 91,000 people to take the pledge to reduce single-use plastics.

b. How will Nat Geo track the effect of this campaign on plastics reduction? National Geographic is sharing metrics on its *Planet or Plastic?* pledge campaign online at www.nationalgeographic.com/environmentlplasticpledge.

Our goal is to prevent 1 billion items from reaching the ocean by 2020, and we are using our global media platform to provide updates on the campaign, share resources, and help inspire others to reduce their use of single-use plastics.

In addition, we will be advancing education initiatives, citizen science efforts, and innovation challenges. We will also be developing a global framework to enable better selection and siting of interventions. Finally, we will be hosting convenings and will continue to shine a light on plastic pollution through our unparalleled storytelling and global media platforms.

c. What role can these voluntary campaigns play in changing consumer and producer or retailer behavior?

These campaigns can play a large role in changing consumer, producer, and retailer behavior. For more than 130 years, National Geographic has sought to further our understanding of the planet and empower us all to create a healthy and sustainable future for generations to come.

By leveraging our brand, we are able to reach millions of people in 172 countries and educate them on this issue. We are also able to share tips for consumers to take actionable steps in order to reduce plastic waste.

13. How do voluntary actions by corporations and individuals support marine debris reduction?

About 40 percent of the nearly 500 million tons of plastic produced annually is only used once before being discarded. Voluntary actions by corporations and individuals can play a major role in reducing this number.

This is why to kick off our multiyear *Planet or Plastic?* initiative. National Geographic is conducting an internal audit of our headquarters in Washington, D.C., to assess how our own

operations and supply chain use plastic. From the plastic wrappers in which our magazines were formerly distributed to the materials we use in our cafeteria, we're working to identify and eliminate single-use plastics. Actions such as these are an essential first step toward the broader nationwide and international reform needed to combat the global plastic crisis.

14. As we work on the next iteration of the Save Our Seas Act, do you have any additional recommendations or comments you would like to share to inform our development of this bill?

The Save Our Seas Act is a powerful piece of legislation. I am glad to see that Congress is taking bipartisan steps to address the global plastic crisis and understands the need to improve international waste management practices to reduce the influx of plastics into our world's oceans.

I recommend a companion piece of legislation that would focus not only on reforming international plastic waste management, but also on addressing what the United States can do within our nation's borders. This legislation could further study the impacts of plastics-at both the micro and macro scales-on our waterways; invest in private-sector and entrepreneurial innovation around better material design and recapture in the United States; and provide incentives and mandates-carrots and sticks tor states to adopt the right recycling protocols and provide national standards tor plastic import, manufacturing, and use.

* * *

Senator BARRASSO. Thank you so much for your testimony and your leadership on this.

Mr. DOOLEY.

STATEMENT OF HON. CAL DOOLEY, PRESIDENT AND CEO, AMERICAN CHEMISTRY COUNCIL

Mr. DOOLEY. Thank you, Mr. Chairman and members of the Committee. I am delighted to be joining you all.

ACC represents a diverse set of companies engaged in the U.S. business of chemistry, and the chemical industry is at the forefront of developing the innovations, the technologies, and the products that are essential to advancing global environmental sustainability.

If you look at the increased fuel efficiency in our vehicles, they are really a function of the plastics and the composites that are contributing their light-weighting.

When we look at the enhanced energy efficiency of our built environment, our homes, our offices, and our factories, it is the products of chemistry that are increasing their energy efficiency and reducing greenhouse gas emissions. Even when we look at the plastic packaging that is reducing the weight of consumer products, that is reducing emissions.

So, there is a lot of really positive contributions that the products of chemistry are making to enhance sustainability. Unfortunately, we have too many plastics that are entering into the environment where they clearly do not belong.

As many of you already noted, the first step to ending plastic waste in the environment starts with understanding the sources. Twenty countries account for 83 percent of plastic waste entering into the ocean. The largest sources are rapidly developing economies, mainly in Asia, where basic plastic waste management infrastructure has not kept pace with the rise in demand for consumer goods.

Studies by The World Bank and McKinsey have identified that the most cost-effective investments to reduce plastic waste in the environment are the implementation of waste collection infrastructure and improved processing of collected waste in source countries.

ACC applauds the efforts of Senator Sullivan and Senator Whitehouse for leading efforts to secure the passage of the Save Our Seas Act. It is a good first step.

But ACC and our value chain partners are excited about the opportunity to provide private sector support that would complement a bigger, bolder, and more effective Save Our Seas Act 2.0.

There is a unique opportunity to build bipartisan congressional and Administration support for increasing the U.S. global leadership in advancing policies that will significantly reduce man-made waste from entering into the environment.

We would encourage your consideration of policies that would include encouraging The World Bank and international development banks and USAID to prioritize waste collection and management. According to the International Solid Waste Association, waste management accounts for only .3 percent of development aid assistance.

We also would encourage promotion of public-private partnerships and business-led efforts to fund waste management in the developing world. We encourage the Department of Defense and other agencies to fund waste management pilot projects at their facilities, particularly in the Asia Pacific region, to transform plastic waste into fuels, feedstocks, and infrastructure materials.

In addition to policies designed to reduce waste in the developing world, there are domestic policies that can enhance waste management systems in the U.S. and also contribute to the development and implementation of new technologies that can capture the value in plastic waste. Plastic waste has more captured energy than coal, and many of ACC's companies are investing in developing technologies that can unlock the captured energy, transforming non-recycled plastics into alternative fuels and feedstock materials for new manufacturing. But current regulations do not specifically recognize these emerging technologies as recycling, which is an impediment to capturing the value of plastic waste.

Some specific opportunities to address this issue include: providing guidance to States recognizing pyrolysis and gasification facilities which take waste plastics and convert them back to chemicals or fuels as manufacturing and not hazardous waste facilities; revise EPA's guidelines for the assessment of environmental performance standards and equal labels for Federal procurement to prefer products and services that utilize recovered plastics as recycled content; partner with the Department of Energy and Department of Transportation and other appropriate agencies to research opportunities that utilize plastic waste and innovative construction materials in transportation and water infrastructure projects nationwide; and, finally, designating fuel derived from plastic waste as a renewable fuel.

We have a great opportunity to create a global public-private initiative to eliminate man-made waste in the environment. ACC and our partners in the plastic value chain are committed to working with you and environmental organizations to identify the policies and

the most cost-effective investments of public and private resources that will eliminate man-made waste from entering into the environment.

Thank you.

[The prepared statement of Mr. Dooley follows:]

AMERICAN CHEMISTRY COUNCIL, STATEMENT FOR THE RECORD, SENATE COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS, "CLEANING UP THE OCEANS: HOW TO REDUCE THE IMPACT OF MAN-MADE TRASH ON THE ENVIRONMENT, WILDLIFE, AND HUMAN HEALTH," SEPTEMBER 26, 2018

The American Chemistry Council (ACC) is pleased to submit this Written Testimony to the Senate Committee on Environment and Public Works regarding the September 26, 2018 hearing, "Cleaning Up the Oceans: How to Reduce the Impact of Man-Made Trash on the Environment, Wildlife, and Human Health."

While marine debris is a huge problem, it is also a solvable one. ACC, together with America's Plastics Makers®, are committed to ending plastic waste in the environment. We are proud to have partnered with governments, NGOs, and the private sector to deliver sustainable solutions to marine debris. Hundreds of projects are underway or already completed, but we know that there is much more to be done.

ACC represents a diverse set of companies engaged in the U.S. business of chemistry, a $768 billion enterprise that is helping to solve the biggest challenges facing our country and the world. Chemistry touches 96 percent of all manufactured goods, and the use of plastics in modern automotive, building and construction, and food packaging industries is helping to create a more sustainable society.

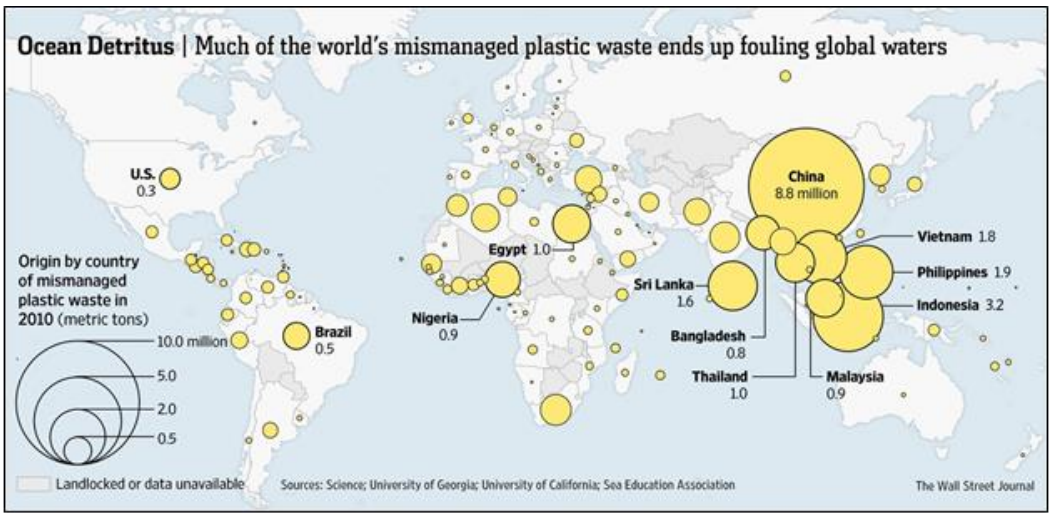
- *Automotive*: Today's plastics make up 50 percent of the volume of new cars but only 10 percent of the weight. Lighter cars are more fuel efficient, and as a result, emit fewer CO2 emissions.
- *Building and Construction*: Architects and designers rely on plastics to help maximize energy efficiency, durability and performance of our homes, offices, and schools.
- *Food Packaging*: Plastics help keep our food fresh and clean with less packaging while reducing food waste. Reducing food waste is important because EPA estimates that more food reaches landfills and incinerators than any other single material in our everyday trash, constituting 22 percent of discarded municipal solid waste.

Although plastics provide important benefits to society, plastics and other trash don't belong in our waterways or the environment. That's why ACC and our members are actively engaged in concrete, well-researched, and sustainable actions to reduce litter and prevent marine debris.

The first step to ending plastic waste in the environment starts with understanding the sources. A number of scientific studies have concluded that plastic litter in the ocean is the result of poor or insufficient waste management and lack of sufficient collection, recycling and recovery facilities infrastructure in rapidly developing countries.

Twenty countries account for 83 percent of the mismanaged plastic waste available to enter the ocean.[31] The largest sources are rapidly developing economies, mainly in Asia, where basic waste management infrastructure has not kept pace with the rise in demand for consumer goods. Over half of land-based plastic waste leaks from just five countries: China, Indonesia, the Philippines, Thailand, and Vietnam.

A recent World Bank study confirms that root cause. Human trash in Indonesian rivers includes 53 percent organic waste, 13 percent diapers, 29 percent plastic and the remainder other debris.[32] Although consumer plastics are a large fraction of the waste stream, holistic solutions are needed to keep plastics out of our oceans and waterways.



The World Bank finding is also consistent with McKinsey's analysis for Ocean Conservancy's Trash Free Seas Alliance® which identified the need to immediately accelerate implementation of waste collection infrastructure, plug post collection leakage, and improve processing of collected waste in source countries.[33]

S. 756, "The Save our Seas Act," first passed in the Senate in 2017 and later by the House in 2018, is a well-designed and thoughtful piece of bipartisan, bicameral legislation. ACC and our members have long supported S. 756 for three important reasons:

- first, it emphasizes greater engagement with the key source countries;
- second, the bill would help ensure that precious waste management resources, technologies and investments are allocated to where they are needed most; and

[31] J. R. Jambeck, R. Geyer, C. Wilcox, T. R. Siegler, M. Perryman, A. Andrady, R. Narayan, and K. L. Law, "Plastic waste inputs from land into the ocean," Science, 2015, Volume 347, Number 6223.

[32] The World Bank Group, Indonesia Marine Debris Hotspot Rapid Assessment Synthesis Report, April 2018.

[33] Ocean Conservancy, McKinsey Center for Business and Environment, "Stemming the Tide: Land-based strategies for a plastic-free ocean," Oct. 2015.

- third, it reauthorizes the Marine Debris Act, which provides provisions to further study land-based waste management solutions and causes of marine debris, as well as increase investment and technical assistance to help expand waste management systems and best-practices in rapidly industrializing nations.

We are pleased that Senate passage of S. 756, with House amendments, is imminent and will allow for expeditious passage in the House before being sent to the President for his signature.

Legislation is one part of the answer. ACC and our members are working with governments, NGOs, and our industry peers to deliver sustainable solutions to marine debris. In 2011, ACC helped lead the development of the Declaration of the Global Plastics Associations for Solutions on Marine Litter.[34] The Global Declaration obliges signatories to commit to action in six areas: education, research, public policy, best practices, recycling/recovery, and product stewardship.

Attached to this Testimony are two documents which highlight work both completed and underway on several marine debris projects across each of the six focus areas:

- "America's Plastics Makers Contribute to Solutions on Marine Litter" (*See Appendix A*);
- "The Declaration of the Global Plastics Associations for Solutions on Marine Litter, 4th Progress Report – Executive Summary" (*See Appendix B*).

75 plastics associations in 40 countries have signed the Declaration since its launch. More than 355 marine litter projects are planned, underway, or have been completed around the globe. Each of them helps to forge cooperation and continuous progress to prevent, reduce, and improve understanding of marine litter.

One of those projects involves a unique partnership between ACC and Circulate Capital, an investment management firm dedicated to financing innovation, companies, and infrastructure that prevent the flow of plastic waste into the world's oceans. Starting initially with Southeast Asia, Circulate Capital will provide capital investments to improve collection, sorting and recycling markets, particularly across the plastic value chain. This emphasis on international cooperation on waste management in the largest source countries is critical to reducing trash in our oceans.

ACC and America's Plastics Makers® are also taking important steps in the United States. In May, ACC's Plastics Division announced three ambitious goals that crystalize U.S. plastics resin producers' commitment to recycle or recover all plastic packaging used in the United States by 2040 and to further enhance plastic pellet stewardship by 2022.

Specifically, members of ACC's Plastics Division have set the following goals for capturing, recycling, and recovering plastics:

- 100 percent of plastics packaging is re-used, recycled or recovered by 2040.
- 100 percent of plastics packaging is recyclable or recoverable by 2030.

[34] www.marinelittersolutions.com.

Circularity at its core is about reducing waste, promoting reuse, increasing recycling rates and developing new recovery technologies. Achieving a more "circular economy" for plastics will enable society to continue to harness plastics' essential benefits, like enhancing the safety and sanitary packaging of food and personal care products, while helping to protect and restore the environment for future generations.

It's important to note that achieving a circular economy does not mean eliminating plastics, since plastics serve an essential role in helping to accomplish ambitious goals in sustainability across all three pillars – social, environmental, and economic. Many proposals to restrict or eliminate certain plastics fail to consider how they can help reduce environmental costs.

For example, a July 2016 study by Trucost found that replacing plastics in consumer products and packaging with a mix of alternative materials with the same function would increase environmental costs from $139 billion to $533 billion annually.[35] The higher environmental cost of alternatives to plastic is a function of the increased quantity of materials needed to fulfill plastic functions. Every material has a cost, including plastics, but the Trucost study tell us that using alternatives to plastic has costs that are almost four times higher.

Another important feature of plastics that is not commonly known is that their "captured energy" is greater than wood, paper or even coal. Today, an emerging set of technologies has begun to unlock that captured energy, transforming non-recycled plastics into alternative fuels and feedstock materials for new manufacturing. In fact, established energy recovery facilities can reduce by 80 percent the volume of waste that goes to landfill.

Plastics that go on to become fuel and other forms of energy are plastics that do not end up in our oceans. Some of the most widely used and rapidly emerging technologies include plastics-to-fuels, pyrolysis, gasification, solid recovery fuels, and waste-to-energy. With the rise of these new technologies comes new jobs and economic growth.

Together with the Trucost findings, the increased recycling and the potential shift toward greater recovery of plastics serves as another example for why product-specific restrictions do not advance social, environmental, or economic goals. Contrary to popular opinion, product bans are not sustainable solutions. Legislation like the "Save Our Seas Act" recognizes this important fact.

Innovations in plastics have helped improve the lives of billions of people around the globe. At the same time, the problem of marine debris is one that businesses, environmental groups, policymakers, and citizens around the globe have become all too familiar with.

ACC believes that awareness, deep appreciation and understanding of the marine debris problem now serves as the necessary prologue to embark on a journey toward creating and implementing sustainable solutions.

We thank you for the opportunity to testify today and look forward to our continued partnership in protecting and restoring the environment for future generations.

The American Chemistry Council (ACC) represents the leading companies engaged in the business of chemistry. ACC members apply the science of chemistry to make innovative products and services that make people's lives better, healthier and safer. ACC is committed to improved environmental, health and safety performance through Responsible Care®;

[35] Trucost, "Plastics and Sustainability: A Valuation of Environmental Benefits, Costs and Opportunities for Continuous Improvement," July 2016.

common sense advocacy designed to address major public policy issues; and health and environmental research and product testing. The business of chemistry is a $768 billion enterprise and a key element of the nation's economy. It is among the largest exporters in the nation, accounting for fourteen percent of all U.S. goods exports. Chemistry companies are among the largest investors in research and development. Safety and security have always been primary concerns of ACC members, and they have intensified their efforts, working closely with government agencies to improve security and to defend against any threat to the nation's critical infrastructure.

SENATE COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS, HEARING ENTITLED, *"CLEANING UP THE OCEANS: HOW TO REDUCE THE IMPACT OF MAN-MADE TRASH ON THE ENVIRONMENT, WILDLIFE, AND HUMAN HEALTH?"*, SEPTEMBER 26, 2018, QUESTIONS FOR THE RECORD FOR THE HONORABLE CAL DOOLEY

Chairman Barrasso

l. Experts seem to agree that stopping the flow of debris into the ocean is a bigger priority than cleaning up the debris already in the ocean. Is this an assessment that you agree with? If not, why not?

Yes, we agree that priority should be given to stopping the flow of debris into the ocean. While there is a need to clean up debris that is presently in the ocean, the highest priority is to stop leakage of additional plastic into the ocean.

A broad coalition of non-governmental organizations, academics, and private sector stakeholders brought together by the Ocean Conservancy, supported the development of *Stemming the Tide*[36], and *The Next Wave.*[37] The reports looked closely at all options to reduce marine debris and concluded that waste management is the critical measure, while finding that bans on plastic products would not solve the problem.

The reports identify solutions for reducing plastic inputs to the ocean and recommend a program for global action to solve the problem. Here are some of the major recommendations from *Stemming the Tide:*

- Close leakage points within local collection systems by optimizing transport systems to eliminate illegal dumping
- Close or improve dump sites located near waterways, and increase waste collection rates by offering expanded services
- Keep leakage points closed by increasing the value of waste, and manually sort waste in rural areas to extract high value plastic waste for recycling

[36] Ocean Conservancy, 2015. http://www.oceanconservancy.org/our-work/marine-debris/mckinsey-report-files/full-report-stemming-the.pdf.

[37] http://www.oceanconservancy.org/wp-content/uploads/2017/05/the-next-wave.pdf.

2. Five countries in Asia China, Indonesia, the Philippines, Thailand, and Vietnam bear much of the responsibility for the increase in ocean plastic. What steps is the private sector taking to reduce plastic waste coming from these countries?

The private sector is engaged in various efforts across the Asia-Pacific region to address leakage of waste into the ocean. Some of these efforts include supporting Circulate Capital, developing of a new global effort to eliminate plastic waste, and engaging in the Asia Pacific Economic Cooperation Forum.

Circulate Capital

In 2017, ACC and the World Plastics Council joined a coalition of partners to support Closed Loop Ocean (CLO), a $150 million fund to support waste management in key source countries. The objective is to absorb some of the risk associated with waste management infrastructure projects in developing countries. The fund also serves as catalytic capital to attract other investors such as multi-national development banks, sovereign wealth funds, and private investors.

In 2018, CLO became Circulate Capital, a separate entity to manage investments in waste management infrastructure in developing countries. Initially, Circulate Capital will focus on waste infrastructure solutions in Southeast Asia. Circulate Capital investments will be provided to improve collection, sorting and recycling markets, particularly across the plastic value chain.

Circulate Capital recently announced the creation of an Incubator Network in partnership with Second Muse to rapidly scale the number of innovators in the sector in South and Southeast Asia, building out their capacity, and providing acceleration support for those innovations.

Asia Pacific Economic Cooperation (APEC)

ACC has been engaged in the APEC for over 20 years and specifically on marine debris issues since 2015. Our work has focused on prioritizing investment in waste management. In 2016, ACC helped to organize a high-level APEC workshop with the Government of Japan on de-risking investment in waste management infrastructure. From the workshop, we helped develop policy and practice recommendations for overcoming barriers to financing waste management systems and reducing marine litter. Since 2016, we have continued to work through various APEC working groups to support investment in waste management, as well as support the development of projects to address marine debris.

3. We know that five countries in Asia bear much of the responsibility for the increase in ocean plastic. That does not mean that we in the United States do not have a critical role to play. What additional steps can local and state government, as well as the federal government, take to address this problem?

The United States has an important role to play in addressing sources of marine debris. The Save Our Seas Act is an excellent step and positions the United States to lead in the global effort to address marine debris. There are many domestic companies with technology solutions that can be deployed to help address the lack of waste management infrastructure and especially waste treatment infrastructure.

Focusing on domestic efforts, the Congress can encourage investment in technologies that repurpose used plastics into a wide range of raw materials, chemical and plastic feedstocks, and lower carbon fuels. The U.S. EPA can communicate a more circular vision of plastics materials management via the use of new technologies and strategies for converting plastics to raw materials, feedstocks, and fuels. If Congress reauthorizes the alternative fuel tax credit and alternative fuels mixture credit, it can make promote fairness by including fuels derived from post-use plastics. If Congress decides to reauthorize the Renewable Fuels Standard, it should also look at the role alternative fuels derived from plastics play in conserving energy and water and reducing greenhouse gas emissions and reducing waste to landfill or litter. Local and state governments can support programs to address littering behavior, improve recycling access and infrastructure, and optimize public services such as collection and street sweeping to reduce littering.

Local governments can also work with groups such as The Recycling Partnership[38] to take advantage of funding to move communities into lidded recycling carts. The program reduces littering, while increasing the volume of recycled material collected.

Plastics recycling and recovery is not only good for the environment but also creates manufacturing jobs. A critical missing piece of the puzzle is our lack of good infrastructure. As part of any infrastructure discussion, Congress should consider how it can incentivize investment in collection (especially in more rural areas) as well as investment in newer and more technologically proficient sortation and processing of post-use materials including plastics.

4. In an answer to one of my questions, you said that:

> "'there are some simple things that Congress could do that would not treat plastic waste and the recovery of it as a 'hazardous waste' because that is stemming the flow of investment dollars in the development of new technologies that could advance the value of that waste stream and recapture some of the value..."

Are there specific provisions in statute or specific regulatory provisions that Congress should re-examine and consider addressing? If so, please identify those provisions.

At the federal level, we could ensure that regulations do not impede innovative, readily available alternative uses to non-recycled plastics. We already know that plastics can be reused in a wide variety of ways, from use as fuel to reinforcing roadways and making decking and fencing. One simple change would be to classify plastics used as fuel to make cement as categorical non-hazardous secondary materials (NHSM), which would ease regulatory burden and cost for these facilities. To make this simple change, Congress could direct EPA to add plastics to an existing regulatory list of authorized alternative fuels.

In the past year alone, states such as Florida, Wisconsin, and Georgia overwhelmingly approved legislation to treat post-use plastics as valuable feedstocks for manufacturing and ensured that newer technologies such as pyrolysis and gasification would be regulated as manufacturing and not waste disposal. Congress should work to understand what these states have done and sec if there is equivalent legislation at the federal level. Led by Senators Wyden and Cassidy, S. 1460 Energy and Natural Resources Act of 2017, included a provision

[38] https://recyclingpartnership.org/.

for the Department of Energy to study how to utilize post-use plastics as a valuable source of domestic energy and raw materials for remanufacturing.

5. On January 24, 2018, the *Financial Times* published an article, by Clive Cookson, that was entitled, "The problem with plastic." It explained that:

> "While the personal-care industry is phasing out microbeads, concern is growing about another ubiquitous micro pollutant: plastic fibers. Analysis shows these to be present in streams, rivers, lakes and seas worldwide, as well as household drinking water. Their main source seems to be clothing and textiles made from synthetic fibers, which become detached in washing machines and are not filtered out by water-treatments plants."

Do you agree with this assessment? lf so, how do we begin to address this issue?

Effective filtration- and then treatment- of wastewater is important. New technologies are now being developed to help washing machines more effectively remove microfibers from home laundry wastewater, and that's a great start. In the meantime, we need to continue work to better understand potential sources of microfibers; how to detect them with accuracy; and to evaluate whether they pose a risk in drinking water or to humans from seafood.

With respect to foods, Congress could ask the Federal Food and Drug Agency and U.S. Department of Agriculture experts in understanding whether water or food are safe- to confirm that seafood's arc currently safe for human consumption while undertaking targeted studies. Congress could also direct USDA to increase its inspection of shellfish and to specifically document any incidence of microfibers. With respect to drinking water, Congress could direct EPA to review the state of technology for dirt and particle removal from drinking water, and provide additional funding if needed to improve technologies for coagulation and f1occulation (removes dirt and other particles through the addition of alum (or other metal salts) to form coagulated masses called f1oc that attract other particles); sedimentation (sifts coagulated, heavy particles through gravity to the bottom of a basin) and filtration (channels water after sedimentation through sand, gravel. coal, activated carbon, or membranes to remove smaller solid particles not already removed).

Ranking Member Carper

6. Stopping the f1ow of marine debris into the ocean and mitigating its impacts- are not problems that can be solved overnight. Long-term. thoughtful, and collaborative solutions are necessary in order to address the full scope of the issue. Solutions that have been discussed included: improving recycling, incentivizing the use of recycled materials in the global supply chain, developing more biodegradable alternatives, and changing manufacturing protocols are potential solutions to help address this issue. What steps can this Committee and the Congress take now to advance these potential solutions here in the United States?

We agree that improving recycling is important. Congress could encourage investment in recycling and modernizing materials recovery facilities to capture more recyclable materials. Congress could also incentivize innovative technologies that convert plastics to monomers or fuels through including fuels made from otherwise unrecycled plastics in the renewable fuel tax credit.

Congress can signal that technologies that convert plastics to raw materials, chemical and plastic feedstocks and lower carbon fuels are part of the circular economy. As noted in response to question 4, Congress could work with EPA to classify plastics used as fuel to make cement as categorical non-hazardous secondary materials (NHSM), which would ease regulatory burden and cost for these facilities. This would be done through the addition of plastics to an existing regulatory list of authorized alternative fuels.

Congress could also work to understand state legislation to treat post-use plastics as valuable feedstocks for manufacturing and see if there is equivalent legislation at the federal level to ensure that newer technologies such as pyrolysis and gasification would be regulated as manufacturing and not waste disposal.

7. Now that China has implemented its “Green Fence Policy," a ban on importing plastic waste, our market for these materials in the United States is flooded. China previously accepted 30% of our plastic waste. Local municipalities are now having even more trouble breaking even when collecting and recycling this waste. In your opinion, what are the best ways for the United States to address this new challenge?

Education of Consumers

There is a need to better education consumers regarding how to properly recycle used plastics to reduce contamination and increase value. A good example is the How to Recycle® label placed on many packages. which provides easy-to-follow directions on proper recycling and disposal of used packaging. Other programs such as ACC's Wrap Recycling Action Program[39] (WRAP) educate consumers on how to properly recycling used bags and films by taking them back to over 18,000 locations across the United States.

Investment in Technology and Infrastructure

Investing in the domestic recycling industry will help to create new market opportunities for material previously sent to China for recycling. By upgrading sortation equipment at material recovery facilities, we will reduce contamination and increase value of used plastics. Chemical recycling and recovery of used plastics through pyrolysis, gasification, and other technologies will create additional markets for used plastics. Increased consumer and brand demand for recycled content in plastic packaging will help to drive investment in needed technologies.

[39] https://www.plasticfilmrecycling.org.

Senator Whitehouse

8. ACC has been a leading voice among the plastics industry in support of both domestic and international marine debris efforts. What are some of the initiatives ACC is leading or supporting that aim to reduce the flow of plastic from land into the oceans?

ACC appreciates the compliment and takes great pride in helping to address the sources of marine debris. The following fact sheet. America's Plastic Makers Contribute to Solutions on Marine Litter[40], and the 41 Progress Report for The Declaration of the Global Plastics Associations for Solutions on Marine Litter[41] provide additional information on ACC's domestic and international efforts to address marine debris. Below are some examples of projects.

Save the Bay Narragansett Bay, Rhode Island

America's Plastics Makers® are partnering with Save the Bay® on the City of Warwick Shoreline Trash Reduction & Prevention project. This initiative aims to reduce littering behavior with a combination of cleanups, community engagement and education. The project is utilizing lessons learned from other efforts to reduce litter and marine debris.

Keep It Beachy Clean (Virginia Beach)

Keep it Beachy Clean is an education and outreach program aimed at reducing beach litter. Clean Virginia Waterways developed the program, which provides Virginia Beach's resort community with anti-litter messaging. Following a successful first year, the program is expanding to capture a wider segment of the Virginia Beach resort community.

Circulate Capital

In 2017, ACC and the World Plastics Council (WPC) joined a coalition of partners to support Closed Loop Ocean (CLO). In 2017 WPC and CLO announced the creation of a $150 million fund to support waste management in key source countries. In 2018, CLO became Circulate Capital, a separate entity to manage investments in waste management infrastructure in developing countries. Initially, Circulate Capital will focus on waste infrastructure solutions in Southeast Asia. Research indicates that the majority of plastic debris originates from five fast growing economics in Asia-Indonesia, the Philippines, Vietnam, Thailand and China. Circulate Capital investments will be provided to improve collection, sorting and recycling markets, particularly across the plastic value chain.

9. Is there a role for materials innovation in reducing the risks plastics pose to wildlife and ecosystems if they enter the oceans?

Material innovation may play a role in reducing the impact of marine debris on wildlife. For example, a marine degradable part was developed for a crab trap to cause a pot to stop catching crabs if it is lost for a significant period of time. Innovations like this arc important

[40] http://plastics.americanchemistry.com/fact-sheets-and-infographics/Americas-Plastics-Markets-Contribute-to-Solutions-on-Marine-Litter.pdf.

[41] https://www.marinelittersolutions.com//wp-content/uploads/2018/04/Marine-Litter-Report-2018.pdf.

but arc challenging and must still meet the original function of the product. Plastics makers continue to look for new materials as well as improvements to existing materials in an effort to balance material use, performance, and recyclability.

10. Is there a role for materials innovation in increasing the value of plastics to ensure they are collected for reuse and recycling?

We are already seeing great potential for increasing the value of used plastics through the development of compatibilizers. This family of technologies improves performance of recycled resins; enables recycling of multi-material, multi-layer pouches; and allows for higher incorporation rates of post-consumer recycled resin into new products.

11. Is ACC investing in any cleanup solutions, including improving wastewater management to better collect microplastics and microfibers from water supplies?

ACC staff participate in annual International Coastal Clean-up events. although our investments related to address marine debris are focused on infrastructure and education. For example, we have provided funding for trash and recycling bins; consumer education regarding proper recycling of plastics wrap, bags, and film; and anti-litter and litter reduction programs; among other projects. As noted above, the following fact sheet, America's Plastic Makers Contribute to Solutions on Marine Litter[42] and the 4'" Progress Report for The Declaration of the Global Plastics Associations for Solutions on Marine Litter[43] provide additional information on ACCs domestic and international efforts to address marine debris.

ACC has not invested into projects to better collect microplastics and microfibers from wastewater management systems. We are aware of several efforts to address the issue of microfibers such as the Cora Ball[44] and Guppy Friend[45] That said, industry efforts to improve the technology to filter microfibers at the source-in wastewater from washing machines-is well-documented.

12. As we work on the next iteration of the Save Our Seas Act, do you have any additional recommendations or comments you would like to share to inform our development of this bill?

We welcome the opportunity to work with you and your staff as the next iteration of the Save Our Seas Act is developed. The Save our Seas Act of 2018 included new language to coordinate with developing countries which are the largest sources of marine debris. This assistance will be the highest priority to solve this problem for the foreseeable future. Unfortunately only a very small fraction of development finance goes to improve waste management (0.3%). There may be a way for the US to encourage development financing agencies to prioritize waste management.

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[42] https://plastics.americanchemistry.com/fact-sheets-and-infographics/Americas-Plastics-Makers-Contribute-to-solutions-on-Marine-Litter.pdf.

[43] https://www.marinelittersolutions.com//wp-conten/uploads.2018/04/Marine-Litter-Report-2018.pdf.

[44] https://coralball.com/.

[45] https://guppyfriebs.com/en/.

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Senator BARRASSO. Thank you so much for your testimony, Mr. Dooley.
Mr. KARAS.

STATEMENT OF BRUCE KARAS, PRESIDENT OF ENVIRONMENT AND SUSTAINABILITY, COCA-COLA NORTH AMERICA

Mr. KARAS. Chairman Barrasso, Ranking Member Carper, and members of the Committee, thank you for the opportunity for inviting me before you to discuss the very issue of marine debris.

Our world has a waste problem. According to the Ocean Conservancy, scientists estimate that more than 8 million metric tons of plastic is entering our ocean every year. From our perspective, it is unacceptable that packaging ends up in the wrong place, in our oceans and waterways or littering the communities where we work and live.

As a total beverage company, we bring people drinks that make life's everyday moments more enjoying, create a shared opportunity for people and communities we call home. While growth is important, we cannot grow at any cost. We believe in doing business the right way, not just the easy way. For us, that means continuously working to reduce our environmental impact by collecting and recycling our packaging footprint, providing access to clean drinking water, supporting women's economic empowerment, and strengthening local communities.

We are a global company operating in more than 200 countries and territories, but through our bottling partners we also have deep, local connections and relationships that offer a unique ability to make a meaningful difference. The key areas where we strive to lead are clean, sustainable water for communities and women's economic empowerment.

A third area we launched just this year is our new packaging vision, World Without Waste. The goal is to rethink how bottles and cans are designed and made, as well as how they are recycled and repurposed. The centerpiece is a bold, ambitious goal to help collect and recycle the equivalent of every bottle or can we sell globally by 2030. The Coca-Cola system intends to back World Without Waste with a multiyear investment that augments ongoing work to make our packaging 100 percent recyclable by 2025.

When it comes to PET, we believe that every package has value and a life beyond its initial use, and should be collected and recycled into either a new package or another beneficial use. We aim to be part of collaborative solutions that prevent waste from getting to the ocean in the first place.

Regardless of where it comes from, we want our packages to have more than one life. To date, all 17 of our geographic business units have developed local plans to address our three strategic pillars: design, collect, and partner.

Design means we aspire to create packaging that is at least 50 percent recycled material by 2030; continue working to make all consumer packaging 100 percent recyclable by 2025.

Collect means to collect and recycle the equivalent of 100 percent of the primary packaging we sell by 2030. Partner means we will work together to support a healthy debris-free environment at both the land and the sea.

In the context of design, our research and development team is working with chemical recycling technologies toward future piloting or partnerships. Additionally, our procurement team is working with our suppliers to advance progress on and increase availability of recycled PET, known as rPET.

In Mexico, our bottled water brand seal is now available in 100 percent rPET bottle as a result of strong collection and conversion infrastructure that our system has partnered in over the past decade. We are also looking at 100 percent rPET bottle in the Hong Kong market later this year. We will pilot the use of rPET in several other Asia Pacific markets in 2019. The increased use of rPET is crucial to accelerate a transition to a true circular economy for plastics.

In the innovation space, we have expanded our package-less delivery model for beverages with both our freestyle touchscreen operated dispenser and our innovative Dasani pure fill.

In the context of collect, marine plastic is driven in larger part by limited collection and waste management infrastructure in many emerging markets. That is why our second strategic pillar centers on improving packaging collection.

We are working around the world to have an up-to-date understanding of collection recycling data and approaches. Where systems do not exist is where we are focusing. Cities with a very active informal sector, like unofficial, small-scale businesses, have high rates of collection. There are correspondingly lower rates of collection for recycling in more developed cities where there is less incentive for small-scale collectors.

We will use the data we collect to partner with government, industry, civil society, and local communities to tailor, co-create, and roll out the type of collection recycling models that have been successful in developing markets in other parts of the world and scalable models that will improve collection rates.

Last is partner. We recognize that although we are part of a problem, we cannot solve the packaging waste problem alone. It is for that reason we have established, joined, and expanded cross-sectoral partnerships around the world. We intend to do all of this not just in a cross-sector way, but in a scalable way that drives systemic change.

We are working with groups from the international level to the very local level, from the Ocean Conservancy Trash for Seas Alliance, the Ellen MacArthur Foundation, to the Closed Loop Fund and local chambers of commerce.

Thank you for your time. I look forward to answering your questions.

[The prepared statement of Mr. Karas follows:]

TESTIMONY OF BRUCE KARAS, VICE PRESIDENT OF ENVIRONMENT AND SUSTAINABILITY, THE COCA-COLA COMPANY NORTH AMERICA, BEFORE THE UNITED STATES SENATE, ENVIRONMENT AND PUBLIC WORKS COMMITTEE, HEARING ON MARINE DEBRIS, SEPTEMBER 26, 2018

Good Morning Senators. Thank you for inviting me before you today to discuss the very real issue and concern of Marine Debris. Our world has a waste problem. According to the Ocean Conservancy, scientists estimate that more than 8 million metric tons of plastic is

entering our ocean every year. From our perspective, it is unacceptable that packaging ends up in the wrong place, in our oceans and waterways or littering the communities where we work and live.

As a total beverage company, we bring people drinks that make life's everyday moments more enjoyable, to create shared opportunity for the people and communities we call home. While growth is important, we cannot grow at any cost. We believe in doing business the right way, not just the easy way. For us, that means continuously working to reduce our environmental impact by collecting and recycling our packaging footprint, providing access to clean drinking water, supporting women's economic empowerment, and strengthening local communities.

We may be a global company operating in more than 200 countries and territories, but through our bottling partners we also have deep, local connections and relationships that offer a unique ability to make a meaningful difference. The key areas where we strive to lead are clean, sustainable water for communities, women's economic empowerment, and to working to create a world without waste by collecting and recycling the equivalent of our bottles and cans in the marketplace.

In 2010, The Coca-Cola Company made a commitment to be water neutral by 2020- a goal we achieved in 2015. Through projects implemented by the end of 2017, The Coca-Cola Company is replenishing an estimated 248 billion liters per year through community and watershed projects globally. We are also working to improve our water-use ration 25% over 2010 levels. To date we have made a 15% improvement and continuing to do more.

At the same time, our commitment to strengthening communities by empowering women has never been stronger. Our 5by20 initiative, also launched in 2010 to enable economic empowerment to 5 million women by 2020 has to date enabled more than 2.4 million women entrepreneurs across 75 countries.

This year, to help tackle the world's packaging problem, we launched our new packaging vision, World Without Waste. It involves rethinking how bottles and cans are designed and made, as well as how they're recycled and repurposed within our system around the world. The centerpiece of this vision is a bold, ambitious goal to help collect and recycle the equivalent of every bottle or can we sell globally by 2030. The Coca-Cola system intends to back World Without Waste with a multi-year investment that includes ongoing work to make our packaging 100 percent recyclable by 2025.

When it comes to PET, we believe every package has value and life beyond its initial use and should be collected and recycled into either a new package or another beneficial use. We aim to be part of collaborative solutions that prevent waste from getting to the ocean in the first place.

Regardless of where it comes from (i.e., whether the package is made by Coca-Cola or one of our competitors), we want it to have more than one life. To date, all 17 of our geographic business units have developed local plans to address our (3) strategic pillars - Design, Collect and Partner:

- Design - Aspire to create packaging that is at least 50% recycled material by 2030; continue working to make all consumer packaging 100% recyclable by 2025
- Collect - Collect and recycle the equivalent of 100% of the primary packaging we sell by 2030
- Partner - Work together to support a healthy, debris-free environment (land & sea)

Design

Our Research and Development team is working with chemical recycling technologies towards future partnerships or piloting. Additionally, our Procurement team is working with our suppliers to forward progress on and increase availability of rPET.

In Mexico, our bottled water brand, Ciel, is now available in a 100% rPET bottle, which builds on the extremely strong collection and conversion infrastructure that our system has financed over the past decade. In Australia, our Mount Franklin water brand is also now available in 100% rPET, and we are launching our water brand in Hong Kong in 100% rPET later this year. We will pilot the use of rPET in a number of ASEAN markets beginning in 2019 and we believe this is going to be very important to accelerate a transition to a true circular economy for plastics.

In the innovation space, we have expanded our "package-less" delivery model for beverages, our innovative Freestyle technology to more than 50,000 machines serving 14 million drinks daily, with continued expansion in North American and moving to Europe and Latin America. Freestyle is a touchscreen-operated dispenser that uses "micro-dosing" technology to deliver nearly 200 beverage options - including 117 low/no-calorie beverages and more than 100 varieties, with only a cup.

Collect

We are keenly aware from research conducted by the Ocean Conservancy and others that marine plastic is primarily a land-based issue, driven in large part by limited collection and waste management infrastructure in many emerging markets. That's why our second strategic pillar centers on improving packaging collection. We are working around the world to have an, up-to-date understanding of existing collection and recycling data and approaches, as you would expect some parts of the world have much stronger systems in place than others. Where the systems do not exist is where we are focusing our efforts.

Data on solid waste management collection and recycling is a significant challenge in ASEAN (governments and civil society don't have this kind of date on PET collection rates) and so we've commissioned a Singapore- based circular economy advisory and action company to conduct baseline research for us in six markets across Southeast Asia. What we've seen from the research is high rates of collection for recycling for PET in cities like Myanmar (estimated 82% collection for recycling rate for PET in Mandalay, 74% in Yangon) or Jakarta (estimated 69% collection for recycling rate for PET) with a very active informal sector (i.e., unofficial small-scale businesses such as street scavengers, recycling pickers and junk shops) and correspondingly lower rates of collection for recycling in more developed cities like Kuala Lumpur (estimated 23% collection for recycling rate for PET) where there's less incentive for waste collectors and recycling pickers with higher incomes to collect recyclables.

As ASEAN develops, driving source segregation will be the only way to capture recyclables. This will require government conviction that segregation is necessary and government commitment to implement and enforce source segregation initiatives. We will be using the data to tailor, co-create and roll-out with government, industry, civil society and local communities the type of collection and recycling models that have been very successful

in developing markets in other parts of the world (e.g., Mexico) and scalable models that improve collection rates by empowering and strengthening the informal collection sector. We see extensive opportunity to do this starting in major cities and tourist destinations in the ASEAN region and we already have a number of industry alliances set up in ASEAN. A few examples are:

- *Indonesia* is the second largest global contributor to issue of ocean plastic, with plastic waste a serious threat to the economy (particularly industries like tourism and fishery) as well as the environment. In 2017, the government launched a *National Plan of Action Against Marine Debris,* developed by a task force led by the Coordinating Ministry for Maritime Affairs. Ahead of National Waste Awareness Day in February, and as part of industry efforts to step up and contribute to solutions, six companies, including Coca-Cola Indonesia, jointly announced the formation of the Packaging and Recycling Alliance for Indonesia Sustainable Environment (PRAISE). The vision of PRAISE is to establish best practices that support sustainable and integrated packaging waste management solutions in Indonesia. This approach to waste management aligns with the government's move towards a circular economy and PRAISE has engaged on a regular basis with the Ministry of Environment and Forestry and the Coordinating Ministry for Maritime Affairs. By contributing to research, education, collaboration and engaging stakeholders in government and civil society, PRAISE is an important industry platform to help advocate for a circular economy and drive industry efforts to reduce impacts of packaging waste on the environment. PRAISE was represented last year at the World Ocean Summit in Indonesia, the first-ever ASEAN Conference on Reducing Marine Debris held in Thailand, the APEC High-Level Meeting on Accelerating Waste Management Solutions to Reduce Marine Litter in Bali and the Responsible Business Forum on Sustainable Development in Singapore. This year, as part of an agreement with the Coordinating Minister of Maritime Affairs, PRAISE commissioned a study in Bali to look at the value chain of Plastic Waste in order to obtain better data on plastic waste and subsequently to implement a pilot project on Sustainable Plastic Waste Management using the data collected. In parallel, this year PRAISE is also starting to implement a Drop Box program in Jakarta, through which 100 drop boxes are being distributed in public as well as retail spaces.
- *Philippines* - Coca-Cola Philippines is a member and active supporter of The Philippine Alliance for Recycling and Materials Sustainability (PARMS) a non-profit industry organization incorporated in 2015 to bring together stakeholders in the recycling value chain, including manufacturers, industry groups, retail groups, waste consolidators and haulers, recyclers and non-government and government entities. The objective of PARMS is to develop and implement holistic and comprehensive programs to increase waste recovery and reduce landfill dependence towards zero waste through strategic partnerships. PARMS recently entered into a Memorandum of Understanding with the Local Government Unit of Paranaque (a fully urbanized city in Metro Manila) to build a plastics recycling facility towards the implementation of a Full Waste-Recovery Program within this year. Once fully operational, this facility will be able to process approximately 365 tons of waste per annum - waste diverted from landfills, from water streams, and the like. During the

Earth Day celebration in April 2018, PARMS formally turned over the proposed recycling facility design to the chief executive of Paranaque City. The project is supported by 8 leading fast-moving consumer goods companies including Coca-Cola and many of our competitors and leading local industry organizations.

- *Vietnam* - we are now advancing a partnership with Unilever, Dow and the Vietnam Chamber of Commerce and Industry to implement a circular economy approach in Ho Chi Minh City. The partnership is still in its infancy, but we will be working together to strengthen recycling infrastructure. We are also getting ready to launch a new community-focused program focused on the iconic tourist area of Ha Long City, working with a local NGO called Green Hub to empower 250 women waste pickers and raise awareness across stakeholder groups and decision-makers about the importance of tackling plastic waste.

Partner

We recognize that although we are a part of the problem, we cannot solve the packaging waste problem alone. It is for that reason we have created, established, joined and expanded cross-sectoral partnerships around the world. We intend to do all of this not just in a cross-sector way but in a scalable way that drives systemic change - to do so will take catalytic funding. Some of our other partners in driving this change are:

- The European Union Beverage Industry: Beverage packaging is already the most collected packaging in the European Union, but the beverage industry just announced a new set of EU-wide ambitions:
  - By 2025 100% of soft drinks primary plastic packaging will be recyclable;
  - By 2025 soft drinks PET bottles will contain a minimum of 25% recycled material on average;
  - Collection rates of soft drinks primary plastic packaging for recycling will be further increased and optimized in all EU markets in collaboration with other packaging recovery actors;
  - Soft drinks primary plastic packaging will be reused - including refillable bottles - where it makes environmental and economic sense.
- The Ocean Conservancy/Trash Free Seas Alliance: A partner since 1995, our partnership activities range from participation in the annual International Coastal Cleanup to supporting recycling infrastructure. Together with the Ocean Conservancy and others, we launched the Trash Free Seas Alliance in 2011 to advance the scientific understanding of marine debris globally, including research to determine the regions contributing the most waste to the oceans.
- Closed Loop Fund: We have worked with the Closed Loop Fund since its inception, predominantly through Coca-Cola North America (as most of its first efforts were focused in the U.S.). More recently, we joined a new initiative of Closed Loop Partners called Closed Loop Ocean that will target innovative approaches on funding waste management and recycling solutions in Southeast Asia.

- Circulate Capital we are an early-stage supporter of the just-announced Circulate Capital initiative established to advance the circular economy- it builds on our long partnership in North America with the Closed Loop Fund. Circulate Capital is focused on catalyzing investments in ocean plastic solutions in Southeast Asia and India. Priority markets are Indonesia and India. Secondary markets are the Philippines, Vietnam and Thailand.
- *Ellen MacArthur Foundation (EMF)* New Plastics Economy initiative: The Company is a founding partner of the New Plastics Economy initiative, which includes leading consumer goods companies and other plastics users and producers. The Company continues to work closely with EMF on the development of our packaging strategy and metrics. EMF is a leading voice supporting the circular economy and pushing for private sector led innovations and solutions to the addressing plastic waste.
- *World Economic Forum Platform for Accelerating the Circular Economy (PACE) initiative* is a public-private multi-stakeholder platform focused on shaping global policy and accelerating targeted action towards the circular economy. Plastics is a core focus area with specific project work underway in ASEAN. We are actively engaged in shaping the agenda at both the strategic and tactical levels. Our CEO James Quincey serves on the PACE Leadership Board.

SENATE COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS, HEARING ENTITLED, *"CLEANING UP THE OCEANS: HOW TO REDUCE THE IMPACT OF MAN-MADE TRASH ON THE ENVIRONMENT, WILDLIFE, AND HUMAN HEALTH?"*, SEPTEMBER 26, 2018, QUESTIONS FOR THE RECORD FOR MR. BRUCE KARAS

Chairman Barrasso

l. Experts seem to agree that stopping the flow of debris into the ocean is a bigger priority than cleaning up the debris already in the ocean. Is this an assessment that you agree with? If not, why not?

Agree as The Coca-Cola Company, we believe that the most effective way to impact change is to address the problem of waste before it reaches waterways and ultimately, oceans. That is where our efforts are focused.

2. Five countries in Asia- China, Indonesia, the Philippines, Thailand, and Vietnam- bear much of the responsibility for the increase in ocean plastic. What steps is the private sector taking to reduce plastic waste coming from these five countries?

The Coca-Cola Company is one example of one private sector organization, and our efforts are focused around our World Without Waste vision. We've set an ambitious goal to help collect and recycle the equivalent of a bottle or can for everyone we sell by 2030 and to make all of our packaging I 00% recyclable by 2025.

We are working to accomplish this industry-first, ambitious goal through 3 main pillars:

- Design - Aspire to create packaging that is at least 50% recycled material by 2030; continue working to make all consumer packaging 100% recyclable by 2025
- Collect- Collect and recycle the equivalent of 100% of the primary packaging we sell by 2030
- Partner- Work together to support a healthy, debris-free environment (land & sea)

Our goal is not only a worldwide goal, but also a local goal. We have strategies, partnerships and engagements in each of the five countries listed above that are tailored to the not just the country, but the needs of the cities within each of those countries as the needs vary depending on locality. Across the Asia-Pacific region our local teams are focused on addressing PET collection systems, raising public awareness, creating and promoting public-private partnerships and establishing recycling infrastructure. We won't reach solutions overnight or by ourselves, but they must come, and we aim to play an important role in achieving progress.

China

Using cutting-edge facial recognition technologies and a voice interactive system, the new VenCycling machine both dispenses beverages and collects used packaging for recycling. In exchange for returning used cans or plastic bottles into the machine, consumers receive credits, primarily via mobile devices, for products made from recycled plastics.

These innovative vending machines are helping to build a circular pathway for our packaging while educating and incentivizing recycling behaviors. We have also partnered with the One Foundation in China to work on public education around recycling.

Indonesia

We are an early-stage supporter of the just-announced Circulate Capital initiative that is focused on catalyzing investments in ocean plastic solutions in Southeast Asia and India. It is an initiative to advance the circular economy and builds on our contribution to and work with the Closed Loop Fund over the last few years in the United States.

In 2007, Coca-Cola Amatil lndonesia and Quiksilver Indonesia initiated the Bali Beach Clean Up (BBCU), a daily clean-up program in five main beaches of Bali. Ten years later, the Bali Beach Clean Up teams have removed more than 34 million kg of rubbish from 9.7 kilometers of shoreline in Bali. The program is powered by 150 new bins per year, 4 beach tractors, 2 barber surf rakes, 3 garbage trucks, and 78 crews from local communities surrounding the beaches. BBCU crews also act as ambassadors to promote the importance of taking care of the environment. Complementing the daily BBCU program, Bali's Big Eco Weekend is an annual festival to drive more support from everyone to keep Bali beaches clean and green.

The Coca-Cola Company is contributing our marketing and communications expertise to public campaigns that bring awareness to the importance of recycling and circular economy solutions. For example, in Jakarta, Coca-Cola Indonesia has launched a program called “Plastic Reborn: Give your Plastic Bottle A Second Life" targeted at millennials and showing that “Recycling can be cool”. Plastic Reborn is increasing awareness about responsible waste

management and the importance of a circular economy. The program has deployed I 00 bins in 77 schools/universities/public places in Jakarta and already collected 1.8 tons of post-consumer PET bottles. These bottles are then shredded into PET flakes and then the process of upcycling turns the flakes into fashionable multi-function bags. 5000 bags already produced through this program.

Philippines

Coca-Cola Philippines is a member and active supporter of The Philippine Alliance for Recycling and Materials Sustainability (PARMS), a non-profit industry organization incorporated in 2015 to bring together stakeholders in the recycling value chain, including manufacturers, industry groups, retail groups, waste consolidators and haulers, recyclers and non-government and government entities. The objective of PARMS is to develop and implement holistic and comprehensive programs to increase waste resource recovery and reduce landfill dependence towards zero waste through strategic partnerships. PARMS recently entered into a Memorandum of Understanding with the Local Government Unit of Paranaque (a fully urbanized city in Metro Manila) to build a materials recovery facility towards the implementation of a Full Waste-Recovery Program within this year.

Once fully operational, this facility will be able to process approximately 365 tons of waste per annum-waste diverted from landfills, from water streams, and the like. During the Earth Day celebration in April 2018, PARMS formally turned over the proposed recycling facility design to the chief executive of Paranaque City. The project is supported by leading fast-moving consumer goods companies including Coca-Cola, Liwayway Marketing Corporation, Monde Nissin, Nestle Philippines, Pepsi-Cola Products Philippines, Procter & Gamble Philippines, Unilever Philippines, and Universal Robina Corporation. Other alliance members are Philippine Chamber of Commerce and Industry. Philippines Business for the Environment, the Zero Waste Recycling Movement. Department of Trade and Industry, Department of Science and Technology, Department of Environment, Philippine Plastics Industries Association, Philippine League of Environment and Natural Resource Officers.

Thailand

Coca-Cola supported the first-ever ASEAN Conference on Reducing Marine Debris hosted by the government of Thailand, the ASEAN Secretariat and UN Environment with participation from ASEAN member governments, civil society and private sector. The theme of partnership was pervasive throughout the conference-. "The Time Is Now" with an emphasis on partnerships across the plastic value chain.

Vietnam

We are now advancing a partnership with Unilever. Dow and the Vietnam Chamber of Commerce and Industry to implement a circular economy approach in Ho Chi Minh City. The partnership is still in its infancy. We are also getting ready to launch a new community-focused program focused on the iconic tourist area of Ha Long City, working with a local NGO called Green Hub to empower 250 women waste pickers and raise awareness across stakeholder groups and decision-makers about the importance of tackling plastic waste.

3. We know that five countries in Asia bear much of the responsibility for the increase in ocean plastic. That does not mean that we in the United States do not have a critical role to play. What additional steps can local and state governments as well as the federal government take to address this problem?

The Coca-Cola Company has long championed public-private partnerships, or what we call the Golden Triangle consisting of private companies, non-profits and government working together to solve a problem. Recycling systems and infrastructure need to be improved throughout the US which is the best way to impact national recycling rates. We believe that working with the broader waste management sector in transitioning from a land filling approach for all waste to a more refined system that builds an infrastructure that increases recycling is key to the success of recycling. Additionally, education on proper waste management, and innovation when it comes to our packaging are areas where we as a Company will continue to focus our efforts.

4. In an answer to one of my questions, you mentioned "waste infrastructure 2.0." How would you (Coca-Cola) design "waste infrastructure 2.0" to ensure that we in the United States collect materials that are not currently recycled?

Waste infrastructure 2.0 is a transition to an infrastructure that reflects more of a circular economy that captures commodities that do have intrinsic value and can be re-used. It is a closed loop. The 2.0 system will have an increased capability to identify, select, sort and bale commodities that can be re-used in a meaningful way. In today's system we are essentially retiring the carbon consumed in making materials in the ground, when recycling and reuse can significantly reduce our carbon emissions. In the beverage sector, glass PET and aluminum arc highly recyclable commodities that represent future supply in a circular economy. If we have an updated system, a steady supply of used PET and high demand for recycled PET is achievable.

Ranking Member Carper

5. Stopping the flow of marine debris into the ocean- and mitigating its impacts- are not problems that can be solved overnight. Long-term, thoughtful, and collaborative solutions are necessary in order to address the full scope of the issue. Solutions that have been discussed included: improving recycling, incentivizing the use of recycled materials in the global supply chain, developing more biodegradable alternatives, and changing manufacturing protocols are potential solutions to help address this issue. What steps can this Committee and the Congress take now to advance these potential solutions here in the United States?

The Coca-Cola Company believes that we need to focus on designing better packaging, collecting the packaging that we sell and partnering in order to achieve both of those goals. Improving recycling infrastructure both in the US and globally is an immediate first step toward improving recycling rates which will ultimately reduce the flow of marine debris into the ocean. It is absolutely critical that in considering the necessary infrastructure thought is given to the economic incentive of creating fully functional end markets that convert the

recycled commodities into future products. These end markets represent green jobs and incentivize the infrastructure as it is a viable business.

Now that China has implemented its "Green Fence Policy," a ban on importing plastic waste, our market for these materials in the United States is flooded. China previously accepted 30% of our plastic waste. Local municipalities are now having even more trouble breaking even when collecting and recycling this waste. In your opinion, what are the best ways for the United States to address this new challenge?

Recycled PET (rPET) has a value on the market. One reason The Coca-Cola Company has set the target of making our bottles out of 50% rPET by 2030 is that will further drive the rPET market. The challenge in the US is that our recycling stream has high levels of contamination that impacts the productivity of the recovery facilities (MRFs). When we improve and update MRFs, we will have a rPET product for which there is a market, one that is in fact growing and becoming more competitive. This is one example of the importance of a viable end market that has a demand for the recycled material.

6. In your testimony, you mentioned Coca-Cola's three strategic pillars for recycling as part of the "World Without Waste" campaign: one of them is "collect." I agree that improving our nation's recycling practices is vitally important. However, many local communities and states do not have sufficient waste management and recycling infrastructure. From your perspective, what can local and state governments do to build this capacity? How might this Committee and the federal government support them?

Business must collaborate with governments, communities, the private sector and NGO's to establish or further develop recycling programs. One example of such is our partnership with the Closed Loop Fund. We are working to advance recycling technologies and partnering with local communities to improve their recycling infrastructure. Aluminum and PET are the two highest value commodities in the waste stream today. There is an opportunity to created viable businesses and green jobs that harvest recyclables. Our work with the Closed Loop Fund has shown that often the ROI for investments in the local infrastructure is positive. We welcome the opportunity to participate in more such partnerships.

Senator Markey

7. The Ocean Plastics Charter was agreed to in June 2018 by five of the G7 countries to encourage a more sustainable approach to the management of plastics. The United States and Japan did not join. But on September 20, Coca-Cola and several other large corporations pledged to help reduce plastic pollution in support of the Ocean Plastics Charter.

a. How important is it for the United States and the private sector to get on board with reducing plastic waste if we are truly going to address the marine debris crisis on a global scale?

As a company headquartered in the United States, we know that our actions, commitments and progress impact movement on a global scale. For our part, as a global business operating in 200 countries and territories, we will continue to work to address our

contributions on a global scale. Our World Without Waste commitment is a global commitment because marine debris is a global issue. We believe it is of vital importance that this continue to be an area of international focus.

Senator Merkley

8. To achieve your goal of recycling all your bottles, you have commented that you will need help collecting them. What challenges has Coca-Cola faced in increasing collection rates abroad?

Today, in some countries, our packaging and collection rates are high while in others, there is still a lot of work to be done. It's a huge challenge and there are a lot of moving targets. We operate in 200 countries and territories, and each of them has its own government, regulatory system, and collection and recycling infrastructure challenges. Measurement and data that pertain to recycling is also lacking in many parts of the world. Certain parts of the world have no infrastructure for collecting and recycling packaging, and others have minimal infrastructure. In these cases, sometimes the market for recycled material doesn't exist. Creating a valuable "end market" component is key. To achieve our goals, we will need the support of many cross-sector partners.

9. Will Coca-Cola support bottle deposit arrangements in the United States, to work in conjunction with curbside recycling?

For many years, we have favored a holistic recycling approach.

We are open to further looking at deposit programs that are non-discriminatory and fair, on a case-by-case basis, to determine whether they may be the most effective and sustainable option for some communities.

Despite our belief that deposit programs are not suitable for every society or economy, and are not always the most efficient solution, we have organized some fair and efficient deposit programs in some of our markets. The rate of recycling has not increased which is why it is time to try new solutions, deposit programs are one of multiple way to do this.

Senator Whitehouse

10. What lessons has Coca-Cola learned through its other sustainability and resource management work within its supply chain and distribution network that can be applied to plastics?

From our water stewardship work, we know that business must collaborate with governments, local communities, the private sector and NGO's to establish or further effective programs. It takes many partners to be successful. We are also acutely aware that when we harness our entire supply chain we have the ability to affect real change with compounded results. That is our goal with World Without Waste.

11. This year, Coca-Cola launched its "World Without Waste" initiative which sets the goal of collecting and recycling every bottle and can Coca-Cola sells by 2030. It also aims to make all Coca-Cola products recyclable by 2025.

a. What types of initiatives are Coca-Cola programs already undertaking or likely to undertake to implement the World Without Waste goals?

The Coca-Cola Company is building partnerships, creating initiatives and joining collaborations for localized programs around the world. Two good examples of collaboratives are Closed Loop Partners ("CLP") and The Recycling Partnership ("TRP"). CLP includes the Closed Loop Fund, a $100 million fund delivering solutions that remove recycling bottleneck with technology. TRP is a collaboration of over 40 companies and enterprises that is working on increasing curbside collection in the U.S. There is no one solution or silver bullet to improving recycling, which is why we are working within cities, towns and counties to find the solution that best tits, but also sharing best practices around the globe.

b. What investments is Coca-Cola making in improving collection and recycling infrastructure in the over 200 countries and territories it operates in?

The Coca-Cola Company has become and is becoming financial partners with organizations that are focused on recycling: from Keep America Beautiful, The Recycling Partnership and Ocean Conservancy, to the Closed Loop Fund and Circulate Capital, a venture loan fund established to address plastics in the Asia-Pacific oceans. Every country in which Coca-Cola operates will make investments to support World Without Waste.

c. What is the role of the private sector, especially in developing countries, in improving waste infrastructure?

The role of the private sector is to work with the local governments. Because we are a global company, The Coca-Cola Company can bring expertise and best practices to contribute to the expansion (or creation) of recycling infrastructure.

d. As part of this initiative, does Coca-Cola plan to redesign its packaging to reduce plastic composition?

By 2030, we will have no single use primary packaging, as we will collect 100%, incorporate at least 50% recycled content in each package, and ensure the balance is recycled in other ways. We will also continue to innovate to lightweight the plastic that is used to further reduce the amount of virgin plastic in our bottles.

12. What other areas of materials innovation or packaging design is Coca-Cola investigating to minimize its waste production?

Our focus is on our packaging materials, primarily PET plastic. We are innovating new packaging that will include 50% recycled PET (rPET) by 2030. We have pledged that our packaging will be I 00% recyclable by 2025. Additionally, we are looking at future process

innovations such as chemical recycling and package-less delivery of beverages. We will continue to design and innovate new packaging that meets our goals.

13. Does China's new prohibition on imported waste alter Coca-Cola's ambitions at all?

No, our World Without Waste goals exist independent of governmental action. In order to meet our World Without Waste goals, we need to collect and reuse PET. Having a strong local supply of pure rPET is an asset to our business in the U.S. and throughout the world.

14. As a user of plastic materials, what pressure can Coca-Cola exert on plastic producers and prod net developers to innovate and minimize the harm these materials can cause if they end up in the oceans?

We are working with Global Procurement and our mainstream suppliers to phase in rPET content globally. As a leader in the industry, our goal is by creating this demand developers will further innovate, and it will encourage greater waste collection to grow the supply of rPET.

15. What role has consumer demand played in encouraging Coca-Cola to be an active participant in marine debris work?

We are always working to listen to consumer points of view, particularly where our business intersects with an opportunity to do good in the communities that we serve- we are a part of them too.

We are committed to marine debris work because we, like others, have a responsibility to help solve the world's packaging problem. We know it's time for us to help lead the way and do more- and we are, through our World Without Waste initiative.

Our work is not reactionary. We have supported recycling and packaging recovery programs throughout the world for decades. We have partnered with the Ocean Conservancy since 1995 on research and action to cleanup marine litter, including being a lead sponsor of the International Coastal Cleanup. Together with the Ocean Conservancy and others, we launched the Trash Free Seas Alliance® in 20 I I to advance the scientific understanding of marine debris globally.

16. As we work on the next iteration of the Save Our Seas Act, do you have any additional recommendations or comments you would like to share to inform our development of this bill?

We look forward to working with the Senate in the creation of the next iteration of Save Our Seas Act.

* * *

Senator BARRASSO. Thank you very much, Mr. Karas. Dr. LAW.

STATEMENT OF KARA LAVENDER LAW, RESEARCH PROFESSOR OF OCEANOGRAPHY, SEA EDUCATION ASSOCIATION

Ms. LAW. Good morning. Thank you, Chairman Barrasso, Ranking Member Carper, and members of the Committee for the invitation to testify at this important hearing on man-made debris in the marine environment. My name is Dr. Kara Lavender Law, and I am a Research Professor of Oceanography at Sea Education Association, or SEA.

Since 1971, SEA has taken undergraduate students to sea on tall sailing ships to study the open ocean firsthand as navigators, sailors, shipmates, and scientists. More than 8,000 SEA semester students, some of whom are in the room today, have contributed to our 30-plus year data set on floating plastics in the ocean, assembled by towing plankton nets from our sailing research ships twice a day, every day, and hand-counting their contents.

Trained in ocean physics, I first learned about ocean plastics in 2003, when I joined SEA, where the distribution of floating plastic debris was common knowledge based upon decades of student research. In contracts to misconceptions about immense floating islands of recognizable items of plastic trash, often referred to as garbage patches, SEA scientists knew that the most numerous type of plastic debris are microplastics, particles smaller than your pinky fingernail that are not readily visible even from the deck of a ship. I have a sample here.

Since 2010, I have carried out scientific research on ocean plastics to better understand their sources, abundance, distribution, and transformation in the marine environment not only to advance scientific understanding, but also to inform solutions to this global problem.

Of all the man-made debris in the marine environment, we focus on plastics because of their ubiquity, persistence, and the risks they pose to wildlife and, potentially, human health. To date, wide-spread encounters of more than 800 species of marine wildlife with plastic debris have been well documented, and scientific evidence clearly demonstrates physical harm that can lead to death of individuals from entanglement or ingestion of large debris.

Laboratory studies have also provided evidence of harm from animal uptake of microplastics and their associated chemicals. However, because experiments are carefully controlled to test single outcomes, it is impossible to generalize results across species of debris types, or from the laboratory to populations in nature. Further research into the ecological impacts of contamination by microplastics is sorely needed.

However, we must not wait for all scientific questions to be comprehensively and definitively answered before taking action to eliminate plastic debris from our oceans. In the short-term, the most important action is to stop uncontained plastic waste from entering the ocean from land.

It is estimated that of the 5 to 13 million metric tons of plastic trash entering the ocean annually, nearly half originates from four countries in Southeast Asia, where inadequate infrastructure cannot keep pace with the rapidly increasing waste generation.

However, here in the United States, the amount of plastic waste generated per capita outranks that in each of those four Southeast Asian countries, and the amount of plastic waste generated each day in the coastal United States is the highest of any country in the world.

In the U.S. we are fortunate to also have a robust waste management system. But even the relatively small amount of waste that is accidentally lost or intentionally littered adds up to a large amount available to enter the ocean. Global investment in waste management,

especially where no formal system currently exists, but even where it does, is the first line of defense in keeping trash out of the ocean.

Cleaning up litter on land, especially in rivers and on coastlines, will continue to be an important "last chance" strategy to capture waste before it enters the ocean. Cleaning up debris in the sea itself is more challenging and resource-intensive, but can be effective when targeting large items in nearshore areas or collecting floating trash before it can move offshore and break apart into millions of microplastics.

Waste collection and cleanups are imperative in the short-term, but long-term sustainable solutions to ocean plastics pollution must address the increasing amounts of plastics in use. We must act to eliminate unnecessary usage and waste; increase demand for recovering and recycling, perhaps through product design that ensures material value at the end of product life; and identify suitable material alternatives where possible.

In summary, to reduce the impact of man-made trash on the oceans, wildlife, and human health, it is imperative that we prevent debris, especially that made of plastics, from entering the ocean. There is an immediate and critical need to assist countries that have inadequate waste management systems and there is much more to do in our own communities here in the U.S. as well.

No matter where in the world we choose to work, a necessary first step is to clearly identify and measure the local sources of ocean debris, as well as the drivers behind each source, which could be a lack of infrastructure, a consequence of product design or use, or factors influencing human behavior. With this information in hand, we can best focus our time, attention, and resources to design appropriate interventions that will reduce input from each source. These actions should always be appropriate to place. There is no silver bullet or one-size-fits-all solution.

Ocean plastics pollution is an environmental problem that is global in scope, in impact, and in responsibility. We all have a stake in a clean and healthy ocean. Whether in towns, cities, or States in the United States, or through international partnerships, we must work together toward short-term and long-term solutions.

Thank you for the opportunity to testify. I look forward to the day when our oceans are clean because of the work we have accomplished together.

[The prepared statement of Ms. Law follows:]

WRITTEN TESTIMONY OF: KARA LAVENDER LAW, PHD, RESEARCH PROFESSOR OF OCEANOGRAPHY, SEA EDUCATION ASSOCIATION, WOODS HOLE, MASSACHUSETTS, BEFORE THE U.S. SENATE COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS, HEARING: "CLEANING UP THE OCEANS: HOW TO REDUCE THE IMPACT OF MAN-MADE TRASH ON THE ENVIRONMENT, WILDLIFE, AND HUMAN HEALTH?", SEPTEMBER 26, 2018

Introduction

Good morning. Thank you Chairman Barrasso, Ranking Member Carper, and members of the Committee, for the invitation to testify at this important hearing on man-made debris in

the marine environment. My name is Dr. Kara Lavender Law, and I am a Research Professor of Oceanography at Sea Education Association (SEA), a non-profit educational organization based in Woods Hole, MA. Since 1971, SEA has taken undergraduate students to sea on tall sailing ships to study the open ocean, first-hand, as navigators, sailors, shipmates and scientists. More than 8,000 SEA Semester students have contributed to our 30+-year data set on floating plastics in the ocean, assembled by towing plankton nets from our sailing research ships twice a day, every day, and hand-counting their contents. In 2016, for our work in, "fostering scientific discovery and stewardship of the world's oceans," the National Science Board awarded SEA its Public Service Award.

As an oceanographer trained in ocean physics, I knew little about marine debris before joining the faculty at SEA 15 years ago. More seasoned faculty quickly taught me that the "plastics project" was a popular independent research topic for our students who were concerned about ocean pollution, and also one of the most reliable because, unlike projects focused on particular marine organisms, one could guarantee that along certain cruise tracks small bits of plastic debris would be found floating at the sea surface. It wasn't until 2007 when I first heard the term "garbage patch," a term rife with misconceptions about immense floating islands of recognizable plastic trash. In fact, the most numerous type of plastic debris in the ocean, and what we typically collect in our plankton nets, are "microplastics," particles smaller than your pinky fingernail that are composed of a variety of synthetic polymers (Figure 1). These microplastics are not readily visible from the deck of a ship, let alone from an aircraft or satellite. At SEA, we recognized the need to shift the focus away from mythical floating islands of trash and towards a scientifically informed description of ocean plastics pollution. To this end, in 2010 we published an analysis of our unprecedented data set, which now consists of more than 10,000 measurements of the concentration of floating plastic particles in the Atlantic and Pacific Oceans. For the past 10 years, I have carried out scientific research on ocean plastics to better understand their sources, abundance, distribution and transformation in the marine environment, not only to advance scientific understanding, but also to inform solutions to this global problem.

It is important to remember than not all marine debris is plastic, and not all is found floating at the sea surface. Trash on beaches and shorelines is marine debris (Figure 2). Litter on the seabed is marine debris, whether close to land (Figure 3) or in deep and remote places (Figure 4). Debris can be tens of meters long, such as lost fishing nets (Figure 5) or derelict vessels, or it can be microscopic in size. Man-made debris is composed of paper, glass, aluminum or other metals, as well as plastics. Yet the debris of greatest concern and focus, both by scientists and citizens, is that made of plastics.

Plastics: Ubiquitous, Long-Lived and Harmful to Wildlife

We focus on plastics in the marine environment because of their ubiquity, and the risks they pose to wildlife and potentially human health. In a 2017 study[46] led by Roland Geyer of University of California, Santa Barbara, we estimated that since the start of mass production, 8.3 billion metric tons of plastics have been produced, more than most other man-made

[46] Geyer, R., J. R. Jambeck and K. L. Law, 2017. Production, use, and fate of all plastics ever made. *Sci. Adv.* 3, e1700782.

materials, with the exceptions of steel and cement. Further, we estimated that 90% of these plastics still exist on the planet, with only a small fraction of plastic waste having been incinerated, and the majority of waste either residing in landfills or in the environment. Plastics are designed for strength and durability and do not biodegrade; thus, once in the environment, plastics persist for years to decades or longer. Other man-made materials such as glass and metals are also persistent in the environment; however, unlike glass and metals, the light weight of plastics makes them easily transportable. As plastics are carried by wind or water in the environment, they are weakened by sunlight and fragment to smaller and smaller pieces. Their chemical composition changes as well, as additives leach out and contaminants already present in seawater transfer to plastics. Thus, as plastic debris moves around in the oceans, its size, shape, and chemical makeup change. As these debris characteristics evolve, so too do the potential impacts on wildlife that encounter these plastics.

More than 800 marine species have been affected by man-made debris[47] through interactions such as ingestion, entanglement (including ghost fishing), and displacement of species that drift with, or upon, floating debris. Further, plastic debris accounts for 92% of reported encounters with individual organisms.[48] Large whales have ingested items as large as flower pots and meters-long lengths of rope and plastic sheeting;[49] bottle caps and cigarette lighters have been found in the guts of dead albatross chicks;[50] and small microplastics particles contaminate a multitude of species, including fish and shellfish we consume as seafood.[51] Plastics ingestion has now been documented for more than 200 marine species, including all species of sea turtles, 59% of whale species, and 59% of seabird species.[52,53] Further, for particular populations, such as the northern fulmar seabird population in the North Sea, as many as 95% of individuals studied have ingested plastics.[54] The direct consequences to wildlife of ingesting relatively large plastic debris can include physical injury and gut obstruction, ultimately leading to death, whereas the consequences of ingesting microplastics remain less well understood. An area of intense scientific inquiry asks whether or not chemicals associated with ingested microplastics transfer into animal tissue and cause physiological damage. Some laboratory studies have demonstrated that for particular animal-plastic-chemical combinations, physiological harm does occur,[55,56] yet more work remains to determine whether or not these impacts are occurring in nature and, if so, at what scale.

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[47] *Marine Debris: Understanding, Preventing and Mitigating the Significant Adverse Impacts on Marine and Coastal Biodiversity*. Technical Series No. 83. Secretariat of the Convention on Biological Diversity, Montreal, 78 pp.

[48] Gall, S. C. and R. C. Thompson, 2015. The impact of debris on marine life. *Mar. Poll. Bull.* 92, 170-179.

[49] de Stephanis, R. et al., 2013. As main meal for sperm whales: Plastics debris. *Mar. Poll. Bull.* 69, 206-214.

[50] http://www.chrisjordan.com.

[51] Rochman, C. M. *et al.*, 2015. Anthropogenic debris in seafood: Plastic debris and fibers from textiles in fish and bivalves sold for human consumption. *Sci. Rep.* 5:14340.

[52] Kühn, S., E. L. Bravo Rebolledo and J. A. van Franeker, 2015. Deleterious effects of litter on marine life. In: *Marine Anthropogenic Litter*, Bergmann, M., L. Gutow and M. Klages, Eds. Springer: Heidelberg, Germany, 447 pp.

[53] Wilcox, C., E. van Sebille and B. D. Hardesty, 2015. Threat of plastic pollution to seabirds is global, pervasive, and increasing. *PNAS* 112, 11899-11904.

[54] van Franeker, J. A. *et al.*, 2011. Monitoring plastic ingestion by the northern fulmar *Fulmarus glacialis* in the North Sea. *Environ. Pollut.* 159, 2609-2615.

[55] Rochman, C. M. *et al.*, 2013. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Sci. Rep.* 3, 3263.

[56] Oliveira, M. *et al.*, 2013. Single and combined effects of microplastics and pyrene on juvenils (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). *Ecol. Indic.* 34, 641-647.

Although recent scientific research has largely focused on impacts of microplastics ingestion, evidence clearly demonstrates impacts of debris on marine wildlife through entanglement and species transport. Entanglements, now reported for 344 marine species, most commonly involve components of derelict fishing gear, such as plastic rope and netting, as well as looped packing or strapping bands, which may cause severe injury and death.[3,7] In 29 years of surveying the critically endangered North Atlantic right whale, 83% of individuals showed evidence of entanglement.[57] The most recent demonstration of species transport on floating debris was the delivery of 289 living marine species from the coast of Japan across the Pacific Ocean to North America for six years following the 2011 Tohoku earthquake and tsunami.[58] Although we don't yet know whether these species will become established and threaten native species, the six-year duration of the invasion is unprecedented, and is likely due to the persistence of plastics in the ocean.

To date, widespread encounters of marine wildlife with plastic debris have been well documented, and scientific evidence clearly demonstrates harm from interactions with large debris. Laboratory studies have also provided evidence of harm from animal uptake of microplastics. However, because experiments are carefully controlled to test single outcomes, it is impossible to generalize results across species or debris types, or from the laboratory to populations in nature. Further research into the ecological impacts of contamination by microplastics is sorely needed.

Identifying the Sources

The most effective way to reduce the impacts of plastic debris on wildlife and the marine environment is to prevent plastics from becoming ocean debris in the first place. This can only be accomplished by first understanding the origins of the debris, and the pathways by which it enters the ocean. Plastics can enter the environment at any point in their life cycle, starting from losses of industrial resin pellets (the material feedstock for plastic products), to accidental loss during product use, such as with fishing and aquaculture gear, to accidental or deliberate discard of plastic waste into the environment. A 2015 study[59] led by Jenna Jambeck of University of Georgia estimated that between 5 and 13 million metric tons of plastic trash generated in coastal regions worldwide enters the ocean in a single year because the waste is not properly captured and contained. We estimated that nearly half of this mismanaged plastic waste originates from four countries in southeast Asia (China, Indonesia, Philippines and Vietnam), countries that have experienced rapid economic growth accompanied by an increase in the amount of plastic waste produced by large coastal populations. When waste generation rates outpace the capacity of existing waste management systems, uncaptured plastic waste can flow into the oceans from rivers and waterways, or wash out to sea during storms or with the tides.

The United States also has a large coastal population, and the amount of plastic waste generated per capita outranks that in each of the four southeast Asian countries. According to

[57] Knowlton, A. R. *et al.*, 2012. Monitoring North Atlantic right whale *Eubalaena glacialis* entanglement rates: a 30 yr retrospective. *Mar. Ecol. Prog. Ser.* 466, 293-302.

[58] Carlton, J. T. *et al.*, 2017. Tsunami-driven rafting: Transoceanic species dispersal and implications for marine biogeography. *Science* 357, 1402-1406.

[59] Jambeck, J. *et al.*, 2015. Plastic waste inputs from land into the ocean. *Science* 347, 768-771.

our analysis, the amount of plastic waste generated each day in the coastal United States is the highest of any country in the world. We are fortunate to also have a robust waste management system – garbage and recycling collection, material sorting, and treatment either in sanitary landfills or by incineration. However, because of the sheer amount of plastic waste we create, even the small amount that is accidentally lost, or intentionally littered, adds up to a large amount available to enter the ocean.

Not all plastic debris in the marine environment originates from improperly managed waste on land. Abandoned, lost or otherwise discarded fishing gear is also a very large source of plastic debris, although no estimate of the global input yet exists. Natural disasters, such as hurricanes, floods and tsunamis, can inject a tremendous amount of debris of all materials into the ocean in a single, short-term event. And microplastics from a variety of sources – including microbeads in cosmetics, dust from tire wear, fragments of agricultural films, and fibers from synthetic clothing – can enter the ocean by pathways such as runoff into waterways, and stormwater and wastewater outflows.

From Sources to Solutions

Ocean plastics pollution is a global problem that has grown in size and scale since the 1950s as an unintended consequence of rapidly increasing production and use of these innovative and, in many cases, indispensible materials. Plastics were never intended to contaminate our oceans, rivers, lakes and soils, posing risks to wildlife and potentially even human health. Yet we are faced with ever increasing applications and use of these materials, without a clear strategy for management at the end of their useful life. Preventing plastics from becoming marine debris requires a suite of actions from local to global scales, carried out by individual consumers as well as material and product manufacturers; by municipal, state and national governments as well as international bodies. These actions should always be appropriate to place – there is no silver bullet, or one-size-fits-all solution.

Contain the Waste

The most pressing short-term need to prevent marine debris is to ensure that no waste is left uncontained in the natural environment, whether terrestrial, freshwater or marine. This requires global investment in waste management systems, especially where no formal waste management currently exists. In the 2015 Jambeck study, we estimated that if plastic waste generation were capped and total waste management achieved in the top ten emitting countries by 2025, then the annual input into the oceans would be reduced by 77%.

Even in countries like the United States, where we have established collection and processing of waste, we must ensure that trash cans are lidded and emptied before they overflow, and that the stray candy wrapper is caught before it blows away in the wind. We must invest in infrastructure that streamlines collection and processing of recyclable materials. The design of waste management systems anywhere in the world must be tailored for the needs of a particular community, understanding how, where and what kind of waste is generated, as well as existing social and cultural practices, to ensure community buy-in and long-term success in managing waste.

Clean Up Uncontained Waste

Cleaning up litter on land, especially in rivers and on coastlines, will continue to be an important strategy to prevent waste from entering the ocean. Seemingly simple interventions can be extremely effective, such as Baltimore's Mr. Trash Wheel, a solar- and hydro-powered river trash collection device with a personality, which has prevented 1.7 million pounds of debris from flowing into the Chesapeake Bay. Cleanups are also effective ways to engage and educate citizens as volunteers, as demonstrated by the nearly 250 million pounds of trash collected by nearly 13 million volunteers around the world since 1985 in Ocean Conservancy's International Coastal Cleanup.

Cleaning up debris in the sea itself is more challenging and resource intensive, but can be effective when targeting large items in nearshore areas, or collecting floating trash before it can move offshore and break apart into millions of microplastics. Fishing for Litter programs engage those in the fishing industry to remove litter from the sea in the course of normal fishing activity, at the same time raising awareness of the importance of keeping trash and derelict fishing gear out of the ocean in the first place. Project AWARE's Dive Against Debris program engages scuba diving enthusiasts to participate in underwater debris cleanups to not only report and remove trash on the seafloor, but also to serve as citizen scientists, collecting data on types and amounts of debris in order to inform prevention efforts.

Sustainable Solutions

Improved waste collection and cleanup of uncaptured waste are imperative in the short term, but long-term, sustainable solutions to ocean plastics pollution must address the increasing amounts of plastics in use by employing a variety of strategies to: eliminate unnecessary waste; increase demand for recovery and recycling; and identify suitable alternatives where possible. In some instances, where plastics are unnecessary for function (or are known to be particularly harmful), a mandated ban may be appropriate. The Microbead-Free Waters Act passed by the U.S. Congress in 2015, banning plastic microbeads in rinse-off cosmetics, is an example of this type of action. In other cases, a reduction in use can be incentivized by government policies (municipal, state, or national), such as mandated fees for single-use retail bags. A complimentary positive incentive to reduce single-use bags is provided by individual businesses that give a discount to customers who bring their own bags. Municipalities and institutions can promote waste reduction not only by offering separate recycling and food waste collection, but also by making improvements to infrastructure, such as installing water bottle refill stations to facilitate use of reusable, rather than single-serve, water bottles, thereby reducing plastic waste. Finally, campaigns by individuals or organizations can influence changes in behavior that will reduce waste. For example, the plastic drinking straw movement has recently grown from a simple "Skip the Straw" pledge to refuse an unneeded drinking straw, to an all-out social media blitz promoted by conservation organizations and celebrities alike, driving strong responses from major consumer goods companies down to stand-alone restaurants and individuals. As consumers, we all make innumerable decisions every day about products that we buy, use and throw away. Although we bear ultimate responsibility for our own decisions, good choices can be made easier by employing a variety of strategies to encourage waste reduction.

Perhaps with an ultimate long-term goal of zero waste, steps should be taken to bring value to products and materials at the end of their useful life. This will ultimately increase demand for reuse, recovery and recycling over disposal. Manufacturers engaged in product

stewardship agree to share responsibility for their end-of-life product, such as through take-back and recycling programs, for example. A successful example of cross-brand product recovery and recycling is demonstrated in communities with container deposit schemes for beverage containers including plastic bottles, glass bottles and aluminum cans. Because of the uniformity in materials used across brands, and efficient recovery incentivized by the deposit, recycling of these materials is more cost effective and the waste material more valuable. With attention at the product design stage to ensure material value at the end of life, market incentives can reduce the number of one-way trips most items eventually take to the landfill or incinerator, or the environment.

Finally, continued scientific research to inform evidence-based decision-making is essential to addressing the problem of ocean plastics pollution, so that limited resources can be efficiently and effectively deployed to the greatest benefit. This does not mean that we must wait for all scientific questions to be definitively answered before taking action. Rather, prevention efforts will be most successful when research is first undertaken to understand the local sources and driving factors behind them. For example, Virginia Coastal Zone Management Program and Clean Virginia Waterways used data to identify balloon litter as items of concern, with nearly 9,000 balloons collected on Virginia beaches during 9 years of International Coastal Cleanups[60]. In response, they conducted public surveying and interviews to understand the who, where, when and why behind mass balloon releases in Virginia and, with this knowledge, designed a community-based social marketing campaign to discourage balloon releases and provide context-specific alternatives, a more targeted and informed strategy than a simple information-sharing education campaign alone.

Concluding Remarks

In summary, to reduce the impact of man-made trash on the oceans, wildlife and human health, it is imperative that we prevent debris, especially that made of plastics, from entering the ocean. There is an immediate and critical need to assist countries that have inadequate waste management systems, and there is much more we need to do in our own communities as well.

No matter where in the world we choose to work, a necessary first step is to clearly identify and measure the local sources of ocean debris, including item types and locations, as well as the pathways the debris follows from the source to the sea. Second, we must determine the drivers behind each source, which could be a lack of infrastructure, a consequence of product design or use, or factors influencing human behavior. With this information in hand, we can best focus our time, attention and resources to design appropriate interventions that will reduce input from the source. Interventions may be relatively simple and inexpensive, such as installing and servicing lidded trash cans. Interventions may be engineered, such as debris traps in waterways or storm drains. Interventions may involve technological design innovations to ensure effective recovery and recyclability, and they may aim to influence human behavior away from a "disposable" mentality to reduce unnecessary

[60] Witmer, V., K. Register and L. McKay, 2017. *Balloon Release Research in Virginia and Reducing Balloon Debris through Community-Based Social Marketing.* Virginia Coastal Zone Management Program (Virginia Department of Environmental Quality), 117 pp.

usage and waste. Interventions may be legislated to mandate or incentivize actions by plastics producers, product manufacturers and consumers that will ultimately reduce the input of plastics to the ocean. Finally, we must continue to increase waste literacy and raise public awareness of this problem in order to drive action by consumers, producers, and governments alike, to both reduce unnecessary consumption and revolutionize our management of waste.

Ocean plastics pollution is an environmental problem that is global in scope, in impact, and in responsibility. We all have a stake in a clean and healthy ocean. Whether in towns, cities or states in the United States, or through international partnerships, we must work together towards short-term and long-term solutions as citizens, scientists, businesses, governments and people. Thank you for the opportunity to testify. I look forward to the day when our oceans are clean because of the work we have accomplished together.

Figure 1. Microplastics collected from surface seawater in the North Pacific Ocean by towing a plankton net. Microplastics are typically defined as particles smaller than 5 mm in size, and are composed of a variety of plastics. Microplastics may be manufactured at this size, such as the industrial resin pellet indicated by the orange arrow, but most are generated when larger plastic items break apart upon exposure to sunlight. Photo credit: Jessica Donohue/Sea Education Association.

Figure 2. Litter piled high and wide on Marquez Beach, Peru. Photo credit: Nicholas Mallos/Ocean Conservancy.

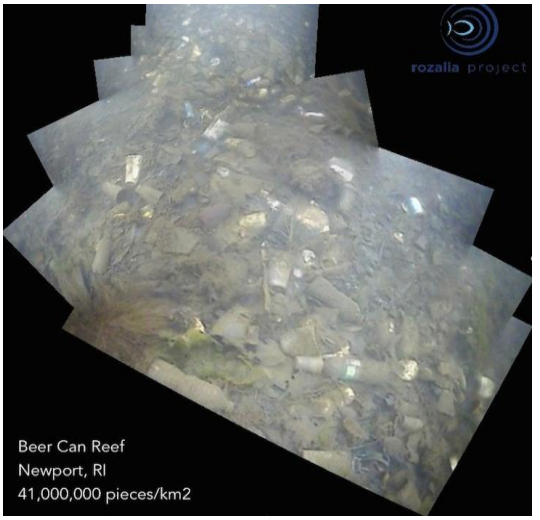

Figure 3. Beverage cans, bottles, food wrappers and other trash in a composite of underwater photos of the seafloor in the harbor in Newport, RI. Photo credit: Rachael Miller/Rozalia Project.

Figure 4. Plastic bag at a depth of 2500 m (8202 ft) at HAUSGARTEN, the deepsea observatory of the Alfred Wegener Institute in Fram Strait in the Arctic. Photo credit: Melanie Bergmann/OFOS, Alfred Wegener Institute.

Figure 5. A NOAA diver removes derelict fishing gear from a reef habitat at Midway Atoll. Photo credit: NOAA Marine Debris Program.

SENATE COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS, HEARING ENTITLED, *"CLEANING UP THE OCEANS: HOW TO REDUCE THE IMPACT OF MAN-MADE TRASH ON THE ENVIRONMENT, WILDLIFE, AND HUMAN HEALTH?"*, SEPTEMBER 26, 2018, QUESTIONS FOR THE RECORD FOR DR. KARA LAVENDER LAW

Chairman Barrasso

1. Experts seem to agree that stopping the flow of debris into the ocean is a bigger priority than cleaning up the debris already in the ocean. Is this an assessment that you agree with? If not, why not?

Yes, I agree. Cleaning up debris already in the ocean treats the symptom of the problem and not the cause. Even if it were possible to clean up all the debris in the ocean at one moment in time, more and more continues to flow in every day the task would never be complete. Further, scientists still do not know where the vast majority of trash entering the oceans from land is located in the ocean, making efficient and effective removal a virtualy impossible task. Targeted, local cleanups are worthwhile, especially if they take place on coastlines, beaches or in nearshore waters, but cleanups alone cannot solve this pollution problem. Stopping debris from entering the oceans is the top priority.

2. We know that five countries in Asia bear much of the responsibility for the increase in ocean plastic. That does not mean that we in the United States do not have a critical role to play. What additional steps can local and state governments as well as the federal government take to address this problem?

Yes, the United States population also contributes large amounts of debris to the ocean through leakage of waste from land, as well as through other sources (such as lost recreational and commercial fishing gear; losses due to natural disasters; losses of microplastics in wastewater and stormwater/runoff). Governments at all levels can take steps to help mitigate the problem. At the federal level, incentives (or disincentives) to reduce unnecessary plastics usage could serve to reduce the amount of plastic waste generated that needs to be managed. The Microbead Free Waters Act of 2016 is a good example of this kind of action. Further, the federal government could stimulate growth in the industries that collect and process materials for recycling. State governments could evaluate and improve waste management services, especially by streamlining recycling collection and processing for efficiency and consistency across municipalities. In particular, container deposit policies have demonstrated success in improving capture and recycling rates. Municipal governments can take steps to identify; specific "leakage" points of waste to the environment, and action directly mitigate those potential sources of waste to the ocean. For example, they could ensure the presence of public waste and recycling receptacles that are regularly serviced and designed to prevent/asses in heavy weather (e.g., wind). At all levels, funding will be necessary for research and implementation of mitigation actions.

3. On January 24, 2018, the *Financial Times* published an article, by Clive Cookson, entitled, "The problem with plastic." It explained that:

> "While the personal-care industry is phasing out microbeads, concern is growing about another ubiquitous micropollutant: plastic fibres. Analysis shows these to be present in streams, rivers, lakes and seas worldwide, as well as household drinking water. Their main source seems to be clothing and textiles made from synthetic fibres, which become detached in washing machines and are not filtered out by water-treatments plants."

Do you agree with this assessment? If so, how do we begin to address this issue?

Synthetic fibers have been detected in a variety of water bodies, as well as in marine organisms, food and drinking water, and as airborne particles. They appear to be the most ubiquitous of the "microplastics." It is reasonable to assume that many of these fibers originate from clothing and textiles, but there may be other sources, such as synthetic ropes used in marine applications. Fibers shed from synthetic clothing during laundering have been identified as a source f pollution in wastewater that, if not captured, could enter the environment in effluent from wastewater treatment plants.

There is no easy solution to the shedding of micro fibers- they cannot simply be phased out like cosmetic microbeads because of a much greater reliance on synthetic textiles. Individuals can reduce the frequency of clothes laundering and install filters on washing machines to trap these particles before they enter wastewater, and perhaps wastewater treatment plans could develop additional methods to filter out: synthetic fibers. Further up the chain, textile manufacturers could work to engineer fabrics that are less susceptible to shedding. Most importantly, we must better understand the impacts of exposure to these microfibers to both wildlife and humans, especially through ingestion and inhalation, to evaluate the risks posed by microfibers. Only then can we properly evaluate the overall benefits and risks of synthetic textiles.

4. What regions of the world are the least studied when it comes to plastic pollution?

On a global scale, we have barely scratched the surface in our scientific understanding of plastics pollution. There are perhaps a handful of regions of the ocean in which plastic debris has been measured extensively and over long periods of time, but this is only the case for plastics in a specific size range floating at the sea surface. In other regions, a single study to document the presence of plastic debris may have been carried out, and, in most 4the world's oceans, plastic debris has not been studied at all. We have only a relatively coarse assessment of the size and distribution of one major source of plastic debris to the oceans (waste generated on land), whereas there exist very few direct measurements of the movement of trash or debris from land into the sea to inform targeted mitigation efforts.

Ranking Member Carper

5. Stopping the flow of marine debris into the ocean- and mitigating its impacts are not problems that can be solved overnight. Long-term, thoughtful, and collaborative solutions are necessary in order to address the full scope of the issue. Solutions that have been discussed included: improving recycling, incentivizing the use of recycled materials in the global supply chain, developing more biodegradable alternatives, and changing manufacturing protocols are potential solutions to help address this issue. What steps can this Committee and the Congress take now to advance these potential solutions here in the United States?

The most pressing short-term need to solve the problem of plastics pollution is to stop the flow of debris into the environment. The science to date suggests that the biggest source of debris to the ocean is likely waste entering from land, including from the coastal United States, where more plastic waste is produced per day than in any other country in the world. The Committee and Congress could commission a study into the state of waste generation, collection and processing (including recycling) in the United States, as well as research to determine the dominant pathways by which lost waste enters the oceans (e.g., via rivers and waterways, from beaches, transported by wind, stormwater/runoff) to gain a baseline understanding of both waste generation and waste leakage to the environment. Using this informed baseline, which will reveal "hotspots" of extremely high waste production, waste leakage, and flows of leaked waste into the ocean, direct intervention strategies can then be designed and implemented at multiple levels (federal, state, local).

6. Now that China has implemented its "Green Fence Policy," a ban on importing plastic waste, our market for these materials in the United States is flooded. China previously accepted 30% of our plastic waste. Local municipalities are now having even more trouble breaking even when collecting and recycling this waste. In your opinion, what are the best ways for the United States to address this new challenge?

I am an ocean scientist by training, and as such cannot comment with any expertise on how to reinvent or reimagine the domestic recycling industry. However, as a citizen and producer of' waste (including recyclables), I can attest to the many challenges at the household and municipal/eve/ in managing individual items. The "Do/Do Not Recycle" list often varies from municipality to municipality, making it extremely challenging for a consumer to know whether any particular item (especially those made of plastics) belongs in the trash or recycling collection. It is my understanding that contamination of recycling collection by items that do not belong (e.g., plastic bags) causes major problems for materials recovery facilities (MRF), which do not have the technical or human power (as China did for so long) to remove every such item. We must revolutionize current systems, starting from the redesign of products and packaging to facilitate recovery of disposed items. Container deposit schemes (e.g., for glass, plastic and aluminum beverage containers) are one example of a successful design and recovery strategy, in which uniform material use across brands, together with recovery incentivized by the deposit, allow recycling of these materials to be more cost effective, and the collected waste material more valuable.

7. Dr. Law, in your testimony, you mentioned that the United States produces more waste - per person, per year- than the Asian countries responsible for most of our world's marine debris. So while the United States may not be contributing as much to the problem as other countries, we can clearly do better. If we are going to do better, we need to fully understand how this waste from the U.S. makes its way into our oceans. What do we already know about this? If we have successfully identified sources, what can we do at each source to better prevent more waste from entering the ocean?

Yes, according to our research, the coastal population of the United States produces more plastic waste per year than any country in the world. In no country has the actual amount of waste entering the oceans from land, or the pathways it takes (e.g., rivers and waterways; storm water/runoff; wind transport; dumping) been directly measured. Before we can design effective interventions, we must better identify the "hotspots" of waste generation, waste leakage (i.e., that escaping the waste management system), and flows of waste from land into the ocean. Ultimately, we need direct measurements of leakage resulting from litter, improper handling, or accidental loss ·- on a local scale.

Senator Markey

8. There is clear consensus that the number one way to reduce the impact of marine debris-from tiny microplastics to piles of plastic water bottles-is to prevent plastic from getting into the ocean in the first place.

a. Can you speak to the importance of grassroots movements that begin at the local or small-business scale when it comes to bringing attention to the marine debris issue and cutting off marine debris at the source? Can you provide some examples?

As a scientist studying ocean plastics pollution, I think that grassroots movements and the concerns of individual citizens have propelled this issue into the spotlight not only for the public and policymakers, but also for scientists themselves. The level of public concern and willingness of individuals and small businesses to take action to solve this pollution problem are absolutely critical. An excellent example is the recent attention to reducing the use of single-use plastic straws. In 2011, a 9-year old in Vermont (Milo Cress) started a local campaign to "Be Straw-Free." encouraging restaurants to provide straws only upon request, and to educate the public about the impacts of plastic waste on the environment. Since then, momentum on the straw/single-use plastics reduction movement has steadily grown through other grassroots campaigns (e.g., StrawFree.org, The Last Plastic Straw, One Less Straw, ReThink Disposable), eventually drawing the attention of celebrities (e.g., Lonely Whale-For a Strawless Ocean), and most recently reaching policymakers who have proposed and/or implemented bans and/or reduction policies at municipal levels (e.g., Seattle, WA; Davis, San Luis Obispo and Malibu, CA), national levels (e.g., France, Scotland, Taiwan, UK) and international levels (e.g., European Commission, G7 Ocean Plastics Charter (without the U.S. and Japan)). Ocean plastics pollution is a problem that/he public cares about, and grassroots movements have propelled not only the science forward, but also the demand for and action on solutions.

Senator Merley

9. In 2010, a total of 270 million metric tons of plastic was produced globally, an estimated 4.8 to 12.7 million metric tons of which escaped into the oceans. How can we scale recycling systems to keep up with the expanding production of plastics?

Plastics are innovative and, in many cases, indispensible materials that were never intended to contaminate our environment or to pose risks to wildlife and potentially even human health. Yet, our development, production and use of these materials continue to grow without a clear strategy for management at the end of their useful life. While scaling and revolutionizing recycling systems must be part of the actions to address accelerating plastics use and waste production, recycling merely delays (through a limited number of additional uses) the ultimate need for disposal. Manufacturers must play a role, starting at the design phase, in planning for recovery, recycling, and eventual disposal of the materials and products they produce. Ultimately, all plastics must be prevented from entering the ocean it doesn't matter to marine wildlife whether the plastic debris they encounter is made from recycled or virgin feedstock.

10. What tools and/or technologies arc available to help remove the marine debris that is already in the ocean? What can the federal government do to support the development of these technologies or scale up technologies that already exist?

Although the top priority is the prevention of debris from entering the oceans, removal of debris from coastlines and nearshore areas is important to not only clean up those areas, but to prevent debris from moving offshore where efficient cleanup is essentially impossible, not least because scientists do not yet know where most of the plastic waste is located in the open oceans.

Current innovations for coastal cleanups include Baltimore's "Mr. Trash Wheel," a river trash collection device that prevents debris from flowing into the Chesapeake Bay. Other trash collectors in development include "Seabin" and "Trash Shark," which gather floating debris in harbors. In my opinion, before investing in cleanup technology, the federal government should first invest in research to clearly identify and measure the largest sources of debris, including the item types and locations and the pathways the debris follows from land to the sea. Armed with this information, the government can then invest in the infrastructure, incentives and/or policies that directly address these sources.

11. As Oregon's experience with the aftern1ath of the tragic 2011 tsunami in Japan shows, natural disasters can introduce a tremendous amount of debris of all materials into the ocean in a single, short-term event. What can we be doing to minimize the introduction of debris into the ocean from such events?

Indeed, natural disasters such as tsunamis, floods and hurricanes can deposit a tremendous amount of debris of all material types into the ocean in a single event. Aside from disincentivizing or banning coastal development, the best approach to minimize losses (human, material and debris alike) due to such catastrophic events may be to build structures designed to withstand extreme winds and flooding events. A very recent example is the "Sand Palace" home in Mexico Beach, FL (https://www.nytimes.com/20181I0114/us/hurricane-

michael-florida-mexico-beach-house.html), which was constructed to withstand 250 mph winds far exceeding local building code requirements, and which was one of very few homes left standing after Hurricane Michael. Not only was the home still standing, but because the roof, doors, and essentially all windows remained intact, it appears nothing, from inside the house was lost to the sea as debris.

Senator Whitehouse

12. What are some of the risks plastics in the ocean pose to wildlife? To humans?

Scientific evidence clearly demonstrates physical harm to marine wildlife, including severe injury and death, due to entanglement in looping materials such as plastic rope or netting, and plastic strapping bands. There is also clear evidence of internal injury and death due to ingestion of plastic debris, especially large debris. The consequences and harm due to ingestion of microplastics (particles smaller than 5 mm in size) by marine wildlife remain less well understood, although there is laboratory evidence of risks associated with physical impacts of ingested debris, as well as with potential transfer of chemicals associated with plastics into animal tissues. We do no/yet know if these impacts are occurring in nature, or if there are measurable risks to humans from eating seafood that may be contaminated with plastics and the chemicals associated with them.

13. What are the current gaps in understanding, or emerging areas of research, we must invest in to better understand and combat the marine debris crisis?

Some of the most basic questions about marine debris remain the largest gaps in our scientific understanding. We have a coarse estimate of only one source of debris to the oceans (plastic waste leakage from land), with very limited direct measurements of the flow of debris from land to the sea (e.g., in rivers. Stormwater/runoff; carried by wind or waves/tides, etc.). In order to design effective interventions, we must first identify and measure inputs at a local scale.

Second, we do not know where in the ocean most of the debris is located. We can only account for -1-3% of the plastic waste estimated to enter the ocean in a single year from our (fairly rudimentary) measurements of floating microplastics. We do not yet know whether most of the debris is residing on coastlines, on the seafloor or in marine wildlife.

We must invest in robust new technologies to efficiently detect and quantify debris of all sizes (nanoscale to large debris) in all marine reservoirs.

Third, we do not know how plastics are transformed and transported within the marine environment, including pathways between the major reservoirs. We have a very limited understanding of the chemical degradation and physical fragmentation of plastics from large items to microplastics and nanoplastics, including the time scales of these processes, and we do not yet know the ultimate fate of plastics (and their chemical additives) in the oceans.

Finally, we cannot fully evaluate the risk of marine debris to wildlife because of the gaps described above. For any particular species or cohort, we cannot yet quantify; the exposure to marine debris because we have little understanding of where the debris is located and the form (size, shape, chemical composition) it takes. For microplastics (and potentially

nanoplastics) in particular, there is some laboratory evidence of harm due to ingestion or uptake into marine animals, butji1rther research into the ecological impacts of contamination by plastics occurring in nature is sorely needed.

14. What marine species are particularly vulnerable to ham1ful marine debris interactions, either from ingestion or entanglement?

Scientific evidence of encounters and/or contamination by marine debris is steadily increasing, with documentation of debris interaction existing for more than 800 species. However, contamination may not always result in harm to an individual, and harm to an individual can only be determined if the animal was encountered, recovered and examined. Thus far, there is clear evidence of harm due to entanglement for animals including marine mammals, sea turtles, seals and sea lions, seabirds, fish and some invertebrates. Many entanglement reports are cases of "ghost fishing" by derelict fishing nets. For ingestion of plastics, clear evidence of harm has been documented for marine mammals, sea turtles, seabirds, fish, shellfish, some invertebrates and even zooplankton, although it should be noted that some of this evidence of harm due to microplastics ingestion comes from controlled laboratory studies. The current scientific understanding of risk due to marine debris is limited to those marine species that have either been encountered or targeted for study, and current understanding of harm occurring in nature due to encounters with microplastics is severely limited.

15. How widespread are micro plastics in the environment?

The abundance of microplastics in the environment (marine, freshwater, soils, air) appears to inversely scale with particle size. That is, the smaller the particle, the more widespread the occurrence seems to be. For example, microfibers (such as those shed from synthetic textiles) seem to be ubiquitous in the environment, including in our homes, workplaces and in scientific laboratories. This makes careful control against procedural contamination by airborne particles in the field and/or laboratory absolutely essential for scientific study of these particles. More generally, because research into environmental contamination by microplastics is still in very early stages, and is severely limited by a lack of robust, efficient and consistent identification and measurement techniques, we cannot yet make broad generalizations about their abundance and distribution in the environment.

16. As we work on the next iteration of the Save Our Seas Act, do you have any additional recommendations or comments you would like to share to infom1 our development of this bill?

Thank you for your dedication to and hard work on this legislation, and congratulations on the successful passage of the Save Our Seas Act of 2018 into law. The reauthorization of the Marine Debris Program (MDP) within the National Oceanic and Atmospheric Administration is instrumental to continuing critical scientific research that informs the design of solutions including, most importantly, the prevention of marine debris. An increase in the MDP budget has been sorely needed, especially as this is the only federal agency substantially investing in scientific research in this field. I think the program will continue to

experience strong and growing competition even for these expanded resources, given the increase in scientific inquiry in this field.

I strongly support the Act's promotion of international engagement and action, especially related to expansion of waste management systems, as this is a critical need to stop the flow of debris to the oceans. I would also like to see attention to waste generation and management in the United States, to better quantify challenges and weaknesses in the current waste and recycling systems that result in leakage to the environment. The coastal United States is the largest producer of plastic waste of any country in the world, thus even proportionally small losses can add up to large inputs of waste into the oceans. In particular, we need to invest in research to identify and measure local sources of ocean debris so that we can design appropriate local interventions. Finally, we need to focus ji1rther upstream to avoid unnecessary use of plastics through reduction and/or redesign, and to ensure a safe and sustainable end-of-life plan for these manufactured materials.

* * *

Senator BARRASSO. Well, thank you all for your testimony. Very thoughtful, very insightful.

We are going to start with some questioning. I am going to ask each of you, and maybe we want to start with Dr. Law and then work down the panel. You talked about training in ocean physics, undergraduate degree in math. Obviously, very thoughtful on these topics.

There are a couple of articles that were in The Economist that ran 2 weeks ago on this specific topic, in the science and technology section. One was entitled "On the Plastic Highway." Road makers are using waste to create harder wearing surfaces, the idea of using some of the recyclables not just for some of the things that you mentioned, Dr. Baillie, but actually for hardened surfaces on the roads.

The second title, which made me think to start with you, was a teenager in California. This article is called "Sweeping the Ocean: A Teenager's Plan to Troll for Plastic in the North Pacific Becomes Reality," and you are familiar with what he is doing out there.

I would like each of you to describe what you think are some of the most promising areas of innovation that are taking place right now. And if it is OK, we can start with you, Dr. Law.

Ms. LAW. Sure. Thank you for the question. I actually think that some of the most promising innovations and interventions are actually quite simple. So, thinking about communities in the United States, in Portland, Maine, where I live, there has been an initiative to replace open garbage cans with lidded garbage cans. So, this is a way that we simply prevent waste from blowing out on a windy day.

In Baltimore there is a river trash catching device called Mr. Trash Wheel that captures litter that is floating down the river before it can enter the ocean.

These are relatively simple interventions. Mr. Trash Wheel runs on solar and hydropower, and actually has a great personality if you follow his Twitter feed. So, raising awareness, as well.

Senator BARRASSO. You are paying close attention to the personality.

Senator WHITEHOUSE. Mr. Trash Wheel. OK. Senator BARRASSO. Let the record reflect.

Ms. LAW. So, I think these innovations are critical because those are acting to trap the trash before it enters the environment and ultimately the ocean.

I think it is laudable that we are thinking big about trying to clean up the open ocean, but I have some concerns about trying to go out in the middle of the ocean to collect particles, most of which are as small as those found in this little vial. So, I do applaud all kinds of innovation and hope people will continue thinking big about how to solve this problem.

Senator BARRASSO. Mr. Karas?

Mr. KARAS. It is a great question. I think in the area of innovation, one of the things to consider is really thinking about innovation in process, how we do things. At least where we sit, as a company that makes beverages, it is very challenging for us to even get PET plastic back into our packages. So, we have partnered with groups like the Closed Loop Fund, which is focused on innovative solutions and they have done some great things in different geographies, basically applying technology to enhance the collection and recycling of materials.

I think we have looked at things like the water wheel. We have looked at partnering with aquariums on different innovations like that. The key is how can you get close to the source of generation, and the innovation would be how do you recycle better.

We can do carts in the street, and we work with the Recycling Partnership. We have a coastal communities grant that we had with the Recycling Partnership, exactly as you talked about in Portland. So, we are learning, as a company, what are some of those next steps that we can take that would be innovations that would improve collection, recycling.

But I would say what we see where we sit today is the recycling infrastructure is very challenging for us to negotiate even in the U.S. It is a combination of privately owned companies, small municipalities, and each one is a little bit separated. What we would like to see longer term is really thinking through what is the waste infrastructure 2.0. What does it need to be to properly collect materials? And then you enable end markets to really function properly.

Senator BARRASSO. Mr. Dooley?

Mr. DOOLEY. Thank you, Mr. Chairman. What I guess I would start off is that there is not one silver bullet that will solve this issue. But one of the things I think is very, very important is how do you establish policies that will result in adding value to the waste stream. And by that I mean how can you see an incentive for the investment in technologies that can ensure that waste plastic is more easily recyclable or the captured energy is more recoverable.

How do you ensure, too, that there could be added value by finding new uses and applications of that plastic waste stream, as you mentioned, in terms of enhancing the infrastructure, the asphalt, and adding, even actually enhancing performance attributes there.

That is where I think there are some simple things that Congress could do that would not treat the plastic waste and the recovery of it as a hazardous waste, because that is stemming the flow of investment dollars and the development of new technologies that could advance the value of that waste stream and recapture some of the value.

A lot of our companies are making investments in pyrolysis. Actually, one company is about ready to launch, where they can take a mixed plastic waste stream that they can break down, run it through pyrolysis, basically, and break it down into a new feedstock that can be recycled into the plastic manufacturing and conversion stream.

Senator BARRASSO. I will get to you in a second, Mr. Baillie, to answer that same question.

I think, Mr. Dooley, in your comments you earlier talked about a public-private partnership. There has to be a profit motive for this, and some of these new advances may lead right to that, it sounds like.

Mr. DOOLEY. Absolutely. If you increase the value of it, you are going to have individuals be more willing to play a role in recovering, picking it up. You could have more incentives for the investment in water wheels in the developing world if there was a greater value to it. National Geographic is doing a lot of work looking at these scalable technologies as well, which we are very supportive of the work they are doing there.

Senator BARRASSO. Well, then, to National Geographic. Mr. Baillie.

Mr. BAILLIE. Thank you. We have been thinking a lot about innovation and we have actually developed an impact investing fund with Sky. We have also developed a number of global challenges which we will be launching to promote innovation.

In our community, we have some interesting projects which are being developed. The one I highlighted there with Heather Koldewey is very interesting, where you take nets from the ocean, which are basically floating around and capturing and killing a broad range of species. The local community benefits, but then we also get a recycled product in the end, so it is an interesting win-win-win scenario.

We also have other explorers that are working on technology to take plastics locally and then convert that into building materials that people can actually use.

In addition to that, we have another woman we are working with who is taking plastics that can't be recycled and looking at how that can be converted into fuel.

So, there are lots of exciting things on the horizon, but I would agree with my colleagues that the greatest innovation is more about process and it is more about incentives. Here in Washington, DC, the five-cent charge on bags made a world of difference, so why don't we have that across the entire United States? Norway has 90 percent of its bottles being recycled with a simple incentive program around that.

We are behind, actually, China and Europe in recycling, so let's look to the others. We are about 9 percent of our plastics being recycled. Let's look to the others and see these basic measures that we can put in place first, and then make sure that that innovation catches up with us as we move forward.

Senator BARRASSO. Thank you. Senator Van Hollen.

Senator Van Hollen. Thank you, Mr. Chairman, for holding this hearing.

It is great to see all of you.

I want to commend my colleagues, Senators Sullivan and Whitehouse, for the SOS Act. I heard you, Mr. Chairman, say we might pass that by unanimous consent today. I am proud to be a co-sponsor.

Great to see Cal Dooley. We served together in the House.

So, as I see it and listening to the testimony, we have to do a couple things. No. 1, we try and reduce upfront the amount of plastic packaging; No. 2, we need to significantly increase our recycling and improve our waste management, and not just here at home, but as has been discussed, you have major sources overseas.

Dr. Law, thank you for giving a shout out to Mr. Trash Wheel. Baltimore is home to the National Aquarium, and the Aquarium works hard to try to educate people throughout the Country and around the world about the importance of protecting our environment and ocean environment.

I should say that since we gave a shout out to Mr. Trash Wheel in the Baltimore Harbor, Canton, Maryland has Professor Trash Wheel. These are actually really important innovations to try and prevent trash that does get into our waterways and rivers from going out into the Chesapeake Bay and, of course, ultimately into the ocean. So, we want to prevent it from getting into rivers in the first place, but, when they get there, it is easier to catch it there than when it disburses.

My question really relates to some of the testimony we have heard this morning on how plastics, as it breaks down, can get into the food chain.

There was a story in Science Alert that just came out a few days ago, September 22d, saying that plastic pollution is now spreading from ocean food chains to land mammals via mosquitos. I don't know if you saw that article.

Dr. Baillie, you say in your written testimony here, "Research has demonstrated that many of the fish and shellfish humans eat are consuming microplastics. It has also tied plastics to issues ranging from weight gain to brain development impairment."

So, if our two doctors here today could comment on the issue of plastics breaking down into microplastics and getting into the food chain, the animal food chain, and then what risk is there currently to the human food chain?

Ms. LAW. Thank you for the question. So, scientific studies have indeed found contamination by microplastics in a wide variety of species who have ingested them, and when you look at the size of these particles, you can see how we can be entering the bottom of the food chain.

When we look at the percentage of individual animals that have microplastics when they are captured, the percentage may be around 20 or 30 percent, but this can add up if we eat a lot of seafood. Of course, it depends on the animal. We eat invertebrates whole, so if there is plastic in the invertebrate, we will be eating that. If it is a fish, likely, the plastics are in the gut, and we don't typically eat the guts of those animals.

I think there is reason to be concerned, of course, because the amount of plastics that we are producing that are leaking into the ocean are going up over time, so the amounts we may ingest can go up over time as well. But there is so much remaining to be learned about what actually happens when an animal eats plastic or when a human eats plastic, including how long is it spending in the body. Are chemicals transferring from those particles to the organism? Do those chemicals bioaccumulate? And we simply don't have those answers yet.

One thing to consider, there was one study looking at plastics ingestion in invertebrates, I believe, mussels or shellfish, and, as a side part of the study, they put out a Petri dish at a typical dinner table, and the number of plastic particles that landed in that Petri dish, sort of equivalent to on your dinner place, was much higher than what was in the seafood itself.

So, I think we need to think beyond just seafood. Think about our drinking water, our bottled water, sea salt, all these other studies that are finding microplastics far beyond just the fish and shellfish.

Mr. BAILLIE. I would agree with all of that. With the number of plastics increasing in the oceans, we are going to see more and more of this. There is plastics breaking down into their tiny fragments, but then there are nanoplastics as well; and the science is at a very early stage in terms of understanding the implications, but we do know with these plastics that the organic pollutants do bind with them in the ocean, which will probably make things even worse.

But even if it turned out that there wasn't major implications for our health, which I don't think will be the case, I don't think future generations will be grateful for having plastics as a large part of their diet.

Senator Van Hollen. I agree. I just wanted to get the most recent State of the science on this, as I said.

Mr. Chairman, if I could ask unanimous consent to put in the record the article that appeared recently, because my understanding is, as you said, in fish, plastics seem to concentrate in the gut. But if you have mosquitos, then passing this on to land mammals, then there are questions about the food chain there. But there are all sorts of reasons to try and want to reduce this huge volume of trash. Obviously, this is one of them.

Thank you.

Senator BARRASSO. Without objection, it is entered into the record.

[The referenced information follows:]

G7 INNOVATION CHALLENGE TO ADDRESS MARINE PLASTIC LITTER

Plastics are one of the most revolutionary inventions of the past century and play an important role in our economy and daily lives. They are used in almost everything from cars, appliances and construction to packaging and food services, because they are low cost, durable and versatile, This Challenge provides an opportunity to spur innovation while promoting both environmental well-being and economic prosperity. The Challenge will also help retain the significant value, resources and energy lost in plastic waste, as well as minimize threats to the environment.

All countries face difficulties in addressing marine plastic pollution. G7 members are well positioned to share their expertise and promote innovations that can be used elsewhere, including among countries that are large sources of marine plastic litter. This G7 challenge is designed to stimulate innovations, raise awareness of how to address marine plastic liner or facilitate much needed improvements to the management of plastic, especially plastic waste, in developing countries. Scalable solutions are needed to foster a more sustainable use of plastic products and reduce plastic waste and marine plastic pollution including technological and social innovations in plastics design and production, use, reuse, as well as management of plastic waste.

G7 members are part of a larger global community committed to addressing marine plastic pollution. We acknowledge the essential role that the private sector, innovators and entrepreneurs play in developing innovative alternatives and solutions for increasing resource efficiency and circular economy in the use of plastics and plastic products by using their expertise, knowledge, and relationships.

'Innovation challenges' are a recognized and effective mechanism by which solutions can be developed and implemented in an economically viable way, as well be responsive to countries' needs and target recipients.

While respecting each participating member's expertise and reflecting national priorities. G7 members commit to undertake international and/or domestic initiatives, individually or jointly, in support of a common objective to promote innovation in addressing marine plastic pollution by managing plastics more sustainably throughout the whole life cycle. G7 plastic

initiatives will respond to varied individual country needs accordingly. For example, domestic initiatives could focus on plastic design or recycling questions in accordance with national needs, while international efforts could respond to the need for support in improving waste management systems or creation of secondary markets. G7 members commit to sharing information their activities in support of this Challenge through the G7 Alliance on Resource Efficiency.

In implementing the Challenge, G7 initiatives will aim to:

- Leverage, build on, and complement existing initiatives throughout the plastics lifecycle,
- Leverage the strength of a diversity of expertise, including entrepreneurs, innovators, small to medium enterprises, researchers, not for profit organizations, and/or large multi-national companies.
- Support gender equality, women's empowerment and women's leadership.
- Encourage innovative solutions that are sustainable, feasible, lasting, economically viable, and scalable (scaling up of an existing initiative; or developing new initiatives that can be scaled up *through* mechanisms such as blended finance) as well as reflect local and regional circumstances and gender dimensions.
- Develop and maximize *effective* relationships *by leveraging* implementation mechanisms including international financial institutions such as the World Bank, Inter-American Development Bank, and Asian Development Bank.as well as pursuing alternative approaches including philanthropic foundations.

The overall objective of the Challenge is to incentivize the development of innovative social or technological solutions for a more sustainable management of plastics throughout their lifecycle in order to increase resource efficiency and to reduce marine plastic pollution including by finding innovative ways to enhance waste management of plastics that may become marine litter. More specific objectives to encourage innovation could include:

- Product Design and Waste Prevention:
  - Developing new product designs and management processes to increase resource efficiency and the durability, reusability and recyclability of plastic products, in particular those that are not currently recycled.
  - Supporting technologies for repair, refurbishment and remanufacturing of plastic products.
  - Developing processes to incorporate recycled content in local manufacturing processes and products to create markets for collected and recycled materials.
  - Developing and using more sustainable plastics and environmentally sound alternatives within a context of science-based and lifecycle decision-making and in consideration of environment, social and economic factors. For those G7 Members that choose to do so, single-use plastics may be an area of focus.
  - Developing solutions that reduce microplastics in products and reduce by design, to the extent possible, unintentional release of microplastics by wear and tear of products during their use.

- Improving production *processes* to minimize loss of plastic materials, including pellets and maximize resource efficiency in the use of the materials.

- Waste and Wastewater Management and Clean-up:
  - Supporting major source countries to manage waste (e.g., collection, sorting, treatment recovery, refurbishment and recycling, disposal, infrastructure, legal frameworks) in a manner that is cost-effective and transferable in order to prevent plastics from entering the environment.
  - Developing new cost-effective technologies and infrastructure to collect, recycle and *treat plastic* waste, including mobile and small scale technologies.
  - Promoting technologies to improve collection and facilitate recycling or recovery of single-use plastics.
  - Using technology to *make it easier for* remote *and* small island developing states to prevent and manage plastic waste.
  - Developing new and utilizing existing technologies and processes to prevent plastic litter and microplastics entering water bodies through improved storm water and waste water management, effectively cleaning-up marine plastic litter from waterways and shorelines in an environmentally sound manner.
  - Strengthening measures to prevent plastics entering the sea from fishing (including ghost gear) and shipping, and to ensure adequate reception facilities in to collect and manage the waste from ships and facility users (including fished waste old/derelict fishing gear).
  - Creating new technologies and processes or improving existing technology to recycle mixed plastic wastes.
  - Developing cost-effective processes to reduce contamination during the collection and recycling process.
- Markets, education and awareness:
  - Developing business models and approaches to establishing new markets and value for used and recycled to achieve environmental, social and economic benefits, including supporting local entrepreneurs in major marine sm1rce countries.
  - Supporting the development of markets for recycled plastics through greater use of secondary plastics into finished products.
  - Creating innovative partnerships along the plastic value-chain to reduce plastic waste and plastic pellets losses.
  - Supporting community-based approaches to changing behaviours towards reducing, reusing plastics, or recycling plastic wastes.
  - Supporting plastic waste mitigation approaches through socially innovative solutions (e.g., education, innovative alliances, connectors, enabling conditions. etc.).
  - Supporting: local and indigenous solutions and initiatives of women and youth, that can be leveraged to support plastic waste mitigation approaches.
  - Forging public-private partnerships to improve plastic waste management in major marine plastics source countries.

- Developing, harmonizing, and sharing methodologies for monitoring and assessing marine litter and microplastics, including their amount and distribution, as well as related environmental and human health impacts.

Examples of Implementation Mechanisms

- Public private partnerships (domestic and international).
- Domestic mechanisms within each G7 member.
- Multilateral organizations efforts such as World Bank Problue: new umbrella multi-donor trust fund in support of the Bank's Global Blue Economy Strategy through consultations with their governing bodies.
- Third party organizations - external private organizations who leads leveraged, incentivized prize competitions with ambitious goals, that target market failures, that can be won by small groups and ultimately that is achievable.

* * *

Senator BARRASSO. Senator Ernst.

Senator ERNST. Thank you, Mr. Chair. I appreciate the discussion today. I think a number of us are in and out, but it does tend to be a very bipartisan issue, so I want to thank you all very much for being here.

Mr. Dooley, a lot of the testimony that we have heard today has been about how to better manage the plastic waste that ends up in our oceans. However, I also think it is important to touch on the work being done to make plastic more eco-friendly. I think a number of us have discussed this before.

The Iowa Corn Promotion Board was one of the first groups to fund research on polylactic acid, PLA, which is compostable and made from corn; and PLA is the most widely used bioplastic and is used to make straws, cups, plates, cutlery, and other items. After composting, PLA doesn't contain any hazardous byproducts that we see from other plastics and doesn't release toxic chemicals into the environment.

Though advancements will need to be made for PLA to become more widely used, do you see that PLA or other bio-based plastics could be part of the solution to the problem that we are facing right now?

Mr. DOOLEY. Yes. Thank you. Like I said before, there is not going to be one silver bullet, and what you have identified is where there is going to be an opportunity, and our companies are investing a lot of money in research and development of some of the bio-degradable plastic alternatives that are out there.

We have to, though, be also concerned in terms of making sure that we do a comprehensive evaluation in terms of how that can be managed through the waste stream as well, because some of these compostable plastics can also contaminate the recycling, the more traditional and other plastic waste streams as well. So it has a role in the marketplace and it is where we see a lot of investment going into.

There is also an increasing demand, as Bruce said in terms of Coca-Cola, with an increased demand that they are making a commitment to 50 percent recycled content. That,

again, is going to add value to that plastic waste stream. The industry, the resin producers and the plastic producers, have to do a better job in investing the technology that facilitates the recovery of this material so that it can go more easily into that recycled content. So that is also going to be a component.

But we have to be careful that we don't go down a path that goes all to biodegradable that then when a consumer or a homeowner mixes it into the recycling bin and then it creates another sorting opportunity and, if not sorted appropriately, then it contaminates the recycled material that some of the consumer product companies need.

Senator ERNST. And we obviously know that there is a multipronged approach to really wrangling with this issue, so certainly I think that composting type products should be part of that discussion.

But then would you all agree maybe then that we need to be looking at additional research and development opportunities for all of these? I think opportunities really is there. With Iowa and the resources that we have, many of our other States as well, through the various commodities that we have, we can produce a number of those types of materials, and that might be one part of the solution, and I do hope that we will take an opportunity to look at that.

Any other comments on those biodegradables?

Mr. BAILLIE. I would just add, and maybe to build on what Cal had mentioned, is I think as we design things for use and they are plastic, you have to design with the end in mind. I think one of the reasons why we are where we are today is many plastics are designed to—a bag is designed to carry materials, but the thought isn't given to the end market, and I think that is a key piece of development in the process, is understanding what we are building and does it actually have value later.

If it has value, it is going to come back and create that circular economy that we want. But if it is very difficult to recycle, we don't have technologies, as Cal mentioned, to actually solve for, then that is when we run into problems. So really focusing on making sure we design with the end use in mind when we make things.

Senator ERNEST. Absolutely. I think that is very smart. So looking at, perhaps, straws, there is the big debate about plastic straws right now, how many plastic straws are actually recycled. I don't know that many of those are, so that could be a potential stream or opportunity for something that is biodegradable and something that would be composted in a landfill, perhaps. So I think that is a very smart approach, is understanding what is recoverable later on and what is actually put into our landfill system.

Did you have a comment as well, Dr. Lavender Law?

Ms. LAW. Yes. Thank you for bringing up straws, because I think that is a really good example of if we don't need a straw, not creating the waste in the first place is actually a higher level strategy so that we don't even have to worry about managing it.

But from the perspective of the oceans, I just wanted to comment on the biodegradable plastics as well. Specifically, the PLA is designed to biodegrade in an industrial composting facility.

Senator ERNST. Composting, correct.

Ms. LAW. So, if you are in a municipality that doesn't have access to one of those facilities, your PLA is trash, and the ocean doesn't really care if it is PLA or polyethylene or polypropylene. So that is something just to keep in mind when we talk. We talk broadly about

these materials, but really we need to think carefully about which specific material we are talking about.

Senator ERNST. Right. Exactly. I will make another plug for it, though. With the PLAs, we still do have to look at other research and development, and understanding where perhaps this particular stream of PLA may not be appropriate for oceans, but certainly if there is additional research that can be done that does support another biodegradable product that is ocean-friendly, we certainly should be looking at all those opportunities.

So, thank you. Oh, excuse me, yes, Dr. Baillie.

Mr. BAILLIE. Just on the straws, Americans are using 500 million straws every day, and there are alternatives. You can use a straw like this and keep it with you at all times. But with a lot of these plastic items that we simply don't need, there is an opportunity to take leadership and just say we are going to ban certain items. You have the French, which have made a commitment to banning plastic cups and plates by 2020. You have many countries that have banned the use of plastic bags. We have about a trillion plastic bags per year being produced. While I gave my testimony, I think about 10 million plastic bags were used, with an average lifespan of about 15 minutes.

So, there are many kinds of interventions that we can make immediately if we are truly serious about addressing the small waste issues like straws.

Senator ERNST. Well, I appreciate it.

Thank you very much, Mr. Chair.

Senator BARRASSO. Thank you, Senator Ernst.

Senator WHITEHOUSE.

Senator WHITEHOUSE. Thank you, Chairman, and thank you again to you for this hearing and to the witnesses for appearing here.

In my opening comments I mentioned a couple of topic areas. One is cleaning up in the oceans, and particularly cleaning up in the rivers that feed the oceans, where much of this plastic comes downriver; trying to find ways to pick the plastic out of the flow points before it hits the ocean. Once it is out there floating around in the great Pacific Garbage Patch, it is really hard, really inefficient, really expensive to deal with it. To try to get it upstream is more significant.

I think to prevent that we need to have a strong focus on requiring countries with whom we have trade relationships to meet elementary standards of upland waste disposal responsibility. We have never been very good at enforcing pollution control standards overseas as a part of trying to balance our trade, so there has been a lot of cheating, where an American company has to keep its junk out of the river and the competitor doesn't, so the price of the competitor's product can go down, but we all pay the price when it ends up in the ocean.

And I think there are technical ways to go about doing that. Even in Newport Harbor we have a little basically a sunk dumpster with a solar pump that keeps pumping the water out so that there is constant inflow and there is enough inflow the plastic flows in, it gets trapped in the dumpster and you can clear it out.

Rivers have similar catchment technologies. But until there is a revenue basis for doing that, it is hard to get it done, so that is something I think that we can work on.

Biodegradability we have talked about, so I don't need to add to that.

Again, on entanglement, if a fisherman loses a long-line rig, for instance, first of all, it may be hard to find, but it is not really hard to put pingers on things these days. Supporting

people in trying to make fishing gear more traceable once it is lost could be a good strategy. Having a bounty so that if you are out fishing and you come across somebody's gear, you take the trouble to bring it in and bring it home and take it out of the ocean.

Fisherman work incredibly hard and it is a very uncertain world out there. It is even more uncertain with oceans warming and populations of fish moving around, so to expect fishermen to stop what they are doing and become the people who are responsible for cleaning the oceans themselves I don't think is really fair unless all of us have found a way to help make that a productive use of their time.

And then I think the human health research is the other point. We really, I think, need to know pretty quickly what risks this poses so we can know with what degree of urgency and alacrity we need to take on the problem.

So, I would just like to ask each of you to comment on those four topic areas for our 2.0, and if there is anything further you think I have completely missed, please throw it in.

We will start with Dr. Baillie. This will be my only question, so if we could just run it out, that would be fine.

Mr. BAILLIE. Sure. So, on the initial map that I showed, it showed the major river systems where plastic pollutants are getting into the oceans and, of course, you commented on the top 20 being in Asia and being a significant problem.

At National Geographic we are committed to doing an exploration looking at some of the major rivers around the world and understanding where the plastics are actually coming from and looking at the social issues and the political issues, but also looking at innovation that we can help promote so that that process can be addressed.

When it comes to fishing gear, you saw the short video in terms of using the nets. It would be wonderful if we could look at creating more of a market for those nets, as many of them are made of nylon, which is actually quite a valuable material that can be reused for things like the carpet tile right here. So, I think it is about exploring some of those innovative approaches, but also putting more pressure on the fishermen to actually keep track of those nets when they are out there.

I very much like your idea of the tracking device. Again, at National Geographic, we are developing a whole bunch of sensor systems, so this is just the type of thing we could explore, sensors to try and keep greater tracking.

Mr. DOOLEY. Thank you. You know, I think that what we are interested in and we are trying again to really establish a private sector initiative that would represent constituents throughout the value chain. Part of our interest is how do we do a better job of identifying those initiatives and those investments that are going to be the most cost-effective in making a meaningful impact on reducing plastic waste in the environment.

We think the public agencies, the U.S. Government, has an opportunity to help and facilitate that as well.

I think when we look at, again, the Asia region, which is the primary source for certainly the Pacific driver, we have the opportunity to focus on cities. Ocean Conservancy has just launched an initiative called Cities, and cities on rivers, because, as you said, the rivers are the source that enters into the ocean. If we focus on developing a comprehensive waste management assistance program for cities on rivers in the Asia region, I think you would be able to see significant private sector resources that would complement public sector investments as well.

You can even get that a little more granular because you also, in order to have a sustainable waste management system, you have to add more value to that waste stream; and that is where I think the U.S. Government also has an opportunity to make investments in pilot programs in that region. We have a number of military bases there that could make an investment in a pilot program that could be able to demonstrate and capture the value in the waste stream not only from their own operations, but perhaps even extend it to the communities.

You can even see it with fishing nets. If you had a pyrolysis unit that could develop syn fuel or diesel fuel from a plastic waste stream, as well as fishing nets, it could be a source of income for the local community that was playing a major role in trying to be a collection center or providing the collection of unused fishing nets.

So we think that there is just a wonderful opportunity for the U.S. to show leadership that would complement and encourage private sector involvement in meaningful initiatives that really focus on making waste management more effective.

Senator WHITEHOUSE. My time has long expired, so I should probably invite the other two witnesses to make their responses for the record, if you would do that.

It is up to the discretion of the Chair, but I think we probably need to be thinking about bounties, as well, because I doubt there is enough of a resource there to make it self-sustaining without some help.

Senator BARRASSO. Senator Inhofe.

Senator INHOFE. Thank you, Mr. Chairman.

Senator Whitehouse, you opened your great comments about the fact that we don't have the control over some of the foreign countries that we would otherwise have. I want to give another side to that because in some of these countries they actually have ideas that we haven't gotten. One of my close friends on the continent of Africa is Paul Kagame, who is head of Rwanda. He made a decision to really cleanup his country. And I have to tell you guys that of all 54 countries, and I have been to all of them, in Africa, you go in there, that is the clean, pristine country, Rwanda.

Don't get me wrong on this, I am not suggesting this, Mr. Karas, but the first thing he did was outlaw plastic bags. Then he went on. Now when you go from the airport to the headquarters, you just see a pristine country. I think we ought to really sit down and look and see some of the things that he has done successfully and emulate those.

I am kind of surprised not many people talked about the meeting that took place just last week. Maybe it is because it is so soon afterwards, but it was in Nova Scotia, where the G7 people, and our participant there, of course, was Andrew Wheeler, who worked for me for 14 years, who was very active on this Committee. He attended that. It was a meeting where they discussed the very thing that we are discussing here.

The objective, and I am going to read this and then I am going to be asking to put the outline in the record, is to "incentive the development of innovative social and technological solutions for a more sustainable management of plastics throughout their life cycle in order to increase resource efficiency and to reduce marine plastic pollution, including by finding innovative ways to enhance waste management of plastics that may become marine litter."

They have excellent suggestions, and I ask, at this point in the record, you include this. With no objection. Thank you.

[The referenced information follows:]

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To: The Senate Committee on Environment and Public Works
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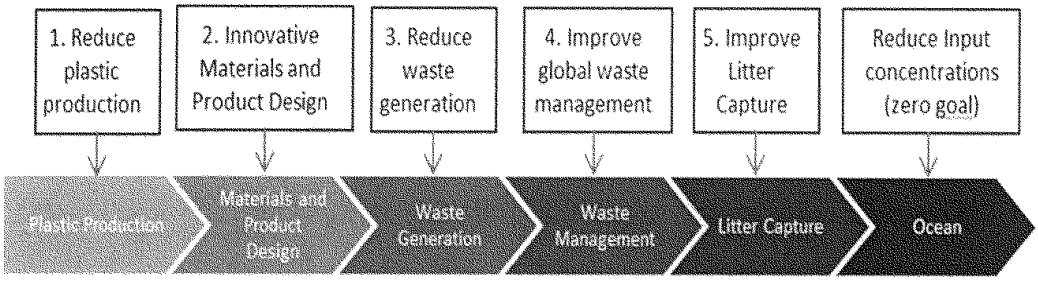
Date: October 9, 2018

Re: Further comments on the hearing titled, "Cleaning Up the Oceans: How to Reduce the Impact of Man-Made Trash on the Environment, Wildlife, and Human Health?"

Thank you for the opportunity to provide comment on the hearing titled, "Cleaning Up the Oceans: How to Reduce the Impact of Man-Made Trash on the Environment, Wildlife, and Human Health?" I am environmental engineering professor at the University of Georgia and have been working in solid waste management for 22 years and on marine debris since 2001. I testified on May 17, 2016 to the subcommittee on this topic. I am also now currently in the International Informational Speakers Program with the US State Department and have been to nine different countries since 2017 working on the issue of marine debris in public environmental diplomacy (Philippines, Indonesia, Japan, South Africa, Vietnam, Jordan, Israel, and India).

As all the witnesses testified to, there is no *one* solution to this issue, but an integrated approach is needed to make headway on reducing plastic entering the ocean. I developed the framework below for my 2016 testimony and would like to submit it again with some ideas, further explanation and answers to some of the questions posed by the senators in this hearing. This framework provides intervention points (1through 5) and then a list of potential (but not all encompassing) interventions that may occur at the various points. In general, this represents a hierarchy of interventions. However, the most "bang for your buck" interventions will depend on the needs of the specific geography addressing the issue, however, in many cases, all geographies have points along the entire framework that will help reduce debris and plastic going into the ocean. Some interventions can be immediate and some will take more time. The framework starts on the left with the most "upstream" interventions and ends with a last chance to capture the material before it enters the ocean. In many cases the interventions offer the opportunity for economic innovation and growth. The USA could be a leader in several of these categories of interventions.



(1) Reduction in demand

a. Consumers demanding less packaging or no packaging (some markets)

i. Not everyone has access to clean water, for example, so can't always make the choice of a reusable bottle, but these choices taken collectively where possible do make a difference

b. Local initiatives (e.g., bans, taxes)

i. These are often very local-specific, but are also becoming more common

ii. Mass of items removed may be relatively small, but numbers of items are also important- there is more than one way to measure debris (e.g., mass, count, etc.)

c. Voluntary industry actions

i. Industry has become more engaged on this issue I wonder if they will volunteer some changes to help in the future as well?

d. The reality is that all signs point to further growth in waste generation, as well as plastic use, especially where economic development is occurring or predicted to occur in the future

(2) Innovative materials and product design

a. Sustainable packaging associations (pre-competitive collaborations)

i. E.g., Sustainable packaging coalition, Green-Blue: These pre-competitive environments could help to standardize packaging and help packaging retain value so that it is easier to recycle and less leakage will occur if it has value.

b. Truly biodegradable alternatives (e.g., PHA)

i. PLA was mentioned in the hearing and while it is bio-based and industrial compostable, it will not biodegrade in the ocean. It will not biodegrade if littered on land. It has to reach a high temperature (reached in industrial composting) to be able to biodegrade.

ii. One polymer that may biodegrade in the ocean is PHA. This polymer is expanding in the market in the USA and will be creating economic value in the future (new facility opening in Kentucky- one open in Georgia already). While it may biodegrade if littered in the environment, it should still be managed in the solid waste system, and be thoughtful about where used (in currently non-recyclable items, for example). But it has the possibility of being home-composted as well. The USA is currently a leader in the development of this material and other bio-based and biodegradable materials.

c. Packaging with more value (e.g., single, homogenous materials, design for recycling/end-of-life)

i. This can be helped by collaborations between industry, brands and waste managers/experts

d. Design out problematic items/materials (e.g., caps/lids)

i. Similar to how aluminum can opening was changed to a tab that stayed on (so the pull tabs did not get littered), we can innovate design for items that leak into the environment (if data is collected on them- see point S.c.).

(3) Reduce waste generation- *how can we decouple waste generation from economic growth?*

I believe we can through creativity and innovation- and I get very excited to see what my students will create in this category one day.

a. Sharing, Collaborative Economy concepts

i. Bike shares, car shares, tool shares, clothing rental, etc. these all reduce the need to purchase and create waste (facilitated by technology), but still meet people's needs and can still create revenue for the companies providing the services.

ii. How can these concepts be related to packaging? (see b.ii.)

b. Decouple waste generation with economic growth (facilitated by technology) Reuse programs (using mobile phones, which many people have globally, especially where rapid economic growth is occurring)

ii. RFID, mobile phones, smart-labels, etc. (e.g., RFID water refill stations exist for both Coca-Cola and PepsiCo products, but are not yet widely distributed yet)

(4) Improve global solid waste management

This has some basic similar concepts, but solid waste collection can be a hyper-local activity and can look different in each country, city and even neighborhood. Global resources are needed to further develop the infrastructure needed. Waste needs to be managed globally no matter what the materials it is composed of. Plastic has made it a more complicated and created a rapid change in the waste stream that we were ill prepared for. It creates a waste stream that is more varied and dynamic than we have ever experienced before. It has proved to be quite a challenge for waste operators and municipalities to manage. Improving infrastructure is especially needed in rapidly developing economies with high population growth.

a. Collect: May be traditional, on-demand, or decentralized waste collection

i. Collection innovation is needed- revers logistics may play a role

b. Capture: Material Recovery Facilities, waste depots, waste banks, community centers (e.g., "punto limpio" in Chile)

c. Contain: Recycling or engineered disposal

d. Important to keep in mind: ***Context and Culture-*** *these can "make or break" the success of a potential intervention. The local community and stakeholders need to be engaged and involved from the start through the end of any project.*

(5) last-chance cleanup

a. Engineered, mechanical systems

i. Mr. Trash Wheel or other engineered devices

b. Manual (by hand)

i. Cleanups (e.g., ICC by Ocean Conservancy)

ii. Use of ocean-bound plastic can catalyze the development of infrastructure since the material now has value- often a much higher value than it did previously (e.g., Parley, Dell, NextWave plastics)

c. Data to feed back to Interventions 1through 4

i. E.g., Marine Debris Tracker developed by NOAA and UGA (or other apps) to collect data

ii. Could make upstream choices/changes based upon what is leaking into the environment

Specific questions asked:

1. What are some of the most promising innovations?

In my opinion some of the most interesting and promising innovations are the ones that decouple waste generation from economic growth. How can we meet people's needs and increase livelihood without creating more waste to manage? Sharing and collaborative economy concepts, RFID cups, using technology to connect people and facilitate sharing and reuse programs all lead to potential interventions. Reduce waste generation in the first place.

2. What is role of PLA and other bio-based plastics?

I think there is a role for material and product innovation and bio-based and biodegradable (truly) polymers will be a part of the solution. However, these materials are being produced at relatively low quantities right now, so they are not going to be a big market for some time- if ever (also see 2.b., above).

3. Fisherman incentives

I think incentives for fisherman to collect or bring back gear would be a way to get some of the most deadly gear out of the ocean and marine environment. I think also supplying a place for fisherman to put used gear is important (e.g., dumpster or recycle bin at the port). Tracking and transparency of nets- and really all plastic (as much as feasible) could help keep the material out of the ocean because we would have a better inventory of it.

4. Root causes

Responsibility- while not particularly popular in the USA, product stewardship is an important concept to discuss here. From an engineering standpoint, when a company wants to build a development/civil engineering project, there often is a partnership with the community. One example, I live near an above ground storage tank farm, and trucks come and go from it regularly. There were likely road improvements needed to be able to build the tank farm and the company who constructed it may have contributed to that infrastructure since they were building at this site. In some ways, this can be analogous to selling products in a country or location that does not have infrastructure to manage the waste created from those products. I don't think companies knew the issues this would bring. And I think they want to help based upon new awareness, but we are certainly playing "catchup" with the issue now. I have seen some good examples of shared responsibility in South Africa and Norway. And some companies are doing this individually, but many still don't know how to help with infrastructure. I think that facilitating this in some way could be significant- maybe it will all

be individual public-private partnerships, but some thought could go into how to facilitate companies engaging in shared responsibility. Ultimately it will take shared actions by industry, municipalities, and citizens to make significant positive change on this issue.

Thank you again for your attention to this issue and for the opportunity to offer my thoughts.

* * *

Senator INHOFE. I would ask you, Mr. Dooley, I think you are familiar with this. Nothing much has been said about this, but I would like to know what thoughts you have about the recommendations that have come from the G7 talks that took place. Actually, that meeting took place in Nova Scotia right up until last Saturday, I think they concluded it, so your thoughts about what has happened there and how that coordinates with the recommendations that we are making with this excellent panel that we have.

Mr. DOOLEY. Well, ACC was very pleased that Administrator Wheeler attended that meeting and also committed, along with the balance of the environmental ministers there, to really support moving forward on how could they collectively create greater incentives for the development of innovations that would contribute to the elimination of plastic waste.

It really was building upon one of the commitments that Trudeau made at the G7 principals meeting, where he committed to doing $100 million innovation grant that was really trying to provide public sector investment that would be matched oftentimes by private sector commitments that was focused on trying to eliminate plastic waste in the environment. That was further developed under the challenge program that was talked about at the Halifax meeting of the G7.

We think those are opportunities to really leverage the private sector funding and to ensure, again, that we have a collaborative effort to identify what are going to be the most cost-effective investments of both taxpayers' dollars, as well as private sector funds. We are very supportive.

Senator INHOFE. That is good. I think we need to become more familiar with that. A lot of heavyweights were involved in those decisions and that discussion in Nova Scotia.

Mr. Karas, I think we may share one philosophy that I kind of picked out of your opening statement, and that is it has been my experience over the years—and I have been on this Committee since, I don't know, a lot of years—that some of the solutions to problems are best handled by the private sector.

I can remember when we had a lot of the Superfund problems. I was actually chairing this Committee at that time. What happened, and I won't mention the oil company, it was an oil company, though, that was working at that time in Louisiana, and they did have a spill, and it was a pretty serious one. So they went in and evaluated what it would take to clean that up and what it would cost to clean that up.

I am going from memory now, but I think it was something like it would take 13 months if this oil company were allowed to do it at the cost of $7 million. EPA rejected it at that time. We were kicking and screaming about that, but they did. They took this on and it ended up taking not 13 months, but 4 years; and not $7 million, but $15 million.

I guess what I would ask you, your thoughts on the things that can be done through private sectors that cannot be done through the public and what your experience has been.

Mr. KARAS. Thank you, Senator. I think the way I would answer that question would be in really looking at this space. We are good at making beverages and marketing beverages and selling; that is our core business, we make beverages. And what we find when we get into a space like waste is we have to rely, in my comments I talked about the critical importance of partnerships, we have to rely on others.

What we have learned in the course of we had a water stewardship goal to replenish the volume of water that we put in our products by 2020. We met that early. We met that because one of the reasons was public-private partnerships. We have a public-private partnership with USDA, U.S. Forest Service. For us, it gives us resources that we thought we would never have, knowledge and information that we wouldn't have access to, and, really, we learned a lot and advanced quickly.

So I think our learning in these spaces has been it is absolutely mandatory for us to really reach out and engage with trade groups like ACC, with technical people that are knowledgeable, to really come to a great solution collectively. I think that is what ultimately wins the day.

Even when we do partnerships through our foundation, we have something called a Golden Triangle partnership, and what we learned when we link business, civil society, and the government together to look at a problem, we usually get to a really good place, a place that we wouldn't have gotten to by ourselves.

Senator INHOFE. Thank you very much.

Thank you, Mr. Chairman.

Senator BARRASSO. Senator Carper.

Senator CARPER. I am told by my colleagues and our staff that you all are doing a good job. As you know, we serve on a bunch of different committees and, unfortunately, all my committees are meeting right now, so I am trying to be three or four places at once. I am not doing really well at it, but now I am with you and look forward to asking a couple questions.

First, I want to ask unanimous consent, Mr. Chairman, to enter a letter from MERR and supplemental materials be entered into the record from the Marine Education, Research & Rehabilitation Institute in Delaware, as well as some other supplemental materials.

Senator BARRASSO. Without objection.

[The referenced information follows:]

* * *

The Honorable Thomas R. Carper
Ranking Member
Committee on Environmental and Public Works
United States Senate
Washington, D.C.

Dear Senator Carper,

The Marine Education, Research & Rehabilitation Institute, Inc. (MERR) has been providing stranding response for marine mammals and sea turtles in the state of Delaware for over 18 years. As Delaware's leading authority on marine animal health and welfare, we have

observed first hand the negative impacts of marine debris on the marine environment, and on the creatures that reside there.

MERR responds to approximately 300 stranded animals per year. the majority of which have stranded due to some type of human impact These impacts include fisheries, dredging, underwater noise pollution, oil spills, marine debris, and more. Marine debris with all of its various components causes constricting entanglements, and is ingested when it is mistaken for prey. Marine animals become entangled in the copious amount of material that we have introduced into their habitat, such as ribbons from balloons, discarded six pack rings, fishing line, fisheries gear, packing straps, etc. The effect of this type of debris on marine animals is slow and painful debilitation as the constricting debris cuts into the animals tissues, leading to infection, strangulation, osteomyelitis, and ultimately death.

The components of marine debris are far too many to list, but plastics pose an especial threat to the quality of ocean habitat. In their intact form, they can appear to be food sources, as in the case of plastic bags, which are accidentally ingested by marine animals, leading ultimately to starvation. Plastic bags and balloons resemble jellyfish in the water, a favored food source by the endangered leatherback sea turtle, and the loggerhead sea turtle. Pieces of plastic large and small have the capacity to photo-degrade, causing smaller and smaller particles to emit into the ocean, ultimately to be consumed by fish. Micro-plastics are consumed by the smaller organisms, which leach into their tissues. The smaller organisms are ultimately consumed by the macro-organisms, such as dolphins, whales, seals, and humans. The prey source for these endangered species is contaminated, as is the water in which they live, due to the inundation of micro-plastics in the ocean and the inland waterways that ultimately lead to the ocean.

Our stranding response team has witnessed the excruciatingly painful suffering of beautiful and majestic whales, dolphins, seals and sea turtles as a result of human based trash and debris in their habitat. A loggerhead sea turtle on the Delaware Bay that had over 65 different types of plastic in its stomach, whales and dolphins maimed and ultimately drowned by Jishing gear, a young pilot whale that had mistaken shredded packaging from beef ramen noodle soup as squid, a helpless one month old seal pup barely able to breath because of constricting fishing net, ribbons and plastic bags, and a bungee cord wrapped around its throat-these are just some of the heart breaking examples of how these animals suffer due to the impacts of marine debris. Our organization invests thousands of hours in an attempt to inform and educate students and the public about the harm caused by marine debris, in hopes that this information will lead to consumer choices that minimize the amount of trash in the ocean.

We know of the astounding statistics surrounding the Pacific Garbage Patch, now believed to be twice as large as the state of Texas on the surface, with depths surpassing that size. The Marianna trench, the deepest known area of the ocean, has been identified as 50 times more toxic than the most polluted rivers of China, due to the cumulative and concentrating effects of plastic in the marine environment. Phytoplankton, which makes up the basis of the food web for all other organisms, is being impacted as algae accumulates on both large and micro sized particles of plastic, interfering with sunlight penetration to stimulate photosynthesis. Plankton is the foundation species for all life forms in the ocean, without which the entire ecosystem is destined to collapse.

Reducing the quantities of marine debris and its toxicity in the oceans is imperative to the health of the marine ecosystem. Our organization strongly supports any initiatives to reduce

the presence of marine debris in the ocean environment. We greatly appreciate any efforts that are put forth to accomplish this important goal.

Sincerely,

Suzanne Thurman
Executive Director

* * *

Senator CARPER. Thanks very much.

Dr. Law, if I could just aim my first question at you. I am told by my staff that you spent a lot of time at sea. They said more than probably anybody in the room. I was in the Navy for 23 years, mostly in airplanes; some time on seas, but I would be happy to give that honor to you today.

They tell me you spend a lot of time at sea observing firsthand the impact of marine debris on the environment, but also especially on wildlife. Do you want to share with us some specific examples of some things you have seen with respect to marine debris' harm to wildlife, including marine mammals, sea turtles and birds?

In addition to minimizing the amount of debris that ends up in our oceans, what else should we be doing to mitigate these impacts?

First some examples.

Ms. LAW. Sorry.

Senator CARPER. First some examples.

Ms. LAW. Some examples, yes.

Senator CARPER. And then some ideas on what we ought to be doing to mitigate these impacts.

Ms. LAW. Sure. So, most of my sailing experience has been in the open ocean, much of it in what are called the subtropical gyres, which are areas of the "garbage patches." These are areas of the ocean that are actually quite nutrient poor and not——

Senator CARPER. Most people probably try to avoid those.

Ms. LAW. Sorry?

Senator CARPER. Most people try to avoid those.

Ms. LAW. Most people try to avoid those, that is right; there are not a lot of wind. They are not places many people spend a lot of time, but we do spend time doing our science there.

I think the most important observation I have had is that many of the descriptions we hear about this problem are not what you observe out at sea on the boat. I don't see very large objects going by, but when you do it is very surprising to see a shoe or a bucket or a teapot or a toothbrush drifting by thousands of miles from land.

When you look over the side, you see these little bits of plastic. And in terms of interaction with wildlife, what I have observe personally is sea birds who are feeding at the sea surface where these little bits of plastic are floating. So while you can't actually see it hanging out of their mouths, you know that the birds are eating these plastics.

Similarly, we have captured in our nets, at one point we captured a five-gallon bucket that did have a fish swimming under it. That fish, we brought aboard and it had 42 pieces of

microplastic in its gut. We also tow a fishing line. We brought a mahi-mahi on-board and it had a piece of plastic——

Senator CARPER. Was the fish dead or alive?

Ms. LAW. Just a line with a hook.

Senator CARPER. OK.

Ms. LAW. We brought a mahi-mahi on board for dinner and it had a piece of plastic this big in its stomach. So we were faced right then, do we want to eat this fish or not? We did. It was delicious.

Senator CARPER. Did you? OK.

Ms. LAW. But I have not seen marine mammals entangled myself, so I don't have that personal experience.

I really think I come back to we have to keep this out of the ocean to solve it. The rescue efforts, when people spend lots of time and resources to help these animals when they are entangled are critical, and when we have access that is very important, but we just have to stop it from entering.

Senator CARPER. All right.

I like to focus on root causes. I just came from a Homeland Security business meeting; we are marking up about 20 different bills.

One of the things we focus on in Homeland Security is border security, including border security with the border with Mexico. We spend a lot of time, effort, and energy trying to keep people from getting into our country from places like Honduras, Guatemala, El Salvador. The root cause of all that, though, is the lives that they live in those three countries are miserable; and we are complicit in their misery given our dependency and addition to all kinds of drugs, narcotics.

But part of the root cause in solving that problem, all these people trying to get into our Country from our southern borders, is to help make sure that their lives are less miserable in those three countries; they have some hope of opportunity. That is the root cause.

Is there a root cause in this case? Is there a similar root cause we ought to be focused on, rather than just focusing on the symptoms of the problem? I have a great photograph of Coast Day in Delaware this last weekend, but we are addressing the symptoms of problems. There is always trash that washes up on the east coast, including in Delaware, so we focus on the symptom of the problem.

If we looked for the root cause, where should we be looking? Because we are really good at spending a lot of time and energy and resources on symptoms of problems, not always on the root cause.

Mr. DOOLEY. Senator, the way that I would respond to that, if you look at where the major source of the problem, it is in the developing economies; and in those developing economies they have a host of public needs. Some of it is nutrition, some of it is education, some of it is healthcare, and some of it also is waste management systems. When they prioritize them, oftentimes the waste management investments come down very low on the list, and that is why, oftentimes, they lag behind in developing the waste management systems. As their economy develops, it becomes the source of a lot of the waste plastic and other waste that is getting into the environment.

That is where I think we have an opportunity in the industrialized world in the private sector to allocate resources to help prioritize waste management, and part of that help in prioritizing investments in waste management is providing public and private sector support.

We think if you do that in the developing world, we can make a tangible difference in reducing the amount of plastic waste in the environment.

Senator CARPER. Good. Thank you.

Mr. Karas, please.

Mr. KARAS. Just an add-in. I think we maybe touched on it around the fringes in the conversation here, but infrastructure is absolutely important. But the layer over that, and we have learned this as we have done projects, it is the level of I guess you would call public education and awareness. How do people value that material? Obviously, if it is all in the ocean, it is a throwaway, and we are not thinking about it. Even in the developed world it takes time to change sort of the hearts and minds, and that has to accompany all the work that we are doing. So, it maybe starts with PSAs, but maybe ingraining into younger generations what should you do with materials when they are in your hand.

I would suspect that if you asked most of the public about recycling, recycling is the act of putting something in a specific container, and not thinking about the circular economy and where it needs to go in the long-run. So, I think some of that cultural piece is important to mix in with that.

Senator CARPER. All right, thanks.

Senator BARRASSO. Senator Whitehouse, now that Senator Inhofe has completed, did you want to get a continuation of the line of questioning?

Senator WHITEHOUSE. Sure, if Mr. Karas and Dr. Law. I don't know if you recall the question; it had to do with the various recommendations about trying to stem the flow into the seas, particularly in the big rivers; about trying to invest in biodegradability; about trying to make it a better revenue proposition for fishermen to keep and recover lost fishing gear; and the last one was research on the human health effects of ingesting microplastics. Are those areas we should be working on and are there other areas you would recommend? That was the question.

Mr. KARAS. Senator, I think those are all good areas that you highlighted there. I think what we have learned in the Asia Pacific region as our business units work is we are having to form some of these cooperative partnerships. We are doing it in Indonesia and the Philippines, starting some of that in Vietnam to really start to look at where do you start. I think that is sometimes the challenge. Here we can look at our infrastructure that exists. Maybe it is not well connected, but it is like where do you start, what is ground zero, and we have to do that collaboratively.

So, what we are seeing is brands are engaging with different NGO's and governments to say, OK, how do we move the needle in that. I think those will help in the long-run, but it takes time to build those out.

Senator WHITEHOUSE. And in this case it takes incentives.

Mr. KARAS. Correct. Correct. But I would add that in terms of incentives, and maybe it is something that Cal has already touched upon, I think we have to really think closely about the end markets and the value of the materials.

What often is lacking, you could sort out polypropylene, but there may not be a market there; and if that doesn't happen, if the economics aren't there, it just isn't going to work. But if we can work to build the proper end markets, it really starts to close the loop. To me, when a business has an incentive to get that material and put it into something else, that is going to be a powerful driver in that space.

Senator WHITEHOUSE. You will agree that there is a discrepancy between the recovered value of waste plastic and the value to humanity of not having an ocean in which there is more plastic than fish.

Mr. KARAS. I would agree. I would agree.

I guess in terms of waterways, one of the interesting experiences we have had here, I talked about partnerships earlier, a group called Living Lands and Waters works our own Mississippi River system. Actually, six barges collecting materials, anything from cars, tires, drums, and plastic.

We have had the opportunity to be able to really create a circular economy with those kinds of activities, actually bring material out of the Mississippi. We had an example, last year sorted 9,000 pounds of PET hand-sorted by our own bottler there, and it was turned into bottles for a product 30 days later. That is a circular economy, but we had to force it.

So, ultimately, I think it is how do we really have a vertically integrated waste management system that really allows it to pull through where there is economic viability to that activity. If you have to prop it up because it doesn't have economic viability, it is going to collapse sooner or later, or you are always going to be feeding it funding. So, to get the 2.0 system it really requires some thinking and thoughtful examination of where we want to go.

Senator WHITEHOUSE. Dr. Law.

Ms. LAW. Thank you. I do agree with all of your priorities, especially the impacts on human health, and I think we need to expand that conversation more broadly into impacts of plastics and fresh-water and soils and agriculture, and things like that.

Other opportunities, though, that I would like to raise are the idea that we can try to make less waste; and this is falling on the previous question as well, starting to think about a cultural shift away from disposable, away from I use it, I put it in the garbage can, and it goes somewhere that is no longer my problem. So, encouraging reuse programs.

One really simple intervention we can all do is put in refillable water stations into our public spaces to encourage people to carry a reusable bottle, as opposed to using something a single time. So I would just like to point out not just information campaigns, sort of your traditional education campaigns, but thinking about targeted interventions in spaces that are locally defined about quite simple interventions that will cause us to just simply make less trash that we then have to deal with.

Senator WHITEHOUSE. Mr. Chairman, shall I tell a brief sailor story that Dr. Law's testimony called to my recollection?

Senator BARRASSO. Please.

Senator WHITEHOUSE. Newport, Rhode Island is probably the sailing capital of the world; we claim that, anyway. Delaware may have a disagreement, but I am sure we have Wyoming beat.

[Laughter.]

Senator WHITEHOUSE. A lot of sailboat racing goes through Newport, including what is now called the Volvo Ocean Race, which is perhaps the most dangerous and demanding sporting event on the planet; and it is around the world, very fast race, very high-tech boats going very fast. Racing boats have, for generations, had to learn a man overboard drill.

You don't go offshore racing without having drilled and drilled on the man overboard situation; who is the spotter, how quickly do you turn the boat. You know, the whole routine is just drilled until you can, as soon as somebody yells overboard, everybody knows exactly what they are supposed to do.

For the first time these racing boats have to have a new and different drill, and that is a keel clearing drill. They sail through the South Atlantic on their course and they sail near the place that is farthest from land anywhere on the surface of the earth; and even out there they are still doing these keel clearing drills. When the boats came into Newport from Brazil on their leg that ended with us, you could see the boats in Newport Harbor as they came in within sight of each other.

They had sailed all the way from Brazil and these races are still so close that they end up within minutes of each other, within sight of each other as they finish, so you really, really need to make sure that your vessel is operating at peak performance. And they have enough computers to know when it is off performance, so they then have to deploy—they know what is wrong, the keep clearing drill; and somebody has to go over the side real quick, with goggles and a knife and whatever else they need, to get the junk, the plastic junk, usually, that the keel has swept like a single comb tooth out of the ocean and get their boat operating back at speed again.

So, it is an interesting physical comparison to the longstanding, ancient, well established man overboard drill. It is only now, only in the last few years that ocean racers now have to come up with a whole new drill that they have to practice, keel clearing, even in the farthest corners of the South Atlantic.

Senator BARRASSO. Thank you, Senator Whitehouse.

Senator CARPER.

Senator CARPER. Mr. Karas, I don't know a whole lot about Coca-Cola's World Without Waste campaign, but I am told that the goals are ambitious. I am told that it will really make a difference to improving international recycling practices and reduce waste in our oceans, and that is encouraging. What are the biggest challenges that your company faces in implementing these goals domestically and what can Congress do to support your efforts in this Country?

Mr. KARAS. I think domestically the biggest challenge that we are trying to work through now, and I mentioned this in some of my earlier comments, at times I am dealing with sort of the waste infrastructure 1.0. I may have five different entities, public and/or private. One might be hauling, one might be operating the material recovery facility or the MRF. My end market might be somewhere off in the distance, and it is very disconnected.

So, for us to be able to deliver 50 percent recycled content, I have to do it in a way that I have an adequate supply, adequate a good quality material, so I think the challenge is I am looking to see what the next 2.0 waste management system will be in the long-term.

We have a combination of different efforts that we are doing to really work on vertically integrating that system, so, from a business perspective, if you are one and the same entity, I just toured a MRF, material recovery facility, earlier this week. They are integrated with making cardboard boxes, so they pull the cardboard out of the materials coming into this site, drive across the parking lot and they are making brand new cardboard boxes. When they do it that way, it works; it has value. I think that is sort of the area that we are seeing as the biggest challenge, is how do we really get that system to work.

I think the second piece is we have probably, it is something I mentioned earlier, about the culture right here in this Country. I really believe that people don't understand the concept of the circular economy and we have very much a culture that is throwaway, so we are working on that space as well.

Senator CARPER. Any question you have not been asked that you would like to be asked?

Mr. BAILLIE. I just wanted an opportunity to respond to the systemic question that you asked.

Senator CARPER. Oh, good.

Mr. BAILLIE. I think there is nothing inherently bad about plastics, but in 1950 we were producing 2.3 million tons.

Senator CARPER. How much?

Mr. BAILLIE. Two point three.

Senator CARPER. In what year?

Mr. BAILLIE. In 1950. And now it is 500 million tons. So that is a massive increase. And we haven't moved to that closed loop economy, so we are producing these plastics without a full cycle of what will happen to them going forward, and there is just too much of it.

But when you talk about the source, it is really working with the industries and saying how can we produce plastics that can definitely be recycled. When you have multiple plastics, say, in a tooth-brush—there are three types of plastics, often—it makes it much more difficult to recycle. So how can we create conditions where it is easy to recycle things? And things like coffee cups, we have plastics being mixed and layered with wood and with aluminum. Again, it makes it extremely difficult to recycle that. How do we simplify that process?

If we can do that and then we develop more standardized approach across the United States in terms of recycling, we can bring recycling to scale. The things you do in D.C. are different than you might do in States across America. We have to standardize this process so we can work at scale and we can innovate at scale.

Then we talked about innovation. I mentioned the Impact Investment Fund we are promoting, but there are much larger funds out there, and I think there is a real opportunity for Government to work with the private sector to develop these large funds to actually drive innovation.

Then, finally, incentives. We talked about the five cents on a plastic bag, which makes a world of difference, or the five cents to collect a bottle, which makes a world of difference. We have to explore and deliver on these standards.

Then, I think that the United States can then play a much stronger global role. We are now only recycling 9 percent of our plastics. Some of these other countries that we are talking about that are putting more waste into the world are actually recycling more than we are. So, we should set a target of going from at least our 9 percent to what Europe is doing, which is around 30 percent, to ensure that we can then move into a leadership position in this space and lead with our innovation as well.

Senator CARPER. Congressman Dooley.

Mr. DOOLEY. If I may respond. I would say that the fact that we have seen an increased use of plastics, that has been a significant contributor to enhancing global sustainability. A few years ago, UNEP, the United States Environmental Program, at the request of one of their members, did a study in terms of trying to identify the environmental costs of plastics. They hired a firm called True Cost that went out and did this study, and they came back and they said, OK, it is about $90 billion a year.

From a policymaker's perspective, I said, what would you respond to a study that said that? You could be led down to say, well, then we ought to eliminate the use of plastics. What we did at ACC, we said, you know, you need to do a more comprehensive assessment.

We went back to True Cost, we said, not only should you do an assessment of the environmental cost of plastics, but what would be the environmental costs of the alternatives. They did that and they came back to us and they said, it is a good news, bad news story. It is not 90 billion, it is 139 billion for the environmental cost of plastics. But the good news is, from a plastic manufacturer, is that the environmental cost of using alternatives to plastics was four times as large.

So I think we have to be careful here when we are trying to develop policies that are going to ultimately enhance global sustainability, that we should do so in a manner that is really based on doing comprehensive assessments of the life cycle impact of the various materials. When you take that approach based on the more comprehensive study, it sends a signal to us as manufacturers that we have to do a better job of ensuring that the plastics that are increasingly being used are more easily recycled, that their energy and their chemicals can be more easily recoverable so that we can minimize their impact in terms of the environment, but still capitalizing on the positive environmental benefits from their use.

Senator CARPER. Fair enough. Thank you. Thank you, Cal.

Could I have one more question, Mr. Chairman?

I am trying to remember the name of the new program they have over in China. I think it is called the Green Fence policy. It is a ban on importing plastic waste. As you know, China was our market for these materials and for a long time they previously accepted about 30 percent maybe a third of our plastic waste. In our Country, local municipalities are having more trouble now breaking even when collecting and recycling this waste.

Anybody have an idea what are some of the best ways for the U.S. to address this new challenge? Any thoughts?

Mr. DOOLEY. Again, I think that there are some real opportunities to use this as an inflection point where we could see opportunities to increase the value of this accumulating plastic waste. In my opening remarks, we identified some policies that we currently have in place in the United States that are impeding the flow of investment capital in developing the innovations that can transform that mixed waste or plastic waste stream accumulated into energy as well as into going through a pyrolysis where you can turn it into feedstocks.

Now, if you want to try to get a permit for a pyrolysis unit to do waste plastic, sometimes it is subject to being permitted as a hazardous waste facility versus what could be a recycling center. If you make that small change, you could, again, create a greater incentive for the flow of that investment capital to develop those new innovations.

The same thing, if you could develop diesel or a syn fuel from a plastic waste stream, it doesn't qualify for the alternative fuels treatment or renewable fuels. Making simple changes like that are going to encourage a lot of investment from a lot of startup companies, as well as a lot of large companies, to make investments that can add value and capture the energy and the value in that plastic waste stream.

Senator CARPER. Were you this smart when you were a Congressman?

[Laughter.]

Mr. DOOLEY. I was smart enough to leave.

[Laughter.]

Senator CARPER. I will say in closing, for myself, this is a really important issue and I again want to thank Senator Whitehouse and Senator Sullivan for their great leadership on this, and our Chairman for holding this hearing and for Senator Inhofe's strong interest in this issue as well. This is one I care about enormously. I am sorry there is so much other stuff going on that hasn't allowed me to be here.

One of my favorite witnesses, the guy who is the controller general of the Country, the head of GAO, General Accountability Office. His name is Gene Dodaro. I don't know if you have ever met him, but he comes and testified fairly regularly on different committees. Sometimes he will be sitting right where you are, Mr. Karas, and he speaks opening statement, no notes. Answers every question, no notes. Finally, I noticed 1 day that when he would speak there was a woman sitting right behind him and her lips were moving when he spoke.

I have been watching you and your responses, and there is a lady in a red dress right behind you, and I noticed that her lips were moving when you speak. She looks very familiar and I just want to say to Missy, welcome to our hearing; it is great to see you. You have another career ahead of you if you can take this show on the road.

Thank you very much.

Senator BARRASSO. Thank you, Senator Carper.

Thank you all. This was very productive.

Senator Whitehouse, thank you for your leadership. Thank Senator Sullivan as well.

If there are no more questions today from the panel, members might submit written questions for up to 2 weeks, so the record will stay open for that period of time.

But I really do want to thank all of you for being here, as well as what you are doing on this vital, vital issue. Thank you to National Geographic for your leadership and for putting visually something that I think really caught the attention of the Country and the world.

With that, this hearing is adjourned.

[Whereupon, at 11:52 a.m. the committee was adjourned.]

[Additional material submitted for the record follows.]

PLASTIC POLLUTION IS NOW SPREADING FROM OCEAN FOOD CHAINS INTO LAND ANIMALS, THANKS TO THIS INSECT

Just when you thought it couldn't get worse.

DAVID NIELD

SEP 201

We know that plastic pollution is a major problem for the world's oceans, but scientists just discovered a new way that discarded microplastics are making their way out of the water and into other food chains through mosquitoes.

What's happening is mosquito larvae are ingesting microplastics as water- dwelling larvae, and those plastic particles are sticking around as they transition into flying mosquitoes.

Those adult insects provide tasty snacks for birds and bats in the air above, which means microplastics are now ending up in the stomachs of land animals, not just marine creatures.

This process is technically known as ontogenic transference, meaning it happens as the organism matures and moves habitats.

Once the plastic-carrying mosquitoes have been eaten by birds and bats, the pollution can then make it further into other food chains and ecosystems, according to the researchers from the University of Reading in the UK.

"This is eye-opening research, which has shown us for the first time that microplastics are able to navigate several life stages in flying insects, allowing them to contaminate all kinds of living creatures who would not normally be exposed to them," says one of the researchers, biological scientist Amanda Callaghan.

"It is a shocking reality that plastic is contaminating almost every corner of the environment and its ecosystems."

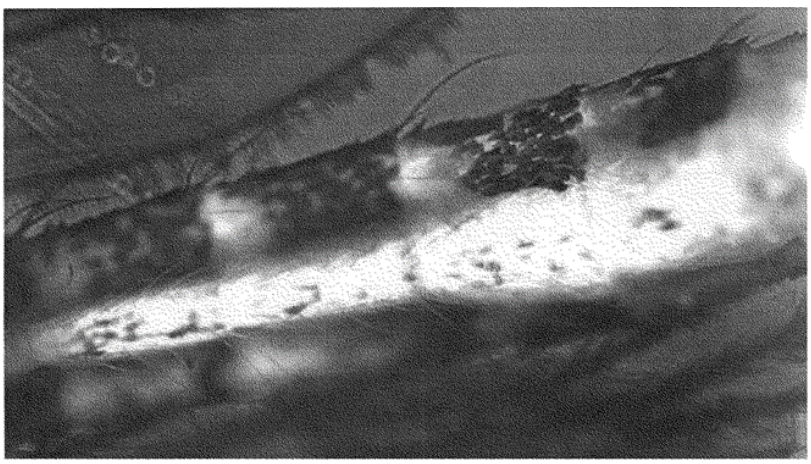
Callaghan and her colleagues fed fluorescent plastic microbeads to mosquito larvae in lab conditions then monitored their growth through a microscope.

The plastic remained in place through the non-feeding pupal stage and into the adult insect, via the Malpighian tubules (structures equivalent to the human kidneys).

Microplastics like these can take hundreds of years to be broken down in the environment, and the study showed that the smaller the plastic beads were, the more likely they were to stick around in the bodies of the mosquitoes.

We already know that plastics polluting our oceans and waterways can have a damaging effect on wildlife and make progress through the food chain.

Now we know these harmful, microscopic fragments stick around as mosquitoes change form and make their way out of the water too.



Fluorescent microplastic in a mosquito abdomen. (University of Reading).

Friends of the Earth plastics campaigner Emma Priestland, who wasn't involved in the research, said the findings were "disturbing" "Knowing that plastic can be transferred from the larval stage to the adult mosquito, which then serves as food to a multitude of larger animals, highlights the urgency with which we need to drastically reduce our use of plastic," Priestland told the BBC.

As well as mosquitoes, insects like mayflies, dragonflies and midges start life in ponds and puddles before making their way into the outside world, where they're often eaten by bigger creatures - so the problem could go way beyond the boundaries of this study.

We know that as plastic seeps into the natural world it can get passed further and further between animals and ultimately have serious consequences for natural ecosystems.

Let's hope this new study helps underline the potential scale of the problem and spurs efforts to cut back on plastic use.

"Much recent attention has been given to the plastics polluting our oceans, but this research reveals it is also in our skies," says Callaghan.

The research has been published in *Biology Letters.*

STATEMENT FOR THE RECORD, OF THE SEPTEMBER 26, 2018, SENATE COMMITTEE ON ENVIRONMENT AND PUBLIC WORKS PUBLIC, PUBLIC HEARING, ON THE SUBJECT: "CLEANING UP THE OCEANS: HOW TO REDUCE THE IMPACT OF MAN-MADE TRASH ON THE ENVIRONMENT, WILDLIFE, AND HUMAN HEALTH"

http:/!www.epw.scnatc.gov/public/index.cfm/2018/9/clcaning-up-the-oceans-how-to-reduce-the-impact-ot man-made-trash-on-the-em·ironment-wildlife-and-human-health

"Food waste is a multi-billion dollar problem" or *"opportunity"* according to Rethink Food Waste through Economics and Data (ReFED) www.refed.com/-ReFED and others are working diligently and successfully to reduce food waste.

However, when a consumer discards food, the packaging that accompanies the food is also discarded. The Animal Digestible Food Packaging Initiative (ADFPI www.adfpi.org) is an effort to *encourage/convince/inspire representatives of grocery stores, quick service restaurants, food manufacturers and food packaging manufacturers to establish a public-private partnership (PPP) with the objective of funding research necessary to identify new food packaging materials that not only meet food safety, integrity and quality requirements but that are also digestible by animals so that grocery and quick service restaurant waste may be sent to a renderer for manufacture into anima/feed instead of being sent to a landfill.*

Because food packaging that meets food safety, integrity and quality requirements and that is digestible by animals either does not exist or is not readily available, and because the United States does not have an economically robust recycling industry, food waste along with its accompanying packaging generated by quick service restaurants and grocers, that is not beneficially used, is sent to a landfill for disposal.

But if grocery stores and quick service restaurants were to utilize food packaging that meets food safety, integrity and quality requirements and that is also digestible by animals, food waste along with its accompanying packaging could be sent to a renderer for manufacture into animal feed http://www.nationalrcndcrers.org/publications/prcss-kit/nra-releases-industrv-vidcorcndering-the-sustainablc-solutionl-Recovered would be all the energy and natural resources that went into producing and manufacturing the wasted food and its inherent packaging, including its inherent animal nutrition value and its economic value as a feed source.

No one industry, be it food or package manufacturers, quick service restaurants or grocers, is willing or financially able to assume the responsibility to fund millions of dollars of research necessary for the identification of new food packaging materials that would meet food safety, integrity and quality requirements and also be digestible by animals.

If such food packaging is to be found, a food industry cooperative research effort will be required through creation of a public-private partnership (PPP) comprised of key stakeholders (including food and package manufactures, grocers and foodservice companies, renderers, pet food manufacturers, their trade associations, government agencies, foundations, think tanks and universities).

Funding for such a PPP can be found through the Foundation for Food and Agriculture Research (FFAR)

https://toundationfar.org/ and the National Science Foundation (NSF) *"Innovations at the Nexus of Food, Energy and Water Systems"* program https://www.grants.gov/web/grants/view-opportunitv.html'?oppld=JO1132.

- FFAR and/or NSF funding would *unleash the genius of students and professors at tbe Nation's land grant universities and other research organizations*- Let us put them to work on the challenge to discover animal digestible food packaging that meets necessary safety, integrity and quality requirements!

- FFAR" *... builds unique partnerships to support innovative and actionable science addressing today's food and agriculture challenges. The Foundation was established by the Farm Bill passed* in *2014 and charged with complementing and furthering the important work of the U.S. Department of Agriculture. Leveraging public and private resources, FFAR will increase the scientific and technological research, innovation, and partnerships critical to enhancing sustainable production of nutritious food for a growing global population."*

- The NSF grant program " *... seeks to support research that conceptualizes FEW systems broadly and inclusively, incorporating social and behavioral processes (such as decision making and governance), physical processes (such as built infrastructure and new technologies for more efficient resource utilization), natural processes (such as biogeochemical and hydrologic cycles), biological processes (such as agroecosystem structure and productivity), and cyber-components (such as sensing, networking, computation and visualization for decision-making and assessment) ...*"

This public hearing is one example of the public's demand that manufacturers take responsibility for the after- life environmental effects of single use packaging as exemplified by the Earth Day site, titled *"Initiatives to Ban or Reduce Consumption o(Single-Use Plastics"* at https://www.earthdav.org/plasticban/ and the Linkedin posts of Jack Cooper, ADFPI Executive Director, at https://wVIw.linkcdin.com/in/juck-coopcr-21474b14/detail/rccent-activitv/sharcs/.

- Further, "*... increased consumer and regulatory concern toward single-use plastic and other packaging materials* is *a critical investment theme, especially for asset managers with an Environmental, Social and Governance (ESG) mandate ..."* according to an extensive August 2018 Citi Global Perspectives and Solutions (GPS) Report, titled *"Rethinking Single-Use Plastics: Responding to a Sea Change in Consumer Behavior"* at https://ir.citi .com/ *II* A 9tF7xva3Sl J 5qL6ghGCV oXt4Wi3eDYs3Nd/tXPY7srxTM21 ihP4orVMVpvfvOW.

Another reason to support such a PPP is presented in a September 2018 University of Cambridge Institute tor Sustainability Leadership report: "*... The significant challenges presented by plastic packaging waste can only be solved through collaborative actions from*

business, government and society ... We do not know if it will even ever be achievable to totally eliminate plastic packaging waste. However, there is *a need to act now, before all of these unknowns can be addressed, and to set a high level of ambition, even if* it *seems hard to achieve in the current context ..."*- The report is available here: hllQs://www.cis/cam.ac.uk/resources/circular-cconom\'/towards-sustainable-packaging-a-plan-to-eliminatc-plustic-packaging-waste-from-uk-bottlcd-watcr-and-sort-drinks.

- As well, according to a June 28, 2018 Sustainable Brands http://www.sustainablebrands.com/ report, when individual food companies and their trade associations take a stand on issues the public cares about, like the after-life environmental effects of single use plastic packaging, consumers are "... *ready to reward brands that take stands ... Consumers want brands to stand up for issues* in *their areas of expertise ... {Consumer;] believe companies should provide ongoing support for issues that align with the types of products or services they offer companies {should] look to their primary business first to refine their purpose and prioritize relevant social issues ... It is clear that brands have much to gain from taking stances that fit with their organization's purpose and product ... The key is effectively communicating those stances* ..." - The SB report is here: https://www.sustainablebrans.com.com/ncws_and_views/marketing comms/sustainablebrands/consumers_ready_reward_brands_take_stands/utm_source=newsletter&utm_medium=brandsweekly&utm_camnaign=jun28.

- Also, according to an August 20, 2018 Boston Consulting Group report, titled *"Tackling the 1.6-Billion-Ton Food Loss and Waste Crisis""... no one group, government, or company can ... [solve the food waste crisis on its own but] ... Companies that play a role in the food value chain stand to reap tangible business benefits ...*

[and] can burnish their brand ..." by addressing the issue: https://www.bcg.com/en-us/publications/2018/tackling-1.6-billion-ton-food-loss-and-waste-crisis.aspx

Finally, plastic waste recycling is not projected to be effective and efficient in the United States and the EU for a decade or two:

- The U.S. Plastics Resin Producers have set the following goals: I 00% of plastics packaging to be re-used, recycled or recovered by 2040 with 100% of plastics packaging to be recyclable or recoverable by 2030, according to a May 9, 2018 The American Chemistry Council's (ACC) news release at httos://www.americanchemistry.com/Media/PressReleasesTranscripts/ACC-news-releases/US-Plastics-Producers-Set-Circular-Economy-Goals-to-Recycle-or-Recover-100-Percent-of-Plastic-Packaging-by-2040.html.

- The European Union proposed EU plastics strategy calls for"... *all plastic packaging ... [to be] recyclable by 2030* ..." according to a January 16, 2018 European Commission News Release at http://europa.eu/rapid/press-release IP-18-5 en.htm.

- Many cities are abandoning recycling programs because of soaring costs in the wake of new waste acceptance policies adopted by China, according a September 14, 2018 Bloomberg Environment story at https://www.bna.com/curbside-recycling-threat-n730l4482530/.

- *"More Recycling Won't Solve Plastic Pollution"* is the title of an August 6, 2018 Scientific American "Observations" which argues that "... *It's a lie that wasteful consumers cause the problem and that changing our individual habits can fix* it ... *The real problem is that single-use plastic ... is an incredibly reckless abuse of technology ... At face value ... {Keep America Beautiful and other] efforts seem benevolent, but they ... shift the onus of*

environmental responsibility onto the public ... A better alternative is the circular economy model, where waste is minimized by planning in advance how materials can be reused and recycled at a product's end of life rather than trying to figure that out after the fact ..."

https://blogs.scientificarnerican.corn/observations/more-recycling-wont-solve-plastic-pollution/.

- Whether or not food and other packages become "recyclable," are actually recovered and "recycled" (that is, actually manufactured into new products), the plastics industry will continue to be robust according to this story: *"Plastics Industry Flourishes in Response to Market Demand'* is the title of an article in the Third Quarter 2018 issue of the Area Development Magazine which notes that"... *It's a good time to be in the plastics manufacturing business. Plastics manufacturers in the US, are continuing to enjoy increased demand for their products ... along with decreasing production costs ... Packaging ... is showing especially strong growth ...*"- The article is here: http://www.areadevelopment. com/Plastics/03-2018/nlastics-industry- flourishes-in-response-to-market-demand.shtml.

END

Submitted on behalf of the Animal Food Packaging Initiative www.adfpi.org by Jack Cooper, Executive Director, 33 Falling Creek Court, Silver Spring, Maryland 20904- JLC@adfpi.org- Text to: 301 384 8287.

* * *

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October 7, 2018

RE: Hearing: Cleaning Up the Oceans: How to reduce the impacts of man-made trash to the environment, wildlife, and human health?"

Dear Chairman Barrasso and Ranking Member Senator Carper,

Thank you for providing me with the opportunity to submit testimony. I also want to thank you for giving time to this important issue.

I am Dr. Chelsea Rochman, a professor in Marine and Freshwater Ecology at the University of Toronto, have been researching the issue of plastic pollution for more than a decade. During this time, I have watched the state of the evidence grow tremendously. Today, there is no doubt that anthropogenic debris of all shapes and sizes litters our oceans and freshwater ecosystems. This debris is found in hundreds of species of wildlife, including in the species we consider seafood. We know that plastic pollution harms individual organisms, wildlife populations and communities. These impacts, combined with evidence for accelerating plastic production and emissions into the environment, suggest the international community should come together to limit future environmental emissions of anthropogenic

litter now, before they transform ecosystems irreparably. Below, I will speak specifically to microplastics followed by marine debris in general.

Microplastics

My research mainly focuses on microplastics (plastics 5mm in size) and demonstrates that microplastics are ubiquitous in the environment, including in seafood. My research has also shown that microplastics are associated with a cocktail of toxicants, including 78% of those we currently consider priority pollutants under the US EPA. It also demonstrates that microplastics can be toxic to fish and invertebrates.

By weight, large plastic debris such as fishing nets, make up the largest percentage of plastic floating in our oceans. However, by count, microplastics by far exceed the number of plastic in our environment. Thus, as we design policies aimed at plastic pollution, we must be mindful to include policies specific to rnicroplastics. Because some sources of microplastics arc unique (e.g., microfibers, tire dust, pre-production pellets), policies will also be unique to larger items of plastic waste. As such, it is important that we invest time thinking about creative and effective solutions for mitigating microplastics.

Although we often think of microbeads when we think of microplastics, the term microplastic incorporates a large diversity of plastic types, including those that were produced as microplastics (e.g., microbeads, pre-production pellets often referred to as "nurdles") and those that are literally degraded bits of larger plastic products (e.g., tire dust, microfibers and fragments of bottles, bags and film). The former is called primary microplastics and the latter is referred to as secondary microplastics. Secondary microplastics are the most common type of microplastic waste found at sea. Still, we must not forget the primary sources of microplastics as well as the sources that emit secondary microplastics into the oceans (e.g., microfibers). These particles, specifically microfibers, are some of the most common microplastic types found in global ecosystems.

Researchers estimate that there are between 15 and 51 trillion microplastic particles floating around in our oceans, reaching from the poles to the equator. Microplastic particles are found in large concentrations in Arctic sea ice and are also present in sediments and wildlife from the deepest parts of the ocean. Consequently, this widespread contamination has led to the contamination of 100s of species of wildlife across all trophic levels. Laboratory studies demonstrate that microplastics can lead to mortality, reduced growth, and decreased reproductive output in marine animals. Although we do not yet understand how they may affect human health, they are also found in sea salt, seafood and drinking water.

Although policies that mitigate large plastic debris also reduce microplastic debris, we need to make sure we consider microplastics when we consider all of the policy options for plastic pollution. Policies specific to microplastics may include, but are not limited to, emissions standards for microplastics (e.g., from washing machine effluent, wastewater, storm water, etc...), filters on washing machines to trap microfibers, increasing participation for operation clean sweep and extend this model to textiles, material innovation, and banning microbeads.

The above mitigation strategies are simple solutions to combat some sources of microplastics. Still, when it comes to plastic pollution, we know the least about sources, fate and effects of microplastics. As such, while we begin implementing policies now related to

known sources of microplastics, we must continue to put resources into research that helps us better understand what some other sources of microplastics are and which may be prioritized for policy based on quantities and risk.

Marine Debris in General

Today's estimates suggest that about eight million metric tons of plastic enters the oceans annually from land. If we continue business as usual, this number is expected to increase by an order of magnitude by 2050. In the US, I think we have at1 opportunity to lead in this space. The US can and should be a large part of the solution, and show other countries that reducing emissions of plastic is possible.

Last year, I co-Jed a paper in the journal *Proceedings of the National Academy of Sciences* titled, "Why we need an international agreement on marine plastic pollution." Like many other contaminants, plastic is not constrained by borders. It migrates via air and water currents in and out of parts of the oceans that are beyond national jurisdiction. Because plastic pollution does not observe borders, neither should policy. At this time, there are no international agreements for plastic pollution. I recognize that the Clean Seas Initiative is a great first step, but I think it is time to move to something similar to the Paris Agreement and at a faster pace than in took to get to the Paris Agreement. To measurably reduce emissions of plastic pollution, we need defined reduction targets, signatories, methods of reporting progress and a global fund.

I envision an agreement where countries sign on as signatories with a defined reduction target. For example, in the US we might agree to reduce 25% of our emissions by 2025. To meet reduction targets, each conn try needs to come up with strategies to do it. Because there is no one-size-fits-all solution, each country may take on its own set of unique solutions to reach its target. For example, countries may adopt container deposit schemes to improve recycling rates, eliminate the use of some single-use plastic items that are unnecessary (e.g., microbeads, straws), improve waste collection and management infrastructure, and agree to market only plastics that are recyclable and/or reusable in their region. For some countries, particularly in the developing world, aid is necessary to build new infrastructure for waste. These countries need access to a global fund, similar to the UNFCCC's climate fund. To build this fund, an extended producer responsibility program can be implemented. If the fund pulled in one penny for every pound of plastic produced, the fund would build by over $6.8 billion per year. Finally, each year countries would report on their success measuring the reduction of plastic emissions globally over time and ensuring signatories reached their goal.

With more than a decade of experience researching plastic pollution, I have a vast knowledge base on this issue, I have published many papers about the topic and have advised managers and policy-makers in several countries. For example, I presented at the Our Ocean Conference at the US State Department and in front of the UN General Assembly in New York City. I have also had one-on-one discussion with the offices of several congressmen and women in the United States. I would be more than happy to sit down with you and discuss the state of the science and how it might inform policy around this important issue both nationally and internationally. Please feel free to connect with me anytime.

Many thanks for your time. Sincerely,

Chelsea M. Rochman
Assistant Professor

* * *

GOVERNMENT OF THE DISTRICT OF COLUMBIA
Deportment of Energy and Environment

October 10, 2018

Senator John Barrasso
Chairman
Committee on Environment and Public Works
410 Dirksen Senate Office Building
Washington, DC 205IO

Senator Tom Carper
Ranking Member
Committee on Environment and Public Works
456 Dirksen Senate Office Building
Washington, DC 20510

Subject: Written Testimony for recent U.S. Senate hearing entitled *Cleaning up the Oceans: How to reduce the impact of man made trash on the environment, wildlife, and human health*

Dear Sen. Barrasso and Sen. Carper:

Thank you for your leadership to reduce the impacts of trash on our nation's waterways. Trash is one of the great environmental challenges of our time and will not be solved without robust participation from federal, state, and local governments. We welcome this opportunity to provide written testimony and stand ready to work with you on further efforts to address this issue.

The District has been a leader in the nation for over a decade on addressing trash in our local waterways. According to a 2015 article published in the journal, *Science*, researchers estimate over 8 million metric tons of trash enter the oceans, annually from land-based sources.[61] In a heavily urbanized area like the District, a large portion of that trash comes from litter being conveyed via storm sewer and combined sewer systems to waterways like the Potomac and Anacostia Rivers. In response to this problem, the District, in collaboration with U.S. Environmental Protection Agency (EPA) and upstream jurisdictions, established a

[61] Jambeck, J.R., R. Geyer, C. Wilcox, T.R. Siegler, M. Perryman. A. Andrady, R Narayan, & K.L Law. 20L"L Plaslic waste inputs from land into the ocean. Science 347: 768-771.

total maximum daily load (TMDL) for trash in the Anacostia River. As a result, the District is compelled by EPA and our own priorities to reduce trash in the Anacostia River as part of our efforts to make the Anacostia fishable and swimmable once more. Given that the District has declared 2018 the Year of the Anacostia River, it is very timely for us to provide the Senate with a summary of our experiences. We are proud of all of our accomplishments and would like to share details of some of our efforts in hopes that this work can, and will be, emulated elsewhere throughout the nation.

Three important components have helped us to effectively manage trash in our waterways: regional partnerships, using sound science to inform policy, and taking multi-pronged, innovative approaches.

Regional Partnerships

The issue of trash impacting the Potomac River watershed began over a decade ago thanks to a regional collaboration facilitated primarily by the Alice Ferguson Foundation of Accoceek, MD. Since 2005, the Alice Ferguson Foundation has held an annual Potomac Watershed Trash Summit in the DC metropolitan area. This summit brings federal, state, and local government agencies, elected officials, and non-governmental organizations together to discuss efforts to reduce trash in the watershed. This would not be possible without grant funding from the NOAA Marine Debris Program, which has been an invaluable partner in regional efforts to address this issue.

Example of trash conditions in Watts Branch, a tributary to the upper Anacostia River in Washington, DC.

Over the years, participants have implemented key efforts discussed at this summit including a regional anti-littering campaign, innovative local policies for reducing trash, and regional collaborations to establish the TMDL for trash for the Anacostia, one of the most

urbanized tributaries to the Potomac River. The summit brought together leaders from the District, the state of Maryland, local Maryland counties, EPA, and local advocates to reach common ground on establishing the TMDL. Without this regional partnership it is very unlikely this effort would have been successful. As we will highlight more specifically, several new innovative approaches for trash reduction have been implemented since that time.

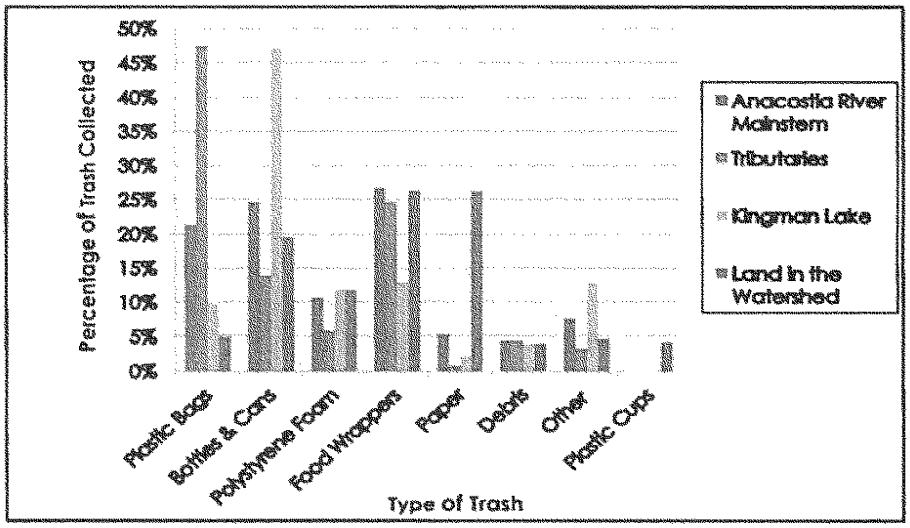
In 2016, the District, Montgomery County, and Prince George's County reaffirmed their commitment to making the Potomac and Anacostia River free of trash by signing the Anacostia River Accord. The accord was signed by the District's Mayor Muriel Bowser, as well as Montgomery County Executive Isiah Leggett, and Prince George's County Executive Rushern Baker. In addition, the Anacostia Watershed Restoration Partnership, housed at the Metropolitan Washington Council of Governments, has convened a trash working group to develop consistent methods for tracking and reporting trash reductions across all three jurisdictions. This is further evidence of the importance of regional partnerships at combatting the issue of trash in our waterways.

The next Potomac Watershed Trash Summit to discuss what the District, other jurisdictions businesses, and non-governmental organizations are doing to address trash in the Potomac River will he held on October 16'h, 2018 at George Mason University in Arlington, VA.

Using Sound Science to Inform Policy

One of the first things the District did to address the problem of trash in the Anacostia River was conduct a two-year comprehensive study of trash conditions. The District Department of Energy and Environment (DOEE) funded the Anacostia Watershed Society of Bladensburg, MD to conduct surveys of litter along the river and its many tributaries and monitor trash loads from storm sewer outfalls. The study provided two important pieces of information: (I) data on the most common types of trash in the Anacostia River and its watershed and (2) data on total weight of trash entering the Anacostia River on an annual basis. The first dataset informed District policies targeted to address specific types of trash most common in our waterways such as single-use plastic bags and expanded polystyrene foam products. The graph below shows the most common types of trash found out of 44 different categories sampled along DC shorelines in the Anacostia River, its tributaries, Kingman Lake (a semi-enclosed lake in the Anacostia River), and land in the Anacostia River watershed. The District utilized the second dataset to develop the trash TMDL and identify and strategically address hotspots in the watershed which are conveying above average amounts of trash to the river.

Since 2016, DOEE has been working with the Metropolitan Washington Council of Governments to sample trash along rivers and streams throughout the District. We provide this data annually to U.S. EPA Region III. This work builds upon a larger monitoring dataset the Council of Governments has been collecting in the Maryland portion of the Anacostia River watershed since the early part of this decade, making it one of the most robust datasets for trash for a waterbody anywhere in the nation. Having this data is imperative to making future strategic decisions for implementation and infom1ing development of new policies.



Graph displaying most common types of trash found by count in 2008 in the Anacostia River, its tributaries, Kingman Lake, and land in the Anacostia watershed.

Multi-Pronged, Innovative Approaches

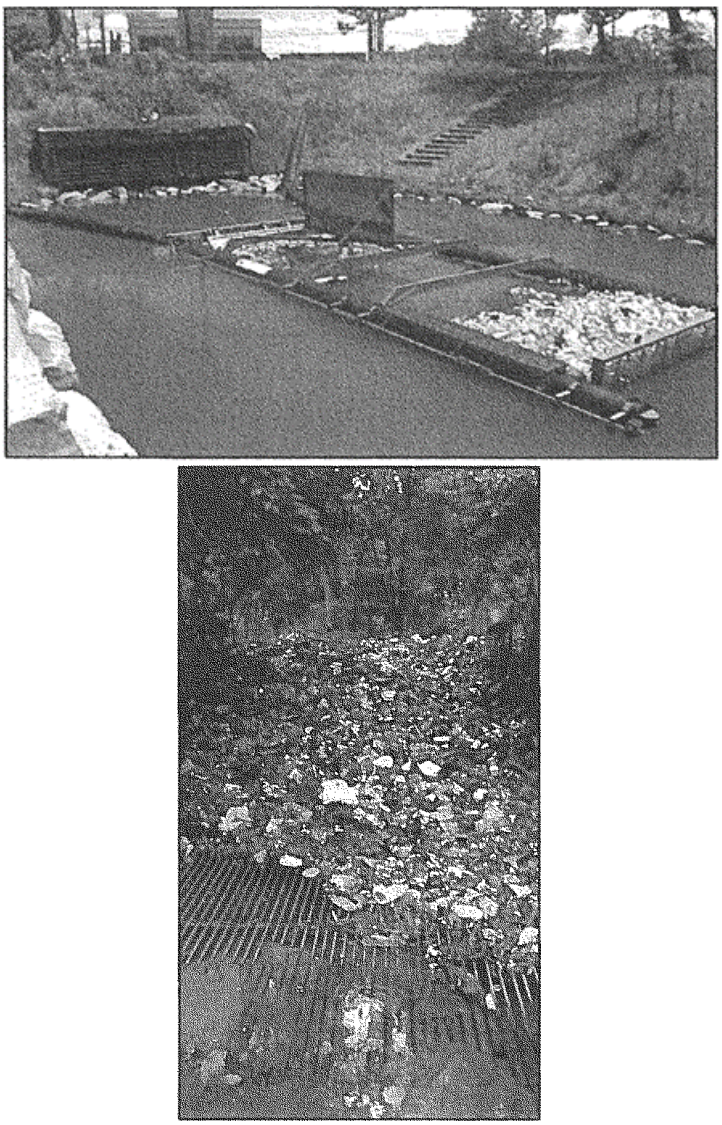
As with most environmental challenges, there is no "silver bullet" for eliminating the harm trash poses to our waterways and wildlife. The District has devised a plan that utilizes innovative policies, trash capture technologies, education and outreach.

As mentioned previously, our monitoring efforts helped us to determine the most common types of trash found in our waterbodies. The District utilized monitoring data described previously to justify the need to reduce three of the most common types of trash found during sampling: single-use plastic bags, expanded polystyrene foam products (commonly referred to as Styrofoam™), and other food service ware.

In 2009, the District enacted the Anacostia River Clean Up and Protection Act (also known as the Bag Law) to create a five-cent fee on single-use plastic bags. Starting January l, 2010, consumers in the District pay the fee at the time of purchase in a restaurant, grocery, liquor, or convenience store. DOEE employs an inspection team to ensure businesses are in compliance with the law. Revenue, fines and other contributions generated by the law goes into the Anacostia River Clean Up and Protection Fund to pay for projects like trash capture devices, stream restoration, stormwater management projects, education, outreach, and administrative costs. In Fiscal Year 2017, the Department found that 76% of businesses inspected were in compliance with the law. More information on the District's Bag Law, including annual revenue and expenditure reports, arc available at https://doee.dc.gov.bags.

In 2014, the District enacted the Sustainable DC Omnibus Amendment Act, which includes restrictions on food service ware packaged and intended for consumption without further preparation. Specifically, a ban on food service ware made of Styrofoam™ (foam) took effect January 1, 2016, and the law requires food service ware to be made of recyclable or compostable materials starting January 1, 2017. As with the Bag Law, DOEE inspects businesses to make sure they are in full compliance with the law. In fiscal year 2017, DOEE

found that 88% of District businesses inspected were in compliance. Further information on the District's food service ware requirements is available at https://doee.dc.gov/foodservice ware.

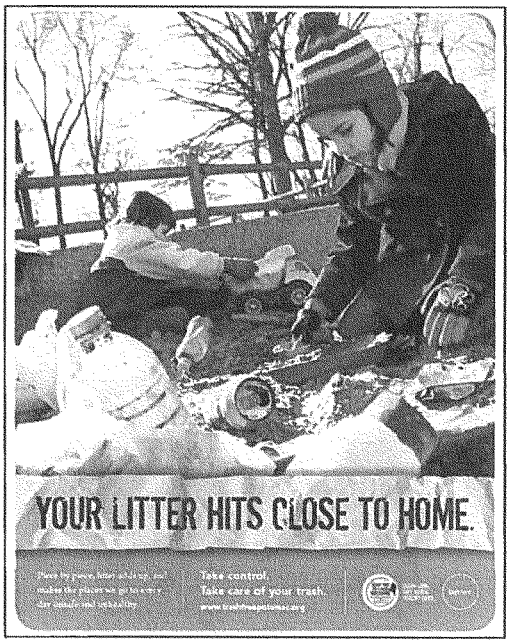


Examples of trash traps installed in tributaries to the Anacostia River in the District. Top: Proprietary device known as a Bandalong Litter Trap™ installed and maintained by Anacostia Riverkeeper. Bottom: custom trash weir designed, installed, and maintained by the Anacostia Watershed Society.

The District has also implemented innovative structural controls for capturing trash. Since 2009, the District has installed nine trash traps in the Anacostia River watershed. These traps have varied from proprietary products to custom devices designed by local non-profits. The pictures below display examples of the devices. These devices have primarily been funded by the Anacostia River Clean Up and Protection Fund and other local funding sources through grants to local nonprofits to design, install, and maintain these devices. The

nonprofits also collect data on trash collected in the traps to further inform policies. Since installation of the first device in 2009, these devices together have helped capture and remove over 60,000 lbs of trash and debris from the Anacostia River and its tributaries.

Lastly, the District has led many education and outreach activities over the years to change behavior. In 2010, DOEE funded the Alice Ferguson Foundation (AFF) to conduct a study of littering behavior that guided the development of an anti-littering campaign throughout the District The campaign's central message, *"Your Litter Hits Close to Home."* was based on AFF's research that found people were most impacted by the effects litter has on their personal space, health, and well-being. Below is an example graphic from the anti-littering campaign. Other local governments in the Potomac River watershed have adopted these campaign materials.



In closing, I want to again thank you and the Senate EPW committee for your interest in this important subject. As the nation's capital, the District has an important role to play in restoring urban waterways. We have set the stage for reducing trash in our rivers and streams using multi- faceted, innovative approaches, but we are not done. We are truly alarmed by the potential impacts microplastics could have on our aquatic resources such as some of our valuable fisheries. A 2014 study published in the journal, *Environmental Science and Technology*, found microplastics to be present in four tidal tributaries to the Chesapeake Bay.[62] 59 of the 60 samples collected during the study contained microplastics. We are actively engaged with the EPA Chesapeake Bay Program, the NOAA Marine Debris Program, and neighboring jurisdictions on research and management to address the issue. We hope Congress will also take a leadership role in finding solutions to this challenging pollution issue.

[62] Yonkos, L.T., E.A. Friedel. A.C. Perez-Reyes, S. Gbosal. & C.D. Arthur. 2014. Microp1astics in four e'tuarine rivers in the Chesapeake Bay. C S.A. Environ. Sci. Technol. 48: 14195-14202.

I would encourage the Senate Environment and Public Works Committee to peruse reports on our monitoring efforts. If you are interested in receiving copies, or have any other questions regarding our efforts to reduce trash in our please contact Matt Robinson of the DOEE Watershed Protection Division at or (202)442-3204.

Sincerely,

Tommy Wells
Director

In: Encyclopedia of Marine Biology (12 Volume Set) ISBN: 978-1-53619-529-3
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Chapter 126

MARINE ENVIRONMENT AND CONTAMINATIONS OF RADIOCESIUM AND ORGANOHALOGENS IN CETACEANS AND PACIFIC COD INHABITING THE COASTAL WATERS AROUND HOKKAIDO, NORTHERN JAPAN

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ABSTRACT

Hokkaido is Japan's northernmost main island and is surrounded by the North Pacific Ocean, the Japan Sea, and the Okhotsk Sea. Some headlands in Hokkaido and their surrounding warm and cold currents restrict the travel and migration of fish and cetaceans and control the dispersal of environmental pollutants throughout the three seas. Three independent stocks of Pacific cod inhabit the three seas, and many cetaceans become stranded along their coasts. In this chapter, we used stranded cetaceans and Pacific cods as indicator species to compare the contamination levels of radiocesium in the three seas shortly after the Fukushima Daiichi Nuclear Power Plant accident in 2011. We assessed the contamination levels of perfluoroalkyl substances (PFASs), classical persistent organic pollutants (POPs), and naturally produced halogenated compounds (NHCs) in cetaceans and Pacific cods from the three seas around Hokkaido. We also compared the contamination levels between the North Pacific Ocean around Hokkaido and other areas around the world.

Keywords: Hokkaido, sea current, cetacean, pacific cod, radiocesium, stable isotopes, perfluoroalkyl substances (PFASs), persistent organic pollutants (POPs), naturally produced halogenated compounds (NHCs)

1. SEA AREAS OF HOKKAIDO

1.1. Introduction to the Sea Areas of Hokkaido

The seawaters surrounding Hokkaido (Figure 1) are complex and rich biodiversity. In particular, the area off Eastern Hokkaido is one of the world's top three fishing grounds. The region consists of four sea currents, which affect the physical seawater characteristics and show significant seasonal variability.

Hokkaido is located north of Honshu, the largest main island, and is therefore Japan's northernmost large island. The Japan Sea is located on Hokkaido's western coast, the Pacific Ocean is located on its eastern coast, and the Okhotsk Sea is located on its northern coast. The Tsugaru Strait lies between Hokkaido and Honshu and connects the Japan Sea and Pacific Ocean, and the Soya Strait connects the Japan Sea to the Okhotsk Sea.

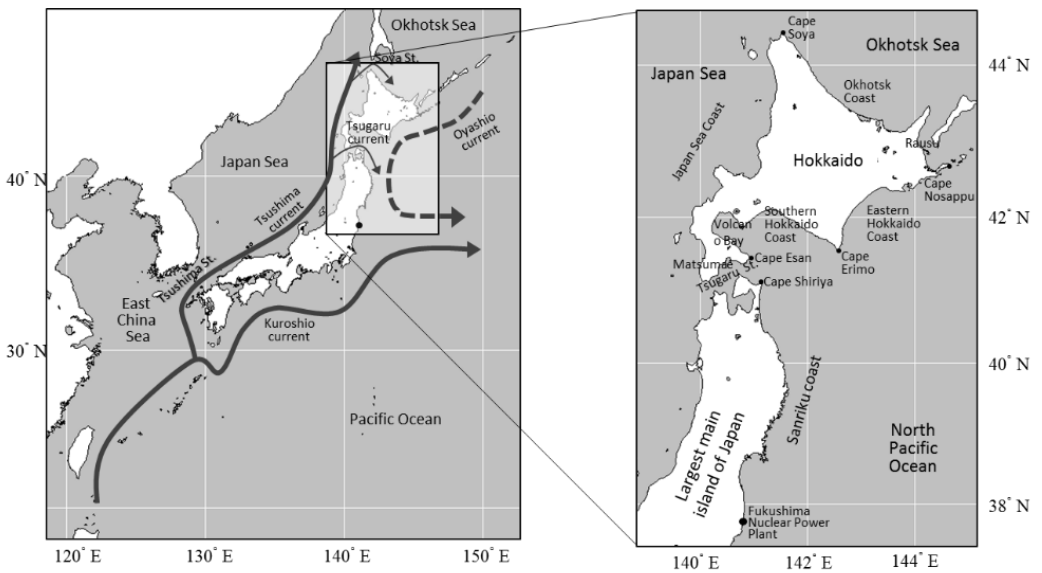

Figure 1. Location, related lands and related sea currents of the sea of northern Japan. This figure was reprinted from Nakamura et al., (2015). Copyright (2015), with permission from Inter Research with slight modification.

1.2. Sea Currents around Hokkaido, Japan

Three sea currents surround the island of Hokkaido. The largest current in the Japan Sea is the Tsushima Current, and the Tsugaru Current in the Tsugaru Strait is a branch of the

Tsushima Current. The Oyashio Current is a dominant cold-water current running from the Kamchatka Peninsula to the Sanriku Coast via the east coast of Hokkaido.

The Tsushima Warm Current is the only source of oceanic water to the Japan Sea; it is sourced from the warm saline waters of the Kuroshio Current, which mixes with freshwater in the East China Sea. The surface current velocity and flow volume in the Tsushima Strait ranges from 0.3 to 0.4 m/s. The majority of the current flows out through the Tsugaru Strait, and the remainder flows northward along the western coast of Hokkaido. Part of the current flows out through the Soya Strait to the Okhotsk Sea, while the remainder reaches the northern Japan Sea (Gallagher et al., 2015).

The Tsugaru Warm Current is a branch of the Tsushima Current, and its outflow into the Pacific Ocean forms two oceanic modes: a gyre mode and a coastal mode (Conlon, 1982). The outflow of the Tsugaru Current forms a warm clockwise gyre in summer. The gyre disintegrates in winter, and the current flows southward along the coast of Honshu to the Sanriku Coast; this results in significant seasonal variability in the south of Hokkaido and the Sanriku Coast (Rosa et al., 2007).

The Oyashio Cold Current originates from the East Kamchatka Current, which is a part of the western boundary current of the subarctic North Pacific. Part of the East Kamchatka Current enters the Okhotsk Sea; the remainder flows along the coast of the Kuril Islands to the Eastern Hokkaido Coast to form the Oyashio Current. The current carries cold, fresh, oxygenated, and nutrient-rich water and has a profound impact on the fishing ground off the Sanriku Coast (Isoguchi and Kawamura, 2006).

1.3. Coastal Zone

The coastal zone of Hokkaido is divided into 4 areas based on the surrounding sea water currents: the Japan Sea Coast, Okhotsk Sea Coast, Eastern Hokkaido Coast, and Southern Hokkaido Coast.

The Japan Sea Coast stretches to Cape Soya from the northern Honshu Island. The coasts facing the Tsugaru Strait (until Cape Esan and Cape Shiriya) also form part of the Japan Sea Coast. The water temperature is warm, especially in summer, due to the influence of the Tsushima and Tsugaru Currents. As a result, warm species of fish, including bluefin tuna (*Thunnus orientalis*), are frequently observed. Strong seasonal winds from the Eurasian Continent result in rough oceanic conditions in winter. In addition to the coastal area, the Musashi Bank located off the west coast of Hokkaido (44° 40’ N, 140° 25’ E) is an excellent fishing ground. According to the official statistics of the Hokkaido Government, the dominant fish in their fisheries include the Okhotsk atka mackerel (*Pleurogrammus azonus*), walleye pollock (*Theragra chalcogramma*), and chum salmon (*Oncorhynchus keta*). The Pacific herring (*Clupea pallasii*) was also abundant until the 1940s.

The Okhotsk Coast stretches from Cape Soya to Cape Nosappu. During winter, drift ice is transported to the coast from the west coast of the Okhotsk Sea. The melting of drift ice in spring due to increased surface temperatures releases abundant nutrients and increases regional primary production. The Japanese scallop (*Mizuhopecten yessoensis*) is cultured on the coast, and chum salmon is also an important fishery resource. The coastline from Cape Shiretoko to Cape Nosappu faces the Nemuro Strait, which connects the Okhotsk Sea to the

Pacific Ocean. Hydrographically, this coast forms a part of the Okhotsk Coast, but the fauna is occasionally strongly influenced by the Pacific Ocean.

The Eastern Hokkaido Coast stretches from Cape Nosappu to Cape Erimo and is strongly affected by the nutrient-rich Oyashio Current. The coastal area is a large habitat for the kelp species *Saccharina longissima*, *S. coriacea*, and *S. angustata*, which are common ingredients in Japanese cuisine.

The Southern Hokkaido Coast stretches from Cape Erimo to Cape Esan where the Tsugaru Warm Current mixes with the Oyashio Cold Current. The Tsugaru Warm Current dominates in summer and autumn, and the Oyashio Cold Current dominates in winter and spring. The aquaculture of Japanese scallop and of the kelp species *Saccharina japonica* and *S. angustata* is actively conducted on the coast. The walleye pollock and Japanese common squid (*Todarodes pacificus*) are also commonly fished.

The sea region off the Eastern Hokkaido Coast is one of top three fishing grounds in the world. The two dominant currents—the cold Oyashio and warm Kuroshio—form a transition zone, which results in a sharp gradient in oceanographic conditions, forming the sub-boreal, cool temperate, and warm temperate and subtropical zones. The high marine biodiversity in this area is attributed to the large topographical, physical, and chemical variability within a relatively small latitudinal range (Kida et al., 2015). Moreover, the high-nutrient Oyashio Current increases primary production and supports the high fish biomass for sustained fishery resources at higher trophic levels. The spatio-temporal variability of physical and biological seawater properties off the Sanriku Coast significantly impacts coastal ecosystems through the supply of nutrients and the transport of planktonic fish and invertebrates (Sakurai, 2007). Pelagic fish, including the Japanese sardine (*Sardinops melanostictus*), Pacific mackerel (*Scomber japonicus*), Pacific saury (*Cololabis saira*), and Bonito (*Katsuwonus pelamis*) are caught by large-scale commercial fishing in this region.

1.4. Cetaceans and Their Migrations

Cetaceans and other marine mammals are apex predators. They have a major influence on the structure and function of some aquatic communities due to their large body size and high abundance (Bowen, 1997).

The Stranding Network Hokkaido (SNH) is collecting cetacean stranding information and specimens from Hokkaido's coasts for providing to a number of cetologists (Matsuishi, 2011). According to the SNH database (http://kujira110.com/), the most frequently reported cetacean species were the harbor porpoise (*Phocoena phocoena*), common minke whale (*Balaenoptera acutorostrata*), and Dall's porpoise (*Phocoenoides dalli*). Most of the cetacean samples presented in this chapter were supplied from the SNH.

Harbor porpoises are common in the coastal areas of the North Atlantic and northeast Pacific, and their migration and population structure in these regions have been extensively reported (e.g., Verfuß et al., 2007). By contrast, their migration and population structure in the northwest Pacific coast is not widely reported. Taguchi et al. (2010) suggested that the seasonal migration of this species occurs along the coasts of the northwest Pacific and the Okhotsk Sea, but the relationship between the populations in the Pacific and the Japan Sea remains unclear.

The common minke whale is also commonly distributed in the seas of northern Japan. According to the Japan Fisheries Research and Education Agency (FRA), the population in the Okhotsk Sea and the west North Pacific Ocean in the 1990's was estimated at 25,049 (13,700–36,600, 95% CI), assuming perfect counting in the sighting survey (Buckland et al., 1992). The population structure of the common minke whale in this region is still under discussion; however, it currently consists of two types: 1) the 'O-type' found primarily in the offshore Pacific Ocean, and 2) the 'J-type' found primarily in the Japan Sea and nearshore waters of Japan's Pacific coast (Wade et al., 2010). Under the JARPN II (the second phase of the Japanese Whale Research Program under Special Permit in the North Pacific), this species has been sampled lethally and non-lethally, and various biological information has been collected and reported (Tamura et al., 2016). The O-type is estimated to migrate northward during April to July and remains in the Okhotsk Sea during summer. The J-type is also assumed to have similar seasonal migratory behavior between the south and north (Hatanaka and Miyashita, 1997).

Dall's porpoises are widely distributed around the seas of northern Japan in both the Pacific and Japan Sea. This species has two color morphologies: the *dalli*-type and *truei*-type. The *dalli*-type is distributed in the Japan Sea and around Hokkaido, while the *truei*-type is distributed from the coastal area of Sanriku Coast to the central Okhotsk Sea. The two types are genetically different and are therefore considered distinct populations (Hayano et al., 2003). The populations of the *dalli*-type and *truei*-type are estimated at 174,000 and 178,000, respectively, based on the official report on the species' stock status published by FRA. This species is not controlled by the International Convention for the Regulation of Whaling, but the Fisheries Agency of Japan (JFA) manages its capture by implementing a catch quota based on the potential biological removal theory (Wade, 1998). In the Japan Sea, the *dalli*-type migrates to the coast of Hyogo Prefecture (southern Honshu) in winter and to the Okhotsk Sea in spring where it remains for breeding in summer. In the Pacific Ocean, the *dalli*-type is distributed off the Sanriku Coast, and the *truei*-type is distributed near the Sanriku Coast in winter; both types migrate to the east coast of Hokkaido during summer (Amano and Hayano, 2007).

Baird's beaked whales (*Berardius bairdii*) are also frequently stranded and commercially caught around Japan under the permission of JFA. According to distribution and morphological information (Kishiro, 2007), three populations around Japan are thought to be distinguished, including the Japan Sea population caught off southwestern Hokkaido; the Pacific-Okhotsk population caught off Abashiri, Hokkaido; and the Pacific population caught off Honshu.

In 2019, Yamada et al. (2019) identified a new species known as the Black Berard's whale (*Berardius minimus*). This species has been confused with the Baird's beaked whale (*B. bairdii*) as a cryptic species (Kitamura et al., 2013). However, its body length is significantly smaller than *B. bairdii*, and the two species show clear genetic and morphological differences. *B. minimus* is thought to be distributed in the Okhotsk and Bering Seas.

1.5. Fishes and Their Migrations

The Hokkaido region has an active fisheries operation, and a wide range of species are caught in both commercial and small-scale fisheries. Most of the fish species migrate seasonally along the coast, and are divided into three stocks: the Pacific stock, Okhotsk stock, and Japan Sea stock. For example, according to the Fisheries Research and Education Agency, the walleye pollock—the most abundant fish species in the region—is divided into at least three stocks with different spawning grounds and population dynamics.

The population structure of the Pacific cod—another common species in the region—is unclear, but it is assumed to be divided into three stocks: the Pacific Ocean stock, Japan Sea stock, and Okhotsk Sea stock (Chimura et al., 2019). In contrast, observations of its life history characters and catch trends point toward the existence of a large number of local stocks (e.g., Bakkala et al., 1984; Mishima, 1984; Kanno et al., 2001). A recent genetic study showed clear differences between the western part of Honshu and other samples around Japan (Suda et al., 2017). Genetic differences between stocks are caused by strict and long reproductive isolation and mutation; however, the spawning ground may change according to abundance, which fluctuates on decadal time-scales. This may explain the population structure discrepancy observed from population dynamics and genetic studies.

1.6. Conclusion

Hokkaido is Japan's northernmost large island and is surrounded by the North Pacific Ocean, the Japan Sea, and the Okhotsk Sea. Some headlands in Hokkaido and their surrounding warm and cold currents restrict the migration of fish and cetaceans and control the dispersal of environmental pollutants throughout the three seas. Three independent stocks of fish—including the Pacific cod—inhabit the three seas and also migrate seasonally along the coast of Hokkaido. Many cetaceans become stranded along the coast of the three seas as they migrate annually from north to south. In the following sections, we assess the contamination levels of radiocesium, perfluoroalkyl substances (PFASs), classical persistent organic pollutants (POPs), and naturally produced halogenated compounds (NHCs) in Pacific cod and cetaceans in the three seas surrounding Hokkaido. We also make comparisons between the contaminations levels in the North Pacific Ocean and other regions around the world.

2. Radiocesium Contamination and Migratory Behavior of Cetaceans and Pacific Cods from the Seawaters of Hokkaido

2.1. Introduction

A magnitude 9.0–9.1 (M_w) earthquake occurred off the Sanriku Coast, the Tohoku district in main island of Japan at 14:46 JST on 11 March, 2011. The epicenter was approximately 70 km offshore, which triggered a large-scale tsunami of 40.5 m. The tsunami caused nuclear

accidents at three reactors in the Fukushima Daiichi Nuclear Power Plant (FDNPP), resulting in the large-scale release of radiocesium (^{134}Cs and ^{137}Cs) and other radionuclides to the atmosphere and across an extensive area of ocean off eastern Japan. The total amount of ^{137}Cs emitted from the FDNPP was 15 PBq (Honda et al., 2012) and a large proportion of these nuclides was transported and diffused eastward by the Kuroshio Current (Buesseler et al., 2011; Honda et al., 2012, see Figure 1). Following the FDNPP accident, the radiocesium contamination in seawater and fish in the vicinity suddenly increased, reaching a maximum (31 kq/kg as ^{137}Cs) between June and August, 2011, followed by a rapid decrease thereafter (Aoyama et al., 2012; Takagi et al., 2014). In contrast, radionuclide contamination in the seawater off northern and western Japan was low (Inoue et al., 2012), likely due to the inhibited transport of radiocesium via the Tsugaru and Oyashio Currents to the coastal areas of Hokkaido in northern Japan (Inoue et al., 2012; Honda et al., 2012).

^{137}Cs is a long-lived artificial radionuclide (half-life: 30.2 years) that bioaccumulates in the marine food chain; it is therefore a strong indicator of radionuclide pollution in the marine environment (Kasamatsu and Ishikawa, 1997). However, ^{134}Cs is detectable in seawater and marine biota shortly after the nuclear accident due to the shorter half-life of ^{137}Cs (2.06 years).

Shortly after the FDNPP accident, high levels of radiocesium (>100 Bq/kg-wet) were detected in many demersal and pelagic fishes in the surrounding western North Pacific Ocean. In contrast, little or no radiocesium contamination was reported in marine biota in the waters off Hokkaido, inferring little contamination in this region. However, radiocesium was detected in stranded cetaceans on the coast of Hokkaido in 2011 and 2012; high levels were also detected in Pacific cod caught in the North Pacific Ocean off Hokkaido in 2011–2015. In this section, we discuss the reasons for the detection of radiocesium in only cetaceans and Pacific cods.

2.2. Contamination of Radiocesium in Stranded Cetaceans on the Coast of Hokkaido

Hokkaido is surrounded by the North Pacific Ocean, the Japan Sea, and the Okhotsk Sea. More than 50 cetacean strandings are annually reported along Hokkaido's coast by the SNH (http://kujira110.com/). We measured the radiocesium (^{134}Cs and ^{137}Cs) in muscle samples of 47 stranded cetaceans on the coast of Hokkaido between April 2011 and September 2012 (Nakamura et al., 2015). The species of odontocetes were the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*; n = 11), harbor porpoise (n = 11), *dalli-type* Dall's porpoise (n = 10), Baird's beaked whale (n = 1), Stejneger's beaked whale (*Mesoplodon stejnegeri*; n = 1), Hubbs' beaked whale (*Mesoplodon carlhubbsi*; n = 1), pygmy sperm whale (*Kogia breviceps*; n = 2), Cuvier's beaked whale (*Ziphius cavirostris*; n = 1), and killer whale (*Orcinus orca*; n = 1). The species of mysticetes were the common minke whale (n = 6) and humpback whale (*Megaptera novaeangliae;* n = 2).

Radiocesium contamination (> 1.0 Bq/kg-wet as ^{137}Cs) was predominantly detected in the cetaceans stranded along the coast of the Pacific Ocean (southern and eastern coasts of Hokkaido, ①–⑭ in Figure 2) between June and October 2011, irrespective of species (Figure 3). The highest level of radiocesium contamination was found in a common minke

whale (19.9 Bq/kg-wet as ^{137}Cs, ③ in Figure 2), which is in disagreement with the notion that contamination levels are generally higher in odontocetes than in mysticetes (Haraguchi et al., 2000; Simmonds et al., 2002). No contamination of radiocesium was found in the cetaceans stranded on the coast of the Japan Sea and Okhotsk Sea in 2011 and 2012. Furthermore, no radiocesium contamination was found in all cetaceans stranded in 2013 (data not shown Figure 3), likely due to the rapid decrease of seawater radiocesium contamination around the FDNPP. The contamination level of radiocesium in cetaceans may reflect that of the seawater along their travel routes and during their sojourn in the contaminated area. Radiocesium levels in marine mammals before the FDNPP accident were approximately 0.2 Bq/kg-wet (Kasamatsu and Ishikawa, 1997; Yoshitome et al., 2003).

Many cetaceans inhabiting the waters off Japan generally migrate from south to north in spring and vice versa in autumn. However, their detailed travel routes remain uncertain, despite the increasing development of research techniques. Figure 4 shows the putative travel routes of the Pacific white-side dolphin (A), *dalli*-type Dall's porpoise (B), and common minke whale (C) that inhabit the waters around Japan. The levels of ^{137}Cs and the date and location of stranding are shown in Figure 4. In addition to the stranded animals, the contamination levels of an incidentally caught (bycatch) Pacific white-sided dolphin (n = 1) and a *dalli*-type Dall's porpoise (n = 1) are shown in Figure 4A and B, respectively.

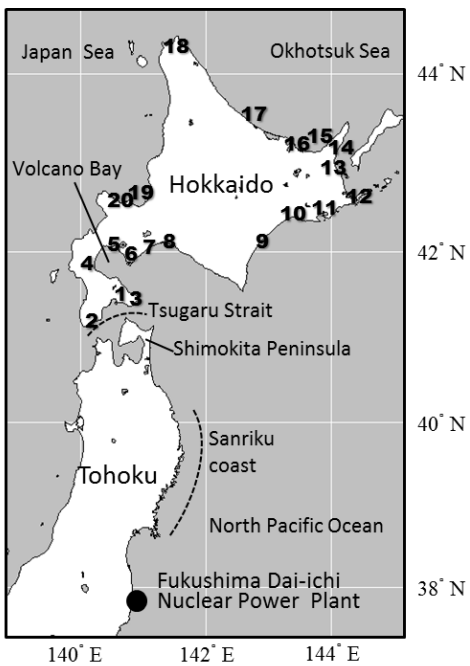

Figure 2. Map of Japan showing the Hokkaido and Tohoku areas. This figure was reprinted from Nakamura et al., (2015). Copyright (2015), with permission from Inter-Research with slight modification.

Radiocesium was detected in all Pacific white-sided dolphins stranded in 2011, six dolphins stranded along the coast of Volcano Bay (southern coast of Hokkaido), and one dolphin stranded on the coast of Rausu (Figure 4A). Furthermore, radiocesium was detected

in a Pacific white-side dolphin incidentally caught in 2011. However, radiocesium was not detected in this stranded species in 2012. The Pacific white-sided dolphin can take two possible travel routes to Volcano Bay: 1) via the Sanriku and Shimokita Coasts (Pacific Ocean route), and 2) via the Tsugaru Strait from the Japan Sea (Japan Sea route) (Tsutsui et al., 2001). The radiocesium contamination of Pacific white-sided dolphins appears to provide direct evidence of their seasonal movements via the Pacific Ocean route.

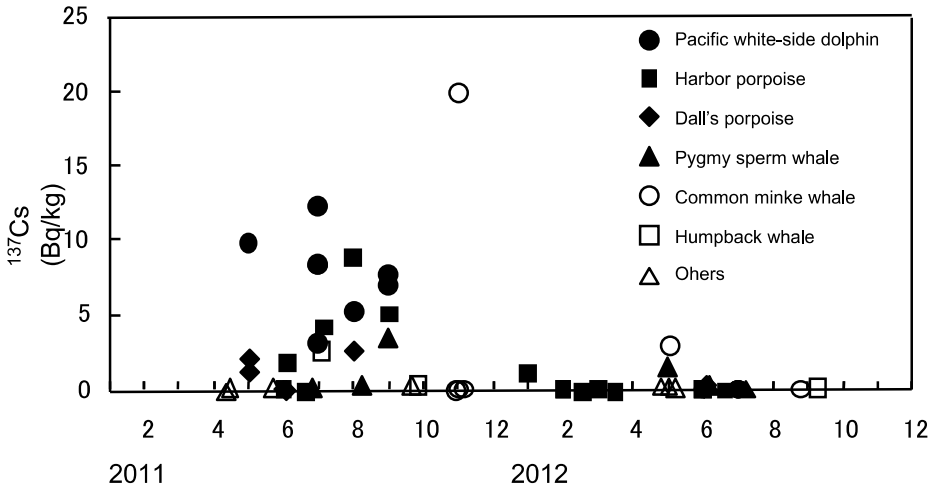

Figure 3. Radiocesium (^{137}Cs) activity in cetaceans stranded along the coast of Hokkaido in 2011 and 2012. This figure was reprinted from Nakamura et al., (2015). Copyright (2015), with permission from Inter-Research.

Radiocesium was detected in 3 out of 5 stranded or caught *dalli*-type Dall's porpoises in 2011 and in an incidentally caught *dalli*-type Dall's porpoise in 2011 (Figure 4B); radiocesium was not detected in this species thereafter. As with the Pacific white-sided dolphin, two travel routes have been postulated for the *dalli*-type Dall's porpoise (Figure 4B): 1) via the Pacific Ocean route, and 2) via the Japan Sea route through the Tsugaru Strait (Hayano et al., 2003; Amano and Hayano, 2007). The ^{137}Cs contamination in *dalli*-type Dall's porpoises suggests that this species seasonally migrates via the Pacific Ocean route.

The ^{137}Cs contamination levels in two stranded common minke whales on the coast of the Pacific Ocean and in the red meat products of this species (supplied from the Japanese Whaling Research Program) are shown in Figure 4C; the caught off area is also indicated. Common minke whales—likely the O-type—may have migrated from the contaminated area of the western North Pacific Ocean (Figure 4C), which is in agreement with the estimated route by Wade et al. (2010).

As described in the previous section, there are three putative Baird's beaked whale populations in the waters around Japan: the Japan Sea population, the Okhotsk Sea population, and the Pacific Ocean population (Kishiro, 2007). A sample of a Baird's beaked whale stranded near Cape Soya in the border between the Okhotsk Sea and the Japan Sea (⑱ in Figure 2) showed no radiocesium contamination. As described later (Figure 7), stable carbon and nitrogen isotope ratios suggest that the beaked whale originated from the Japan Sea population.

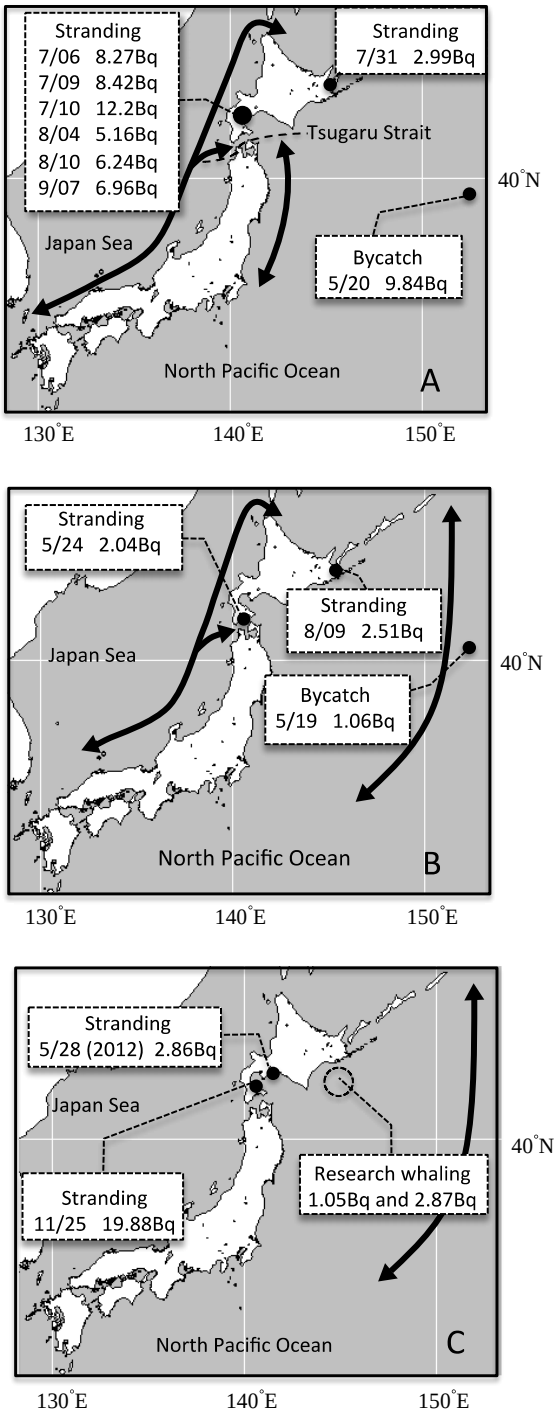

Figure 4. Putative travel routes of the Pacific white-sided dolphin (A), Dall's porpoise (*dalli type*; B) and common minke whale (O –type; C) around Japan, and ^{137}Cs activity in those cetaceans after the Fukushima accident. Solid lines with arrows indicates the putative travel routes of Pacific white-sided dolphin, Dall's porpoise and common minke whale. This figure was reprinted from Nakamura et al., (2015). Copyright (2015), with permission from Inter-Research.

Radiocesium was detected in 4 out of 5 harbor porpoises stranded in 2011 but was undetected in 6 porpoises stranded in 2012. Although the seasonal migratory route of this species is unknown, a possible route may be through the Sanriku and Shimokita Coasts (see Figure 2, Taguchi et al., 2010).

Little is known about the migration of odontocetes inhabiting the waters of Hokkaido, such as the Stejneger's beaked whale, Hubbs' beaked whale, pygmy sperm whale, Cuvier's beaked whale, and killer whale. Among these odontocetes, radiocesium was only detected in pygmy sperm whales stranded in 2011 (n=2), inferring their movement from the radiocesium-contaminated area of the North Pacific Ocean surrounding the FDNPP. In contrast, radiocesium was undetected in a Stejneger's beaked whale and a Hubbs' beaked whale stranded in 2011; this likely rules out their movement across the radiocesium-contaminated area prior to stranding. Furthermore, radiocesium was also undetected in a Cuvier's beaked whale and a killer whale stranded in 2012.

2.3. Contamination of Radiocesium in Pacific Cod Caught Off Hokkaido

The Pacific cod is a bottom-dwelling species and an important commercial food product in Japan. Several individual stocks of Pacific cod are reported to inhabit the waters off the Asian coast; these stocks only have limited migration and inhabit relatively small areas (Bakkala et al., 1984). As mentioned in the previous section, three independent stocks of Pacific cod are thought to inhabit the waters off Hokkaido; the North Pacific Ocean stock, the Okhotsk Sea stock, and the Japan Sea stock (Chimura et al., 2019). Two further stocks of Pacific cod are assumed to inhabit the western North Pacific Ocean off Tohoku, including the area surrounding the FDNPP and the northwestern areas of the Japan Sea off Tohoku (Savin and Kalchugin, 2011).

We measured the radiocesium concentrations in 42 Pacific cod samples purchased from retail outlets in Hokkaido; the cod samples were caught from the North Pacific Ocean (n = 22), the Okhotsk Sea (n = 8), and the Japan Sea (n = 12) between 2011 and 2015 (Figure 5) (Nakamura et al., 2018). The highest ^{137}Cs level measured in our survey was 24 Bq/kg-wet. The highest level reported in another survey was 100 Bq/kg-wet in a Pacific cod caught in location A in the North Pacific Ocean in 2012 (Figure 5).

Radiocesium was predominantly detected in Pacific cod samples caught in the North Pacific Ocean during 2011 and 2015 (A–G in Figure 5) and undetected in samples caught in the Okhotsk Sea (H–J) and Japan Sea (K–M). The regional variability of radiocesium contamination in Pacific cod samples was similar to that of the stranded cetaceans in the three areas. The contaminated Pacific cod caught in the North Pacific Ocean around Hokkaido may have therefore migrated from the FDNPP region. The radiocesium contamination in Pacific cods was measured over 5 years, while contamination in stranded cetaceans was only measured over one year (Figure 3). Demersal fish are suspected to have more persistent radiocesium contamination relative to pelagic fish (Nakamura and Sugisaki, 2015). The difference in contamination term between Pacific cod and cetaceans may be due to differences in inhabiting area, migration route, and physiology.

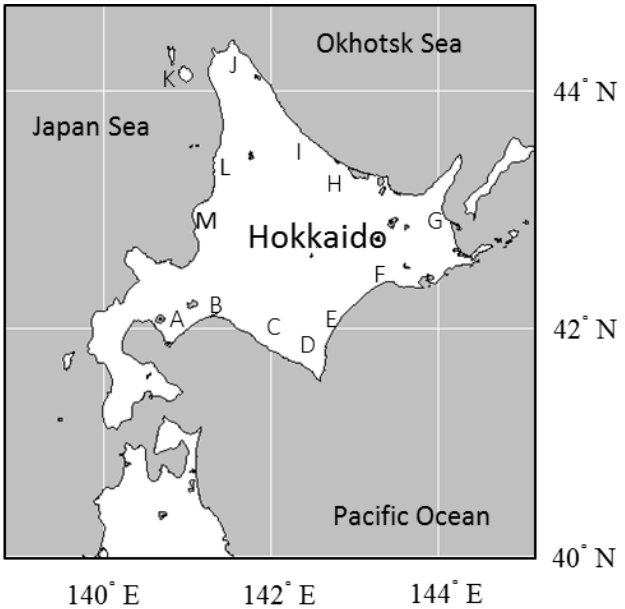

Figure 5. Map showing the sampling locations for Pacific cod (n = 22). This figure was reprinted from Nakamura et al., (2018). Copyright (2018), with permission from Elsevier.

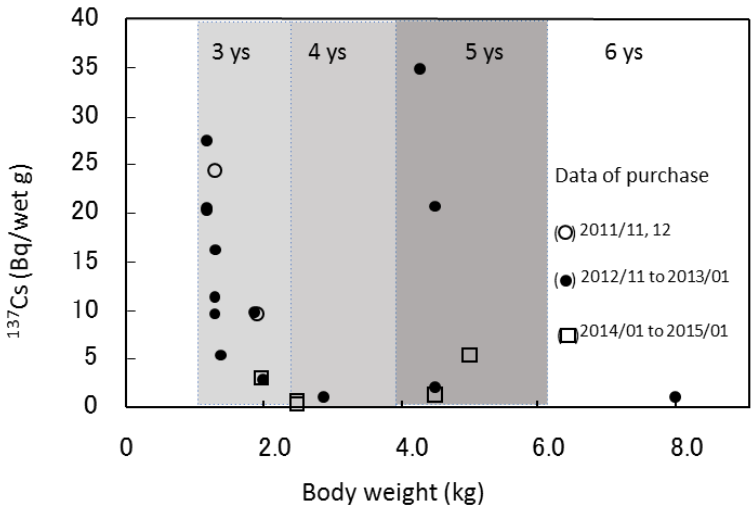

Figure 6. Relation between ^{137}Cs and body weight of Pacific cod purchased in the period between 2011/11 to 2011/12, 2012/11 to 2013/01, and 2014/01 to 2015/01. This figure was reprinted from Nakamura et al., (2018). Copyright (2018), with permission from Elsevier.

We investigated the relationship between radiocesium contamination in Pacific cod and their age (Figure 6) using the Pacific cod growth curve reported by Sakuma (2016). Considerable radiocesium contamination was found in several Pacific cod samples from the North Pacific Ocean around Hokkaido with body weights (BWs) of 1.3–2.0 kg. Two cods, which were estimated to have hatched between 2009 and 2010 (3 yrs) and 2007 and 2008 (5 yrs), respectively, had BWs of 4.3 and 4.5 kg. No Pacific cod hatched after the FDNPP accident (2011) was included in this study. In agreement with our data, Pacific cods caught

near the FDNPP showed highest radiocesium contamination in the 2009 year-class (yc) and the <2009 yc, followed by the 2010 yc (Narimatsu et al., 2017); radiocesium was rarely detected in the 2011 yc. Their results are further evidence that contaminated Pacific cods caught in the North Pacific Ocean around Hokkaido may have migrated from the waters in the vicinity of the FDNPP. Our study suggests the communication between the Pacific cod stocks off Hokkaido and off Tohoku. As of 15 March, 2012, the allowable levels of radiocesium (sum of ^{134}Cs and ^{137}Cs) in foods (excluding milk, etc.) set by the Japanese Government is 100 Bq/kg-wet.

2.4. Stable Carbon and Nitrogen Isotope Ratios in Fish and Cetaceans in the Seawater around Hokkaido

Figure 7 shows the dual isotope plot of Pacific cods and Baird's beaked whales. In agreement with the putative three stocks (Chimura et al., 2019), the stable carbon (δ^{13}C) and nitrogen (δ^{15}N) isotope results discriminated our cod samples between the North Pacific Ocean (n = 22), the Japan Sea (n = 13) and the Okhotsk Sea (n = 8). Similarly, the δ^{13}C and δ^{15}N discrimination of Baird's beaked whale samples between the three areas have also been reported (Endo et al., 2010). The δ^{13}C values in both the Pacific cod and Baird's beaked whale samples occurred in the following order: Pacific Ocean > Okhotsk Sea > Japan Sea. The same δ^{13}C order was also observed in stranded harbor porpoises and common minke whales on the coasts of Hokkaido's three seas (unpublished data). The δ^{13}C value rather than the δ^{15}N value was found to be a stronger indicator of inhabiting area (Kelly, 2000). In the next section, we demonstrate further evidence for the three Pacific cod stocks based on the different patterns of environmental contaminants (section 3.3 and 4.3).

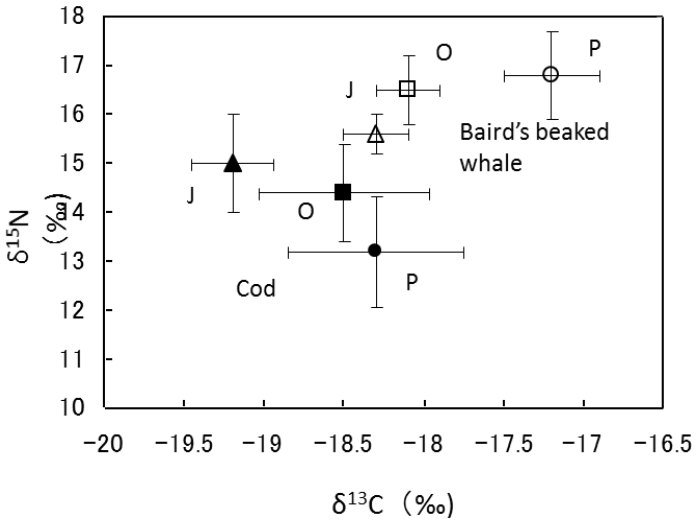

Figure 7. Stable isotope ratios of carbon and nitrogen in Pacific cods and Baird's beaked whales caught off Pacific Ocean (P), Okhotsk Sea (O) and Japan Sea (J). This figure was reprinted from Nakamura et al., (2018). Copyright (2018), with permission from Elsevier.

2.5. Conclusion

The occurrence of a large earthquake in 2011 at the Tohoku district on the largest main island of Japan lead to the Fukushima nuclear plant accident. As a result, the North Pacific Ocean off Tohoku became contaminated with high quantities of ^{137}Cs. ^{137}Cs was detected in cetacean species stranded along the coast of Hokkaido (a northern island of Japan) in 2011, which revealed their previously unknown migration routes. ^{137}Cs was also detected in Pacific cods caught off Hokkaido, inferring their potential migration from the waters off Tohoku. The Pacific cod stocks off Tohoku and Hokkaido are generally believed to be completely independent; however, our results suggest that they are able to migrate and communicate.

3. PERFLUOROALKYL SUBSTANCES IN CETACEANS AND FISH FROM THE SEAS OF NORTHERN JAPAN

3.1. Introduction

Perfluoroalkyl substances (PFASs) that contain both hydrophobic and hydrophilic moieties have been used as surface tension depressants and emulsifiers in industrial applications since the 1950s. They are commonly released into the environment during the fluoropolymer manufacturing process (EFSA, 2008), and perfluoroalkyl sulfonic acids (PFSAs) and perfluoroalkyl carboxylic acids (PFCAs) in particular are widely recognized global environmental contaminants (Gebbink et al., 2016) (see Figure 8). The concentrations of perfluorooctane sulfonic acid (PFOS; C8S) and perfluorooctane carboxylic acid (PFOA; C8A) have slightly declined in marine mammals since the early 2000s following their production phase-out by a major producer. Instead, long-chain PFCAs with 9–14 carbon atoms (C9A–C14A) have been increasingly distributed in the North Pacific. Unlike hydrophobic chemicals, PFASs tend to concentrate primarily in the blood or liver of marine organisms rather than in their fatty tissues (Ahrens et al., 2009). *In vitro* studies have shown that PFASs associate strongly with serum albumin and liver fatty acid-binding proteins (Luebker et al., 2002). PFASs have been shown to accumulate in the livers of marine mammals (Hart et al., 2008a) and in human serum from East Asian and some European countries (Fujii et al., 2017; Glynn et al., 2012; Harada et al., 2011). The seawaters around Hokkaido are one of the most important areas for commercial fishing in Japan, and many cetaceans have also been stranded along its coast. Pacific cod in this area is one of the major dietary fish species consumed by both cetaceans and humans. However, the distribution of PFASs in northern Japan is relatively unknown.

This section first summarizes the profiles and levels of PFASs—including PFOS (C9S), PFOA (C8A), and long-chain PFCAs (C9A to C14A)—in small cetaceans stranded on the coast of Hokkaido (Fujii et al., 2018a and 2018b) and in other coastal regions around the world (Figure 9). Secondly, in order to evaluate the current and historical release, we investigated the contamination levels of PFCAs (C8A to C14A) in Pacific cods from the Pacific Ocean, the Okhotsk Sea, and the Japan Sea around Hokkaido (Fujii et al., 2015 and 2019).

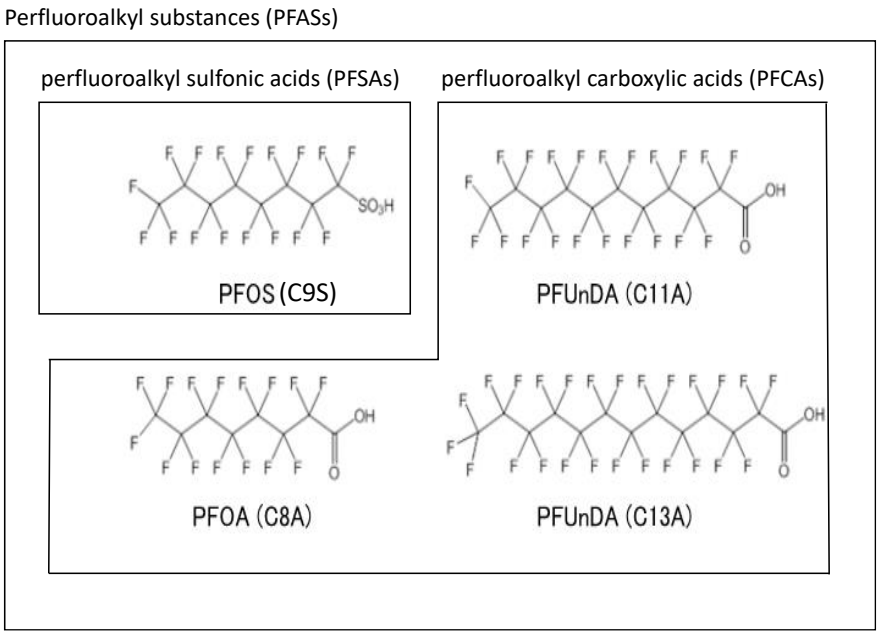

Figure 8. Structures of PFOS (C8S), PFOA (C8A) and long-chain PFCAs (C11A and C13A).

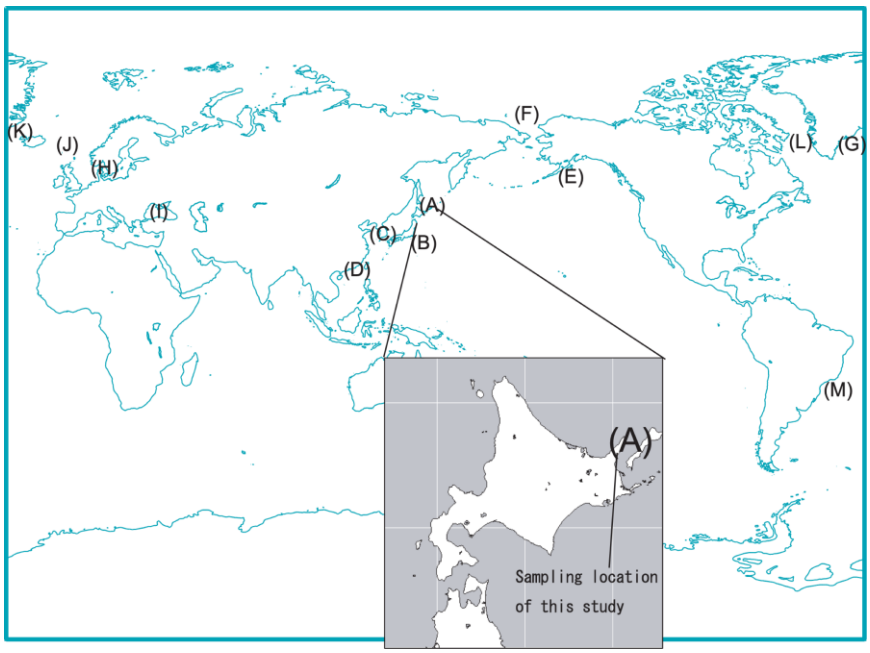

Figure 9. Map of the sampling locations of Dall's and harbor porpoises in Hokkaido (A) and cetaceans from the other regions (B to M), which are listed in Table1. This figure was reprinted from Fujii et al., 2018a, Copyright (2018), with permission from Elsevier, with slight modification.

3.2. PFASs in Cetaceans

3.2.1. Stranded Cetacean Samples for PFASs Analysis

Small cetaceans accidentally stranded in fixed fishing nets in Rausu on the Shiretoko Peninsula between 2012 and 2013 (location A in Figure 9) were collected by the SNH following the standard protocol (Matsuishi, 2011) (section 1). Liver, blood, and blubber samples of Dall's porpoises (n = 10) and harbor porpoises (n = 6) were analyzed for PFOS (C8S), PFOA (C8A), and long-chain PFCAs, including perfluorononanoic acid (PFNA, C9A), perfluorodecanoic acid (PFDA , C10A), perfluoroundecanoic acid (PFUnDA, C11A), perfluorododecanoic acid (PFDoDA, C12A), perfluorotridecanoic acid (PFTrDA, C13A), and perfluorotetradecanoic acid (PFTeDA, C14A). The analytical methods for PFCAs are described by our group provisory in Fujii et al. (2012). The original data is mainly published in Fujii et al. (2018a).

3.2.2. Tissue-Specific Accumulation and Species Differences

PFOS (C8S) and PFOA (C8A)

Among the three tissues analyzed in the Dall's porpoises, the mean concentrations of C8S and C8A were the highest in liver (17 and 7.1 ng/g-wet, respectively), followed by blood, and the lowest in blubber. Harbor porpoises also showed the highest concentrations in liver (6.5 ng/g-wet) and the lowest concentrations in blubber (Fujii et al., 2018a).

Long-Chain PFCAs (C9A-C14A)

The mean concentrations of long-chain PFCAs in the Dall's porpoises were the highest in liver (573 ng/g-wet), followed by blood, and the lowest in blubber (Figure 10). The liver to blood (liver/blood) total PFCAs tissue distribution ratios were > 12, indicating that PFCA accumulation is liver-specific. Although PFOA and PFOS are bind to serum albumin (Jones et al., 2003), long-chain PFCAs are bind to the liver fatty acid-binding proteins (Luebker et al., 2002; Ng and Hungerbühler, 2013).

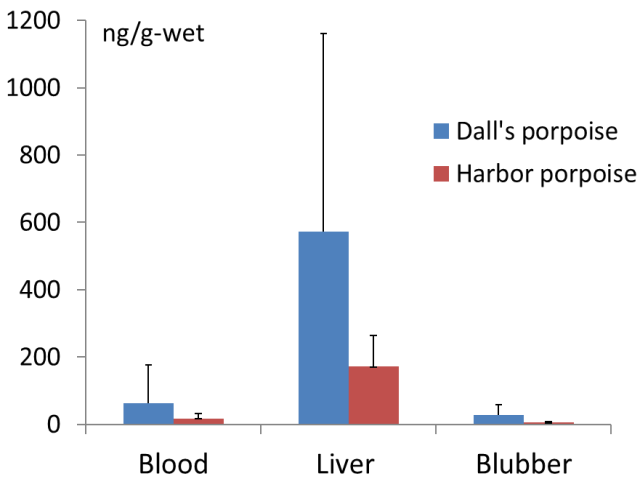

Figure 10. Concentrations of total long-chain PFCAs (C9-C14) in blood, liver and blubber of Dall's and harbor porpoises stranded along the coast of Rause, Hokkaido (Fujii et al., 2018a).

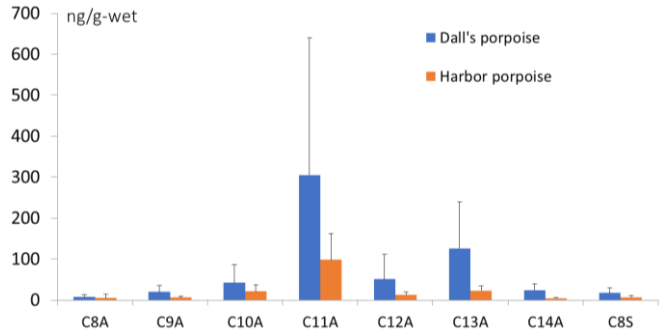

Figure 11. Concentrations of individual Perfluoroalkyl substances in liver of Dall's and harbor porpoises from Hokkaido (Fujii et al., 2018a).

The concentrations of long-chain PFCAs in the livers of harbor porpoises were significantly lower ($p < 0.05$) than in those of Dall's porpoise (Figure 10), despite their similar accumulation patterns. The difference in long-chain PFCA contamination between species is likely due to their different feeding habits as well as the higher biomagnification potency of PFCAs in Dall's porpoises relative to harbor porpoises (Ohizumi et al., 2003).

3.2.3. Congener-Specific Accumulation

Figure 11 shows the profiles of PFOS (C8S), PFOA (C8A), and long-chain PFCAs (C9A to C14A) in the livers of both porpoise species. PFUnDA (C11A) showed the highest concentrations in both species (305 ng/g-wet for Dall's porpoise and 98 ng/g-wet for harbor porpoise), followed by C13A (126 ng/g-wet for Dall's porpoise and 23 ng/g-wet for harbor porpoise). Combined, C11A and C13A contributed 70% of the total PFCAs, whereas the contributions of neighboring even-numbered PFCAs (C10A, C12A, and C14A) were less than 20%. The dominance of odd-numbered PFCAs has been commonly observed in all mammal species in East Asia-Pacific and Alaskan waters (Hart et al., 2008a; Rotander et al., 2012). The high proportion of odd-numbered PFCAs (C11A and C13A) is likely due to increased production during the synthesizing process or their slower elimination from marine biota (Verreault et al., 2005).

3.2.4. Geographical Distribution

Table 1 shows the levels of PFOS, PFOA and long-chain PFCAs in the five species of cetaceans (Dall's porpoise, harbor porpoise, killer whale, Pacific white-sided dolphin, and Baird's beaked whale) stranded on the coast of Hokkaido; this data is also compared with the data of other species in different regions (Location A to M in Figure 9).

PFOS (C8S)

In non-Pacific regions, PFOS concentrations were the highest in harbor porpoises from the Danish North Sea (325 ng/g-wet, Location H, Figure 9) (Galatius et al., 2013), followed by the Black Sea (Location I) (Van De Vijver et al., 2007) (Table 1). In southern Asia, PFOS concentrations of 251 ng/g-wet were measured in Indo-Pacific humpback dolphins (*Sousa chinensis*) from Hong Kong (Location D) (Yeung et al., 2009).

In cetaceans around Hokkaido (Figure 12), the PFOS concentrations ranged from 0.6 to 24 ng/g-wet. Similar PFOS contamination levels were observed in stranded melon-headed

whales (*Peponocephala electra*) from the coast of Chiba on the Boso Peninsula, Japan (Location B) (Hart et al., 2008a); in Beluga (*Delphinapterus leucas*) from Alaskan waters (Locations E, F) (Reiner et al., 2011); and in common minke whales and common dolphins from Korean waters (Location C) (Moon et al., 2010). The lower PFOS contamination in cetaceans around Hokkaido may reflect the reduced or alternative use of PFOS in northern Japan.

Table 1. Mean concentrations of PFOS and PFCAs in livers of cetaceans from different location of world

Location and Species	Area	Sampling Year	N	Mean concentration (ng/g-wet)			
				PFOS (C8S)	PFOA (C8A)	PFUnDA (C11A)	Long-chain PFCA (C9A-C14A)
Japan, Hokkaido[1,2)]	(A)						
Dall's porpoise	NP	2012-2013	10	17	7.1	305	568
	JS	2010-2014	3	14	1.8	163	322
harbor porpoise	NP	2012-2013	6	6.5	5	98	165
	JS	2014	1	24	0.3	410	729
Killer whale	NP	2010-2014	2	10	0.8	225	534
	JS	2014	1	20	5.2	637	1509
Pacific white-sided dolphin	NP	2010-2014	4	15	1.3	164	316
	JS	2014	1	7	1.8	188	408
Baird's beaked whale	OS	2014	1	0.6	nd	18	80
Japan, Chiba [3)]							
melon-headed whale	(B)	2006	2	57	< 5	77	123
Korea [4)]							
common minke whales	(C)	2006	36	69	< 0.1	40	57
long-beaked common dolphin	(C)	2006	31	47	0.7	50	80
Hong Kong [5)]							
Indo-Pacific humpback dolphin	(D)	2003-2007	10	251	1.7	38	58
finless porpoise	(D)	2003-2007	10	151	0.3	21	33
Alaska [6)]							
beluga whale	(E)	1992-2007	27	23	nd	20	50
	(F)	1992-2007	41	9.2	nd	6.7	14
Greenland [7,8)]							
killer whale	(G)	2012-2013	6	115	0.3	67	135
common minke whale	(L)	1998	4	48	0.1	10	17
Denmark, North Sea [9)]							
harbor porpoise	(H)	1999-2002	11	325	< 1.4	7.2	12
white-beaked dolphin	(H)	1999-2002	7	290	1.1	11	25
Ukraine, Black Sea [10)]							
harbor porpoise	(I)	1997-1998	31	210	na	7.3	23
North Atlantic [8)]							
Atlantic white-sided dolphin	(J)	2006	3	107	0.2	52	93
harbor porpoise	(K)	1997	5	39	0.1	20	33
Brazil [11)]							
Tucuzi	(M)			91	1.1		2

(A) to (M) location in Figure 9. nd = not detected, na = not analyzed

1) Fujii et al., 2018a; 2) Fujii et al., 2018b; 3) Hart et al., 2008a; 4) Moon et al., 2010; 5) Yeung et al., 2009; 6) Reiner et al., 2011; 7) Gebbink et al., 2016; 8) Galatius et al., 2013; 9) Van De Vijver et al., 2007; 10) Rotander et al., 2012; 11) Quinete et al., 2009

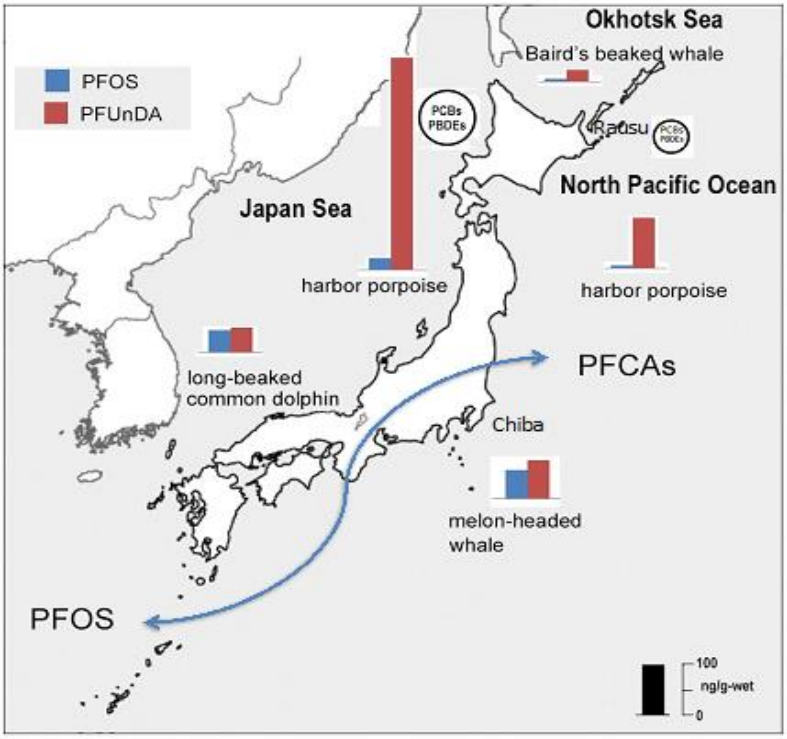

Figure 12. Geographical distribution of PFOS and PFUnDA in cetaceans around Japan. Data from harbor porpoises (Fujii et al 2018a), melon headed whales (Hart et al., 2008a), and long-beaked common dolphins in the east coast of Korea (Moon et al., 2010) are included. POP levels around Hokkido are illustrated in this figure (Fujii et al., 2018b).

PFOA (C8A)

PFOA (C8A) is one of the most abundant PFCAs detected in seawater samples (Yamashita et al., 2008). Interestingly, PFOA was undetectable in 11% of all cetaceans around Hokkaido, indicating its low bioaccumulation potential (Conder et al., 2008).

Long-Chain PFCAs (C9A to C14A)

Of the long-chain PFCAs (C9A-C14A), PFUnDA (C11A) is one of the most dominant congeners measured in all investigated cetaceans globally (Fujii et al., 2018a). We compared their levels between northern Japan and other regions around the world (Table 1). Among the five cetaceans stranded along the coast of Hokkaido, the concentrations of long-chain PFCAs were highest in killer whales (1509 ng/g-wet), followed by harbor porpoises (729 ng/g-wet) from the Japan Sea. Their levels in the same species were three to four times lower in the Pacific Ocean. In previous surveys, lower levels of PFCAs were observed in melon-headed whales (123 ng/g-wet) from Japanese coastal waters (Hart et al., 2008a) and in long-beaked common dolphin (52 ng/g-wet) from Korean waters (Figure 12) (Moon et al., 2010). In non-Pacific regions, PFCA levels were lowest in harbor porpoises (12 ng/g-wet) from the Danish North Sea (Galatius et al., 2013) (Location H in Figure 9). Based on the findings of these reports and our own results, cetaceans from the Japan Sea appear to be the most contaminated with long-chain PFCAs (especially C11A) relative to cetaceans from other sea regions around

the world. In contrast, cetaceans around Hokkaido are less contaminated with PFOS and PFOA compared with Southeast Asian and North Atlantic cetaceans. The different contamination status of PFASs in cetaceans may be attributed to different emission sources of PFOS/PFOA, which are likely derived from fluoropolymer industries (Niisoe et al., 2015).

The concentrations of long-chain PFCAs (C11A) were higher in cetaceans from the Japan Sea compared with those from the North Pacific Ocean (Figure 12, Table 1) ; this suggests that effluents from East Asia to the Japan Sea (a closed ecosystem area) result to high contamination of long-chain PFCAs.

3.3. PFCAs in Pacific Cods

3.3.1. Profiles and Levels of PFCAs in Hokkaido

Pacific cod are widely distributed around the coast of the North Pacific Ocean from California's Santa Monica Bay to Japan and Korea. They commonly inhabit the coastal to continental shelf region (Mishima, 1984). Pacific cods are suitable for monitoring the localized contamination status of seawater due to their limited migration range (Westrheim, 1984).

Pacific cods inhabiting the coastal areas of Hokkaido were divided into three stocks (section 2) to evaluate the geographical trend of PFCAs. The profiles and concentrations of PFCAs were investigated in the muscles of Pacific cods collected from the Japan Sea (n = 5), the Okhotsk Sea (n = 5), and the North Pacific Ocean (n = 12) during October 2012 and January 2013 (Fujii et al., 2015) (Figure 13). The mean concentrations of PFCAs (sum of C8A to C14A) in Pacific cods from the Japan Sea and the North Pacific Ocean were 2144 and 2066 pg/g-wet, respectively, which were both approximately two times higher than the measured concentrations from the Okhotsk Sea (Fujii et al., 2015). Lower PFCAs contamination in Pacific cods from the Okhotsk Sea may be attributed to the lower human use of PFCAs in the surrounding sparsely populated areas

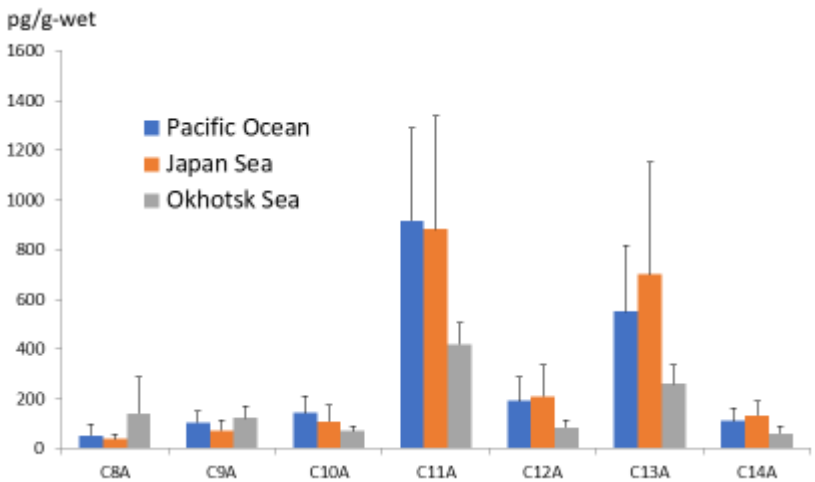

Figure 13. Concentrations of individual PFCAs in muscle of Pacific cod from the North Pacific Ocean (n = 12), the Japan Sea (n = 5) and the Okhotsk Sea (n = 5) around Hokkaido (Fujii et al., 2015).

The long-chain PFCAs profiles in the muscles of Pacific cods were dominated by C11A and C13A, contributing 43% and 27% of the total PFCAs, respectively. Similar compositional profiles in Pacific cods have also been observed in skipjack tuna (*Katsuwonus pelamis*) from the offshore waters of Japan between 1997 and 1999 (Hart et al., 2008b). The profiles in cods were similar to those in cetaceans, suggesting that prey fishes, such as skipjack tuna and Pacific cod, are dietary sources of long-chain PFCAs detected in small cetaceans around Hokkaido.

3.3.2. Geographical Distribution

To compare the PFCA contamination status between the seawater around Hokkaido and other coastal areas, we investigated the profiles and levels of PFCAs (C8A–C14A) in the muscles of Pacific cods collected from 14 coastal sites in the North Pacific Ocean (Figure 14) (Fujii et al., 2019). The mean concentrations of PFCAs in muscles of Pacic cods ranged from 216 to 770 pg/g-wet in the north and east Pacific Ocean (Seattle, Vancouver, Alaska and Russia), from 819 to 1710 pg/g-wet in Japanese coastal waters (three sites of Hokkaido, Aomori, Iwate, Miyagi, and Tottori/Shimane), and from 288 to 892 pg/g-wet in Korean waters (Sokcho, Busan and Yeosu) (Fujii et al. 2019). The long-chain PFCAs (C11A and C13A) contributed 96% of the total PFCA concentration across Japan and only 33% in the USA and Canada. The concentrations of C11A and C13A in cod samples were approximately three to four times higher in Japanese and Korean Pacific cods realtive to those from the USA, Canada, and Russia. Edible clams from five sites in Japan and one site in Canada also showed similar geographical trends in PFCAs (C6A–C15A) contamination, with highest mean concentrations in Japan (mean 1170 pg/g-wet) relative to Canada (48 pg/g-wet) (Fujii et al. 2020). Higher concentrations of PFCAs in fish or shellfish in Japanese coastal waters may be due to the higher industrial discharge near their habitats.

Figure 14. Geographical distribution of PFCAs in Pacific cods from the coast of different North Pacific areas. This figure was reprinted from Fujii et al., 2019, Copyright (2019), with permission from Elsevier.

3.3.3. PFCAs in the Food Chain

Figure 15 shows the percentage composition of PFCAs in tissues of cetacean, fish, human blood, and diet around Hokkaido. Among the three tissues (blubber, liver, and blood) in Dall's porpoises, the sum of C13A and C14A contributed 60% of the total PFCAs in blubber, whereas C11A was the major congener in liver and blood. This suggests that longer carbon-chained PFCAs are more lipophilic and tend to accumulate in fatty tissues. The PFCAs profiles in Pacific cod and cetaceans were similar, indicating that fishes such as cods are the main dietary source of long-chain PFCAs in cetaceans. In contrast, lower-chain PFOS (C8S) and PFOA (C8A) contributed 30% of the total PFCAs in human diet in Hokkaido and 60% in human serum. In our recent report, edible clams in Japanese coastal waters contained abundant PFOA (C8A), contributing 65% of the total PFCAs (Fujii et al., 2020); this was similar to the PFCA concentration ratios reported in the Yodo River basin in Osaka prefecture, Japan (Niisoe et al., 2015). These findings suggest that PFOS and PFOA may come from different emission sources from long chain PFCAs or they may have different bioaccumulation mechanisms (Fujii et al., 2020).

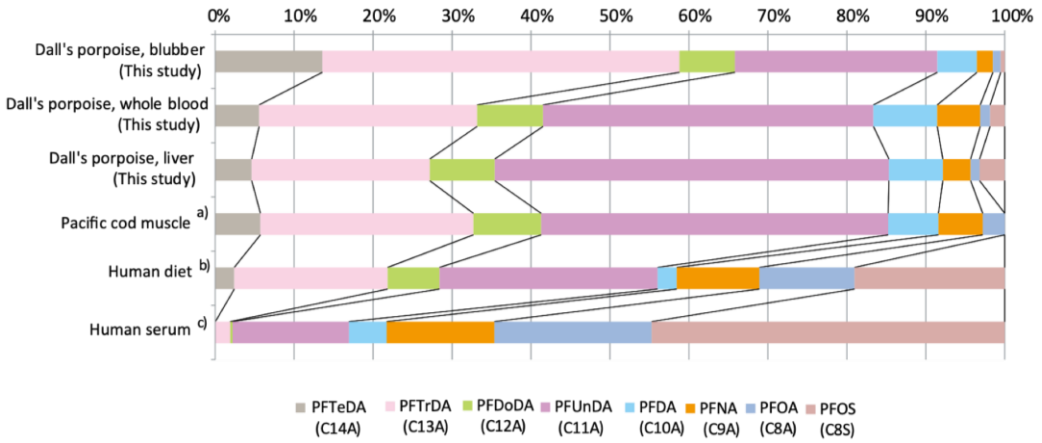

Figure 15. Percentage composition of Perfluoroalkyl substances in tissues from Dall's porpoise (This study; Fujii et al., 2018a), [a)] Pacific cod muscle (Fujii et al., 2015), [b)] the human diet (Karmanet et al., 2009; Fujii et al., 2017), and [c)] human serum (Kato et al., 2016; Fujii et al., 2017).[a)] PFOS is not measured (Fujii et al., 2015). This figure was reprinted from Fujii et al., 2018a, Copyright (2018), with permission from Elsevier, with slight modification.

3.4. Conclusion

We identified lower PFOS and PFOA concentrations (< 24 ng/g-wet) in cetaceans from Japanese coastal waters, accounting for only 5% of the total PFASs. In contrast, long-chain PFCAs (C9A to C14A) showed higher concentration with a dominance of PFUnDA (C11A), followed by PFTrDA (C13A). Further investigation into their dominant sources and exposure routes are necessary for predicting the future change of PFAS contamination.

4. ANTHROPOGENIC ORGANOHALOGENS IN CETACEANS AND FISH FROM NORTHERN JAPAN

4.1. Introduction

Persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), and organochlorine pesticides (OCPs), such as DDTs, have been used in industrial and agricultural applications for more than 50 years. Polybrominated diphenyl ethers (PBDEs) have also been used as flame retardants in plastics, textiles, and electronic appliances. POPs accumulate in marine mammals or humans through their dietary seafood intake (Kajiwara et al., 2002; Nomiyama et al., 2011). This section describes the recent contamination status of selected OCs (such as PCB153, a representative PCB isomer and BDE47, frequently detected PBDE isomer, see Figure 16) in cetaceans stranded on the coast of Hokkaido as well as in Pacific cods from the surrounding three seas. We also compared our findings with POPs contamination in other regions around the world (Fujii et al., 2018a and 2018b).

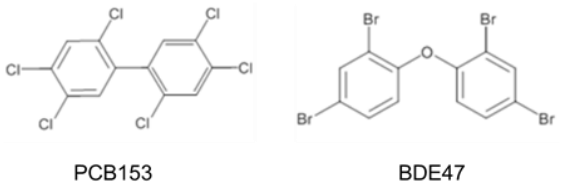

Figure 16. Structures of PCB153 and BDE47.

4.2. POPs Contamination in Cetaceans

POPs in the tissues of cetaceans stranded on the coast of Hokkaido during the period 2010–2014 were analyzed to determine the contamination status of legacy POPs (Fujii et al., 2018b). Table 2 shows the concentrations of POPs in therteen cetacean species from the North Pacific Ocean, six cetaceans species from the Japan Sea and two cetacean species from the Okhotsk Sea around Hokkaido; the POPs include a representative PCB congener (PCB153), a major congener of chlordanes (trans-nonaclor (trans-NC)), β-hexa-chlorocyclohexane (β-HCH), and a representative PBDE congener (BDE47). All analytes showed higher accumulation in cetaceans from the Japan Sea coast compared with those from the Pacific Ocean or the Okhotsk Sea. All POPs also showed highest accumulated concentrations in killer whales, followed by Pacific white-sided dolphins. Lowest concentrations of all POPs were observed in common minke whales. POPs concentrations were in the order of PCB153 > trans-NC > β-HCH > BDE47. As with the toothed whales, high concentrations of PCB153 and BDE-47 were observed in the livers of killer whales and Pacific white-sided dolphins.

Despite the low sample size in this study, POPs contamination was higher in toothed whales relative to baleen whales due to differences in their dietary habits. The concentrations of PCBs and PBDEs were also higher in cetaceans from the Japan Sea compared with those from the North Pacific Ocean (Table 2). This indicates an ongoing source of PCBs and DDTs

in cetaceans of the Japan Sea (closed system). Previous studies have shown that killer whales stranded in Hokkaido were highly contaminated with PCB153 at 17 μg/g-lipid (Haraguchi et al., 2009a)—the highest level among the toothed whales. PCBs levels in Dall's porpoises were also found to be higher than those of other cetacean species (Kajiwara et al., 2002). The PBDEs levels in the blubber of melon-headed whales stranded on Japanese coastlines between 1982 and 2006 (Kajiwara et al., 2008) and in killer whales stranded in Hokkaido in 2005 (Kajiwara et al., 2006; Haraguchi et al., 2009a) ranged from 0.2 to 0.5 μg/g-lipid; this is similar to the PBDEs values observed in ceaceans around Hokkaido in the present survey from 2010 to 2014. Combined, these findings indicate no change in the contamination levels of PCBs and PBDEs in cetaceans throughout the last decade; therefore, POPs are continuing to be discharged from the coastal countries of the Asia-Pacific region.

Table 2. Mean concentrations of POPs in livers of cetaceans stranded in Hokkaido

Species	Sex[a] (N)	Mean concentration (ng/g-wet)			
		PCB153	trans-NC	β-HCH	BDE47
North Pacific Ocean					
Toothed whale					
killer whale	F = 2	4,242	1,817	423	13
Dall's porpoise	M = 1	371	114	107	7.8
	F = 1	987	260	156	3.6
harbor porpoise	F = 4	252	65	24	2.7
Pacific white-sided dolphin	M = 1	1,710	410	94	18
	F = 3	2,071	560	129	27
Baird's beaked whale	M = 2	613	139	30	0.4
dwarf sperm whale	F = 2	735	135	181	0.9
Cuvier's Beaked Whale	M = 1	303	100	19	0.9
	U = 1	656	233	31	0.4
Hubbs' beaked whale	M = 1	1,091	271	60	0.4
Indo-Pacific beaked whale	F = 1	274	64	15	4.6
short-finned pilot whale	F = 1	210	79	20	5.6
sperm whale	U = 1	128	43	4.6	1.2
Baleen whale					
common minke whale	M = 1	232	141	81	0.4
humpback whale	M = 2	101	106	192	0.4
Japan Sea					
Toothed whale					
killer whale	M = 1	13,289	4,701	1,889	3.6
Dall's porpoise	M = 1	1,475	711	86	18
	F = 2	858	201	116	11
harbor porpoise	F = 1	1,866	489	185	12
Pacific white-sided dolphin	M = 1	9,159	1,762	219	1.5
Stejneger's beaked whale	F = 2	763	158	132	3.8
Baleen whale					
common minke whale	M = 1	623	184	177	7.6
Okhotsk Sea					
Toothed whale					
Baird's beaked whale	M = 1	2,667	1,273	238	0.4
Baleen whale					
common minke whale	F = 1	109	71	26	4.1

[a] M = male, F = female, U = unknown

4.3. POPs Contamination in Pacific Cod

Pacific cod can be used as a suitable bioindicator for the global monitoring of environmental pollutants in Pacific Ocean ecosystems. In this study, Pacific cods (n = 22) collected from the three seawaters around Hokkaido were analyzed for legacy OCPs and PCBs (Fujii et al., 2015). Figure 17 shows the concentrations of dichlorodiphenyl dichloroethene (4,4'-DDE; a major metabolite of DDT), hexachlorobenzene (HCB), a representative PCB congener (PCB153), trans-nonaclor (trans-NC), and β-hexachlorocyclohexane (β-HCH) in the muscles of Pacific cods from the North Pacific Ocean, Japan Sea, and Okhotsk Sea around Hokkaido. The concentrations of 4,4'-DDE and HCB were an order of magnitude higher than those of PCB153, trans-NC, and β-HCH. The concentrations of 4,4'-DDE and PCB153 were slightly higher in Pacific cods from the Japan Sea (500 and 100 pg/g-wet, respectively) compared with those from the other two seas. This observation is consistent with the findings of other reports from Asian waters. For example, measurements of OCPs in skipjack tuna from the Japan Sea showed higher concentrations of DDTs and HCHs (Ueno et al., 2003b). Another report also showed higher levels of OCP in Japanese common squid (*Todarodes pacificus*) in the Japan Sea relative to those in the North Pacific Ocean (Ueno et al., 2003a). These findings infer the continuous discharge of PCBs and DDTs (as 4,4'-DDE) into the Japan Sea from the surrounding coastal countries.

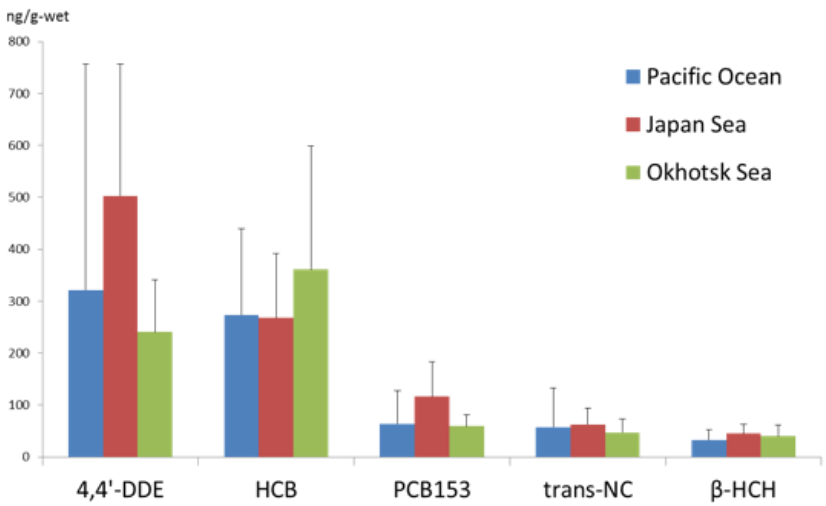

Figure 17. Concentration of selected OCs in muscle of Pacific cod collected from the North Pacific Ocean, the Japan Sea and the Okhotsk Sea around Hokkaido (Fujii et al., 2015).

4.4. Conclusion

The contamination levels of legacy POPs, such as PCBs, DDTs, and PBDEs, in toothed whales around Hokkaido were comparable to those in whale products from Japanese coastal waters. However, pesticide (e.g., trans-NC) levels were higher in toothed whales around Hokkaido relative to cetacean species from other areas. Due to their lower trophic level in the marine food web, OCPs and PCBs concentrations in fish were an order of magnitude lower

than those in cetaceans, such as harbor porpoise, around Hokkaid. Our findings demonstrate that the contamination status of legacy POPs in the seawater around Hokkaido is still impacting the environmental health of both marine animals and humans.

5. CONTAMINATION OF NATURALLY PRODUCED ORGANOHALOGENS IN NORTHERN JAPAN

5.1. Introduction

In recent years, screening for POPs in the marine environment has resulted in the identification of naturally produced organohalogen contaminants (Vetter and Stoll, 2002; Teuten et al., 2005). Some NHCs are halogenated secondary metabolites produced by marine biota, such as marine sponges, algae, and symbiotic bacteria (Gribble, 2004; Cavalleri et al., 1978). At present, the major NHCs detected in whales and dolphins in Japanese coastal waters include two halogenated bipyrroles (3,3',4,4'-tetrabromo-5,5'-dichloro-1,1'-dimethyl-2,2'-bipyrrole (Br_4Cl_2-DBP), heptachloro-1-methyl-1',2-bipyrrole (Cl_7-MBP)) and two methoxylated tetrabrominated diphenyl ethers (6-methoxy-2,2',4,4'-tetraBDE (6-MeO-BDE47) and 2'-methoxdy-2,3',4,5'-tetraBDE (2'-MeO-BDE68)) (Figure 18). These products are known to accumulate in marine mammals and humans, and their concentrations were found to be similar to those of legacy POPs, such as PCBs and PBDEs (Haraguchi et al., 2006; Marsh et al., 2005). This section describes the profile, levels, regional variation, and sources of NHCs consisting of bipyrroles and MeO-BDEs in cetaceans stranded on the coast of Hokkaido during 2010–2014. Additionally, the contamination status of these products were compared with those of anthropogenic PFASs and legacy POPs in cetaceans from the Japan Sea and the North Pacific Ocean.

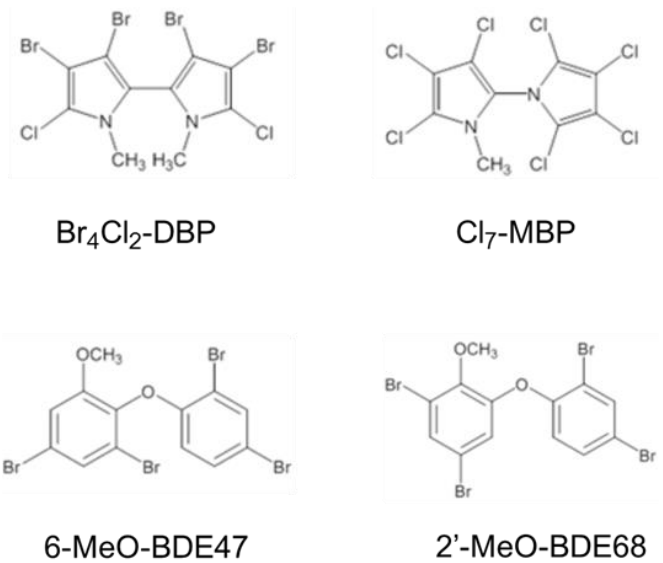

Figure 18. Structures of NHCs investigated (two bipyrroles and MeO-BDEs).

5.2. Profiles and Levels of NHCs in Hokkaido

Of the cetaceans stranded on the coast of Hokkaido during 2010–2014, two baleen whales and twelve toothed whales were analyzed for NHCs. The analyzed species included odontocetes (Dall's porpoises, harbor porpoises, killer whales, and Pacific white-sided dolphins) and mysticetes (common minke whales) from the Japan Sea (n = 7) and the North Pacific Ocean (n = 14). Liver samples of five cetaceans were analyzed for bipyrroles (Br_4Cl_2-DBP, Cl_7-MBP) and MeO-BDEs (6-MeO-BDE47 and 2'-MeO-BDE68) (Fujii et al., 2018a and 2018b). Table 3 shows the mean concentrations of the major congeners in the livers of cetaceans from both the Japan Sea and North Pacific Ocean around Hokkaido.

Table 3. Concentration of NHCs in livers of cetaceans from Hokkaido and the other areas

Location and Species	Area	N	Mean concentration (ng/g-lipid)		
			Br_4Cl_2-DBP	Cl_7-MBP	6-MeO-BDE47
Liver of cetaceans					
Hokkaido[1)]					
killer whale	NP	2	70,100	10,400	2690
	JS	1	19,500	56,600	1470
Pacific white-sided dolphin	NP	4	6,450	17,700	1310
	JS	1	8,630	55,100	1220
Dall's porpoise	NP	3	33,670	6,780	616
	JS	3	17,200	14,500	581
harbor porpoise	NP	4	1,530	889	117
	JS	1	2,110	6,150	361
common minke whales	NP	1	4,290	297	89
	JS	1	638	1,310	268
Atlantic					
harbor porpoise [2)]	NS	2	-	-	43
franciscana dolphin [3)]	SA	11	-	-	2,874
bottlenose dolphin [4)]	SA	3	-	-	19,900
Blubber of cetaceans					
East Asia					
killer whale [5)]	NP	1	5,960	520	107
Dall's porpoise [6)]	NP	5	2,540	-	-
Dall's porpoise [7)]	NP	16	2,860	-	-
bottlenose dolphin [7)]	NP	33	11,800	-	-
bottlenose dolphin [8)]	NP	1	-	-	710
Australia					
bottlenose dolphin [9)]	SP	4	-	450-9,100	-
bottlenose dolphin [10)]	SP	1	-	-	1,910

NP = North Pacific, JS = Japan Sea, NS = North Sea, SA = South Atlantic, SP = South Pacific.
1) Fujii et al., 2018b; 2) Weijs et al., 2010; 3) Alonso et al., 2014; 4) Dorneles et al., 2010; 5) Haraguchi et al., 2009a; 6) Tittlemier et al., 2002; 7) Haraguchi et al., 2006; 8) Marsh et al., 2005; 9) Vetter et al., 2001; 10) Melcher et al., 2005.

Br_4Cl_2-DBP

The major congener of the three detected DBPs was Br_4Cl_2-DBP (Figure 18), which contributed 80–92% of the total DBPs. The Br_4Cl_2-DBP contamination levels of the five cetaceans around Hokkaido and of species from other regions are shown in Table 3. Br_4Cl_2-

DBP concentrations ranged from 0.64 μg/g-lipid in common minke whales from the Japan Sea to 70 μg/g-lipid in killer whales from the North Pacific.

The Br_4Cl_2-DBP values in cetaceans around Hokkaido were higher than those from any other region and similar to those of Dall's porpoises (2.54 μg/g-lipid) from the Asia-Pacific region (Tittlemier et al., 2002). In ondontocetes of Japanese coastal waters, Br_4Cl_2-DBP levels were the highest in the blubber of killer whales (6.4–26 μg/g-lipid) from Hokkaido (Haraguchi et al., 2009a), followed by the blubber of bottlenose dolphins (12 μg/g-lipid) from southern Japanese coastal waters (Haraguchi et al., 2006).

Br_4Cl_2-DBP is widespread in a variety of commercially available fish and seafood in Canada (Tittlemier, 2004) and Japan (Haraguchi et al., 2009a; Haraguchi et al., 2009b). High accumulation of Br_4Cl_2-DBP in toothed whales from Hokkaido is likely due to the consumption of DBP-contaminated prey and/or the lowered ability of this species to metabolize Br_4Cl_2-DBP.

The source of Br_4Cl_2-DBP is still unclear; however, bipyrroles may be produced by a marine bacterium (e.g., *Pseudoalteromonas*) that is known to produce a structurally similar compound called hexabromo-bipyrrole (Andersen et al., 1974). Due to its detection in a variety of fish and seafood (Tittlemier, 2004), it is likely that Br_4Cl_2-DBP biomagnifies in cetaceans through the food chain. High exposures to Br_4Cl_2-DBP may enhance the toxicological risks of hepatic cytochrom P450 (CYP1A) induction in marine mammals (Tittlemier et al., 2003).

Cl_7-MBP

Cl_7-MBP—another type of halogenated bipyrrole—was detected in toothed whales from the North Pacific Ocean and Japan Sea at ranges of 36–1031 and 170–4000 ng/g-wet, respectively (Table 3). The highest concentration was found in killer whales and Pacific white-sided dolphins from the Japan Sea. The concentrations were comparable to those in the blubber of killer whales stranded in the same region in 2005 (Haraguchi et al., 2009a); this suggests a widespread contamination of Cl_7-MBP that has remained unchanged over the past decades.

Currently, only a few studies exist on the global distribution of Cl_7-MBP; however, Cl_7-MBP levels in the blubber of bottlenose dolphins from Australia (Vetter et al., 2001) and in marine mammals from Africa and the Antarctic (Vetter, 2006) were found to range from 0.45 to 9.1 μg/g-lipid. Cl_7-MBP levels in cetaceans around Hokkaido were much higher than those in cetaceans from other regions around the world. Marine bacteria are assumed to be the dominant biogenic sources of Cl_7-MBP, as a structurally similar chlorinated phenyl pyrrole was isolated from *Actinoplanes* sp. (Cavalleri et al., 1978) from around Hokkaido. Since killer whales feed on other mammals that accumulate these contaminants via the food chain, cetaceans or fish prey are likely to be the dominant source of Cl_7-MBP in killer whales around Hokkaido.

MeO-BDEs

The majority of NHC studies in marine biota have focused on two MeO-BDE congeners—6-MeO-BDE47 and 2'-MeO-BDE68—due to their environmental persistence and structural similarity to BDE47 (Figure 18). We measured the concentrations of the two congeners in the livers of five cetacean species from the Japan Sea and North Pacific Ocean around Hokkaido. The concentration of 6-MeO-BDE47 was 2–10-fold higher than that of 2'-

MeO-BDE68 in all cetaceans, except for the common minke whale and Indo-Pacific beaked whale. The concentration of 6-MeO-BDE47 ranged from 89 ng/g-lipid in common minke whales to 2690 ng/g-lipid (Table 3) in killer whales in the Pacific Ocean. The levels of 6-MeO-BDE was higher in toothed whales than in baleen whales and higher in cetaceans from the Japan Sea than those from the Pacific Ocean. Differences in the accumulation of 6-MeO-BDE in different cetacean species may be due to differences in their feeding ecology, migration, and lifestyle.

MeO-BDE levels in cetaceans from Hokkaido were higher than those in harbor porpoises from the Danish North Sea (Weijs et al., 2010). However, more recent surveys have shown that the levels of MeO-BDEs in the livers of bottlenose dolphins in the southwest Atlantic off Brazil are an order of magnitude higher than those from Japan (Alonso et al., 2014, Dorneles et al., 2010; Table 3). Symbiotic cyanobacteria (Malmvärn et al., 2008), marine sponges (Unson et al., 1994; Haraguchi et al., 2011), and marine algae—such as *Sargassum* sp. (Haraguchi et al., 2010) and *Ceramium tenuicorne* (Malmvärn et al., 2008)—are all possible sources of MeO-BDEs. Although the biological effects of MeO-BDEs are still unknown, their O-demethylated products have been reported as endocrine disruptors (Meerts et al., 2001).

5.3. Comparisons between Natural and Anthropogenic Compounds

PFCAs and NHCs

The percentage compositions of selected congeners in the five species are shown in Figure 19. The relative contribution of Br_4Cl_2-DBP was higher in killer whales and common minke whales from the North Pacific Ocean and the Japan Sea, whereas the contribution of PFUnDA was higher in common minke whales from both seas (section 3). These findings indicate that the contamination of NHCs (Br_4Cl_2-DBP, Cl_7-MBP) and PFCAs (PFUnDA) are regionally different and species-specific. We observed no regional differences in the distribution ratios of the two MeO-BDEs, inferring a wide distribution of MeO-BDEs in both seas.

PCBs and NHCs

We investigated the regional differences in NHC and PCBs contamination in the five cetaceans between the North Pacific Ocean and Japan Sea (Figure 20). Br_4Cl_2-DBP was distributed at relatively higher concentrations in cetaceans from the North Pacific, whereas Cl_7-MBP and PCBs were abundant in cetaceans from the Japan Sea. No regional differences were observed in the levels of MeO-BDE. Our findings suggest that the North Pacific Ocean (open sea) is a high souce of Br_4Cl_2-MBP in cetaceans, whereas the Japan Sea is a high source of Cl_7-MBP and PCBs. PCBs were highly corerlated with Cl_7-MBPs ($r = 0.892$, $p < 0.01$) (Fujii et al., 2018b), emphasizing their similar bioaccumulation mechanisms despite their different emission sources (industrial and biogenic).

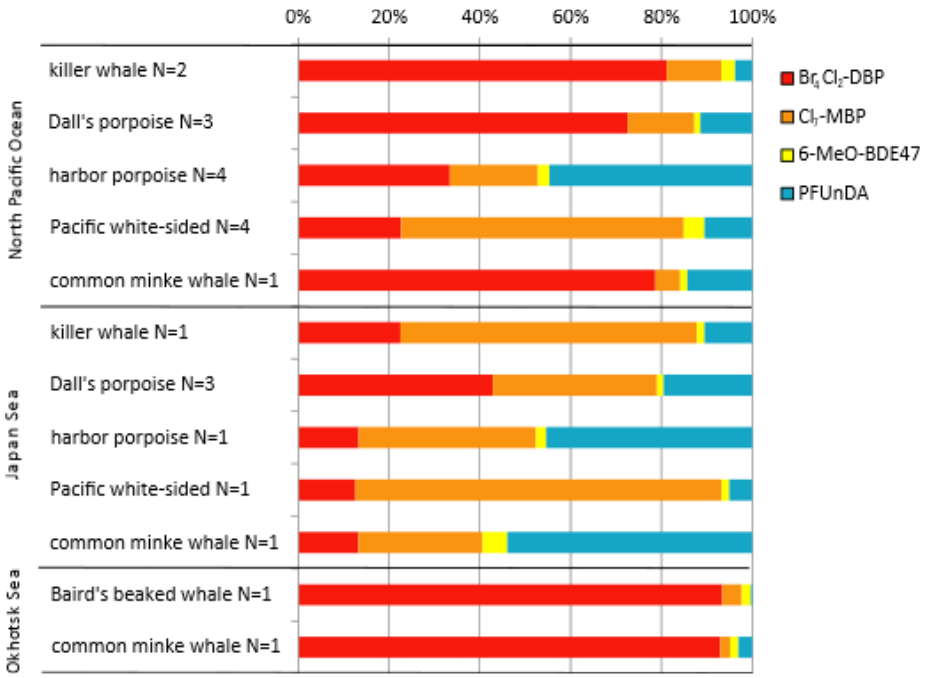

Figure 19. Composition of Br_4Cl_2-DBP, Cl_7-MBP, MeO-BDE47 and PFUnDA in the liver of five cetaceans from the Pacific Ocean, the Japan Sea and the Okhotsk Sea around Hokkaido. This figure was reprinted from Fujii et al., 2018b, Copyright (2018), with permission from Elsevier.

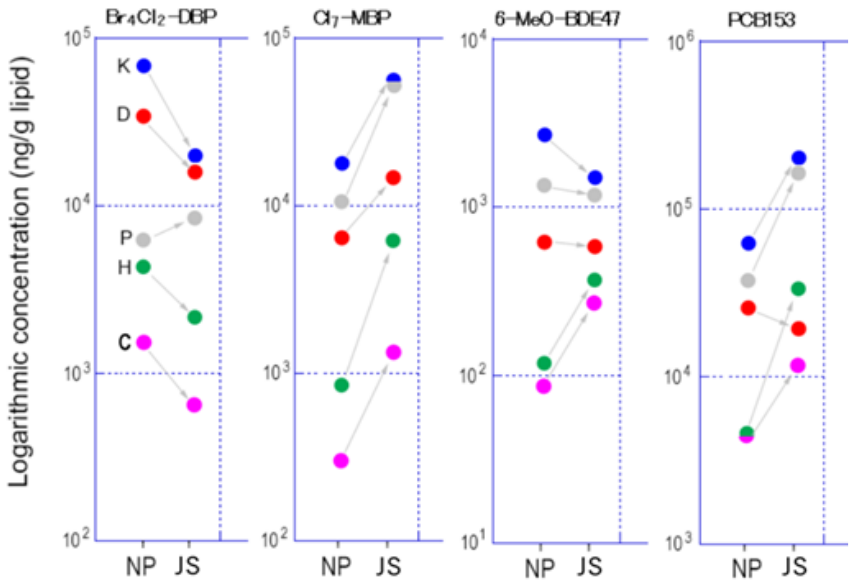

Figure 20. Regional difference in concentrations of NHCs (Br_4Cl_2-DBP, Cl_7-MBP and 6-MeO-BDE47) and PCB153 in liver of five cetaeans (K = killer whale, D = Dall's porpoise, P = Pacific white-sided dolphin, H = harbor porpoise, C = common minke whale between the North Pacific (NP) and the Japan Sea (JS) stranded in Hokkaido.

5.4. Conclusion

Cetaceans around Hokkaido were found to be contaminated with comparable levels of NHCs and long-chain PFCAs based on several cetacean surveys. The contamination of legacy POPs (PCB153 or BDE47) in cetaceans occurred in the order of killer whale > Pacific white-sided dolphin > common minke whale. We observed different NHCs distribution ratios in the livers of cetaceans between the Japan Sea and the North Pacific Ocean. The source of Br_4C_{l2}-DBP is likely localized in the North Pacific Ocean, whereas the sources of Cl_7-MBP are abundant in the Japan Sea. 6-MeO-BDE47 and 2'-MeOBDE68 in cetaceans showed no regional differences and were widely distributed at higher concentrations than PBDEs.

The relative contribution of NHCs to anthropogenic chemicals can serve as markers of localized inputs, prey preferences, or global variation of anthropogenic pollutants in cetaceans and fish around Hokkaido and across the world. In further studies, it is necessary to determine the correlation between high accumulation of contaminants (NHC. PFCA and POPs) and various abnormalities in aquatic mammals and fish.

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Chapter 127

COMPARATIVE STUDY OF HISTOPATHOLOGICAL AND BIOCHEMICAL BIOMARKERS OF TWO BLACK SEA MARINE FISH SPECIES, BELONGING TO DIFFERENT ECOLOGICAL GROUPS

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ABSTRACT

Growing interest in aquatic resources and marine coastal ecosystems is concomitant with depletion of their resources. Human activities in shelf area may contribute to marine environmental degradation due to the domestic, industrial, agricultural and maritime transport effluents that are produced as a consequence of an increasing population and growth of economic development. Regarding the vulnerability of several species in relation to their life history traits of slow growth, maturation at relatively old age and longevity have prompted stock assessment studies and estimates on the effects of environmental and anthropogenic factors impacting fish populations. Marine environment acts as a sink for different kinds of contaminants including heavy metals, organic substances, nanoparticles, plastics, pharmaceuticals, and etc. Together with the climate change, overfishing and anthropogenic pollution of the marine environment result negative consequences to biota, especially fish. Therefore, assessment of the health status of marine fish species forms an important approach that can be applied internationally. Such approaches use combination assessment of externally visible damage of tissues and organs and internal evaluation of pathology, in particular of the biomarkers of oxidative stress. In polluted areas, exposure of fish to xenobiotics leads to interactions between these chemicals and biological systems, which give rise to biochemical disturbances. Oxidative stress is induced by the reactive oxygen species (ROS), associated with the different kinds of pollutants and unfavorable living conditions. Thus, toxic substances,

that enter the fish tissues and organs, directly affect the vigour of the organism. Therefore histological and biochemical biomarkers can serve as a potent indicators of stressful conditions and fish health. Histological and biochemical changes associated with pollutants in marine environment are efficient indicators of water quality and fish health exposed to both natural and anthropogenic stressors. The aim of the present work was to assess health of Black Sea fish species, belonging to different ecological groups. The hystopathological and biochemical biomarkers of the blood, spleen and liver of scorpion fish *Scorpaena porcus* L. and peacock wrasse *Crenilabrus tinca*, caught in the coastal waters of Black Sea, Sevastopol, (Crimea) were studied. The significant differences of the number of melanomacrophage centers (MMCs) in the liver and in the spleen of both fish species were shown. Antioxidant enzyme activities superoxide dismutase (SOD), catalase (CAT), peroxidase (PER) and glutathione reductase (GR) were higher in red blood cells of the *S. porcus* as comparted with *C. tinca*, while glutathione content demonstrated the opposite trend. Antioxidant enzyme activities in fish muscle and liver was not uniform. In muscle of *C. tinca* antioxidants level was greater than in *S. porcus*, in the liver the similar results were shown also with the exception of SOD activity. Our findings demonstrated the interspecies differences of histopathology of liver and spleen and antioxidants content in blood, muscle and liver in both tested Black Sea teleosts, that were associated with different level of their adaptations to the environment and mechanisms of action of pollutants on their physiological functions.

Keywords: histopathology, antioxidant enzymes, Black sea, fish ecological groups, blood, liver, spleen, melanomacrophage centers, oxidative stress, biomarkers

INTRODUCTION

In recent years, water ecosystems is facing a harsh loss of biodiversity that includes climate alterations, nutrient swings, acidification, habitat loss, exploitation, biological invasions and anthropogenic pollution. Marine coastal waters are recognized as highly productive systems with great natural biodiversity. They are important spawning and nursery grounds for many invertebrates and fish species. Aquaculture farms account for substantial contribution to world economy with annual production of 80 million tonnes, which provides livelihood to millions of people (Kolkovski and Kolkovski, 2011). To the other hand, coastal waters are also impacted by multiple anthropogenic stressors, derived from the intense human activities, namely domestic, agricultural and industrial effluent discharges, maritime transport, port activities, bank reclamation, dams and fishery (Fonseca et al., 2011a; Serafim et al., 2012; Franca et al., 2012).

Fishes are very important for commercial purposes, including fishery and aquaculture (Ababouch, 2006). Fish species play the key role in ecosystem because they belong to the vertebrates, which are at the top of the food chain. Fishes are very sensitive to anthropogenic pollution, and some of them may be tested as biomonitors and test-organisms for the evaluating of the effects of contaminant-related stress on marine environment and used widely in aquatic monitoring (Sole et al., 2009). Fish is a well known bioindicators, as presence of the toxicant in aquatic system is capable to alter fish biochemistry, physiology, morphology, behavior; thus serves an appropriate model for ecotoxicological studies, which is also important for human health (Jindal et al., 2019). Fishes are valuable organisms to asses toxicological effects caused both traditional toxicants namely heavy metals, oil, PCB,

detergents, pesticides, and etc. and emerging contaminants (ECs) represent relatively newly discovered groups, such as pharmaceuticals, personal care compounds, plastic and nanoparticules (Ale et al., 2018; Jindal et al., 2019; Vidya et al., 2019).

Many investigations have proved that fish are valuable organisms to asses toxicological effects caused by different toxicants, both traditional and emerging contaminants. Therefore, health parameters of fish, especially physiological and biochemical responses, are evaluated as indicators of habitat quality of coastal marine waters. The relation between environmental variability and fluctuations of fish health parameters and biomarkers of exposure to contaminants could provide important information on their abundance (Pasquaud et al., 2013; Fonseca et al., 2013a; Rudneva et al., 2012;). Indicators of negative effects allow the direct determination of pollutant impact on living organisms in aquatic systems.

Usually the investigators demonstrate the elevated levels of the man-made pollution and its negative effects on marine organisms in all levels of their biological organization. Environmental pollutants transfer to fish organism and accumulate in it, caused early biological effects firstly in cell and molecular levels. Accumulation of these alterations provokes damage of organ structure and function, tissues and systems disfunction, and during some time the pathologies and morphological anomalies are observed. Histopathological analysis of various organs and tissues is an important tool of environmental monitoring of water pollution, which allows assessing of structure changes, and lesions that caused by environmental toxicants and various negative factors (Chalghmi et al., 2016).

Liver is associated with the detoxification and biotransformation processes. It is the most important organ that detoxifies toxicants, including heavy metals and organics both endogenous and exogenous nature and due to its function, location and blood supply it is also most affected by contaminants in the water. Because liver has effective system of detoxification, the hepatic histological changes are the good tool of the evaluation of the negative impact on the organism (Feist et al., 2015; Vidya et al., 2019). The microscopic examination of the liver tissues of the fish inhabiting polluted areas showed mononuclear inflammatory cell infiltration, cloudy degenerations, congestions and also micro- and macrovesicular fatty degenerations. Analysis of histological changes in the fish liver is a highly perceptive and precise way to assess the effects of xenobiotics on aquatic organisms both in field and experimental studies.

The most common pathological changes in the liver of fish as the primary organ for xenobiotic accumulation and detoxication include alterations of the mural architecture (degenerative hepatocyte lesions: hepatocyte hypertrophy, pyknotic nuclei, nuclear pleomorphism and peripheral nucleoli, focal necrosis of hepatocytes that can be noticed by the presence of darkly stained eosinophilic debris as a result of cellular component disintegration). Extensive dilation of sinusoids with blood congestion, hypertrophy and hyperplasia of bill duct cells, fibrosis of blood vessels, increased numbers of Kupffer cells are also often occur in fish inhabiting polluted environments (Abdel-Warith et al., 2011). Among the other pathological alterations it needs to be noted a loss of lipid vacuoles in hepatocytes, pancreatic atrophy characterized by decreased number of acini and appears as remnants around hepatic blood vessels (Kaewamatawong et al., 2013). Subcapsular edema and fat accumulation in liver is a common problem in aquaculture that can be induced by parasites, inappropriate nutrition, pesticides and toxins (Pietsch, et al., 2014) as well as steatosis – fatty degeneration (Feist, et al., 2015). Histopatological lesions also include lymphocytic infiltration, hypertrophied hepatocyte nuclei with coarse chromatin, hepatocellular and

nuclear pleomorphism (Feist, et al., 2015; Al-Mamoori et al., 2015), presence of syncitia characterized by multiple nuclei and granular cytoplasm (Maharajan et al., 2016).

In fish, the spleen and head kidney are the most important immune organs that can trap and detoxify xenobiotics and maintain stable internal environment (Wang et al., 2013). Therefore, spleen and head kidney play a role in haematopoiesis and since the majority of pollutants absorbed by fish are transported within the body by blood. They accumulate in these hematopoietic organs and can induce toxic effects on many physiological and biochemical processes (Kondera et al., 2014), which disturb homeostasis, and defense systems such as the antioxidant system and the immune system of fish (Santana et al., 2018). Therefore, histological alterations of spleen of fish are also good biomarkers of health status.

In polluted areas, exposure of fish to xenobiotics leads to accumulate these chemicals and interactions between them and biomolecules, cells and tissues, which result to biochemical and physiological disturbances. Many toxicants, distributed in the environment, accumulated in tissues and organs of the aquatic organisms cause oxidative stress, characterizing the increase of reactive oxygen species (ROS), accumulation of the oxidized biomolecules in cells and the induction of the components of antioxidant defense system (Livingstone, 2001; Van der Oost et al., 2003; Rudneva, 2012). Homeostasis of the living organisms is characterized a balance between production and elimination providing certain steady-state ROS level. This balance can be disturbed leading to enhanced ROS level and damage to cellular constituents which is attributes with "oxidative stress" (Lushchak, 2011). At this case the parameters of oxidative stress are most informative indicators for unfavorable living conditions (Van der Oost et al., 2003).

At the other hand, for the elimination of ROS products there is antioxidant system in the aquatic organisms, which comprises both—low molecular mass scavengers namely vitamins A, E, C, carotenoids, glutathione, and high molecular weight antioxidants, which involve special adopted antioxidant enzymes (Livingstone, 2001). Among the biochemical biomarkers antioxidant activities in the tissues of aquatic organisms are good tool for the evaluation of the homeostasis (Adams, 2005, Rudneva et al., 2012), because they destroy ROS molecules. The main antioxidant enzymes are catalase (CAT), which exclusively degrades H_2O_2, superoxide dismutase (SOD), which destroys superoxide anion, glutathione-dependent enzymes namely glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST) and some others. Previously we described high diversity of antioxidants in various fish species and the dependence of their activity on fish species, their belonging to different ecological groups, season fluctuations, abiotic (temperature, oxygen concentration, depth, light) and biotic factors (feeding behavior, life cycle, reproduction, development stage, and etc.) (Rudneva, 2012; Rudneva et al., 2014; 2016a,b; 2018).

The investigators demonstrated the correlations between the antioxidant biomarkers and histopathological changes (Gul et al., 2004; Rudneva et al., 2016c) and the pollution impact. In our previous study we use the antioxidants and liver histopathology biomarkers, characterizing fish health in various Black Sea sites, characterizing different pollution levels (Rudneva et al., 2016c). The presence of different kinds of chemicals in estuaries and marine bays trends monitoring and risk-assessment procedures essential to ensure the preservation of their biological function and, especially, biodiversity and has led to the search for improved monitoring methods that can express the biological and ecological implications of pollution beyond environmental health (Fonseca et al., 2011a; 2011b; 2013; Vinagre et al., 2006; Sadauskas-Henrique et al., 2011).

Therefore, both biochemical parameters and histopathological observations can be assumed as sensitive indicators of fish tissues metabolism for polluted areas (Gul et al., 2004), the aim of the present study was to determine the hystopathological indicators in spleen and liver and antioxidant biomarkers in the red blood cells, muscle and liver of scorpion fish *Scorpaena porcus* L and peacock wrasse *Crenilabrus tinca* L caught in the coastal waters of the Black Sea in the region of Sevastopol (Crimea).

MATERIALS AND METHODS

Biological Characteristics of Tested Fish Species

Scorpion fish *Scorpaena porcus* (n = 10) and peacock wrasse *Crenilabrus tinca* (n = 5) are highly distributed teleost species in Black Sea coastal waters.

Scorpion fish *S. porcus* (Figure 1) is among the most common fish species in Black Sea coastal waters. Previously we selected it as biomonitor species for the monitoring of the coastal waters of Black Sea (Rudneva, 2012; Rudneva et al., 2016b). It is demersal termophilic fish species inhabiting bottom waters on the depth of 1-40 m, however it prefers the depth of 1-10 m. Because it is settled species, the fish migrates on a short distances at the bottom, and it is active at night time. Spawning time covers the period of the end of May to the middle of September. Female produces the eggs in twin sacks, which are dissolved in marine water, and the embryos in the eggs are developed in pelagic zone. Scorpion fish is the species with many spawning portions, and the female produces eggs every 1-2 day. Every portion contains 1100- 27200 eggs. Scorpion fish is predator, its preferable prey is small fish. (Svetovidov, 1964).

Figure 1. Scorpion fish *Scorpaena porcus*.

Peacock wrasse *C. tinca* (Figure 2) is omnivorous bentopelagic fish species inhabiting the coastal waters of the sea. It is settled form, the spawning time is covered the period from May to June, female and male are differed each from other. The fecundity is estimated as 12—58 000 eggs. The preferable food is mollusks, polyhaets and crustacean. Its abundance is varied from 1 to 50 m. (Svetovidov, 1964).

Figure 2. Female and male of peacock wrasse *Crenilabrus. tinca.*

Sample Preparation for Histochemical Studies

Scorpion fish (n = 10) and peacock wrasse (n = 5) were caught in spring period in the coastal waters of Black Sea in the region of Sevastopol (Crimea). The animals were immediately placed in the aerated tank, transferred to the laboratory and anesthesy.

The liver and spleen were removed from fish of both species for histochemical studies. Liver samples were fixed in 10% neutral buffered formalin following by dehydration in a graded series of ethanol solutions of increasing concentrations. Fixed specimens were processed to paraffin embedded blocks by accepted, routine methods and sections were cut at 6 μm on a sledge microtome. Tissue sections were stained with Boehmer's haematoxylin and 1% eosin by standard method and mounted into Canadian balm. Samples were examined using light microscope and MICROmed digital camera. To count melanomacrophage centers (MMCs), 8–10 fields were randomly selected on each slide, captured using the camera, and readings were performed at 200X magnification. After each field of liver tissue samples had been photographed, the area of MMCs was measured (μm^2) (MICROmed digital camera software). Quantitative changes in the numbers and size of the liver MMC were determined by counting the mean number of MMCs per field and by measuring of the average absolute (μm^2) and relative (% of the field area) size of single MMC (the percentage of organ area occupied by MMC) and total MMCs per field (Kranz, 1989; Kurtovic, et al., 2008; Manrique et al., 2014).

Sample Preparation for Biochemical Studies and Antioxidants Assay

For biochemical determinations blood was taken by caudal arteria puncture and serum was separated. The red blood cells were processed as we described previously (Rudneva, 2012). The sediment was washed three times with 0.85% NaCl solution and then lysed by addition of 5 vol of distilled water for 24 h at the refrigerator. The enzyme activity was determined in the lysates immediately after preparation. Liver and muscle samples were washed several times by cold 0.85% solution of NaCl, homogenized and centrifuged at 8000 g during 15 min at cool conditions.

Antioxidant activities in the fish liver and muscle extracts were determined according the methods, which we described previously with small modifications. Catalase (CAT) was measured by the method involving the reaction of hydroperoxide reduction (Asatiani, 1969). Superoxide dismutase (SOD) was assayed on the basis of inhibition of the reduction of nitroblue tetrasolium (NBT) with NADH mediated by phenazine methosulfate (PMS) under basic conditions (Nishikimi et al., 1972). Peroxidase (PER) activity was detected by spectrophotometric method using benzidine reagent (Litvin, 1981). Glutathione reductase (GR) was determined according the method of NADPH degradation and glutathione (GSH) concentration was indicated spectrophotometrically used Spectrophotometer Specol–211 (Carl Zeiss, Iena, Germany) (Goldberg, Sparner, 1987). Enzyme activity was estimated in arbitrary units per protein concentration which was determined used the kit of Filicit (Ukraine, Katalog…, 2005).

Statistical Analysis

Biochemical measurements were detected in duplicate for each sample. Descriptive statistics were performed using an ANOVA (Halafian, 2008). P value of 0.05 was used for the determination of statistical significance between the values of both examined fish species. Statistical analyses were performed using Statistica software. The graphs were constructed using Microsoft Office Excel software.

RESULTS

Hystopathological Characteristics of Fish Liver and Spleen

Scorpion Fish

Fish liver tissue that does not exhibit histopathological changes displays only few MMCs in the parenchyma or their absence (Abdel-Warith et al., 2011). Polygonal shaped or classical hexahedral hepatocytes with large spherical nucleolus and variable amount heterochromatin were detected in samples of fish liver (Figure 3). Hepatocytes formed hepatic cell cords locating among blood capillaries (hepatic sinusoids). Erythrocytes were mainly observed in the lumen of sinusoids. Kupffer cells (tissue-resident macrophages with large processes and bean-shaped nucleus) were registered in the space between hepatocytes and on the luminal surface of the sinusoid endothelium.

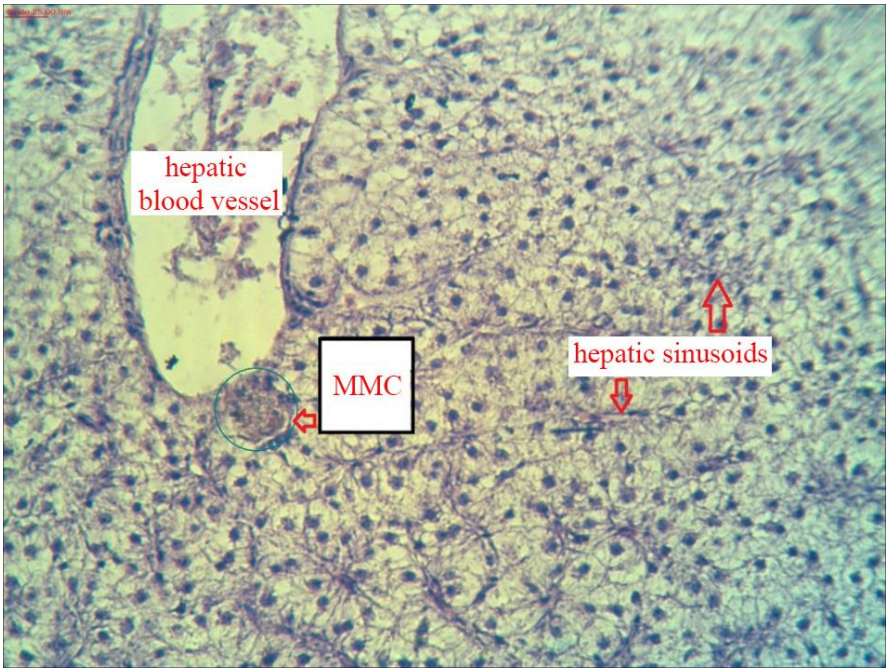

Figure 3. Section through liver of Black Sea Scorpion fish (*S. porcus*) captured in the marine coastal waters showing melanomacrophage center (big arrow), hepatic sinusoids (small arrows) and hepatic blood vessel. H&E, 200X, Bar = 25 μm.

Hepatic MMCs were not detected in 60% (3/5) of tested scorpion fish and occurred very rarely in the rest 40% of the animals, in which they were relatively small and associated with the blood vessels.

Spleen MMCs were indicated in 100% on tested samples of Scorpion fish (Figure 4). The average number of MMCs in spleen was approximately 5.

The microscopic pictures of peacock wrasse *C. tinca* liver and spleen are present in the Figures 5 and 6. In the liver of peacock wrasse *C. tinca* MMC in hepatocytes were large and dark color (Figure 5). High concentration of migrated melanomacrophages was indicated in parenhima as compared with the corresponding data of *S. porcus*. Among 5 tested fish samples high variability of histopathological changes in the spleen was observed: in two samples the hyperemia was shown and the boundaries between white and red pulpa were differed in the majority of fish samples (Figure 6).

The quantitative parameters of the MMC in the liver and spleen in both examined fish species are present in Table 1.

The average number of MMC in spleen was the similar in both fish. However, in *S. porcus* liver the average number of MMC in the field of vision was in 10-fold lower than in spleen ($p < 0.01$), while in *C. tinca* the values were identical (Table 1). The average size of MMC in the spleen of both checked animals were significantly higher in the spleen than in the liver, and in *C. tinca* they were greater as compared with *S. porcus*.

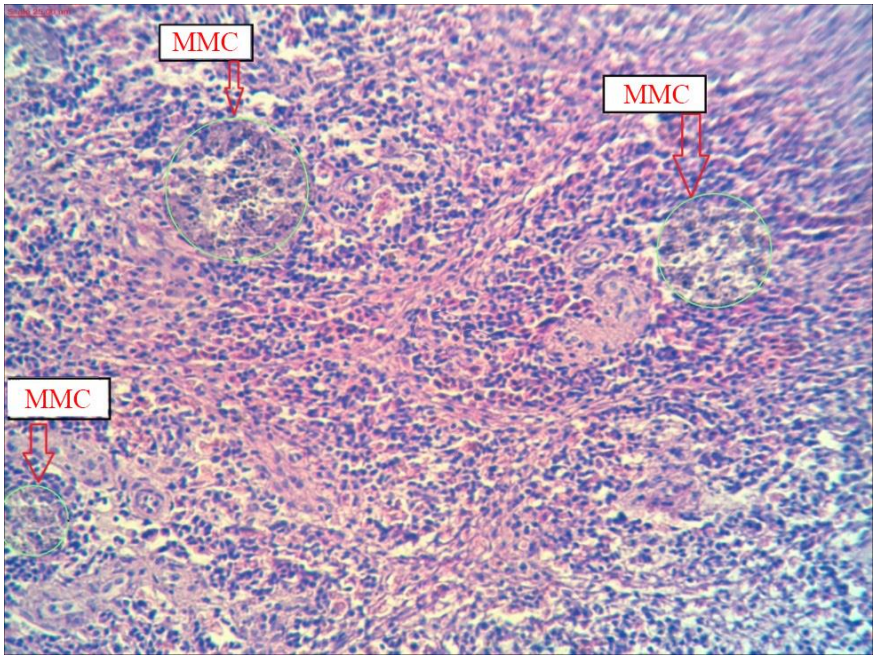

Figure 4. Section through spleen of Black Sea Scorpion fish (*S. porcus*) captured in marine coastal waters showing melanomacrophage center (big arrow). H&E, 200X, Bar = 25 μm.

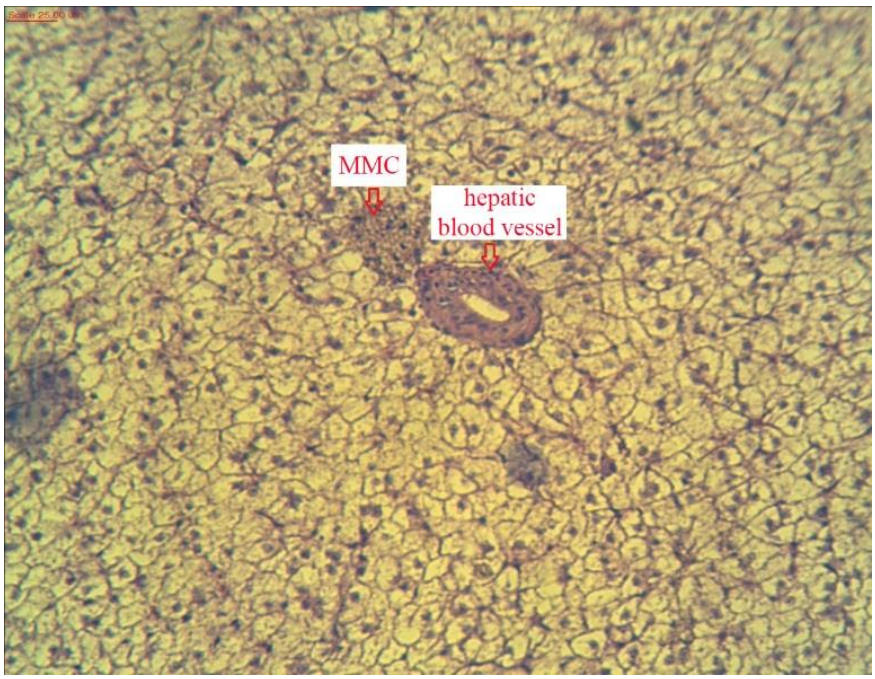

Figure 5. Section through liver of Black Sea peacock wrasse *C. tinca* captured in marine coastal waters showing melanomacrophage center (big arrow), hepatic sinusoids (small arrows) and hepatic blood vessel. H&E, 200X, Bar = 25 μm.

In both tested fish species the similar liver pathology was observed (Figure 3, 5), which characterized the increase of hepatic sinusoids and hyperemia, degenerative changes of hepatocytes. The infiltration of the tissues on leukocytes was also indicated, which was explained by the infections connecting with the increase of the number of Kuppfer's cells and elevated the number and size of MMC. At the same time the animals without any hepatic pathologies and low number of MMC were also were shown in the tested waters.

In the spleen tissues in both examined fish species the MMC contained dark-brown and black pigments, which were identified as hemosyderine and melanin, while in the liver the color was light and yellow, and they may contain hemosyderine and lipofuscin.

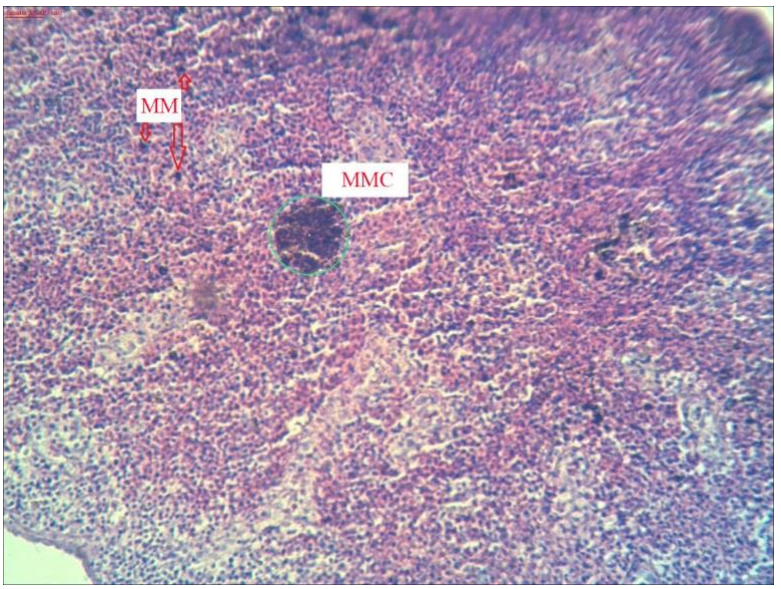

Figure 6. Section through spleen of Black Sea peacock wrasse *C. tinca* captured in the coastal marine waters showing melanomacrophage center (MMC), separate melanomacrophags (MM), capsule with high concentration of lymphoid cells in under capsule zone. H&E, 200X, Bar = 25 μm.

Table 1. Characteristics of the MMCs in the liver and spleen of *S. porcus* and *C. tinca* caught in the coastal waters of Black Sea, Sevastopol (Crimea)

Fish species	Average number of MMC		Average square of the separate MMC			
	Per section	Per field of vision	Separate MMC		Total number of MMC in the field of vision	
			μм^2	% in field of vision	μм^2	% in the field of vision
S. porcus Spleen Liver *C. tinca*, Spleen Liver	17.60 ± 2.70 26.30 ± 5.90	2.52 ± 0.40 0.24 ± 0.19* 2.26 ± 0.27 2.35 ± 0.36	3132.92 ± 790.90 791.31 ± 485.30 6508.6 ± 1456.1 827.62 ± 133.75	2.06 ± 0.52 0.52 ± 0.32 4.3 ± 0.96 0.54 ± 0.09	8774.2 ± 3554.73 791.31 ± 485.30 13836.4 ± 2741. 2081.02 ± 649.4	5.78 ± 2.34 0.52 ± 0.32 2 9.11 ± 1,8 1.37 ± 0,43

MMC- melanomacrophage center; *- significant differences, $p < 0.05$

Antioxidant System of the Fish Species

It is necessary to point out a significant individual variability of MMCs area in the liver and spleen of the fish captured in marine coastal waters. The goal of our further investigations was to study the response of fishes, belonging to different ecological groups, used the biomarkers of antioxidant activity. The results observed the differences between blood, hepatic and muscle biomarkers in both tested fish species. (Figure 7, 8).

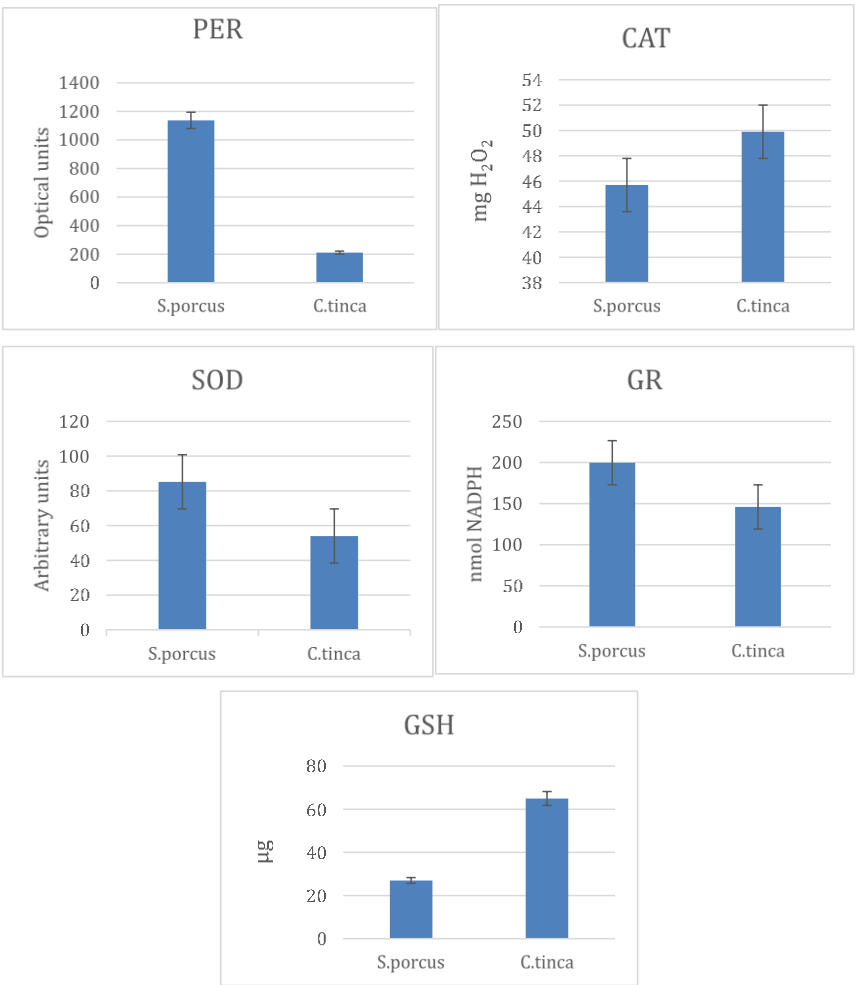

Figure 7. Antioxidant enzyme activities in the RBC (ml^{-1} red blood cells · min^{-1} of *S. porcus and C. tinca*, captured in the coastal waters of Black Sea, Sevastopol (Crimea).

Significant differences of PER activity and GSH content ($p < 0.05$) were shown in both studied fish species red blood cells (RBC) (Figure 7). SOD and GR activities tended to decrease in the RBC of *S. porcus* as compared with the *C. tinca*, while the activity of CAT was insignificantly greater in *C. tinca.*

The antioxidant enzyme activities in muscle and in the liver of two tested Black Sea teleosts are present in the Figure 8.

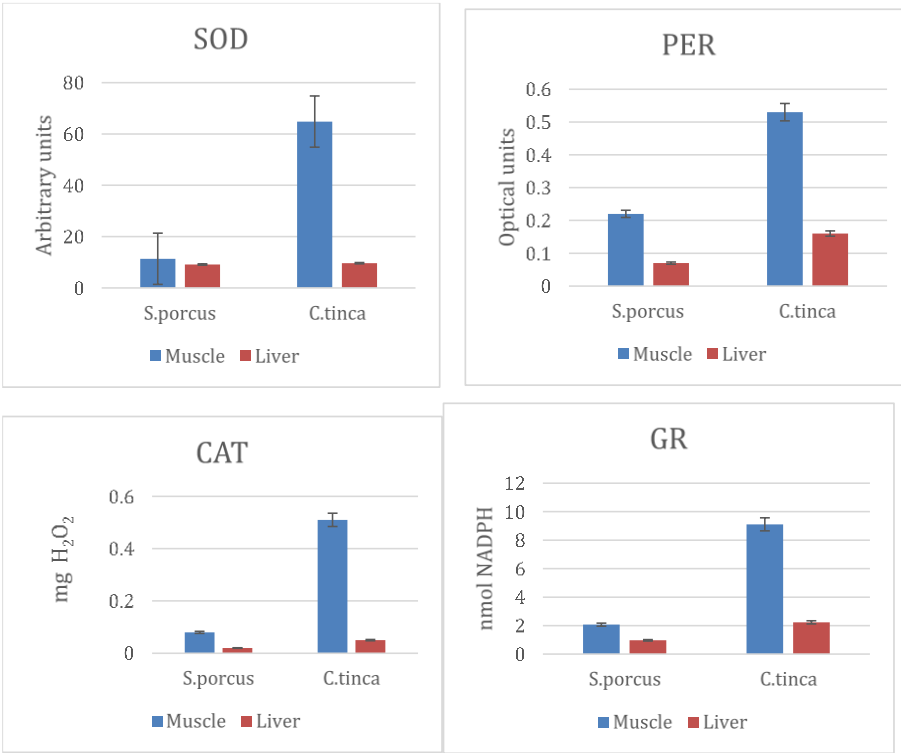

Figure 8. Antioxidant enzyme activities in the muscle and liver (mg protein^{-1} min^{-1}) of *S. porcus and C. tinca*, captured in the coastal waters of Black Sea, Sevastopol (Crimea).

The activity of checked antioxidant enzymes in both fish species were higher in muscle as compared to liver. In all cases the enzymatic activity was significantly higher in the muscle of *C tinca* than in *S. porcus* ($p < 0.05$). In the liver SOD activity was the similar in both fish species, while hepatic PER, CAT and GR activities were significantly higher ($p < 0.05$) in the *C. tinca* as compared with *S. porcus*.

DISCUSSION

The results of hystopathological and biochemical studies of the liver and spleen in both tested Black Sea teleosts *S. porcus* and *C. tinca*, caught in Sevastopol coastal waters demonstrated impact of the negative ecological living conditions in the abundance on fish health. One of the great advantages of using histopathological indicators in the evaluation of environmental status is that this battery of biomarkers allows examining specific target organs of fish and thereby histopathological responses are highly ecologically relevant. The toxicants enter into the body and from blood circulatory system they can easily enter tissues, including liver and spleen, that are responsible for vital functions, such as red blood cells production and the accumulation and biotransformation of xenobiotics in the fish. Histopathological damage may decrease individual fitness through disturbing the homeostasis and proper functioning of vital physiological and biochemical processes (e.g., detoxification, metabolic

rate, oxygen consumption, endocrine functioning, respiration, osmoregulation, nutrient absorption, and etc.).

Taking into account that the liver is the important metabolic organ, in which toxic chemicals are accumulated and detoxified resulted biotransformation processes, parameters of the evaluation of its status could reflect the environment influence and its harmful for fish. Fish hepatic pathology is widely used by histological observations and it is good tool in ecotopxicological studies (Camargo, Martinez, 2007; Trinchet et al., 2011; Vydia et al., 2019).

The degenerative changes of the hepatocytes were shown in the liver of both fish species. The infiltration of liver tissues by leukocytes could be connected with the infection accompanied with the increase of Kuppfer cells number and the elevation of the quality and size of the MMC. The level of water pollution significantly influences on the patterns of fish liver pathology. Lesions in the liver of scorpion fish captured in coastal waters of Sevastopol mainly included extensive dilation of hepatic sinusoids, hyperemia, and degenerative changes in hepatocytes. Marked focal infiltration by lymphocytes in liver tissue possibly indicating infection process was observed and it was associated with increase in the number of Kupffer cells and in the number and size of MMCs. At the same time, fish without significant signs of hepatic pathology and low MMCs count were detected also in the coastal waters. However, large MMCs were found in liver as well as focal infiltration by lymphocytes and hyperemia were detected indicating marked pathological changes in liver and plausible focal inflammatory lesions. The similar histopathological changes were demonstrated in the fish species, caught in the polluted water bodies (Table 2) (Pietsch et al., 2014; Feist, et al., 2015).

It is a common knowledge, that MMCs in fish tissues can contain four types of brown pigments: melanin (catabolic product of fatty acids degradation at low temperatures), lipofuscin (product of the destruction of cellular membranes and the end product of lipid peroxidation which is utilized in the lysosoms), ceroid and hemosiderin (product of red blood cells degradation) (Kranz, 1989; Manrique et al., 2014). Morphologic features of MMCs vary markedly depending on fish species, organ of their location and physiological status (age, physiological stage, starvation, tissue type, iron and hemoglobin metabolism, anatomopathological conditions). The composition and functions of liver MMCs are differ from spleen or kidney MMCs because liver is the key organ for the metabolic state that plays important role in the processes of cell renovation and storage as well as in detoxication and biotransformation processes (Manrique et al., 2014).

The histopathological alterations in aquatic organisms impacted unfavorable environmental and anthropogenic factors were present in Table 2. The researchers had been shown that the number of MMCs was significantly higher in the liver of Sea bass (*Dicentrarchus labrax*) exposed to the various environmental pollutants. It can be used as a bioindicator of environmental stress. Besides that, the biological pollution also plays a role in the organ pathology and causes the tissues defects. Strong correlation between the appearance of MMCs and high level of resistant intracellular bacteria and parasites had been demonstrated. MMCs play important role as the main locations for long-term pathogen retention (Kurtovic et 2008; Biyasheva et al., 2014). It corroborates usefulness of the bioindicator for potential monitoring of fish health and their environment.

Histopathological alterations in the liver of examined Black Sea fish species were mainly characterized by the dilated hepatic sinusoids and marked focal infiltration by lymphocytes, increase in the number of Kupffer cells, hyperemia appeared as congested hepatic blood

vessel and sinusoids. Moreover, vacuolization in the cytoplasm of hepatocytes, which was presumably caused by fat accumulation, was observed. Similar patterns of histopathological changes were shown in many fish species exposed to the toxicants or caught in polluted waters (Ferreira, 2011; Hansson, et al., 2006). At the other hand, we demonstrated the tissue specific differences between the histopathological alterations in the spleen and liver. For instance, in both tested fish species the number and size of MMCs were higher in the spleen as compared with the liver. Besides that we could detect the interspecies differences of the MMCs characteristics in the spleen and liver of both examined fishes. In *C. tinca* the parameters of MMCs higher as compared with the *S. porcus* which was connected with the ecological peculiarities of these animals and the different sensitivity to anthropogenic impact. Therefore, fish liver and spleen histology could serve as a model for studying the interactions between environmental factors and hepatic structure and functions. The parenchymatous hepatic tissue in teleosts has many important physiological functions and also detoxification of endogenous waste products as well as externally derived chemicals. Due to these reasons, the hepatic cells are damaged severely, on chronic exposure to xenobiotics. The liver and spleen exhibited several pathological changes including hyperplasia, degeneration of blood vessels, vacuolisation, hypertrophy; pyknotic nuclei, necrosis, and accumulation of blood vessels.

High concentration of xenobiotics in the water causes oxidative stress in fish resulted their transfer via water and food in the organism. Liver is the main organ of pollutants accumulation and biotransformation resulted ROS-generation and accumulation of their products (Palluel et al., 2018). Growth of lipid peroxidation production in tissues of the animals in contaminated habitats are postulated as the non-specific response of the organism to the stress effects and it was documented at the case of environment pollution by various chemical agents and their complex (Liu et al., 2006; Napierska et al., 2009; Martinez-Gomez et al., 2009; Martinez-Porchas et al., 2011). In polluted areas the exposure of aquatic organisms to xenobiotics results to interaction between these compounds and biological systems, which may give elevation to biochemical and physiological damage or/and adaptive mechanisms via the induction of defense immune and antioxidant systems (Liu et al., 2006). Thus, biochemical and physiological parameters are used as biomarkers for contaminants and could be applied for evaluation of environmental stress and its after-effects. However, biomarkers exposure to environmental stressors vary widely depending on the type of anthropogenic activity, ecological and biological peculiarities of fish species (Adams, 2005).

Antioxidant enzyme activity in the red blood cells was not significant differed in two tested fish with the exception of PER activity, which was higher in *S. porcus* as compared with *C. tinca*. At the other hand the concentration of GSH was greater in RBC of *C. tinca*. Therefore, we could postulate the insignificant interspecies differences of antioxidants content in the red blood cells of both animal species. The interspecies variations of AOA defense may reflect the specific adaptations to the oxidative stress and protective mechanisms against ROS damage. Thus the activity of antioxidant enzymes in fish blood correlated with their swimming capacity. In previous studies the investigators found that CAT activity in fast swimming pelagic fish were significant higher than in blood of slow swimming gobies, scorpion fish and flounder (Filho & Boveris, 1993; Filho, 1996, 2007; Rudneva, 2012).

Table 2. Relationships between histopathological alterations and antioxidant system of aquatic organisms tissues

Fish species	Hystopathology	Antioxidant system	References
Cyprinidae	Liver tissues of the fish living in the polluted area showed mononuclear inflammatory cell infiltration, cloudy degenerations, congestions and also micro- and macrovesicular fatty degenerations.	Glutathione-S-transferase (GST), lactic dehydrogenase (LDH), catalase (CAT), glucose-6-phosphate dehydrogenase (G6PDH), superoxide dismutase (SOD) and malondialdehyde (MDA) were significantly affected in fish at the polluted site.	Gul et al., 2004
Asian sea bass *Lates calcarifer*	Epithelial necrosis, hypertrophy, rupture of gill epithelium and haemorrhage at primary lamellae were observed after 7 days of Cu exposure. Lifting of epithelium, oedema and fusion of adjacent secondary lamellae were conspicuous at 28 days of exposure. The experimental liver tissue showed reduction in the size of hepatocytes, vacuolisation, and hypertrophy. The intestine displayed fused microvilli, sloughing of mucus membrane and swollen cells. Muscle bundles with severe oedema and their thickening and separation were more pronounced in sublethal treatment of copper in the muscle		Maharajan et al., 2016
Clam *Ruditapes decussatus*	Histopathological alterations (haemocytic infiltration, epithelium alteration, ciliary erosion, lamellae alteration, malformation at tip of the gill, fusion of lamellae, fibroma) were observed in the clam gills from metal-contaminated sites	Increase in glutathione S-transferase and catalase activities and lipid peroxidation levels, decrease in acetylcholinesterase activity in molluscs from metal contaminated sites. Significant overexpression of cytochrome c oxidase subunit I, ribosomal RNA 16S, Cu/Zn superoxide dismutase, heat shock protein 70, and metallothioneins.	Chalghmi et al., 2016
Fish *Channa punctatus*	The gills of fishes from metal-contaminated site contained several lesions like necrosis, epithelial lifting, lamellar fusion, hyperplasia, synechin infiltration of lymphocytes, and bridging in gill tissue.	Increased levels of lipid peroxidation (LPO) as well as antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST) were found in the gills of the fish from metal-contaminated site. The level of reduced glutathione (GSH), and enzymatic antioxidant, was quite expectedly lower than that in the reference fish	Javed et al., 2015

Table 2. (Continued)

Fish species	Hystopathology	Antioxidant system	References
Fish *Cyprinus carpio* L	Pathological changes (dissolved parenchymal architecture, vacuolar degeneration, necrosis, hemorrhage and slight inflammatory cells infiltration) in liver tissues could be observed in fish exposed in the microcistine C. Dissolved MC-LR could also result in pathological changes like partial broken epithelial cells, deformed taste buds and loose gill filament and lamella in gill tissues.	Catalase (CAT) activity increased slightly at first and then decreased with exposure of microcistine C period. A time-dependent decrease of reduced glutathione (GSH) with an increase of oxidized glutathione (GSSG) level suggested GSH was involved in detoxification of MC-LR in the liver. Oxidative damage was evidenced by the significant increase of malondialdehyde (MDA) level at 2–6 days.	Jiang et al., 2011
Blackbelly rosefish (*Helicolenus dactylopterus*), greater forkbeard (*Phycis blennoides*), black scabbardfish (*Aphanopus carbo*), orange roughy (*Hoplostethus atlanticus*), roundnose grenadier (*Coryphaenoides rupestris)*	A wide variety of inflammatory and degenerative lesions were found in all deep-water fish species examined.		Feist et al., 2015
Fish *Chalcalburnus tarichi*	The fish from the polluted site of the lake displayed various histopathological alterations, including hepatocyte vacuolization, fatty degeneration, increase in melanomacrophage centers, necrosis, infiltration, congestion, fibrosis, single cell necrosis, bile duct alterations, glycogen depletion and nuclear pleomorphism.	Lipid peroxidation (LPO) and antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) in the fish from polluted site as compared with the fish from reference area were significantly higher, indicating increased oxidative stress.	Kaptaner et al., 2012
Fish *Gobius niger*	Liver hypertrophy was shown in fish collected from Bizerte lagoon as well as many nonspecific lesions, unlike the reference site. The mean intensity (MI) of parasites seemed to be more sensible as an indicator of pollution levels.	Significant GST induction was observed in the fish collected from polluted sites, indicating an enhancement of conjugating activities, compared to fish collected at the reference site GH. EROD activity very weakly reflects hazardous pollution of sediments in ML site. Generally, there is a three-week lag between induction of EROD and GST activity	Louiz et al., 2018

Fish species	Hystopathology	Antioxidant system	References
Yellow Catfish (*Pelteobagrus fulvidraco*)	Cd2+ exposure altered the histological structure of immune organs, characterized by red cell loss in the splenic sinus of spleen, increased multinucleate giant cells and decreased lymphocyte numbers in head kidney, and increased MMCs in both tissues, produced negative influence on normal immune function of fish. Increase of multinucleate giant cells might be an inflammatory response to Cd exposure	The elevation of antioxidant gene transcriptional levels may be attributed to induce of antioxidant-related signaling molecules, such as Nrf2	Sun et al., 2019
Channa striatus and *Heteropneustes fossilis*	Histopathology of the liver, kidney and brain of metal-affected fishes was distorted significantly with its reference fishes. The influenced liver of the fish exhibited tissue vacuolization, pyknosis and distorted artery wall while as reference fishes showed normal histology. The liver of reference fish shows normal histology of liver tissue while the liver of infected fish is marked by vacuolization of the tissue, ruptured vein and hemorrhages. The kidney showed degenerated renal tubules, pyknosis, hemorrhages and glomerular degeneration as compared to its reference with normal histology. Brain histopathology showed spongiosis, neuronal degeneration, macrophages and inflammation.	Low levels of GSH revealed oxidizing state of the cell in both species. In the fish increased hepatic SOD activity was noticed, while in the gill, kidney and brain showed decreased activity, which perhaps shows more oxidatively challenged tissues.	Fatima et al., 2015
Fish *Scorpaena porcus*	Liver histopathology and the increase of MMC in the liver tissues of the fish from marine polluted site as compared with the animals collected in the reference site.	Increase of oxidized proteins, antioxidant activities, LPO in the liver of scorpion fish from polluted bay was observed.	Rudneva et al., 2016c

The researchers suggested, that antioxidant defense in marine species in the liver and blood may be related to the oxygen consumption of the tissues and of the whole organism, while in freshwater fish it may be related to physical and chemical characteristics of the living conditions rather than to physical activity (Filho, 1996). At the same time the changes in the sturgeon blood prooxidant-antioxidant status, as a consequence of adaptation to marine conditions, were not reflected in the liver and other tissues (Martinez-Alvarez et al., 2005).

Liver of the vertebrates exhibits a high metabolism and oxygen consumption and it is the main organ of xenobiotic detoxification. Fish liver displayed the high levels of the key antioxidant enzymes. This could be related to the fact that the liver is the site of multiple oxidative reactions and maximal free radical generation (Gul et al., 2004). Antioxidants level in liver appears to indicate that the active species of teleosts had greater enzyme activity compared with low mobile forms (Filho & Boveris, 1993). The higher activity of antioxidant enzymes in liver of active fish correlated with the higher oxygen consumption in fast swimming species and their high metabolic rate (Filho, 2007; Martinez-Alvarez et al., 2005). In our studies hepatic antioxidant concentrations in active *C. tinca* were greater as compared with sluggish *S. porcus*, with the exception of SOD activity. Animals with high metabolic rate exhibits the high rates of free radical production and caused the induction of antioxidant defense mechanisms (Zelinski & Portner, 2000). Physical activity of fish is often associated with fitness, reproduction or predator avoidance. Therefore, the link between oxidative stress and physical activity may appear to be more complex.

Muscle tissue also comes in close contact with pollutants dissolved in water. Hence, reactions in the histopathology of the muscles are spontaneous. The histopathology of muscle show progressive damage in the structure of the tissues with increasing concentrations of toxicants (Ramesh, Nagarajan, 2013). The alterations in muscle histopathology accompanied with the antioxidant status of the tissues. In our studies the antioxidant enzyme activity in muscle was higher in *C. tinca* than in *S. porcus*. Therefore, their antioxidant status depends on specificity on abundance and complex of living conditions in biotope. Ecological conditions of the habitats play an important role in fish antioxidant status and their adaptation to the living conditions. Previously we described enzyme activities in various tissues in several Black Sea teleost (Rudneva, 2012; Treberg et al., 2003). Therfore, the obtained results supports the great interspecies differences in antioxidants activity in various tissues between examined species, which agrees with the opinion of many researchers studying fish antioxidants (Mukherjee et al., 2017). In our previous studies we showed the differences of low molecular weight scavengers in fish blood.

We could note the possibility of the histological alterations and the specificity response of antioxidant enzymes in fish liver and spleen to different toxicants expose in the water and sediments as well as synergic and antagonistic effects at the case of interactions between xenobiotics and with the water. These interactions could modulate organism response and result histopathological damage and adaptive effect or toxic response of antioxidant defense system.

CONCLUSION

Hystopathological and biochemical biomarkers apparently are useful indicators of the health status of fish in monitoring studies, and they reflect the sensitivity/resistance of the fish

to unfavorable factors. The response of biomarkers in fish tissues such as liver, spleen, muscle and blood is important tool for the evaluation of fish abilities to protect against chemical pollution and keeping life in the polluted environments. The present study provides additional evidence for the usefulness of a set of liver and spleen histological and biochemical biomarkers in assessing the health of aquatic systems. Therefore, it is important to note that the responses of biochemical biomarkers depend on the frequency of release of pollutants and type and degree of contamination. However, in most cases stressed environment corresponds to chronic or subacute conditions. Hystopathological changes in liver and in spleen disturb various biochemical reactions as indicated by antioxidant enzyme activities. Antioxidant enzyme activity changes can be considered as a 'early-warning' adaptive or toxic response, while liver and spleen hystopathologies are 'second' adaptive/toxic response, depending on the level of water contamination and chronic exposure. Therefore, water pollution is capable of inducing both histopathological and biochemical alterations in liver and spleen, which may cause metabolic dysfunction. Histopathological alterations and antioxidant enzyme response can be used as indicators for the effects of various pollutants on fish and their abundance. These biomarkers are closely related to other indicators for stress because many pollutants have to undergo metabolic activation in order to be able to provoke cellular changes and biochemical alterations in the affected organism.

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Chapter 128

DESIGN OF IOT-CLOUD MARINE KNOWLEDGE SYSTEM BASED ON OPEN SOURCE

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ABSTRACT

Recently the research of marine knowledge and information technology has grown rapidly by the increasing interest in the rich repository of natural resources in the sea. For marine knowledge services, accurate marine environmental data must be continuously collected to deeply understand and analyze the marine circumstances. However, there is an insufficiency of research on the observation of marine circumstances in South Korea for the marine knowledge services. The ocean data buoy, a marine environmental monitoring equipment currently operating in South Korea, is large in size and high in production cost because it consumes a lot of power for communication. It also provides only marine data and lacks information on marine knowledge. In this paper, we have proposed a Containerized Marine Knowledge System by means of IoT-Cloud and LoRaWAN to improve marine environment monitoring. The proposed system enables flexible construction of the system and can analyze the marine knowledge through visualizing the gathered data and the knowledge processing with respect to the prediction of red tides. LoRaWAN-based IoT devices are able to collect long-range marine environmental data in an energy efficient manner. Our proposed method is helpful for researching low cost marine monitoring buoy and flexible marine knowledge systems.

Keywords: marine knowledge system, IoT, cloud, LoRaWAN, realtime processing, batch processing

1. INTRODUCTION

In recent years, the ocean has been attracting attention as a valuable resource pool, and the marine environment has been widely studied worldwide. For marine knowledge services through analysis and understanding of the marine environment, information on the marine circumstances must be continuously gathered. Especially in South Korea, there are various marine aquaculture industries in the south coast and the west coast because the three sides of Korea are the coast. The Korea fishery industry is highly influenced by marine circumstances and the natural phenomena. In other words, the damage of fisheries is increasing by changing the marine environment every year. The damage of the fishery industry is growing since typhoons, coastal pollution, an oil seep, and blooms of red tides. The harmful algal blooms (HABs) of South Korea have inflicted massive mortality on fin fish and shellfish, damaging the economies of fisheries for almost every year from 1993 to 2019. Particularly, there were huge harmful algal blooms of Cochlodinium polykrikoides in 1995, resulting in about 95 million dollars in fishery damage [1].

Collecting and analyzing coastal environments data can help to reduce the damage of coast disasters. But there is very limited research on marine knowledge services that collect and analyze environmental information from Korea's sea. Studies on marine knowledge Systems include sea buoys, radar of weather at sea, radio microwave at coast, coast circumstances observation system, and underwater circumstances observation system [2-13]. Tateson et al. studied a marine monitoring communication model using temperature and pressure measurement sensors. In this method, an antenna for transmitting and receiving is installed by fixing the position of a buoy, and a sensor package is installed to connect underwater by wire [2]. Yoon et al. [3] constructed a marine data communication network for water quality monitoring. These methods are composed of 9 sensors that can measure the water environment using Ethernet and CDMA-based web camera, temperature, salinity and dissolved oxygen amount in Gyeongpo Lake of South Korea. In addition, the studies on ocean weather monitoring include the use of radar to detect rainfall and snowfall, and the measurement of sea water temperature using a microwave radiometer [4]. Yoo et al. [5] studied the drift buoy for the marine environment monitoring. These drift units are designed to be able to flow by sea currents and sea wind which the measured data of temperature and salinity are transmitted to the server using a satellite communication model. Lee et al. [6] proposed a prototype of a gateway that can transmit data on the ground by integrating data from sensors using different protocols.

The Korea National Fisheries Research and Development Institute has been operating various surveillance systems to utilize basic data of the fishery area and predicting future fishery resource changes due to climate change [1, 11]. Park et al. [12] proposed a model of marine environmental monitoring and analysis system that automatically collects marine environmental information. Their model can intelligently monitor the marine environment and analyze collected marine data. In addition, they designed the new concept of convergence type maritime devices based on software definition devices in accordance with the new paradigm change [13]. The Korea Meteorological Administration operates 17 ocean data buoys on the coast of South Korea to provide real-time service of marine meteorological data. The ocean data buoy collects wave height, mean wave period, wave direction, wind direction, wind speed, barometric pressure, humidity, temperature, and water temperature. The

transmission of the monitored data at the buoy uses CDMA and satellite communications. The size of the buoys and the high production costs are due to the inclusion of solar panels and batteries to maintain communication. The 3-meter discus buoy for ocean data costs $ 400,000 USD and the 6-meter NOMAD buoy costs $ 800,000 USD. The high production price of ocean data buoys is limited to marine data collection. There is a need for a technical solution to this limitation. In addition, there is a need for research on marine knowledge information services that can generate added value by processing collected data from research currently focused on marine data collection [1, 12, 13].

In order to reduce red tide damage, another study on marine knowledge service is a study on predicting red tide based on Case-Based Reasoning (CRB) and Machine Learning (ML). Song et al. [14] proposed a CRB (case-based reasoning) based red tide monitoring system using past cases and simple classification methods has been proposed. Fdez-Riverola et al. [15] carried out a forecasting system of red tide blooms [15] using CBR by means of multi-inference stage. Rong et al. [16] proposed a forecasting method of red tide events using a neural network based on fuzzy. Our previous studies proposed forecasting models for the predicting of red tides events of the South Korea coast, which uses neural networks [17], fuzzy inference [18], ensemble method [19], and fuzzy reasoning and Naive Bayes classifier [20]. We also proposed the forecasting system of red tides events [1] using integration methods of fuzzy inference and ensemble technique. The CBR method [14, 15] and the classifier methods [16-20] show the red tide prediction results in numerical or categorical type. The categorical prediction has a high accuracy but is displayed as a simple binary number of 1 and 0. The numerical prediction can forecast the density of red tide algae but is less accurate than categorical prediction. Our previously proposed red tides prediction system [1] improved precision of prediction of red tides occurrence by using integration of the categorical type and the numerical type. In this paper, we modified our previously proposed red tide prediction system to design and implement marine knowledge analysis in our marine knowledge system.

In this chapter, the containerized marine knowledge system by means of IoT-Cloud and LoRaWAN is designed for low cost marine monitoring buoy and flexible marine knowledge system. The designed system consists of the hardware of marine knowledge system and the marine knowledge service. The marine knowledge system hardware consists of an IoT device to collect marine data and a marine server to store and process the collected information. IoT devices for collecting marine environment data can support long distance data transmission with low power using LoRaWAN communication module. The marine server can support a private Nano cloud that uses the LXD container to easily manipulate the increasing new infrastructure of marine knowledge service. The marine knowledge service is designed to support real-time marine knowledge processing and batch marine knowledge processing, such as the Lambda architecture. The real-time marine knowledge processing can analyze coastal information from visualizing gathered data by containerized visualization module. The batch marine knowledge processing can predict the red tide events by knowledge processing of data gathered over a period using a containerized prediction module.

2. MARINE KNOWLEDGE SYSTEM

2.1. Proposal System Concept

In this sub chapter, we explain the concept of our containerized marine knowledge system that provides the visualization of collected data and marine knowledge services to collect the coast circumstances data and analyze the coast knowledge. Figure 1 shows a conceptual picture of the marine knowledge system, which consists of Figure 1(1) Marine μBoxes (MB) and Figure 1(2) Marine IoT-Cloud Hub (MICH). The MB is the IoT device of marine data collection, which continuously collects coast circumstances data and transmits the gathered data to the MICH using LoRaWAN communication. The MICH is the marine knowledge server for the marine data visualization and red tide event prediction. The marine data is concentrated through the LoRaWAN gateway (i.e., concentrator) of MICH. It stores the collected marine data in the Hadoop distributed file system for marine big data processing, which it supports a real-time visualization analysis using a Grafana on the stored time series data. It also analyzes red tide occurrences using the proposed red tide prediction algorithm.

2.1.1. Marine μBox (MB)

The hardware and software of Marine μBox (MB) modules is designed, which plays the role of IoT device for marine information collection. The MB transmits the gathered coast circumstances data by the marine sensors to the MICH (Marine IoT-Cloud Hub) using the LoRaWAN communication module.

The Marine Box hardware consists of a battery pack, a LoRaWAN communication module, a sensor and an Arduino. LoRa is an abbreviation for Long Range, which is alarge, low-power long-range wireless communication technology with low stand bypower and low module prices. LoRa can transmit and receive a small amount of data at low power by putting chipset on device without separate base station or relay equipment. The communication speed of LoRa is 0.3 to 5 kbps, and the propagation reaches up to 21 km in a visible range.

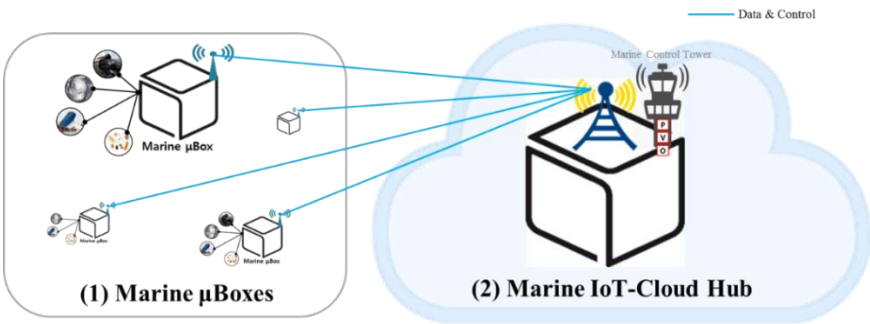

Figure 1. Conceptual diagram of Marine knowledge system.

In South Korea, SK Telecomhas installed the LoRa network nationwide to provide IoT service. LoRa Korea frequencies were announced in LoRa 1.0.2 in October 2016 from 920.9 to 923.3 MHz [21]. LoRaWAN is the communication protocol and the network architecture for the long-range communication link of the physical LoRa layer. The characteristics of

LoRaWAN is a star topology connected to the Marine IoT-Cloud Hub (MICH) in which gateway serves to relay messages between MB and MICH. The gateway module of MICH has two independent communication links with respect to LoRa communication and IP backhaul. LoRa protocol structure consists of MAC and PHY. The PHY includes LoRa modulation for regional LoRa frequency bands. The MAC consists of Class A (baseline), Class B and Class C (optional). Class A is utilized for the low-energy LoRa communication module of MB, which allows bi-directional communication between MB and LoRa gateway module of MICH.

Figure 2 shows the composition of MB hardware. As shown in Figure 1(1), the LoRa Arduino Shield (i.e., Dragino's LoRa communication module) [22] transmits the sensed data from LoRaWAN node (i.e., MB) to LoRaWAN gateway. The frequency band of the LoRaWAN module in Figure 2(1) sets 920.9 MHz, which is allocated to South Korea. Figure 2(2) Arduino Mega 2560 [23] gathers the coast circumstances data by sensors and sends the gathered data through LoRaWAN communication module. DHT11 is a temperature and humidity sensor in Figure 2(3), which senses the digital data of temperature and humidity. DS18B20 is a water temperature sensor in Figure 2(4), which senses the digital data of water temperature. YDDYP01 is a Xiaomi 20000 mAh auxiliary battery in Figure 2(5), which gives power to the MB.

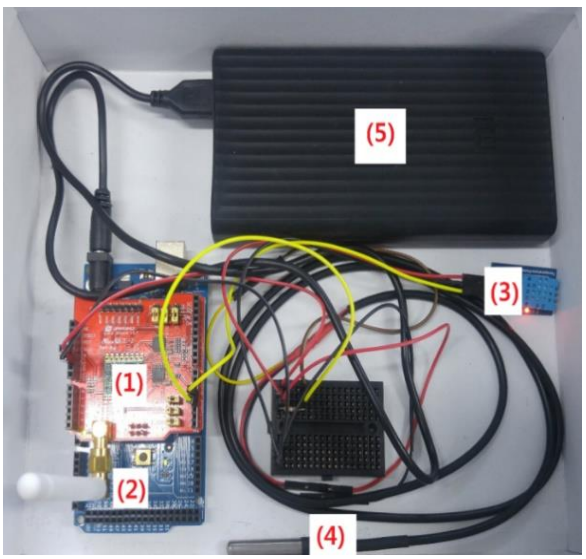

Figure 2. Composition of MB hardware.

Figure 3 shows the MB software module, while Figures 3(1) and 3(2) show the SMDM (Sensing Marine Data Module) and the LCM (LoRaWAN Communication Module), respectively. MB software module is operated in the Arduino. The marine environmental data (i.e., sea temperature, temperature and humidity) is collected by the MDCM (Marine Data Collection Module). The collected marine environment data are transmitted by LoRaWAN communication module to LoRaWAN gateway of MICH. It can also handle data transmission error. The MB software module is implemented using C language. Figure 3(3) shows the example of a software module to send data from LoRaWAN communication module to LoRaWAN Gateway.

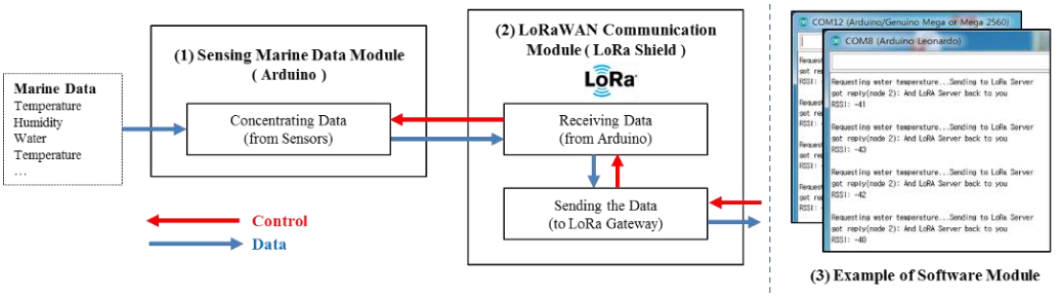

Figure 3. Marine μBox software module.

2.1.2. Marine IoT-Cloud Hub

Marine IoT-Cloud Hub stores coastal environment data as time series data, which serves red tide forecasting and visualization of coast circumstances data for marine knowledge services. The MICH consists of a hardware module and software module.

Table 1. Marine IoT-Cloud Hub (marine server): Container node hardware specification for private nano cloud

CPU	RAM	Sata SSD	m.2 SSD	NIC
Xeon E5-2630v4 x 2	128GB	12TB	1TB	1G*3

Table 2. Marine IoT-Cloud Hub (LoRaWAN gateway): Dragino (LG01-P) hardware specification [25]

Component	Hardware specification
Gateway module	400MHz MIPS Processor 64MB RAM 16MB Flash
LoRa module	ATMega MCU LoRa Chip 2KB RAM 32KB Flash
Functions	10M/100M LAN WiFi LoRa Wireless

The MICH hardware module contains a marine server and LoRaWAN gateway. Table 1 shows the hardware specifications of cluster nodes, which consist of one server with two Xeon CPUs. The cluster nodes' hardware is efficiently used by using virtualized software modules based on resource pooling. The LoRaWAN gateway module is implemented by using Dragino LG01-P [24] LoRaWAN gateway. Table 1 shows the hardware specification of Dragino LG01-P LoRaWAN gateway.

MICH software contains marine server software module and LoRaWAN gateway software module. As shown in Figure 4, the marine server software module is designed based on a private Nano cloud, which can support the virtualization of the LXD container for flexible resource utilization. The Nano cloud of MICH is constructed by clustering compute

nodes, storage and container networking based on LXD containers. Any number of LXD servers share the same distributed database for the compute node of MICH, which can be managed uniformly using lxc client or the REST API. The storage of MICH is used in the LXD's file storage pool. The MICH container networking is supported by virtualizing the network within the container. As shown in Figure 4, the Ubuntu 16.04 Linux is installed on all LXD container nodes for marine server software modules and Ubuntu 18.04 is installed on the host node of the server. Hadoop is a software framework with the Apache open source project that allows distributed applications to run in a cluster by clustering computing resources to handle large amounts of data. Hadoop can store data in the Hadoop Distributed File System (HDFS), which the distributed processing system can handle large amounts of data using MapReduce [25]. LXD is Canonicals' open source lightweight container hypervisor, which improves Linux Containers (LXC) features and provides new features and functionality for handling of LXC [26].

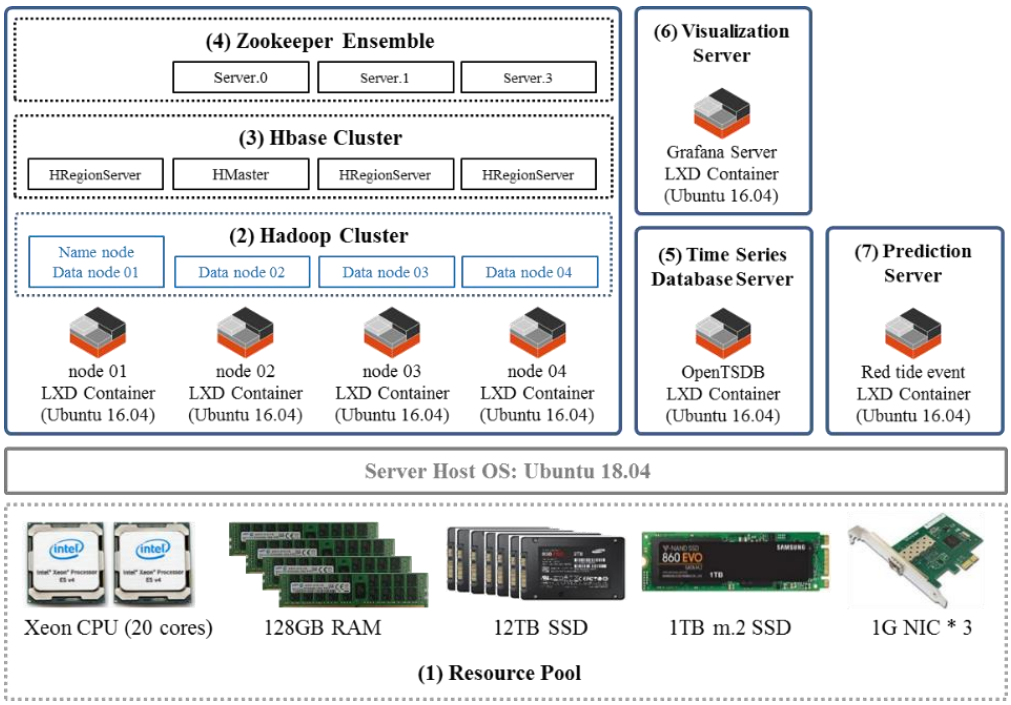

Figure 4. Marine IoT-Cloud Hub: Marine server software modules.

As shown in Figure 4(2), Hadoop 2.7.3 [25] is clustered on the containerized four nodes to share a resource pool. As shown in Figure 4(3), NoSQL database, Hbase 1.2.6 [27], is installed in the upper layer of HDFS (Hadoop Distributed File System), in which a large amount of data can be processed by a distributed file system. HBase is open source software as a NO-SQL distributed database system that runs on the Hadoop platform, which was developed for the Hadoop project of Apache Software Foundation. The data tables of HBase provide input and output for MapReduce operations running on Hadoop, which can be accessed through Java APIs, REST, Avro or Thrift gateways [27]. In this paper, the Zookeeper in Figure 4(4) acts as a coordinator of distributed clusters to the Hadoop cluster in Figure 4(2) and Hbase cluster in Figure 4(3). Zookeeper 3.4.6 [28] is installed on node 2,

node 3 and node 4, which Zookeeper Ensemble is configured. Zookeeper is developed by the Yahoo development team for the Apache project. It is a distributed cluster coordinator that manages and coordinates the application programs composed of multiple nodes. In addition, Zookeeper manages the state (i.e., dead or alive) of cluster nodes such as synchronizing specific data. Zookeeper is low in latency and high in throughput, because it is an in-memory based tool which stores data in memory and snap shots in storage [28]. OpenTSDB 2.3.0 [29] is installed on an OpenTSDB container as shown in Figure 4(5), which the marine circumstances data are saved as time series data to HBase using TSD (Time Series Daemon) of OpenTSDB. OpenTSDB is a scalable time series database based on Hbase. It consists of time series daemons (TSDs). Each TSD is independent and processed without state sharing which uses HBase to fetch and storetime series data. Users can communicate with TSD using telnet protocol, HTTPAPI, built-in GUI, etc. [29]. Grafana 4.4.3 [30] is installed on Grafana server containers as shown in Figure 6(6), which visualizes the marine environment time series data by displaying graphs. Grafana is a dashboard for displaying time series data and measurement information, and it supports visualization of time series data stored in various data sources such as Graphite, Elastic search, OpenTSDB, Prometheus, InfluxDB, and Cloudwatch [37-39].

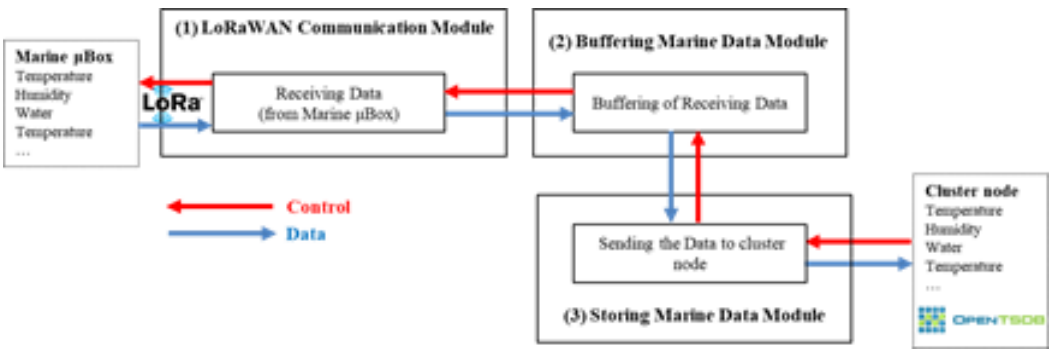

Figure 5. Marine IoT-Cloud Hub: Software modules of LoRaWAN gateway.

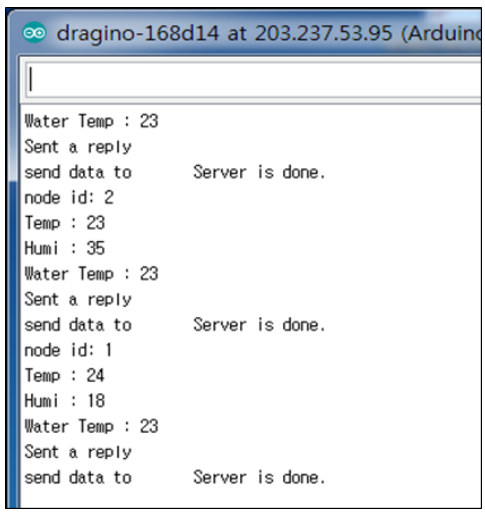

Figure 6. Example of running LoRaWAN gateway software modules.

As shown in Figure 5, the software module of LoRaWAN gateway includes the LoRaWAN Communication Module (RCM), the Buffered Marine Data Module (BMDM) and the Storage Marine Data Module (SMDM), which runs on Dragino LG01-P LoRa gateway. The RCM in Figure 5(1) receives the coast circumstances data from the LoRaWAN communication module of MB. It also requests data retransmission if a transmission error occurs. The BMDM shown in Figure 5(2) temporarily stores the marine environment data before transmitting to the OpenTSDB Server. The SMDM in Figure 5(3) sends the coast circumstances data to the marine server's OpenTSDB and stores the sent data as time series data. Figure 6 shows an example of running the LoRaWAN gateway software module.

2.1.3. Red Tide Prediction Server

The prediction server in Figure 4(7) containerised the revised of our previous red tide prediction system for ocean knowledge service. Generally, results of red tide prediction consist of categorical type and a numerical type. The categorical type is high precision but it represents a simple binary result of 0 or 1. The numerical type can forecast how much density to increase in red tide bloom, but the prediction result is less accurate than the results of the category type. In our previous work [1], we proposed a hybrid-based red tide prediction systemthat uses fuzzy reasoning and the ensemble method (i.e., bagging of decisiontree) to obtain prediction results for the categorical and numerical types. Please refer to [1] for details of our previous red tide prediction system. In this paper, we use Random Forester method [31] instead of the decision tree of ensemble method to improve the performance of our previously proposed method. The revised red tide prediction method forecasts the density of red tide algae and red tide events. As shown in Figure 7, the revised method consists of four phases: Preprocessing, Fuzzy Reasoning, Random Forester, and Postprocessing. Preprocessing phase normalizes training and test data of classifiers for Fuzzy Reasoning method and Random Forester method. The Fuzzy Reasoning phase forecasts numerical prediction values about the algae density of red tide blooms. Random Forester phase predicts the categorical prediction binary value of 0 or 1 for red tide events. Post-processing phase enhances the results of numerical prediction with respect to the Fuzzy Reasoning phase. The revised method uses Python 3.5 and Python machine learning frameworks (i.e., scikit-learn, scikit-fuzzy, tensorflow, and keras) for easy implementation in a short time.

2.2. Test of Designed Marine Knowledge System

In this sub chapter, to evaluate the performance of the proposed system, we evaluate the operational test of the marine knowledge system and the accuracy of red tide forecasting of the prediction server. The marine environmental monitoring system for operational testing is configured as shown in Figure 8. As shown in Figure 8 (1), the Marine μBox consists of two boxes which collect temperature, humidity and water temperature data through two sensors. The collected data are saved as time series data of OpenTSDB in the marine server of MICH in Figure 8(2). The stored time series data are visualized by Grafana to display as a graph.

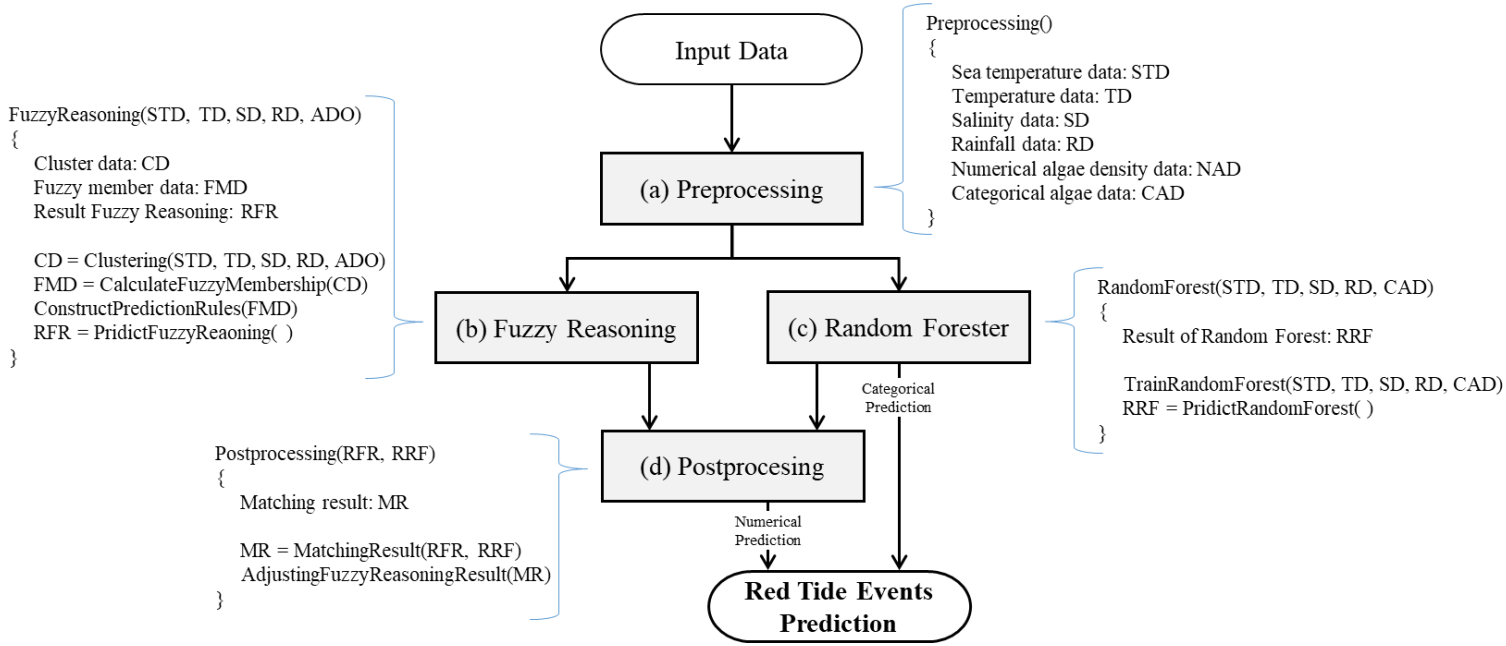

Figure 7. Revision of our previous method for ted tide prediction.

Operational testing of the proposed marine knowledge system consists of visualization of coastal environments data and visualizing workload of cluster nodes. The visualization of coastal environments data visualizes the collected data from MB in real time. The visualizing workload visualizes the usage of the CPU of each clusternode of the MICH in 1-minute and 5-minute increments. The first MB (node 1) is located in the same laboratory 2 m away from the MICH and the second MB (node 4) measures the indoor environment information located in another laboratory 30 m away from the MICH. The MB (node 1) is open in the room, and the MB (node 4) is packed in a paper box for mobility.

Figure 9 is a graphical representation of the data gathered from two MBs in a month (2018). Figure 10 shows the visualization results of the data collected over the last 12 hours. Figure 9(1) and Figure 10(1) are temperature data gathered from two MBs. Figures 9(2) and 10(2) are the humidity data. The Figures 9(3) and 10(3) show the water temperature data. In Figure 9 and Figure 10, the green graph is the MB (node 1) and the yellow isthe MB (node 4).

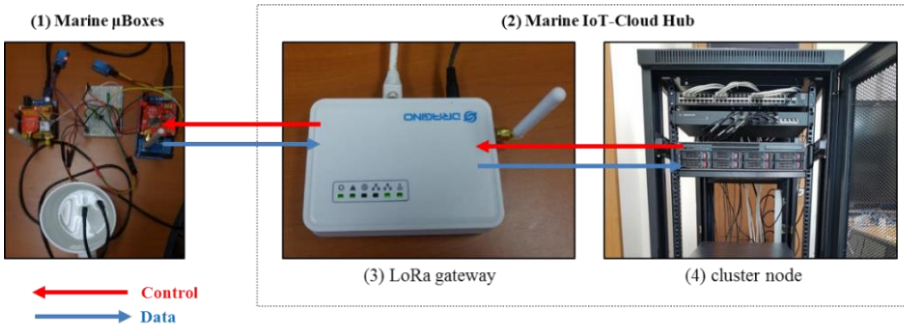

Figure 8. Test configuration diagram of the proposed system.

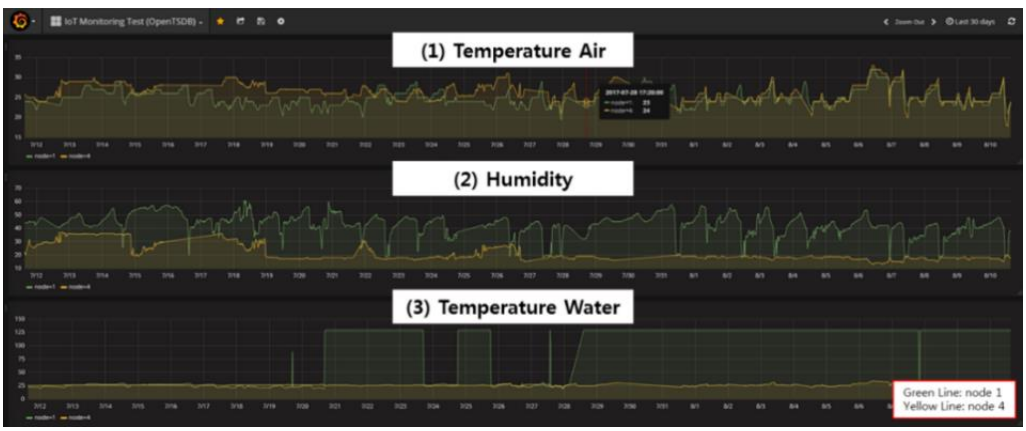

Figure 9. Visualization results of monthly (2018) gathering data from MB.

Figure 11 shows the visualizing of the CPU utilization data gathered from the four cluster nodes of the MICH. Figure 12 shows the visualization results of CPU utilization data collected during the last 3 hours. Figure 11(1) and Figure 12(1) are CPU utilization data collected for 1 minute from 4 cluster nodes of Marine IoT-Cloud Hub. Figure 11(2) and Figure 12(2) are 5-minute CPU usage load data. In Figure 11 and Figure 12, green line is data node 1, yellow line is data node 2, blue line is data node 3, and orange line is data node 4.

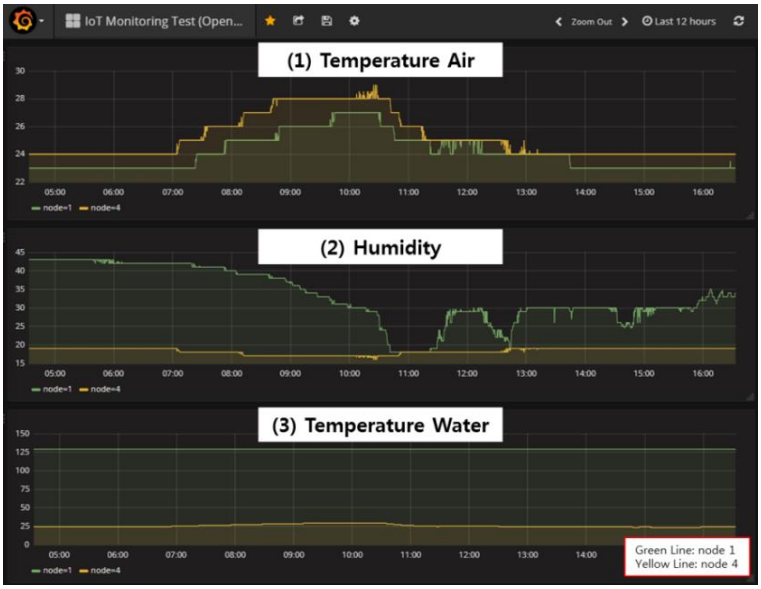

Figure 10. Visualization results of 12-hour gathering data from MB.

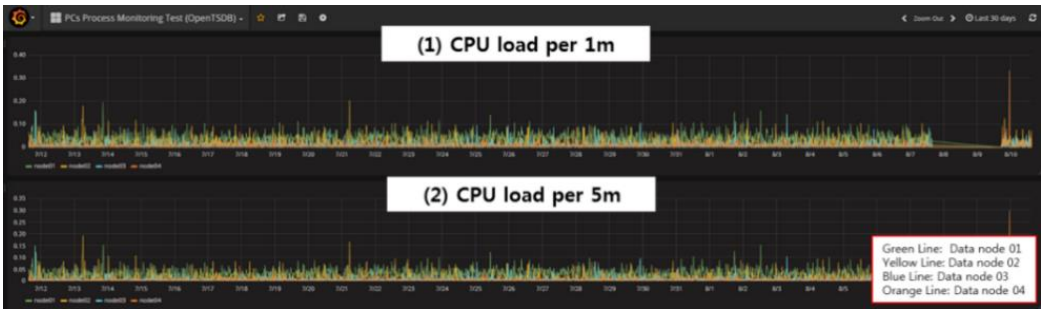

Figure 11. Visualization result of monthly (2018) collection data of CPU load from data node in MICH.

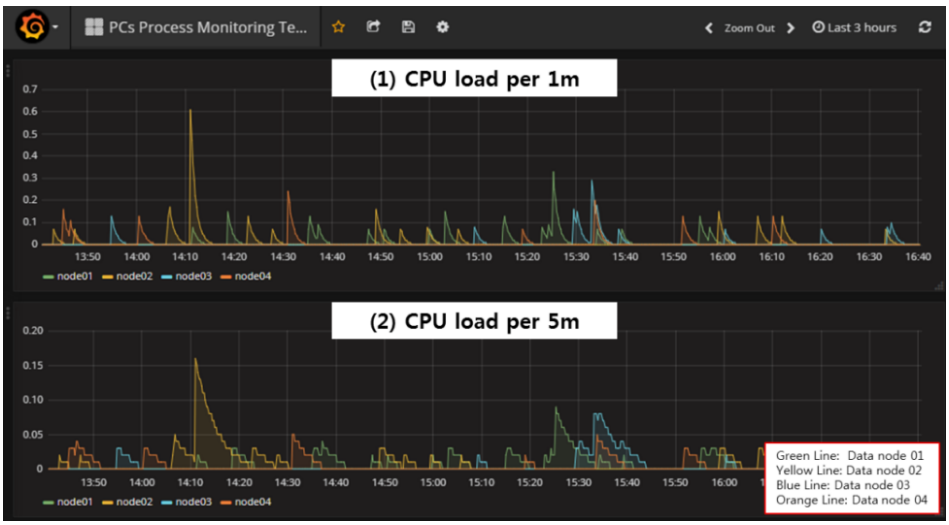

Figure 12. Visualization results of 3-hour collected CPU load data from data node in MICH.

To evaluate the revised red tide prediction method, we used the experimental data of 746 actual events of harmful algal (i.e., Cochlodinium p.) and unharmful algae in the eighteen years from 2002 to 2019 that occurred on the coast of South Korea. Red tide events data was collected from the red tide information system of Korea National Institute of Fisheries Science [32]. Temperature and rainfall data related to red tide events was gathered from the Korea National Weather Service [33]. Water temperature data in connection with red tide events was collected from Korea National Institute of Fisheries Science [34]. The ratio of classifier training data and predictive test data is a cross-validated of 70 and 30, respectively.

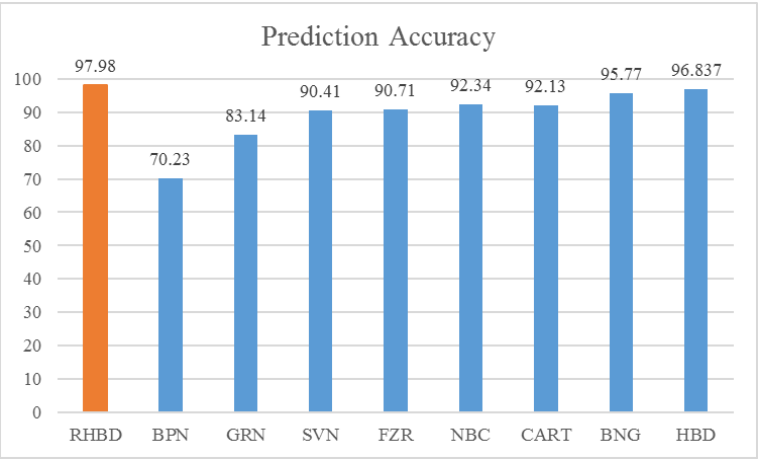

Figure 13. Results of comparison of the classifiers for red tide events prediction.

We compared the average prediction accuracy with respect to the nine methods, RHBD, BPN, GRN, SVM, FZR, NBC, CART, BNG, and HBD. BPN and GRN are the back propagation neural network classifier and the general regression neural network classifier, respectively [17]. SVM is the support vector machine classifier [17]. FZR is the fuzzy reasoning classifier [18]. NBC is the Naïve Bayesian classifier [19]. CART is the decision tree classifier based on the CART algorithm [1]. BNG is a bagging classifier based on the CART decision tree [1]. HBR is our previous method which hybrid-based red tide prediction system uses fuzzy reasoning classifier and CART bagging classifier [1]. RHBD is the proposed method in this chapter by revising our previous method of HBD based on Random Forester. In Figure 13, the average prediction accuracy of RHBD is approximately 27.75% higher than that of BPN, 14.48% higher that of GRN, 7.57% higher that of SVN, 7.27% higher that of FZR, 5.64% higher that of NBC, 5.85% higher that of CART, 2.21% higher that of BNG, and 1.143% higher that of HBD.

CONCLUSION

We have designed the containerized marine knowledge system, which consist of Marine μBox for IoT data collection devices and Marine IoT-Cloud Hub for marine knowledge services. The Marine μBox can gather long-range coast circumstances data at low power by LoRaWAN. The infrastructure of IoT-Cloud hub is designed as a private Nano cloud with

LXD container, which new infrastructure of marine knowledge service can be easily created. The marine knowledge services of IoT-Cloud hub can support real-time processing for visualizing marine information and batch marine knowledge processing with respect to prediction of red tides blooms.

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Chapter 129

METHODS OF DETERMINATION OF MICROPOLLUTANTS IN DIFFERENT MARINE MATRICES

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ABSTRACT

In recent years there has been growing environmental concern, especially regarding the use and discharge of toxic substances. Many organic and inorganic pollutants are detected in marine ecosystems such as pesticides; heavy metals (HM), synthetic musk compounds (SMs), and polycyclic aromatic hydrocarbons (HAPs). Because of their toxicity, bioaccumulation, and persistence in the environment, they cause harmful effects to organisms and human health. Many extraction techniques such as Soxhlet extraction, ultrasonic extraction, supercritical fluid extraction, microwave extraction, QuEChERS, accelerated solvent extraction have been used for the determination of micropollutants in different marine matrices. Extraction techniques in analytical chemistry provide an effective separation of the analytes from the matrix with minimal use of solvent and duration. Many techniques are used for the detection of micropollutants in marine matrices such as high-performance liquid chromatography (HPLC), gas chromatography (GC) for organic pollutants, and atomic absorption spectrometry (AAS), *inductively coupled plasma* (ICP) for inorganic pollutants. In this chapter we investigate a comparison between different extraction and detection techniques used for the determination of organic and inorganic pollutants in environmental matrices such as biota, water, and sediment.

Keywords: micropollutants, marine environmental matrices, extraction, analytical detection techniques

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1. INTRODUCTION

The natural environment continuously receives foreign chemicals due to urban and industrial activities. Pesticides present a huge class of compounds that are used everywhere in the planet for several decades regarding their advantages and benefits. The appliance and release to the aquatic ecosystems of those organic compounds has become a matter of concern (Pinto et al. 2016). Within the last century, thousands of organochlorine pesticides (OCPs) are produced and released into the environment which constitutes one among the foremost important persistent organic pollutants (POPs) (Zhou et al. 2006). Many of those pesticides belong to endocrine-disrupting chemicals and due to their persistence, high lipid solubility, and carcinogenic properties, OCPs threatened the ecosystem, human health and wild animals (Yan et al. 2014; Tang et al. 2008).These compounds and their metabolites were the most explanation for birth defects, system dysfunction, endocrine disruptions, and cancer (Montuori et al. 2016). Many people are poisoned and hundred are dying annually around the world from POP, and a huge number of them live in the developing countries (Sarkar et al. 2008). Their intensive use throughout the agricultural world for crop protection within the past has caused their persistence within the environment. Emerging contaminants are natural and artificial chemicals or micro-organisms that are not commonly monitored within the environment but has the potential to enter the environment and cause ecological and human health effects (Kuster et al. 2008). Synthetic musks (SMs) have recently been classified as a new class of emerging contaminants of the marine environment (Heberer et al. 2002). These compounds became indispensable to our modern society and are utilized in a good range of applications (fragrance additives in perfumes, lotions, sunscreens, deodorants, antiseptics and laundry detergents) (Hu et al. 2011). The SM compounds contaminate the environments with the discharge of effluents into lakes and rivers (Hu et al. 2011). Because of their high octanol–water partition coefficient (Log $K_{OW} \geq 5$) and their lipophilic nature, SMs are easily absorbed by suspended particulate matter and, eventually are deposited and accumulated in sediments. The evaluation of the impacts of anthropogenic activities on marine environment requires the analyses of sediment samples which represent the last sink for hydrophobic pollutants (Zeng et al. 2008). Many researches shows that OCPs are still detected in various media including: Mud (Ju et al. 2009), SPM (Liu et al. 2013), surface sediment (Liu et al. 2013), beverage (Eissa et al. 2013), surface water (Schreiber et al. 2013), ground water (Raposo Junior et al. 2007), even after their use has been banned for the past several decades. The presence of SMs in coastal and marine environments has been previously reported in several environmental matrixes: water, mussels, fish, sludge and sediment. Most of the analytical methods adopted for the analysis of SMs in sediment matrices are supported traditional liquid solid extraction followed by a cleanup procedure. The necessity of huge amounts of organic solvents (200 to 500 ml), the time-consuming extraction steps (8-24 h) and therefore the great deal of sample used (1-10 g) are the most drawbacks of traditional extractions (Sumner et al. 2010). The replacement trend in analytical chemistry is the development of "environmentally friendly" sample pre-treatment methods, which are solvent-free or solvent-minimized and, at an equivalent time, faster and more selective than classic procedures (Pinto et al. 2010). Anastassiades et al. (2003) developed a QuEChERS procedure to extract pesticides from fruits and vegetables. High-quality results with a high sample throughput, low solvent and glassware consumption, little work, low cost and time of study per sample was provided by this method (Pinto et al. 2010).

2. THE ORGANIC POLLUTANTS

2.1. Organochlorine Pesticides (OCPs)

Organochlorine pesticides (OCPs) include various groups of chemicals, which tend to share certain structural characteristics. They have an aliphatic radical or an aromatic ring structure, which is strongly substituted with chlorine atoms (Shen et al. 2005). As a result, most of these compounds are sparingly soluble and semi-volatile. In May 2001, the Stockholm Convention on POPs, which was adopted by the United Nations Environment Program (UNEP) stressed the need to control the global contamination produced by toxic chemicals in the environment.

The convention promotes global regulations on the production and use of persistent OCPs, such as aldrin, dieldrin, endrin, heptachlor, chlordane, hexachlorobenzene, Mirex, toxaphene, PCBs and DDTs. Although most of the developed countries have already banned or restricted the production and use of these compounds, some developing countries still use OCPs in agriculture (Gao et al. 2008). Several studies have shown the presence of OCPs and PCBs in the various compartments of the marine ecosystem: surface water, suspended particles and sediments. It has been proved in other studies the presence of its organic pollutants persistent in the plant matrix and even in living tissues with more or less significant concentrations (DGPA 2008; APEK 2005).

Table 1. Presence of pesticides in different matrices in Tunisia

Location	Matrices	Analysis methods	Concentration of pesticides	References
Northern Tunisia	Breast milk	LLE-GC-ECD	∑3DDT: 0.058–31.325 (ng g^{-1}) HCB: 0.003–3.127 (ng g^{-1}) Dieldrine : ND–0.529 (ng g^{-1})	(Ennaceuret al. 2007)
Bizerte lagoon	Fish	SE-GC-ECD	∑12 PCB: 164–642 (ng g^{-1}) ∑ 7 OCPs: 52.9–265 (ng g^{-1})	(Ben Ameur et al. 2013)
Tunisia	Butter	SE-GC-MS	∑8 PCB: 11.81(ng g^{-1}) ∑6 DDT: 7.8 (ng g^{-1}) ∑3 HCH: 6.41 (ng g^{-1}) HCB: 2.340 (ng g^{-1})	(Kalantzi et al. 2001)
Tunisia	Breast milk	LLE-GC-ECD	∑8 PCB: 1- 154 (ng g^{-1}) ∑3 DDT: 8-7060 (ng g^{-1}) HCB: 1 -727 (ng g^{-1}) Dieldrine : 1-713(ng g^{-1})	(Ennaceur et al. 2008)
City of Bizerte	Breast milk	LLE -GC-ECD	∑3DDT: 125.8–4574.8 (ng g^{-1}) HCB: 24.1–1470.2 (ng g^{-1}) Dieldrine : ND–62 (ng g^{-1}) ∑8PCB: 16.4-1360.2 (ng g^{-1})	(Ben Hassine et al. 2012)
Bizerte lagoon	Sediment	ASE-GC-ECD	Σ10PCB: 0.8-14.6 (ng g^{-1}) Σ 4 OCPs: 1.1-14.0 (ng g^{-1})	(Barhoumi et al. 2013)

Tunisia signed the Stockholm convention on May 23, 2001 and the parliament ratified it on March 9, 2004. Within the framework of this convention, Tunisia is required to prohibit all production and use of POPs and to take the necessary measures to remove and eliminate POPs that are already in the country (on storage sites and in the natural environment). Although OCPs and PCBs have never been produced in Tunisia and their import and use have been banned, these compounds continue to exist in the various matrices of the marine

environment: sediment, biota and even in breast milk. Table 1 summarizes the different pesticides, their levels and their methods of analysis.

2.2. Synthetic Musks

Musk is a secretion from the glands that has been used for centuries as a component in perfumes. The discovery of natural musk dated from ancient China, where the musk deer was killed to obtain the musk gland (Rimkus et al. 2004). At the end of the 19th century, the first synthetic musk was synthesized in the laboratory, "musk Baur," allowing the use of a cheaper synthetic compounds (Heberer et al. 2002) instead of very expensive natural musk which was the main because of the almost extinction of the musk deer (Yang et al. 2003). Polycyclic musks are the most used in cosmetics and detergents and, therefore, those supposed to be found in high concentrations in the environment (Rimkus et al. 2004). The extensive use of SM in cosmetic products results in large quantities going down the drain after being applied to skin, hair and clothing. They enter sewage and wastewater systems and find their way into the aquatic environment through effluents and wastewater, primarily wastewater from treatment plants (Ricking et al. 2003; Stevens et al. 2003).

Once in the aquatic environment, synthetic musks can enter the food chain, being taken by fish and crustaceans. Because of their octanol-water partition coefficient (log $K_{ow} \geq 5$) and their lipophilic nature. The SM is easily absorbed by suspended particles which are then deposited and accumulated in sediments which represent the ultimate location for hydrophobic pollutants (Zeng et al. 2008). Synthetic musks have different chemical structures than natural compounds. The physical and chemical properties of synthetic musks are similar to those of persistent chemicals which are known by their biomagnifications such as PCBs and OCPs (Peck et al. 2004).

Table 2. Concentrations of synthetic musks in the environment

Location	Matrix	Method	Concentration of synthetic musks	references
City of Tainan (Taiwan)	Sludge	SPME-GC –MS	∑6MS: 0.3–10.9 ng g^{-1}	(Wu et al. 2010)
Guangdong (Chine)	Sludge	SE-GC –MS	HHCB: 5.416–21.214 mg kg^{-1} AHTN: 0.715–6.195 mg kg^{-1} DPMI: 0.599–2.870 mg kg^{-1}	(Zeng et al. 2007)
Bay of Biscay (Spain)	Surface water	SPME-GC –MS	DPMI: 22–267 ng L^{-1} ADBI: 24 ng L^{-1} HHCB: 794–2347 ng L^{-1} AHTN: 45–262 ng L^{-1}	(Cavalheiro et al. 2013)
Hai He River (China)	Fish	ASE-GC	∑7 MS: 6.0–18.6 ng g^{-1}	(Hu et al. 2011)
Denmark	breastmilk	GC –MS	∑8MS: 38.0–422 g kg^{-1}	(Olesen et al. 2005)
Portugal	care products	QuEChERS GC –MS	MS: 2–340 ng g^{-1}	(Homem et al. 2013)
Busan City (Korea)	Surface water	LLE-GC –MS	∑4 MS: 0.15–16.72 µg L^{-1}	(Lee et al. 2010)

Due to its properties, synthetic musks are widespread environmental contaminants, particularly in freshwater and marine ecosystems (Tanabe et al. 2005). They have been measured in rivers, lakes, sediments, soils, sewage sludge and effluents from wastewater treatment plants in Canada, the United States, the United Kingdom and Europe. Recent research has shown that levels have increased dramatically in lake sediments in North America over the past 15 years, in keeping with increased US consumption of chemical scents in consumer products (Peck et al. 2006). Due to their use in many consumer products, synthetic musks have also been detected in outdoor air (Peck et al. 2004). Table 2 shows the concentrations of SM in different matrices.

2.3. Triazole Pesticides

In the 1970s, triazoles were introduced as pesticides and exactly from fungicide class due to their relatively low persistence risk and high antifungal activity (See et al. 2010). Triazole contain five-chain heterocyclic compounds in each one three nitrogen atoms and having at least two double bonds. They work by disrupting the biosynthesis of one of the compounds in the wall of fungi. Among the wild range of triazoles studied, there are; Mesosulfuron-methyl, Fludioxonil, Tebuconazole, Cyproconazole, Triticonazole, Penconazole, Bromuconazole, Hexaconazole, Propiconazole, Fluquinconazole, Triadimenol, myclobutanil, and Epoxiconazole. Myclobutanil, for example, is a preventive and curative fungicide with multiple diseases. It acts by inhibiting the demethylation of sterols, thus blocking the synthesis of ergosterol necessary for the proper development of fungi. Tebuconazole is very effective against many cereal diseases. In other side, triazole fungicides can give rise to endocrine-related side effects on humans and fauna (Zam et al. 2003).

Despite the fact that their use provides undeniable benefits by increasing agricultural production, however, pesticide residues can be found in food and constitute a potential risk for consumers. Moreover, owing to their characteristics such as the high chemical and photochemical stability also low biodegradability and easy transport in the environment, gives them back persistent in soil and water (Bromilow et al. 1999; Wang et al. 2011).

3. DETECTION TECHNIQUES FOR ORGANIC POLLUTANTS

3.1. Gas Chromatography

Gas chromatography (GC) is a separation technique that applied to gaseous compounds or compounds that can be vaporized by heating without decomposition (Santos et al. 2002). Since its introduction in the 1960s, GC has been quickly used for the analysis of pesticide multi-residues, thanks to the high selectivities and sensitivity that can be achieved. It is generally used for the analysis of thermostable, volatile or semi-volatile molecules, or moderately polar (Santos et al. 2002). Today, GC is applied to approximately 60% of the pesticides and their metabolites available on the market. The development of capillary columns has greatly contributed to the significant increase in separation power. The

possibility of using sensitive and selective detectors also contributed to the rise of the GC (Van der Hoff et al. 1999).

It is indeed compatible with many detectors: Flame ionization detector (FID), the thermoionic detector (TID) or the electron capture detector (ECD). The FID is considered to be the least selective detector for the analysis of pesticides. The EDC is particularly suitable for the analysis of electronegative compounds including many halogenated pesticides. Detection limits can be up to a hundred times lower than those obtained with an FID detector. The thermionic detector is used for molecules composed of nitrogen and phosphorus atoms such as pesticides from the triazine family and organophosphates (Santos et al. 2002; Tanabe et al. 2005).

3.2. High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) makes it possible to separate the constituents of a mixture based on the selective distribution of the analyses between a liquid mobile phase in which they are soluble and a stationary phase which exerts on them a retarding effect. It allows the separation of a large number of compounds and in particular those which are thermosensitive (Thammana et al. 2016).

To the triazole detection, HPLC seems to be the most suitable separation method for pesticides for the accuracy and precision of detection. Among the different types of existing liquid chromatography, the reverse phase partition one is specifically useful in pesticide analyses.

In general, the choice of the detection system depends closely on the nature of the solutes to be sought and their concentrations. The detection systems coupled to the chromatography are varied and each has specific sensitivities. Only the mass spectrometer offers universal detection of a majority of molecules, it is a powerful tool with high sensitivity and high specificity (Malviya et al. 2010).

3.3. High Performance Liquid Chromatography Coupled to Mass Spectrometry Detector and Quadrupole Time of Flight Analyzer (HPLC-MS-QTOF)

For triazole detection, the best analytical instrument used is the HPLC-MS-QTOF named also HPLC-MS en tandem. It was largely cited in bibliography due to its high selectivity, sensibility and rapidity (Hernàndez et al. 2005; Baker et al. 2004; Jin et al. 2007; Xu et al. 2019).

3.3.1. Mass Spectrometer

Mass spectrometry is a physico-chemical analysis technique used to detect, identify and quantify molecules of interest by measuring their mass. Its principle lies in the separation in the gas phase of charged molecules (ions) according to their mass / charge ratio (m / z). In addition, mass spectrometry allows characterization the chemical structure of molecules by fragmenting them. Mass spectrometry consists in a first step to produce ions of the desired

molecule in the gas phase (Figure 1). We obtain the mass spectrum of the molecule, m / z as a function of intensity, with m the mass and z the charge carried by the ion or the fragment (Wilhelm et al. 2003; Medhe et al. 2018).

The composition of a mass spectrometer is as follows:

- A source of ionization allowing the passage in the gas phase of the sample but also the ionization of molecules
- An analyzer allowing to separate the ions according to their m / z ratio,
- A detector transforming the impact of ions into an electrical signal.

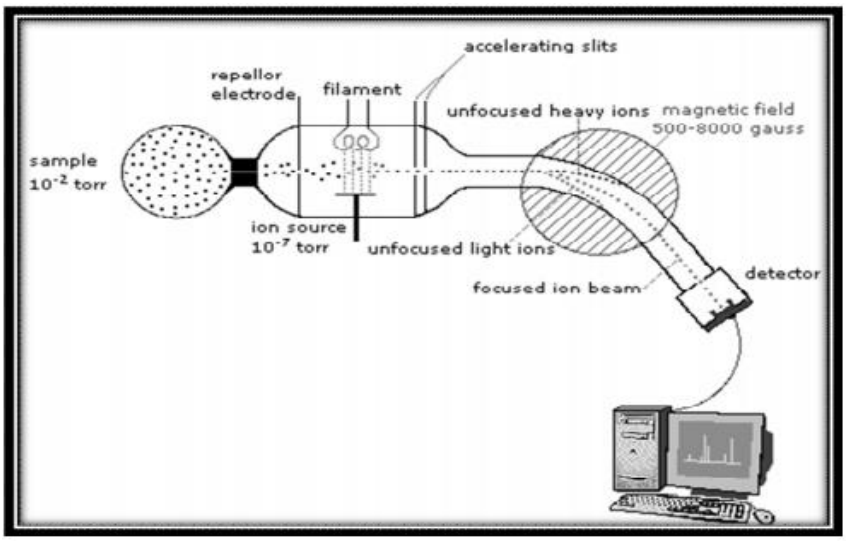

Figure 1. Mass spectrometer (Neetu et al. 2012).

3.3.1.1. Ionization Source: Electrospray Ionization Source (ESI)

Electrospray ionization has become the interface of choice for studies of dissociation in the source and in the collision cell of organic compounds in tandem mass spectrometry, due to its ability to ionize macromolecules and its strong field of application.

3.3.1.2. Electrospray History

The action of an electric field on a liquid is a phenomenon known since the end of the 19th century. Lord Rayleigh was the first to describe the instability of a charged droplet in 1882.

A liquid subjected to a sufficiently strong electric field polarizes. The liquid then containing an excess of charges takes the form of a cone, which emits a jet of liquid at its tip. The liquid jet, then forms a multitude of charge droplets in order to compensate for the instability of the charged liquid by surface tension forces. The first scientific observation of this phenomenon was reported by the physicist John Zeleny in 1914. The interaction between a liquid and an electric field was physically described 50 years later by Taylor. Finally, it was Malcolm Dole's experiments in the late 1960s and early 1970s who suggested that the electrospray mechanism could be used to measure high masses of molecules. Electrospray analysis of polymers such as polystyrenes resulted in the formation of single and multi-

charged ions. It was in 1984 that Yamashita and Fenn successfully reported the coupling of an electrospray ion mass spectrometer with a quadrupole (Yamashita et al. 1984). They demonstrate with this device that ions can be obtained without fragmentation from non-volatile molecules. A little later, the first examples of biomolecule spectra obtained by electrospray appear.

3.3.1.3. Desolvation/Ionization Mechanism

The principle of the ESI is developed in three stages, making it possible to obtain, at the end, single or multi-charged ions in the gas phase.

1. Electrospraying of the liquid in the presence of an electric field creating charged droplets,
2. Coulomb explosion of charged droplets activated by strong desolvation of the liquid,
3. Consecutive disintegration (fission) of the charged droplets leading to highly charged micro-droplets emitting solvated ions in the gas phase, which will be released from the residual solvent in the desolvation zone.

3.3.1.4. Electro-Nebulization of Liquid under Influence of an Electric Field

The solution is injected at a low flow rate (10 µL min^{-1}) via a very fine introduction capillary (0.75 µm internal diameter). This capillary is separated a short distance (3 cm) from a counter-electrode whose opening allows the passage of ions to the analyzer. A potential of 2 to 5 kV is then applied either to the capillary or to the counter-electrode, thus creating an electric field of high intensity at the level of the capillary (Bruins et al. 1998).

The value of this electric field can be evaluated, in the absence of a solution, by equation 1, where Vc represents the voltage applied to the capillary, d the distance separating the counter electrode from the capillary and rc the internal radius of the capillary.

$$E_c = \frac{2Vc}{rc \ln rc(4d)} \quad (1)$$

This relation indicates to us that the field Ec is proportional to Vc and inversely proportional to rc. Under the effect of the electric field (positive for the study of positive ions and negative for that of negative ions), ions of the same polarity migrate to the surface of the liquid.

The polarity of these ions is fixed by that of the potential applied (positive or negative) to the introduction capillary or to the counter electrode. For example, as far as we are concerned, charges in positive mode are redistributed in the liquid, creating an excess of positive ions on its surface (Tang et al. 1999) and negative charges are eliminated. The surface of the liquid is thus deformed by the accumulation of positive charges on its surface.

This phenomenon is the consequence of the opposition of two opposite forces: one of electrical origin attracting positive ions to the surface of the liquid, the second of mechanical origin preventing the ions from escaping from the liquid. The shape of the surface of the liquid thus becomes conical; it is the Taylor cone (Wilhelm et al. 2003; Tsuchiya et al. 1998).

3.3.1.5. Factors Influencing Ionization

Electrospray ionization is a process by which polar and ionic species solution can be converted into ions in the gas phase. A large number of instrumental and experimental factors control the process of ionization.

Despite the debates over the theoretical characteristics describing the theory of the Electrospray Ionization Mass Spectrometry (ESIMS), the experimental dependencies are not concentrated on a factor, but rather on a series of variables. Indeed, the variables surrounding the solution, the gas phase and the transition between them influence the ionization process. The ESI strongly depends on the chemistry of the solution (pKa, conductivity and surface tension), electrophoretic migration, proton affinity and molecular structure of the analyte, as well as the experimental conditions used, such as the composition of the solution (pH, type of additives and the ratio of organic solvent / aqueous solvent), interface parameters (voltage, gas flow rate and geometric configuration), and mass spectrometer parameters (temperature, voltage and type of orifice) (Andries et al. 1988).

3.3.2. QTOF Analyser

The performance of an analyzer is linked to its limit of the mass-to-charge ratio of the analyzable ions (m/z), its ion transmission and its resolution.

The transmission is the ratio between the number of ions arriving at the detector and that produced in the source. For example, a quadrupole (scanning) or time of flight analyzer will transmit ions differently during the analysis. Finally, the resolution corresponds to the ability to provide signals that can be distinguished for two ions of neighboring masses. We admit that two peaks are resolved when the intensity of the valley between these peaks is equal to 10% of the intensity of the weakest peak (we will speak of resolution at 10%). The resolution for an isolated peak is calculated by the ratio between the width of the peak δm at 10% of its maximum and mass.

Q-TOF is the most performant analyzer due to its capacity to analyse higher molecular weight (Allen et al. 2019).

The Q-TOF-MS is a 'hybrid' instrument combining quadrupole technologies with a time-of-flight mass analyzer. It works by accepting ions coming from quadrupole, where they will be reaccelerated inside the ion modulator region of the time-of-flight analyzer and by an electric field ion are pulsed and accelerated orthogonally to their original direction (Chernushevich et al. 2001). Then, ions having captured the same kinetic energy come in the flight tube where ions mass separation will take place which is a field free drift region where mass separation occurs. Ions revealing a lighter mass will acquire a shorter time of flight than the heavier ones. Modern time-of-flight analyzers use a reflective device which cares for adjusting the kinetic energy dispersion and spatial extended of ions present the same *m/z* but different velocities and contribute to the same *m/z* arrival at the detector at the same time.

4. EXTRACTION TECHNIQUES OF ORGANIC POLLUTANTS

4.1. Soxhlet Extractor

In 1879 von Soxhlet developed an extraction system which has long been the most widely used technique (Jensen et al. 2007). Indeed, extraction with the Soxhlet extractor has

been a standard technique for more than a century. It allows the treatment of solid samples of all particle sizes with organic solvents. The solid matrix is introduced into a cellulose cartridge fixed on a solvent container which is surmounted by a coolant (Figure 2). The heat solvent is vaporized then condensed at the level of the tank and remains in contact with the solid. When the volume of solvent condensed in the tank reaches a certain level, the solvent goes back into the tank heater by a siphon phenomenon to be recondensed again while entraining the compounds of interest in the tank where they will finally be concentrated after several cycles of extraction (Wang et al. 2006).

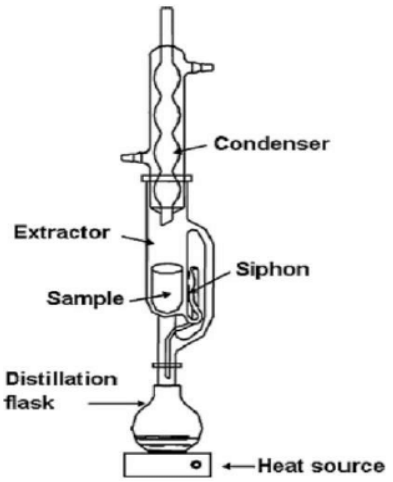

Figure 2. Schematic diagram of soxhlet extractor (Aris et al. 2014).

4.1.1. The Advantages and Disadvantages of Soxhle Extraction

The advantages of the Soxhlet extractor are as follows: the sample quickly comes into contact with a fresh portion of the solvent, which helps to shift the transfer equilibrium towards the solvent. This method does not require filtration after extraction. The Soxhlet is independent of the solid matrix (Luque de Castro et al. 2010).

The most significant drawbacks of this method, compared with other conventional techniques are: the long extraction time and the large amount of solvent consumed, which not only leads to economic losses but also poses environmental problems. As the samples are brought to a high temperature for a relatively long period, the risk of thermodestruction of certain compounds should not be overlooked if the organic matter contains thermolabile compounds.

Given the large amount of solvent used, the subsequent evaporation/concentration step becomes limiting. Local warm-ups are also possible. This technique is limited from the point of view of the selectivity of the solvent and is not easily automated (Luque de Castro et al. 2010).

4.1.2. Example of Study 1: Distribution of Organochlorine Pesticides in Sediment Cores from the Bizerte Lagoon (Tunisia) (Necibi et al. 2019)

The Bizerte Lagoon is one of the most economically important areas in Tunisia, known as a fishery and aquaculture parks related to the presence of three mussel sectors. It is subject

to intensive industrial activities such as oil refineries, metallurgical activities, naval constructions and tire productions (Ben Ameur et al. 2013)(Figure 3).

The analysis of the OCPs in the different sediment layers was carried out in two sediment cores (SCs) results and average compositions of studied compounds are presented in Table 3. Levels of OCPs for SC1 and SC2 ranged between 26.98 ± 0.04 and 11.65 ± 0.02 ng g^{-1} at 3cm depth and varied between 14.48 ± 0.03 and 1.47 ± 0.02 ng g^{-1} at 20 cm depth. The pp' DDT and its metabolites pp' DDD and pp' DDE were higher than other OCPs investigated in this study. The sedimentary profile observation shows low concentrations of OCPs in the deeper sediments notably in SC2. The comparison of the contamination in the deep sediments with that of the superficial sediments in SC1 shows a decrease in the concentrations of the OCPs and the ΣDDT down to the bottom of the SCs. The OCPs concentrations remain relatively height at 9 and 12 cm depth (Table 3). The vertical evolution of the organic matter in sediments shows, on the majority of the SCs, a decrease of the TOC contents with the depth. Higher TOC contents in SC1 than in SC2 could greatly contribute to OCPs accumulation due to their high affinity for organic matter (Table 3). The increase in the organic matter, content promotes the accumulation of hydrophobic pollutants. Therefore, sediments rich in organic matter have a better ability to retain the hydrophilic pollutants.

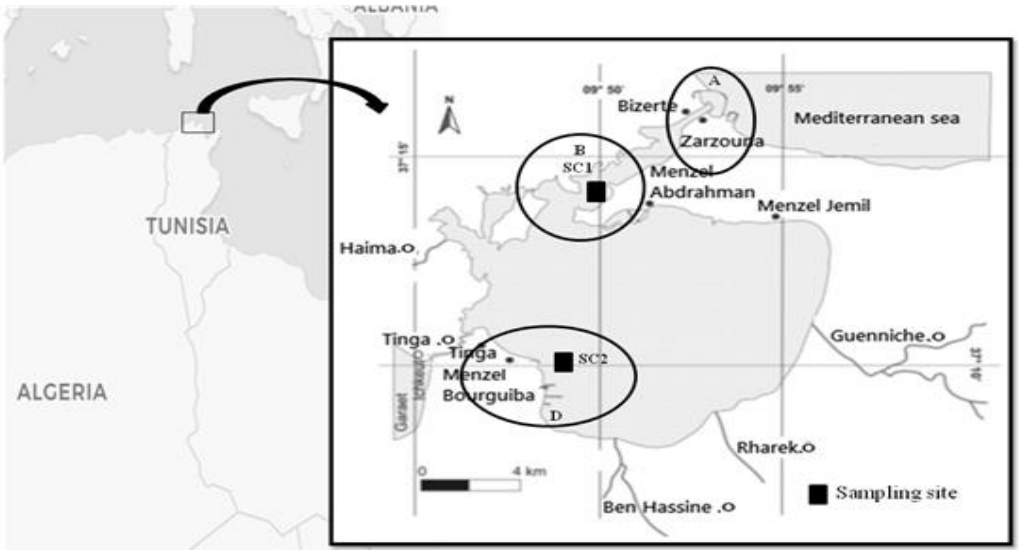

Figure 3. Study areas with sampling locations in the Bizerte Lagoon (Necibi et al. 2019).

Table 3. Levels of OCPs (ng g^{-1}), ΣDDT (ng g^{-1}) and TOC (%) in sediment cores from the Bizerte Lagoon(Mean; expressed in nanograms per gram dry weight±SD; expressed in %; n = 3)

	Sediment core 1			Sediment core 2		
Depth (cm)	∑DDT	∑OCPs	TOC %	∑DDT	∑OCPs	TOC %
3	26.94 ± 0.01	26.98 ± 0.04	3.07	8.41 ± 0.07	11.65 ± 0.05	1.39
6	8.76 ± 0.03	10.23 ± 0.02	2.05	4.57 ± 0.68	6.24 ± 0.34	1.20
9	12.47 ± 0.01	16.79 ± 0.03	2.95	10.18 ± 0.18	11.77 ± 0.11	1.10
12	10.26 ± 0.03	14.90 ± 0.03	2.16	2.64 ± 0.01	3.58 ± 0.01	1.13
15	13.36 ± 0.01	13.42 ± 0.02	2.46	3.93 ± 0.02	6.03 ± 0.02	1.70
20	10.43 ± 0.02	14.48 ± 0.03	2.93	1.01 ± 0.01	1.47 ± 0.02	0.05

4.1.3. Example of Study 2: Distribution of Organochlorine Pesticides in Suspended Particulate Matter and Sediment from the Bizerte Lagoon, Tunisia (Necibi et al. 2019)

4.1.3.1. Residual Levels of OCPs in SPM

The concentrations of OCPs compounds in SPM samples are summarized in Table 4. Levels of total OCPs ranged from 0.91 to 15.15 ng g^{-1} in SPM. The less contaminated stations of SPM were S1, S3 and S5 with ∑OCPs concentration 0.91, 3.65 and 3.30 ng g^{-1} respectively. The most polluted stations of SPM were S9, S10 and S13 with ∑OCPs concentrations 13.74, 15.15 and 12.94 ng g^{-1}, respectively. The highest concentration of OCPs in the SPMsamples was 15.15 ng g^{-1} recorded for station S14, while the lowest one was 0.91 ng g^{-1} observed at station S1. It could be explained for station S1 by the exchange of water between the lagoon and the Mediterranean Sea, which reduced the concentration of such type of organic contaminants. Station S14 was near the city of Menzel Abderrahman which receives direct inputs of untreated urban and industrial sewage (Figure 4). The ∑DDT represented the sum of pp' DDD, pp' DDE and pp' DDT. The ∑DDT were detected in five sampling stations from the Bizerte Lagoon SPM samples. The highest level of ∑DDT was observed in S5 (5.05 ng g^{-1}) near the mouth of Tinja River which represent a dumping site for agricultural inputs.

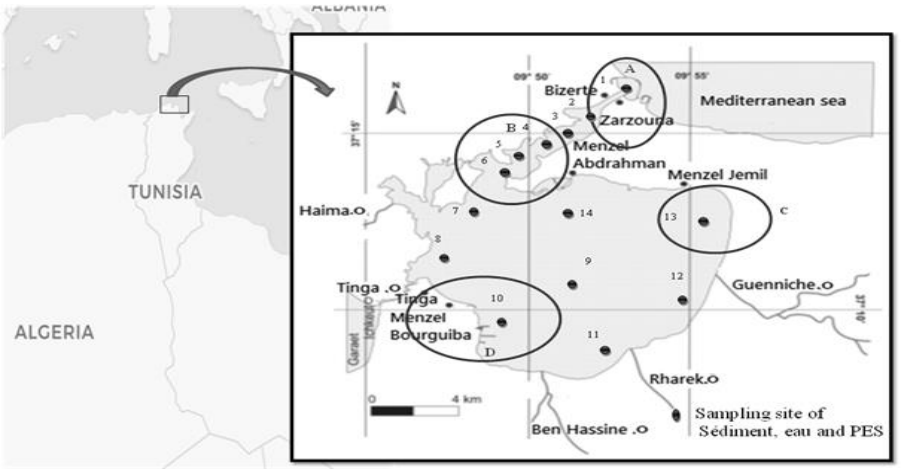

Figure 4. Study areas with sampling station and different types of industrial areas (A, B, C and D) in the Bizerte Lagoon (Necibi et al. 2019).

4.1.3.2. Residual Levels of OCPs in Sediment

Fourteen samples of superficial sediments were analyzed. The results of the OCPs analysis are summarized in Table 4. HCB and pp' DDE were detected in the thirteen-sampling station. The highest concentration of ΣOCPs was registered in station S5 (16.29 ng g^{-1}) while the lowest concentration was recorded at station S3 (0.09 ng g^{-1}). Relatively high concentrations of ΣOCPs (15.96 ng g^{-1}) were also found in station S10 located in Zone D which is characterized by significant industrial and agricultural activities. The levels of pp' DDT and its metabolites in this study varied in the order of pp' DDT> pp' DDE> pp' DDD. The levels of ΣDDT obtained varies between 0.03 and 13.49 ng g^{-1}, between ND and 8.89 ng g^{-1} for pp' DDD and between ND and 3.66 ng g^{-1} for pp' DDE. The highest concentration of pp' DDT (8.79 ng g^{-1}) was recorded in station S5. This station is close to zone B

characterized by industrial activities (cement and metallurgy). The TOC% in sediment ranges from 0.05% recorded in station S3 to 5.22% recorded in station S5. The TOC% found in the different stations are presented in table 4. The lowest percentages of TOC% are founded at stations S12 (0.43%), and S13 (0.47%), these two stations are therefore considered the least rich in organic matter. The TOC % of the rest of the sediment samples are also considered relatively low (<2%).

Table 4. Concentrations of OCPs in SPM and sediment (ng g^{-1}) collected from the Bizerte Lagoon

	matrice	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14
HCB	sediment	0.07± 0.14	0.12± 0.04	0.05± 0.71	0.36± 0.2	1.97± 0.44	0.72± 0.18	2.89± 0.03	0.4± 0.01	0.28± 0.03	3.96± 0.43	ND	2.58± 0.23	1.06± 0.08	1.71± 0.05
	SPM	0.25± 0.14	1.92± 0.14	1.56± 0.15	0.89± 0.03	0.35± 0.15	1.06± 0.33	0.81± 0.01	0.09± 0.01	5.92± 0.19	1.17± 0.71	0.37± 0.01	0.77± 0.05	0.68± 0.33	4.96± 0.65
Heptaclor	sediment	ND	ND	ND	0.06± 0.01	0.16± 0.03	1.22± 0.55	0.25± 0.03	ND	0.65± 0.5	1.4± 0.07	0.04± 0.01	0.69± 0.09	0.05± 0.04	ND
	SPM	ND	ND	2.09± 0.12	ND	ND	ND	ND	ND	ND	ND	3.11± 0.17	1.12± 0.01	ND	ND
Aldrin	sediment	ND	0.04± 0.01	ND	0.03± 0.01	0.58± 0.02	2.3± 0.09	0.23± 0.04	ND	0.01± 0.01	0.84± 0.09	ND	ND	0.22± 0.01	ND
	SPM	0.66± 0.09	3.22± 0.03	ND	ND	ND	ND	ND	ND	6.75± 0.05	2.76± 0.03	2.03± 0.01	ND	1.92± 0.04	1.25± 0.09
Dieldrin	sediment	0.56± 0.18	ND	0.03± 0.01	ND	0.05± 0.01	ND	0.01± 0.02	0.01± 0.02	ND	0.02± 0.01	ND	0.01± 0.01	ND	ND
	SPM	ND	ND	ND	ND	0.24± 0.04	ND	ND	ND	ND	ND	ND	ND	ND	ND
Endrin	sediment	0.26± 0.03	ND	0.01± 0.01	ND	0.04± 0.01	ND	ND	ND	ND	ND	ND	ND	ND	ND
	SPM	ND	ND	ND	1.19± 0.02	0.91± 0.01	2.05± 0.15	ND	0.85± 0.03	1.07± 0.11	6.09± 0.19	ND	3.97± 0.48	2.25± 0.10	1.13± 0.15
Lindane	sediment	ND	ND	ND	0.19± 0.01	ND	ND	0.55± 0..3	ND	ND	ND	ND	ND	ND	ND
	SPM	ND	ND	ND	4.03± 0.13	1.80± 0.09	ND	ND	ND	ND	ND	2.83± 0.15	1.54± 0.91	8.09± 0.03	2.54± 0.09
pp' DDD	sediment	5.93± 0.34	ND	ND	ND	3.04± 0.18	ND	5.23± 0.78	2.84± 0.27	ND	8.89± 0.13	ND	0.13± 0.01	0.13± 0.01	ND
	SPM	ND	ND	ND	ND	ND	1.50± 0.05	1.52± 0.09	ND	ND	3.96± 0.13	ND	ND	ND	ND
pp' DDT	sediment	ND	ND	ND	ND	8.79± 0.73	ND	ND	ND	ND	ND	ND	ND	ND	ND
	SPM	ND	ND	ND	ND	ND	ND	1.60 ± 0.08	ND	ND	1.17± 0.41	ND	ND	ND	ND
pp' DDE	sediment	1.55± 0.31	0.63± 0.09	ND	0.04± 0.01	3.66± 0.24	0.88± 0.36	0.87± 0.16	0.34± 0.04	0.45± 0.05	0.85± 0.02	2.3± 0.13	1.02± 0.02	0.03± 0.01	0.03± 0.01
	SPM	ND	1.48± 0.04	ND	ND	ND	1.10± 0.01	ND	5.05± 0.61	ND	ND	ND	ND	ND	ND
∑DDT	sediment	7.48± 0.22	0.63± 0.03	ND	0.04± 0.01	15.49± 3.07	0.88± 0.12	6.10± 0.31	3.18± 0.10	0.45± 0.02	9.74± 0.05	2.30± 0.04	ND	0.16± 0.01	0.03± 0.01
	SPM	ND	1.48± 0.01	ND	ND	ND	2.60± 0.02	3.12± 0.06	5.05± 0.20	ND	5.13± 0.18	ND	ND	ND	ND
∑OCP	sediment	8.37± 0.08	0.79± 0.01	0.09± 0.10	0.65± 0.03	18.29± 0.51	5.12± 0.13	10.03± 0.10	3.59± 0.02	1.39± 0.08	15.96± 0.09	2.34± 0.01	4.43± 0.05	1.49± 0.02	1.74± 0.01
	SPM	0.91± 0.03	6.62± 0.03	3.65± 0.04	6.11± 0.03	3.30± 0.04	5.71± 0.07	3.93± 0.01	5.99± 0.03	13.74± 0.05	15.15± 0.16	8.34± 0.05	7.40± 0.21	12.94± 0.07	9.88± 0.14
TOC %	sediment	1.99	2.28	0.05	0.66	5.22	4.06	1.73	1.44	1.26	2.02	1.18	0.43	0.47	1.19

4.2. Liquid-Liquid Extraction (LLE)

Liquid-liquid extraction (LLE) is an ancient enrichment technique based on the distribution of analytes between two immiscible liquid phases, usually an aqueous phase, which contains a polar solvent, for example, water, and an organic phase non-polar which contains an organic solvent. The purpose of the extraction is to transfer analytes from the water to a more suitable solvent for injection into the gas chromatograph and at the same time increasing the concentration of the analytes. Depending on the distribution coefficients of the analyte between the two phases, the extraction can be more or less complete. In addition, its selectivity is low and non-polar solvents can extract the analytes from the aqueous matrix, so that interference may occur during the analysis.

4.2.1. The Advantages and Disadvantages of Liquid-Liquid Extraction

The advantages of LLE are its simplicity, which allows rapid development of the method, however the extraction time is quite long, it uses a large amount of solvents (some are very expensive and sometimes toxic), it does not meet the current requirements of green chemistry (Robles-Molina et al. 2013) it has not been automated and the risk of emulsion formation is high.

4.2.2. Example of Study: Distribution and Partitioning of Aliphatic Hydrocarbons and Polycyclic Aromatic Hydrocarbons between Water, Suspended Particulate Matter, and Sediment in the Harbours of the West Coast of the Gulf of Tunis (Tunisia) (Mzoughi et al. 2011).

Harbors of la Goulette, Rades and Sidi Bou Said are the principal largest and most important port within the Gulf of Tunis characterised by an instantaneous influence of various activities (sailing, industry and fishing) to the Mediterranean (Figure 5).

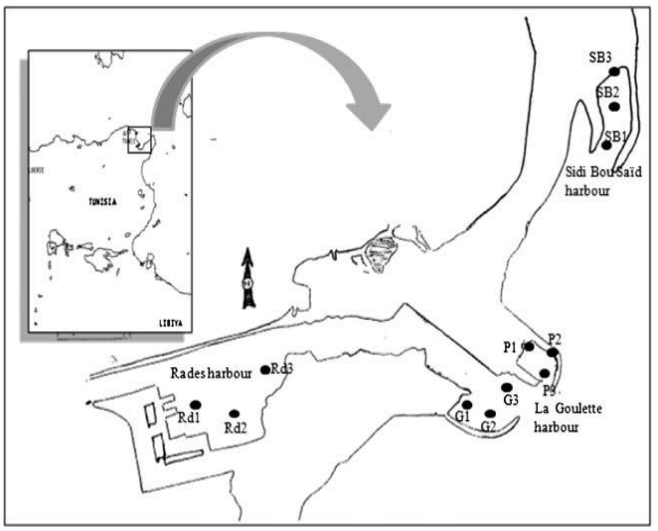

Figure 5. Location map of studied samples (Mzoughi et al. 2011).

Concentrations of total aliphatic hydrocarbons (PAH) in water and SPM samples ranged from 251.4 ng L^{-1} to 1096.5 ng L^{-1} and from 3976 ng g^{-1} to 10 067.7 ng g^{-1} respectively in summer and from 311.5 ng L^{-1} to 1054.3 ng L^{-1} and from 1299 ng g^{-1} to 12 484 ng g^{-1} respectively in winter (Tables 5 and 6). The highest concentrations of PAH in water and SPM in summer and in winter were recorded for station P1 of la Goulette harbour and therefore the lowest level of PAH was recorded for station SB3 of Sidi Bou Said harbour. In fact, the distribution of aliphatic fraction (F1) in water and SPM varied from 24% to 63% of total hydrocarbons (average 50%) and from 42% to 69% (average 60%) respectively in summer and from 46% to 85% of total hydrocarbons (average 66%) and from 38% to 88% (average 65%) respectively in winter. Percentages of aliphatic fraction (F1) in water and SPM found during the summer and winter were statistically significantly different ($p < 0.05$). Total PAH concentrations in water and SPM samples varied from 149.8 ng L^{-1} to 1008.3 ng L^{-1} and from 2573.5 ng g^{-1} to 8222.4 ng g^{-1} respectively in winter and from 139.2 ng L^{-1} to 693.8 ng L^{-1} and from 909.9 ng g^{-1} to 5858.2 ng g^{-1} respectively in summer (Tables 5 and 6).

Table 5. Concentration of aliphatic (AH), polycyclic aromatic (PAH) in sediment (ng g^{-1}) and different ratios characterizing the origin of hydrocarbons

	Season	Rd1	Rd2	Rd3	G1	G2	G3	P1	P2	P3	SB1	SB2	SB3
Pr/Ph	Summer	0.6	0.8	1.2	0.9	0.4	0.9	0.9	0.1	0.6	0.9	1	1
	Winter	0.6	0.9	1.3	0.6	0.7	0.7	0.4	0.7	0.8	0.5	0.4	0.6
	Average	0.6	0.9	1.3	0.8	0.6	0.8	0.7	0.4	0.7	0.7	0.7	0.8
PAH	Summer	306.7	642.7	968	787.7	978	1053.9	1096.5	490.7	761.5	343.6	731.8	251.4
	Winter	540.1	1034.6	708.2	912	675.5	462.4	1054.3	761.2	1020.9	495.4	924.5	311.5
	Average	423.4	838.6	838.1	849.8	826.8	758.2	1075.4	626	891.2	307	382.1	139.2
PPAH	Summer	272.9	185.4	597.3	218.8	326.1	535.7	693.8	561.4	321.2	964.2	475.8	149.8
	Winter	948.2	861.5	755	571.5	556.8	981.6	1008.3	636.5	800.6	635.6	428.9	144.5
	Average	610.5	523.4	676.2	395.1	441.5	758.6	851.1	462.5	560.9	0	0	0
Phe/An	Summer	2.1	1.7	0.5	0	2.1	3.4	2.8	0	1.2	0.6	0.3	0.3
	Winter	0	1	0.3	0.4	0	0.3	0	0	24.8	0	0.1	0.1
	Average	0	1.4	0.4	0	1	1.9	1.4	0	8.3	2.4	0	0
Fl/Py	Summer	0	25.4	1.6	2.9	4.1	3.7	0	7.5	0	1	2.2	1.2
	Winter	0	1.2	1.5	0.5	0.6	0.2	0.9	0	0.7	1.7	0	0
	Average	0	1.3	1.5	1.7	2.3	2	0.4	0	0.3	0	0	0
BaP/BeP	Summer	0	0	0	0	0	0	0	0	0	0	0	0
	Winter	0	0	0.2	0	10	0	0	0	0.5	0	0	0
BaA/Chr	Summer	0	0	0	0	0	0	0.2	0	0	0	0	0
	Winter	0	0	0.7	22.8	0	3.4	0	0.1	0.3	0	0	0
Total TEQ	Summer	6.3	44.9	3.6	0	74.9	8.3	11.4	14.5	18	3.6	3.7	3.3
	Winter	3.4	0	0.2	0.7	0	1.93	2.8	0	0.2	0	15.7	2
	Average	4.9	22.4	1.9	0.3	37.4	5.1	7.1	7.3	9.1	1.8	9.7	2.6

Table 6. Concentration of aliphatic (AH), polycyclic aromatic (PAH) in SPM (ng g^{-1}) and different ratios characterizing the origin of hydrocarbons

	Season	Rd1	Rd2	Rd3	G1	G2	G3	P1	P2	P3	SB1	SB2	SB3
Pr/ Ph	Summer	0.4	0.3	1.5	0.9	0.5	0.5	0.6	0.5	0.5	0.7	0.5	0.9
	Winter	0.3	0.6	1.2	0.1	0.2	0.2	0.2	0.2	0.4	0.1	0.2	0.4
	Average	0.4	0.5	1.4	0.5	0.4	0.4	0.4	0.4	0.5	0.4	0.4	0.7
PAH	Summer	8099.4	5206.9	4989.3	4520.6	7776.1	6009.2	10067.7	9960.6	7180.1	5898.9	7237.9	3976
	Winter	6092	7011	8508	7888.8	5768.3	6550.1	12484	6314.1	6535.4	3994.5	2950.2	1299
	Average	7095.7	6109	6748.7	6204.7	6772.2	6279.7	11275.9	8137.4	6857.7	4946.7	5094.1	2637.5
PP-AH	Summer	3262.2	4193	2629.2	4115	2114.6	2851.5	5858.2	2174.3	2050	4754	2456	909.9
	Winter	4548.5	3738.3	7000.5	5108.8	4293.1	3299.4	8222.4	4456.1	3074.7	5517	3357	2573.5
	Average	3905.3	3965.7	4814.9	4611.9	3171.5	3075.4	7040.3	3315.3	2594.7	5135.5	2906.5	1741.7
Phe/ An	Summer	1.7	5.1	2.5	0.1	1.4	0.9	0.4	1.6	1.9	20.9	1.6	1.3
	Winter	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.9	0	0
	Average	1	2.6	1.3	0.1	0.7	0.5	0.3	0.8	1	8.5	0.8	0.6
Fl/ Py	Summer	0	0.1	0	0.2	0.3	0.1	0.2	0.3	0.3	0.2	0.6	0.3
	Winter	1.1	26.5	0.6	1.1	1.7	1.5	1.4	0	1.3	7.1	1.5	1.2
	Average	0.6	1.3	0.3	0.6	1	0.8	0.8	0.1	0.8	1.7	1	0.7
BaP/ BeP	Summer	0.6	0	0	0	0	0.1	0	0	8	0	0.6	0.1
	Winter	2.3	0	7.1	2.1	5.1	7.1	11.4	7.4	0	1.5	4.5	11.3
BaA/ Chr	Summer	0	0	0	0	0	0.2	1.2	0	0.6	0	0	0.6
	Winter	05	1.1	2.1	0.3	1	0.8	2.4	1.1	0	0.2	0	0
Total TEQ	Summer	1090.3	687	653.8	81.3	363.5	288.1	712.1	639.1	629.1	402.7	277.9	714.5
	Winter	35718	78.6	4261.7	1780.7	3031.9	4261.7	14486	193.4	39225.3	14597.3	6274.4	4261.7
	Average	18404.1	382.8	2457.8	931	1697.7	2274.9	7599	416.2	19927.6	7491	3276.1	2488.1

Pristane to phytane: Pr/Ph, phenanthrene to anthracene: Phe/An, fluoranthene to pyrene: Fl/Py, benz(a)pyrene to benz(e)pyrene: BaP/BeP, benz(a)anthracene to chrysene: BaA/Chr and Total toxic benzo(a)pyrene equivalent: Total TEQ. Rades (Rd), La Goulette fishing (P) and passenger (G), and Sidi Bou Said (SB).

As for AH the highest concentrations of PAH in water and SPM were observed for station P1 in La Goulette harbour and the lowest levels at station SB3 in Sidi Bou Said harbour. The distribution of PAH concentrations in water and SPM varied within the range of 36% to 76% (average 50%) and 26% to 58% (average 41%) respectively in summer and between 19% to 54% (average 34%) and 12% to 62% (average 35%) respectively in winter. Percentages of PAH in water and SPM found during the summer and winter were statistically significantly different ($p < 0.05$). The reduced AH and PAH water concentrations within the least stations could also be explained by the transfer of hydrocarbons to SPM and to decantation and accumulation in the marine sediments. This accumulation might be associated with the sediment's characteristics, seawater flux and other physical parameters.

4.3. QuEChERS Extraction Method

A new trend in analytical chemistry is the development of "ecological" methods of sample pre-treatment, which are solvent-free or minimize the amount of solvent used; they must be at the same time, faster and more selective than conventional procedures (Pinto et al. 2010).

Figure 6. Schematic representation of a QuEChERS procedure applied to environmental samples (Tette et al. 2016).

The QuEChERS procedure, an acronym for the quick; cheap; effective; robust and safe was developed by Anastassiades and his colleagues in 2003 (Anastassiades et al. 2003). It has been used as a new approach to extract pesticides from fruits and vegetables. Since then, it has become a very versatile technique and has been adapted to a wide range of substances and matrices (Lesueur et al. 2008; Norli et al. 2011). This procedure is a simple sample preparation technique based on liquid extraction with an organic solvent followed by cleaning using dispersive solid phase extraction.

The principle of the QuEChERS method reveals three main stages:

1. The first step consists of a liquid / solid extraction of the sample with manual stirring for one minute with an organic solvent.
2. The second step consists in adding a buffer and salts to promote the separation of the two organic and aqueous phases and also the preferential transfer of organic pollutants to the organic phase. This step is particularly important if the sample contains a large amount of water. When analyzing dry sediments, this step may not

be considered. The sample is then centrifuged and the collected supernatant is optionally concentrated.

3. The third step is to purify the extract on different solid phases. It is carried out by adding one or more adsorbents (C18, primary and secondary amine adsorbent (PSA)). The mixture is then stirred manually for one minute. The interfering compounds of the matrix will become trapped on the adsorbents. The supernatant is recovered for analysis (Romero-Gonzalez et al. 2011). Figure 6 shows an example of the QuEChERS procedure applied to environmental samples.

4.3.1. The Advantages of Extraction with QuEChERS

This method offers many advantages. First, it appears as effective and robust as conventional extraction methods (Soxhlet, ultrasound, etc.). The method replaces many complex analytical steps commonly used in traditional methods, and provides high quality results with a short analysis time (10 min as extraction time), low amount of solvent, minimum of glassware used, little of work and low cost of analysis by sample (Pinto et al. 2010).

This extraction technique is considered to be relatively gentle (extraction with an organic solvent and mechanical stirring) thus minimizing the risks of extraction of interference and a priori simplifying the chromatographic analysis. The QuEChERS technique provides good recovery and reproducibility and is much cheaper than other sample preparation techniques.

4.3.2. Example of Study 1: Determination of Synthetic Musks in Surface Sediment from the Bizerte Lagoon by QuEChERs Extraction Followed by GC-MS (Necibi et al. 2016)

The QuEChERS method was then applied for the determination of SMs compounds for thirteen surface sediment samples collected from the Bizerte Lagoon (Figure 7). The concentrations found for SMs were presented in table 7. For HHCB and AHTN, concentrations were 1.09-2.8 ng g^{-1} and 0.31-1.7 ng g^{-1}, respectively. The concentration levels for ADBI, AHTN, ATII, MM, MX and MK were clearly below the LOQ (Table 7).

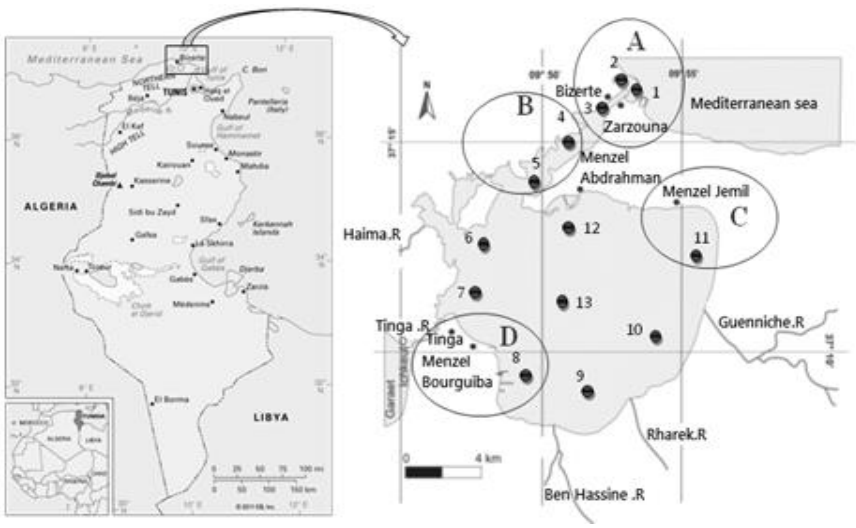

Figure 7. Study areas with sampling locations and different types of industrial areas (A, B, C and D) in the Bizerte Lagoon (Necibi et al. 2016).

Table 7. Concentrations (ng g^{-1}) and TOC (%) of SMs in surface sediment of the Bizerte Lagoon

compounds	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13
ADBI	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
AHMI	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
ATII	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
HHCB	2.3	2.8	1.69	1.50	2.16	1.40	1.26	1.23	1.50	1.10	1.84	1.71	1.09
AHTN	0.7	1.7	0.47	0.32	0.62	0.51	0.42	0.41	0.54	0.31	0.51	0.30	0.31
MM	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
MK	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
MX	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
∑ musks	3.0	4.5	2.2	1.8	2.8	1.9	1.7	1.6	2.0	1.4	2.3	2.0	1.4
HHCB/ AHTN	3.3	1.6	3.6	4.7	3.5	2.7	3.0	2.8	3.0	3.5	3.6	5.7	3.5
TOC (%)	1.99	0.66	2.28	0.55	5.22	4.06	1.73	2.02	1.26	1.18	0.43	0.47	1.19

<LOD: Below limits Of Detection.

The highest concentrations of ∑SMs were found at the station S1 (3.0 ng g^{-1}) and S2 (4.5 ng g^{-1}) in the mouth of the Bizerte channel and were slightly higher along the channel in comparison with the rest of the stations. The concentration of ∑SMs was 2.3 ng g^{-1} at S11 and 1.4 ng g^{-1} at S10 and S13, respectively. The predicted no effect concentration (PNEC) in sediment for marine organisms was set at 8.4 mg/kg dw for HHCB and at 5.2 mg/kg dw for AHTN (HERA 2004). The levels of HHCB and AHTN investigated in this study were much lower that the PNEC. In order to take into account variable input of organic matter in surface sediment from Bizerte Lagoon its influence on SMs concentrations, TOC were measured in the sediment of the different sampling stations (Table 7). The highest TOC % was recorded in sampling site S5 (5.22 %), while the lowest was found in S11 (0.43 %). The SMs in marine environment were subject to different degradation process which could explain the absence of relationship between synthetic musk concentrations and TOC.

4.4. Solid Phase Extraction (SPE)

4.4.1. Definition of SPE

Solid phase extraction (SPE), first introduced in the mid-1970s, is the most widely used technique today when analyzing traces in liquid samples (Sabi et al. 2000). It is a powerful and necessary tool for the development of methods for analyzing organic pollutants at very low concentrations in water. Since its introduction into the sample pretreatment protocols, this method has undergone many modifications and adaptations depending on the nature of the analytes to be extracted and according to the objectives to be achieved moreover (SPE), is certainly the most commonly used extraction and purification technique for aqueous or organic matrices. The goal is to isolate and concentrate the analytes from the matrix and obtain an extract which can be injected on an analytical device. The SPE was developed to replace the long, tedious and hard to reproduce liquid-liquid extraction methods. The basic principle of SPE is that one of liquid chromatography. In chromatography, the migration of the various families of molecules is slowed down by selectively retaining them on an

adsorbent, in SPE these same molecules are blocked on this adsorbent and then they are selectively eluted with a specific solvent.

4.4.2. SPE Protocol

The extraction protocol on an SPE cartridge includes several steps.

- Activation of the adsorbent

This involves percolating through the column a few mL of an appropriate solvent, such as methanol, for adsorbents based on grafted silica, or an acid / base solution for ion exchangers.

- The conditioning of the adsorbent.

In order to obtain an adsorption environment similar to that of the water sample, the activated adsorbent is washed with a few mL of demineralized water or a suitable buffer solution.

- Application of the sample.

It consists in passing a volume of water through the pre-concentration column. The interesting solutes are fixed on the adsorbent.

- Purification

Extraction on a solid phase is unfortunately not always selective enough, which means that there will be other compounds which will be trapped by the adsorbent. Some interferences can be eliminated by applying a washing solution consisting of water and a small proportion of an organic solvent.

- Drying

The drying step is necessary in the case where the solvent for elution of the trapped solutes is not miscible with water. The drying is carried out by a water bath.

- Desorption

It is a question of recovering the fixed solutes by eluting them with a small volume, either of a hydro organic mixture, or of pure organic solvent.

4.4.3. Example of Study 1: Response Surface Methodology Approach for the Preparation of a Molecularly Imprinted Polymer for Solid Phase Extraction of Fenoxycarb Pesticide in Mussels (Atayat et al. 2019)

The SPE was widely applied in various ways for the extraction of different analytes from water (Sanchís et al. 2012; Székács et al. 2015; Binsalom et al. 2016), soil (Yegemova et al. 2015) and environmental matrix (Faraji et al. 2019). The solid-phase extraction was

developed using molecularly imprinted polymer (MISPE) for the analysis of fenoxycarb (Fnx) pesticide from mussel's sample. In this work, the optimization of the MIP synthesis for the recognition of Fenoxycarb was performed toward two factors, VACN and QEGDMA, using the ED approach. The Response Surface Methodology approach demonstrated to be useful for the determination of the optimum polymer that was obtained with VACN of 2.2 mL and QEGDMA of 1.345 mmol. Finally, the optimum MIP showed good selectivity properties compared to other carbamates and was successfully used as sorbent of a MISPE cartridge for the determination of Fnx in real mussel samples. The range of linearity was 0.3–30 mg/L of Fnx (R2 = 0.991), with a LOD value of 0.247 mg/L. The recovery of Fnx extracted from mussel samples of the Mediterranean Sea was around 97% (n = 3) with RSD between 6 and 7%, proving the reliability of the developed method. Therefore, the MISPE device developed in this work can be a promising alternative for the selective extraction and analysis of Fnx from mussel samples.

5. DETECTION TECHNIQUES OF INORGANIC POLLUTANTS

5.1. Heavy Metal

Metals occur naturally within the crust, and their contents within the environment can vary between different regions leading to spatial variety of background concentrations. The distribution of metals within the environment is governed by the properties of the metal and influences of environmental factors (Morais et al. 2010). Of the 92 present elements, approximately 30 metals and metalloids are potentially toxic to humans, Be, B, Li, Al, Ti, V, Cr, Mn, Co, Ni, Cu, As, Se, Sr, Mo, Pd, Ag, Cd, Sn, Sb, Te, Cs, Ba, W, Pt, Au, Hg, Pb, and Bi. Heavy metals are that the generic term for metallic elements having a relative mass above 40.04 (the mass of Ca) (Ming-Ho et al. 2005). Heavy metals enter the environment by natural and anthropogenic means. Such sources include: natural weathering of the earth's crust, mining, erosion, industrial discharge, urban runoff, sewage effluents, and pest or disease control agents applied to plants, pollution fallout, and variety of others (Ming-Ho et al. 2005). Although some individuals are primarily exposed to those contaminants within the workplace, for several people the foremost route of exposure to those toxic elements is through the diet (food and water) (Yuan et al. 2004). The contamination chain of heavy metals nearly always follows a cyclic order: industry, atmosphere, soil, water, foods and human. Although toxicity and thus the resulting threat to human health of any contaminant are, of course, a function of concentration, it's well-known that chronic exposure to heavy metals and metalloids at relatively low levels can cause adverse effects Agency for Toxic Substance and Disease Registry (ATSDR). Therefore, there has been increasing concern, mainly within the developed world, about exposures, intakes and absorption of heavy metals by humans (Che et al. 2002).

5.2. Atomic Absorption Spectrometry

Atomic absorption spectrometry (AAS) was a technique during which free gaseous atoms absorb electromagnetic radiation at a selected wavelength to supply a measurable signal. The absorption signal is proportional to the concentration of these free absorbing atoms within the optical path. Therefore, for AAS measurements the analyte must be first converted into gaseous atoms, usually by application of warmth to a cell called the atomizer. The type of atomizer defines the two main AAS-based analytical techniques: flame AAS (FAAS) that gives analytical signals during a continuous fashion and electrothermal AAS (ETAAS) delivering analytical signals in a discontinuous mode (2–4 min per sample). In both cases, liquid (or dissolved) samples are easily introduced into the analyzer, as an aerosol in the case of FAAS or as well-defined low microliter volumes in ETAAS. Furthermore, the coupling of hydride generation and cold vapor methods allow the introduction of analytes in the atomizer as a gas phase. Also, especially in ETAAS, direct elemental analysis of solids without previous dissolution is possible (Luisa et al. 2005).

The analytical application of AAS was considerably delayed due to the apparent need for very high resolution to form quantitative measurements (typical atomic absorption lines could also be narrower than 0.002 nm, while themonochromators capable of isolating spectral regions narrower than 0.1 nm are rather expensive (Fernández et al. 2006). In 1955 Walsh (Australia) overcame this obstacle with a light source emitting narrow lines. The use of the hollow cathode lamp (HCL) as the light allowed the demonstration of the analytical applicability of AAS measuring the radiation absorbed by atomic vapor during a flame. The idea was pursued independently by Alkemade in the Netherlands and Walsh in Australia, their works being published in 1955 (Fernández et al. 2006). Nowadays, AAS is routinely employed for elemental analysis of about 70 elements. Fundamentals, key components of atomic absorption spectrometers and analytical performance characteristics of AAS-based techniques are briefly described below (Thomas et al. 1994).

5.2.1. Example of Study 1: Assessment of Heavy Metals in Sediment and in Suspended Particles Affected by Multiple Anthropogenic Contributions in Harbors (Chouba et al. 2011).

Metal concentrations in summer and winter found in sediment harbours varied between (1.586–0.242 µg g^{-1}dw (dry weight)) for Cd, (527–70 µg g^{-1} dw) for Zn, (246–12 µg g^{-1} dw) for Pb, (132–70 µg g^{-1} dw) for Cu, (45–25 mg g^{-1} dw) for Fe and (281–173 µg g^{-1} dw) for Mn (Figure 5). Concentration difference test was carried out for the heavy metals in sediment between the four sampling areas. Significant result differences (P\0.05) showed an equivalent evolution profile for the following metals: Cd, Pb, Zn and Cu. On the opposite hand, no significant difference was noticed for the Mn and Fe metals. Generally, the highest levels of all metals analyzed in summer and in winter were registered in sediment for all stations from Rades (RD) (industrial area) and La Goulette (P) (fishing) harbors (Figure 8) indicating the presence of local source of contamination. It might be attributed to many reasons as these harbours are characterized by the discharge materials, activities of fishing, antifouling paint and the two steam electrical power plants situated near the harbour and drainage sewage during this zone. The lowest metal contents were measured in Sidi Bou Said harbor (yachting) in summer and in winter sediment, only copper showed a high level after fishing

harbour (P). The levels of Pb, Cu, Zn and Fe were found in SPM. Whereas Cd and Mn weren't detected (Table 8).

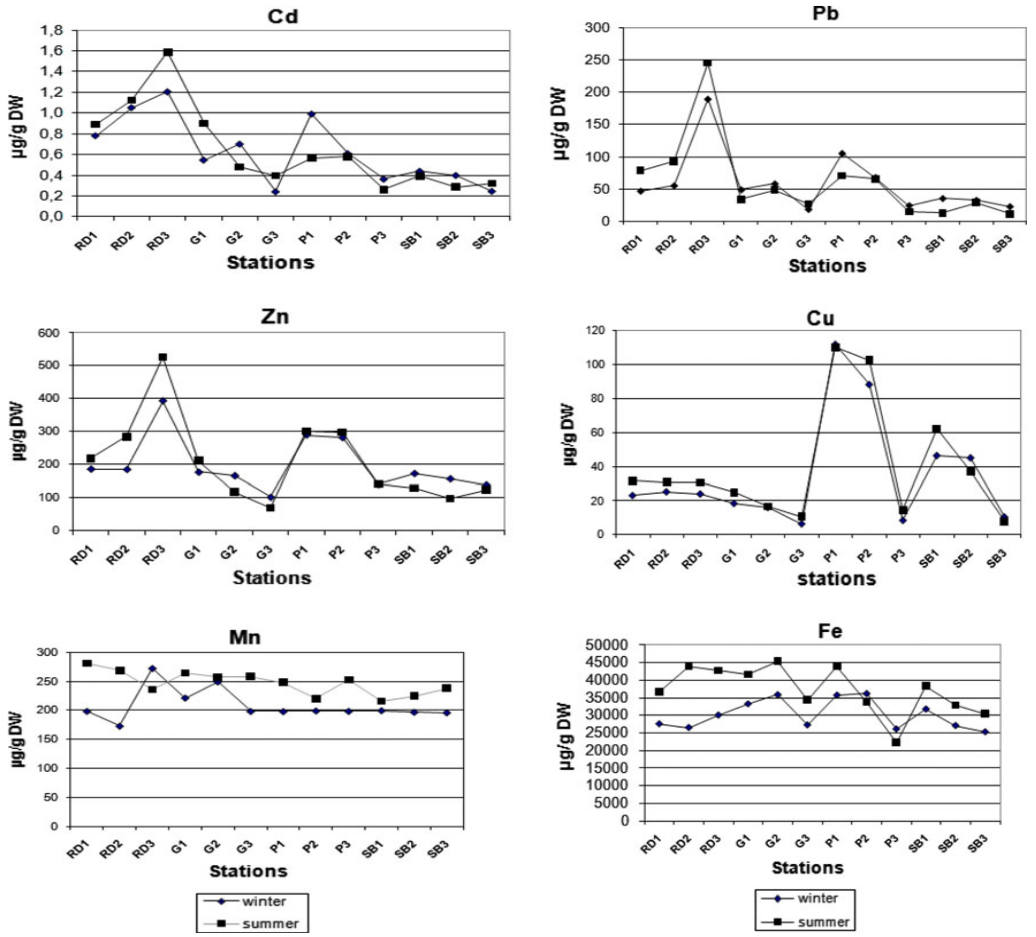

Figure 8. Concentrations (µg g^{-1}dw) of Cd, Pb, Zn, Cu, Mn and Fe in sediment collected from Rades, La Goulette (fishing and passenger), and Sidi Bou Said (yachting) harbors.

Table 8. Concentrations of Pb, Cu, Fe, Zn, Cd and Mn in the SPM (µg g^{-1}) La Goulette (fishing and passenger), Rades (commercial) and Sidi Bou Said (yachting) harbors

	Pb	Cu	Fe	Zn	Cd	Mn
RD1	6.23	2.33	1.22	1.28	ND	ND
RD2	7.93	2.53	1.24	1.27	ND	ND
RD3	14.91	3.47	1.44	1.30	ND	ND
G1	27.49	4.90	1.58	1.45	ND	ND
G2	4.42	1.17	1.54	1.18	ND	ND
G3	3.09	1.15	1.43	1.13	ND	ND
P1	4.41	0.43	1.39	1.01	ND	ND
P2	27.12	0.55	1.73	1.49	ND	ND
P3	9.75	0.48	1.34	1.17	ND	ND
SB1	8.30	11.40	1.43	1.23	ND	ND
SB2	7.69	11.72	1.31	1.19	ND	ND
SB3	5.25	8.53	1.43	1.48	ND	ND

These activities explained the source of Cu and Zn in SPM. Copper and Zinc based antifouling paints and cathodic protection devices are previously identified as important sources of those metals in estuaries and harbor sediments (Turner et al. 2010). These values observed within the SPM are due probably to the fine particles generated by sanding or blasting of boat hulls or that are produced gradually by antifouling paint (Turner et al. 2010). The levels of Pb, Cu, Zn, and Fe in SPM of varied harbours are much but those found in other harbours. Birch and O'Hea (2007) explained that the high levels of Cu and Zn in SPM caused by the difference of metal dissolutions. However, dissolved Cu concentrations increased with the increasing ambient energy, i.e., from quiescent to high precipitation to high rainfall/high wind conditions, and dissolved Zn concentrations the highest during high precipitation.

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Chapter 130

ORGANOCHLORINE PESTICIDES (OCPS) IN COASTAL MARINE ENVIRONMENTS: LEVELS, FATE, BEHAVIOR, AND EFFECTS ON BIOTA

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ABSTRACT

Pesticides have been extensibility used in the control of agricultural and sanitary pests. From World War II onward, more efficient and cheaper synthetic pesticides, such as organochlorine pesticides (OCPs), including DDT, lindane, or endosulfan, replaced the relatively environmentally friendly pesticides. OCPs were widely used worldwide; however, in the 1970s these compounds were began banned due to their harmful effects on both, biota and humans. Nevertheless, half a century later, due to their high persistence and the existence of some active sources, OCPs remain a problem for the health of marine ecosystems.

Once in the environment, their distribution depends on the interaction of several factors -explained in detail in this chapter- such as the physical-chemical properties of

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each compound; interactions with the matrix; characteristics of the environment; and intensity, distance, and seasonality of the source, between others.

This chapter reviews current levels, environmental behavior and fate, and effects on biota of organochlorine pesticides in global marine coastal environments, noting that these compounds are still detectable and could be harmful to marine biota.

Keywords: persistent organic pollutants, organochlorine pesticides, DDT, lindane, endosulfan, coastal environments, marine pollution, environmental fate, environmental behavior

INTRODUCTION

Pesticides have been used to control pests in agriculture for over 4,500 years. Until the mid-1900s, inorganic compounds, such as sulfur and copper, and plant extracts were used. In the first decades of the XX century, new synthetic pesticides such as organochlorine pesticides (OCPs) were developed; for instance, during the Second World War, these substances were used to control insect vectors of disease. At the end of the war, OCPs were adapted for pest control in agriculture and public health, being considered a miraculous pesticide due to their high effectiveness and low cost. However, in 1962, Rachel Carson documented the environmental impacts, especially on bird populations, of the widespread use of DDT in the United States, in the famous book "Silent Spring". This publication highlighted the collateral effects of the inappropriate use of some chemical substances and the controversy between benefits and costs that accompany the industrial growth and the welfare state. The publication raised the alarm of public and political opinion, generating environmental movements and massive protests, which ended with the banning, from 1972, of the agricultural use of DDT and other organochlorine pesticides in many countries (Hayes and Hansen 2017).

After the 1960s and 1970s, the harmful effects of pollutants and their presence in the environment began to be studied, and our knowledge of chemicals is quite extensive. However, new substances that threaten the environment are created daily, and sometimes, the discovery of their harmful effects arise too late. This was the case with the OCPs, which despite having been banned or restricted in almost all countries, they are still being detecting in the environment. Moreover, due to their long-term toxicity, sometimes, current environmental levels could pose a risk to biota (Girones et al. 2020) since even when environmental concentrations are low they can cause damage to the biota (Petrovic et al. 2013; Sosa-Ferrera et al. 2013).

Marine pollution processes are one of the most important problems which humanity is facing at present (Lohmann 2007). Among the most affected marine environments, coastal environments stand out as the heaviest chemically polluted. These are the areas where the main aquatic and terrestrial interactions occur. On the one hand, waste or effluents that come from different sources (urban, industrial, or agricultural) are dumped directly (sewage, landfills) or through rivers due to runoff. On the other hand, many coastal environments, especially semi-closed ones such as bays, estuaries, fjords, or coastal lagoons, have great potential to accumulate organic pollutants because the loss of pollutants by export and dilution is limited by the long residence times of water (Diamond et al. 2005), and also

because the adsorbent materials levels, especially organic carbon, are generally high. Further, coastal areas, including coastal wetlands such as coral reefs, mangroves forest, salt marshes, or seagrass beds, are very important to biota and humans because they provide invaluable ecosystem services, such as refuge, cross-ecosystem nutrient transfer, erosion control, and pollution control, between others (Barbier et al. 2011).

Then, considering this complex scenario, this chapter aimed to comprehensively review the available organochlorine pesticides data about their distribution, production, regulation, main current sources, physic-chemical properties, environmental fate and behavior, documented effects on marine biota and humans, and the current levels in global coastal and marine sediments.

PRODUCTION AND USE

The first organochlorine pesticide massively used worldwide was DDT, beginning in 1939 when Paul Hermann Muller discovered its properties as an insect poison and its low toxicity to humans. DDT was indiscriminately used during World War II (1939-1945), to protect soldiers and civilians against insect-borne diseases such as malaria, dengue, and typhus (Wheeler 1946). After the war, the used amounts increased exponentially (Hayes 1991). The effectiveness, persistence, versatility, and low cost turned DDT massive in agriculture, forestry, and public health for 30 years (USEPA 1975). In addition to protecting crops and kill household insects, the use of DDT for public health avoided the death of more than 5 million people per year worldwide. Thus, for example, in India, there were 75 million malaria cases in 1952 and after using DDT massively, only 100,000 cases have been registered in 1964 (WHO 1971). Regarding the agricultural purposes of DDT, it was used in a wide variety of crops, reaching more than 36,000 tons per year until its prohibition or restriction (Hayes and Hansen 2017). Currently, the World Health Organization recommends the use of DDT in areas where malaria is a public health problem due to its effectiveness in its control (WHO 2006), and so that, about 3,300 tons are produced annually worldwide (Hayes and Hansen 2017). Given the proven insecticidal capabilities of DDT, after World War II, other organochlorine pesticides with similar features were synthesized, such as chlordane (1945); aldrin and dieldrin (1948); heptachlor (1949); toxaphene (1949); endrin (1951); endosulfan (1956); hexachlorocyclohexane (HCH and lindane; 1940), and mirex (1950; Kennish 1997). These pesticides showed several uses and applications depending on their characteristics and regions (UNEP 2003). Following DDT, the most popular OCP worldwide was hexachlorocyclohexane (HCH) as lindane (pure γ-HCH) or technical HCH (a mixture of various isomers, including α-HCH, 55-80%, β-HCH, 5- 14%, and γ-HCH, 8-15%), which was used as a broad-spectrum insecticide in seeds, soils, foliage, trees, fabrics, and wood, as well as a treatment against ectoparasites in veterinary and human applications. Voldner and Li (1995) estimated the global use of 550,000 tons of technical HCH and 720,000 tons of lindane between 1948 and 1993. Another group widely used worldwide was the cyclodienes, which includes aldrin, dieldrin, endrin, chlordane, heptachlor, and endosulfan. Aldrin and dieldrin were used as insecticides (1950-1970) to control soil insects, the corn rootworm, the rice weevil, the grasshopper, and for crops of corn, potatoes, and cotton, and for protect wooden structures from termites. Endrin was used as an insecticide in cotton, rice, sugar cane,

corn, and other cereal crops, and as a rodenticide in orchards (Larini 1999; UNEP 2002). Meanwhile, chlordane was mainly used as an insecticide in rice, oilseeds, corn, sugar cane, and citrus crops, and in the control of domestic pests (e.g., cockroaches, ants, and termites). Heptachlor was used primarily in the control of soil insects and termites, and, sometimes, it was used against cotton insects, grasshoppers, and some crop pests, such as sugarcane, as well as to fight malaria. Endosulfan is a broad-spectrum acaricide and insecticide, for agricultural or domestic use, in a wide variety of vegetables, fruits, cereals, and cotton, as well as ornamental shrubs, trees, vines, and ornamental plants (Ulberth 2000), wood preservation (UNEP 2002), and in the control of vectors of diseases such as the tsetse fly (Li and Macdonald 2005). Another important group of OCPs was the cyclopentadienes, including toxaphene and mirex. The first was used intensively (450,000 tons from 1950 to 1993 according to Voldner and Li, 1995) as an insecticide and acaricide in cotton, cereals, fruits, nuts, and vegetable crops, and in the control of ectoparasites in livestock, such as lice, flies, ticks, scabies, and mites. While the second, mirex, was used mainly for the ant's control and, lesser degree, as a flame retardant in plastics, paint, paper, and electrical equipment (UNEP 2002). Meanwhile, the hexachlorobenzene (HCB) has been a fungicide used since 1945 to treat grain crops and to manufacture inks, fireworks, ammunition, and synthetic rubber. This compound is also a by-product of the manufacture of chlorinated industrial chemicals, such as solvents (e.g., carbon tetrachloride, trichloroethane, and pentachlorobenzene) and various pesticides (e.g., pentachlorophenol, dicloram), and the incineration of chlorinated waste.

MANAGEMENT

In 1962, Rachel Carson published "Silent Spring", the first ecological manifesto against the indiscriminate use of organochlorine compounds. There she denounced that the widespread use of DDT could be the main cause of the population reduction of various bird species, many of them occupying the top of the food chain, such as the peregrine falcon and the bald eagle, symbol of the United States (Baird 2018; D'Amato et al. 2002). Because of the results of this and other studies presented by the scientific community, Sweden, in January 1970, was the first country to ban chlorine insecticides (D'Amato et al. 2002). In the same year, the ex-USSR banned the use of DDT as a household pesticide and, in 1981, as an agricultural pesticide, except for use in disease control programs (Turusov et al. 2002). Between 1970 and 1980, the use of pesticides was banned in most developed countries (Kutz et al. 1991). It took a few more years for developing countries to ban or restrict their use, either by their own regulations or by signing international conventions. In 1998, the UN countries signed the Rotterdam Convention, where guidelines for the transport, storage, and disposition of organochlorines were proposed (Hough, 2000). Years later, in 2001, they signed the Stockholm Convention, which entered into force in May 2004, where the progressive elimination or minimization of the use and production and the safe elimination of an initial list of twelve contaminants was proposed. These compounds and those added later are considered persistent organic pollutants (POPs) due to their high environmental persistence, toxicity, and ability to bioaccumulate and transport long distances. Among the POPs listed in the Stockholm Convention are the following organochlorine pesticides: aldrin, endrin, dieldrin, chlordane, heptachlor, DDT, HCB, mirex, and toxaphene, added to the initial list that entered into force in 2004; α-HCH, β-HCH, γ-HCH (lindane), and Chlordecone,

added in 2009; and endosulfan and Dicofol, incorporated in 2011 and 2019, respectively. However, the Stockholm Convention notes exceptions to the use of some pesticides: DDT can be used in the fight against disease vectors when there are no safe, effective, and affordable local alternatives; endosulfan can be used in specific crop and pest combinations; lindane can be used as a second-line treatment for the control of pediculosis and scabies in humans.

CURRENT SOURCES TO COASTAL ENVIRONMENTS

Although the use and production of organochlorine pesticides have been restricted worldwide since the 1970s, the marine environment continues incorporating them. One of the main sources is the continental exports from obsolete reserves and contaminated areas, which act as secondary sources. In a global report published in 2003 (UNEP 2003) and many current national implementation plans, it was observed that information on OCPs stockpiles in most developing countries is poor, scarce, or null (UNEP 2003, Girones et al. 2020). Anyway, the UNEP global report (UNEP 2003) was able to record the existence of large stockpiles in Europe (80,000 tons, mainly in central and eastern Europe) and in Sub-Saharan Africa (120,000 tons). Moreover, agricultural and urban soils where pesticides were used or produced in the past and river sediments associated with these areas constitute deposits that slowly release pesticides into the marine environment, reaching the coastal waters by processes, which will be explained later.

Another current source for the marine environment, probably less important due to its severe restriction, is the use and legal production of some OCPs. These are contemplated in the exceptions of international treaties and some national laws.

Finally, a potentially important source appears to be the illegal use of pesticides in agriculture in some countries (Van Den Berg et al. 2017). Several authors observed patterns between the parent pesticides and their metabolites (e.g., DDT/(DDE+DDD) and technical endosulfan/ endosulfan sulfate) that would indicate recent use of them (Carvalho et al. 2009; Chen et al. 2020; Girones et al. 2020; Jin et al. 2017; Rizzi et al. 2017a).

ENVIRONMENTAL FATE AND BEHAVIOR OF OCPS

Once OCPs enter the environment, their fate, behavior, and spatio-temporal distribution are controlled by a combination of three groups of factors (Mackay et al. 2006). First are the physic-chemical properties of pollutants such as molecular composition, structure, weight, and volume, and solubility in the different media, which influence the partition and reaction trends between environmental compartments and the rate of degradation in other chemical species. Second are the environmental conditions such as temperature, fluxes, and the composition of the various environmental compartments, i.e., air, water, and solids. Third are the patterns of use/release to the environment, such as the periodicity and intensity of the source.

Factor: Nature and Physic-Chemical Properties of OCPs

Organochlorine pesticides are a group of chemicals that share certain structural characteristics. They have a highly chlorinated aliphatic or aromatic cyclic structure, and in some cases are associated with oxygen or sulfur atoms. Consequently, most OCPs are resistant to biological, physical, and chemical degradation. This makes the behavior profiles of the OCPs in the environment, such as the phase distribution and the transport processes, tend to play a fundamental role in the control of their environmental fate and distribution. The variability in these profiles, usually, is attributed to some key physicochemical properties inherent in each chemical compound: solubility in water, solubility in octanol, vapor pressure, the three partition coefficients between air, water, and octanol (Figure 1), and susceptibility to degradation or transformation reactions. Furthermore, other essential molecular descriptors are molar mass and molar volume.x

Solubility is the maximum ability of a solvent, such as distilled water or octanol, to dissolve chemicals. Since most OCPs exhibit low solubility in water, which is even lower in seawater due to the salty effect, up to 5.8 times lower (Cetin et al. 2006), and high solubility in octanol (a compound assimilable to lipids and organic matter) the octanol-water partition coefficient (K_{OW}) is high, i.e., they are hydrophobic and lipophilic.

Meanwhile, the vapor pressure (VP), which can be considered as "air solubility", is the saturation concentration or solute pressure in a gas phase, and is expressed as mol/m^3. The relationship between VP and the solubility in water (mol/m^3) determines Henry's law constant (H; Pa.m^3/mol) and the partition coefficient between air and water (K_{AW}), which is calculated as $K_{AW} = H/RT$, where R is the ideal gas constant (8,314 J/mol.K) and T is the absolute temperature (K). Vapor pressure affects the atmospheric transport of the OCPs. Compounds with the higher VP reach longer distances to the source (Muir et al., 1999), therefore, the different congeners of the same pesticide but with different VP, such as the α-, β-, γ-HCH isomers, leave the atmosphere at different speeds due to precipitation and air-water gas deposition, resulting in discrimination in long-distance transport (Iwata et al. 1993). However, there is a common misconception that substances with very low VP, i.e., "non-volatile" (or very poorly soluble in the air) such as DDT and other OCPs, cannot partition to the atmosphere (Mackay et al. 2006). This is not necessarily the case because these substances also have low solubility in water, therefore Henry's law constants are relatively high and can be appreciably divided from water to the atmosphere (Mackay et al. 2006). This partition is very sensitive to change in temperature due to the large enthalpy change associated with transfer to the vapor phase, which would explain the global distillation of OCPs, evaporating in warm areas and condensing in cold areas.

Another important partition coefficient to understand the environmental behavior of OCPs is the octanol-air partition coefficient, K_{OA}, which is mainly used in continental environments to evaluate the diffusive exchange between air and foliage, aerosol particles, or soils (Finizio et al. 1997; Mackay et al. 2006), and is calculated experimentally, although it generally follows this equation: $K_{OA} = K_{OW}/K_{AW}$.

In addition to K_{OW}, K_{AW}, and K_{OA} (Figure 1), other partition coefficients are used to model and predict the environmental fate of OCPs, such as K_{OC} (organic carbon-water partition coefficient), K_D (sorption partition coefficient for all the soil on a dry weight basis), K_{OM} (organic matter-water partition coefficient), and K_B or BCD (water-to-fish bioconcentration factor). These coefficients are calculated from laboratory or field

measurements or are estimated from the relationship with other coefficients, for example, K_{OC} is often estimated to be equivalent to 0.35 K_{OW} (Seth et al. 1999), K_{OM} is 0.56 K_{OC} (i.e., 56% organic carbon in organic matter; Mackay et al. 2006), and K_B is 0.05 K_{OW} (i.e. 5% lipid in fish; Mackay 1982). However, these estimates can vary considerably from the real values due to the complexity of the matrices and environmental conditions, either due to the variation in pH, content and type of organic carbon, temperature, pollutant concentration, polarity, and age, or the metabolic capacity of the organisms, and so that, care must be taken when using them.

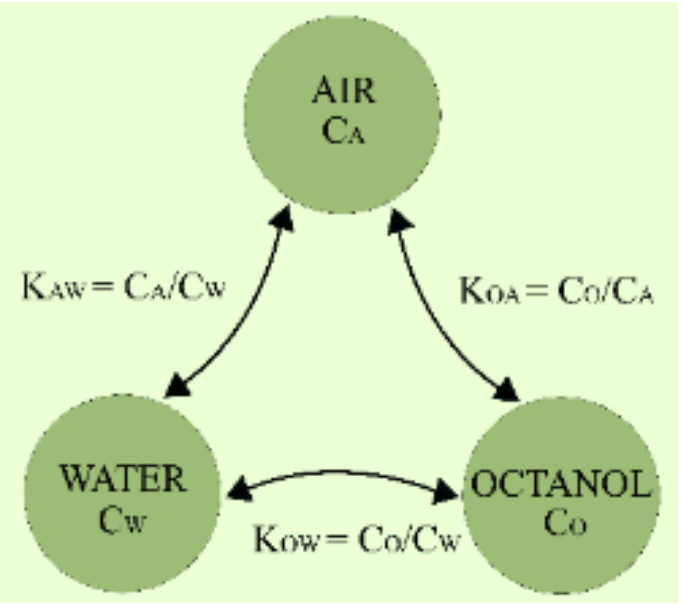
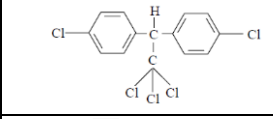
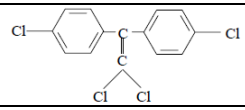
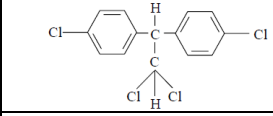
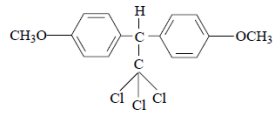
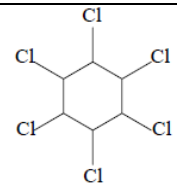


Figure 1. Diagram of relationships between the three solubilities (C_A, C_W, C_O) and the three partition coefficients (K_{AW}, K_{OW}, K_{OA}).

Another characteristic that notably affects the environmental fate and distribution of OCPs is their half-life in the environment. Since this is affected by the nature of the compartments and the environmental conditions, it is impossible to document a single reliable value for this parameter. However, a quite reliable half-life range can be established for each compound. Hence, Table 1 summarizes the ranges for the half-life of the OCPs estimated for average environmental conditions according to Mackay et al. (2006) for the main abiotic media: air (A), sediment (S), and water (W). Given the different exposures to weathering agents, in general, the OCPs half-life class in water is 3 times higher than in air (30 times slower degradation) and the half-life class in sediments is 2 times higher than in water; i.e., if the half-life class in the air is 2, in water will be 5 and in sediments will be 7. This is summarized and shown in Table 1, in conjunction with the structure and physicochemical properties of parent OCPs and their main metabolites.

Table 1. General characteristics and physicochemical properties of parents organochlorine pesticides and their main metabolites: Common name - IUPAC name; CAS number [x-x-x]; molecular formula and structure; molecular weight (MW); melting point (MP); water solubility (Sw, mg/L at 25°C); vapor pressure (VP, Pa at 25°C); Henry's Law constant (H, $Pa.m^3.mol^{-1}$); octanol-water partition coefficient at 25°C (log K_{OW}); octanol-air partition coefficient at 25°C (log K_{OA}); organic carbon-water partition coefficient at 25°C (log K_{OC}); Half-life class in air (A), water (W), and sediment (S): 1 (<10 h), 2 (10-30 h), 3 (30-100 h), 4 (100-300 h), 5 (300-1000 h), 6 (1000-3000 h), 7 (3000-10000 h), 8 (10000-30000 h), 9 (> 30000 h)

p,p'-DDT - 1,1,1-trichloro-2,2-bis-(4-chlorophenyl)-ethane						
	[50-29-3]	$C_{14}H_9Cl_5$	MW: 354.49	MP: 108.5	Sw: 0.149	VP: 0.0005
	H: 1.100	log K_{OW}: 6.39	log K_{OA}: 9.73	log K_{OC}: 5.4	Half-Life. A:4 ; W:7 ; S:9	
p,p'-DDE - 1,1-dichloro-2,2-bis-(p-chlorophenyl)-ethylene						
	[72-55-9]	$C_{14}H_8Cl_4$	MW: 318.03	MP: 89	Sw: 0.251	VP: 0.003
	H: 4.200	log K_{OW}: 6.93	log K_{OA}: 9.70	log K_{OC}: 5.0	Half-Life. A:4 ; W:9 ; S:9	
p,p'-DDD - 1,1-Dichloro-2,2-bis (4-chlorophenyl)ethane						
	[72-54-8]	$C_{14}H_{10}Cl_4$	MW: 320.04	MP: 109.5	Sw: 0.736	VP: 0.001
	H: 0.500	log K_{OW}: 6.33	log K_{OA}: 10.03	log K_{OC}: 5.0		
Methoxychlor - 1,1,1-trichloro-2,2-bis(4-methoxy-phenyl)ethane						
	[72-43-5]	$C_{16}H_{15}Cl_3O_2$	MW: 345.65	MP: 87	Sw: 0.045	VP: 0.001
	H: 0.999			log K_{OC}: 4.9	Half-Life. A:2 ; W:4 ; S:7	
Hexachlorocyclohexane (HCH) - 1,2,3,4,5,6-Hexachlorocyclohexane						
α-HCH	[319–84–6]	$C_6H_6Cl_6$	MW: 290.83	MP: 158	Sw: 1.000	VP: 0.100
	H: 0.872	log K_{OW}: 3.81	log K_{OA}: 7.46	log K_{OC}: 3.8		
β-HCH	[319–85–7]	$C_6H_6Cl_6$	MW: 290.83	MP: 309	Sw: 0.100	VP: 0.026
	H: 0.116	log K_{OW}: 3.80	log K_{OA}: 8.74	log K_{OC}: 3.4		
γ-HCH	[58-89-9]	$C_6H_6Cl_6$	MW: 290.83	MP: 112.5	Sw: 7.300	VP: 0.027
	H: 0.149	log K_{OW}: 3.70	log K_{OA}: 7.74	log K_{OC}: 3.0	Half-Life. A:5 ; W:8 ; S:9	
δ-HCH	[319–86–8]	$C_6H_6Cl_6$	MW: 290.83	MP: 141.5	Sw: 8.000	VP: 0.027
	H: 0.073	log K_{OW}: 4.14				

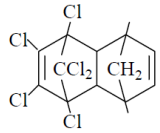
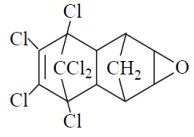
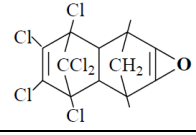
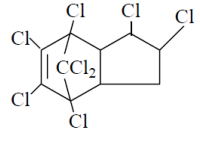
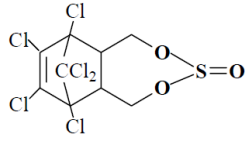
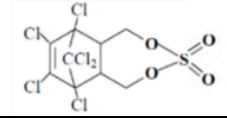
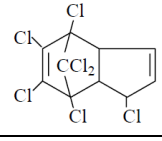
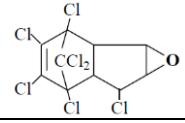
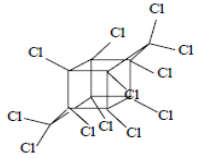
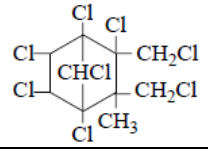
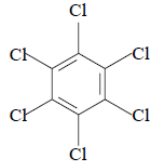
Aldrin - 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endoexo-5,8-dimethano-naphthalene						
	[309-00-2]	$C_{12}H_8Cl_6$	MW: 364.9	MP: 104	Sw: 0.985	VP: 0.064
	H: 23.000	log K_{OW}: 6.24	log K_{OA}: 8.26	log K_{OC}: 2.6	Half-Life. A:4 ; W:8 ; S:9	
Dieldrin - 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-exo-1,4-endo-5,8-dimethanonaphthalene						
	[60-57-1]	$C_{12}H_8Cl_6O$	MW: 380.91	MP: 175.5	Sw: 4.952	VP: 0.014
	H: 1.100	log K_{OW}: 5.48	log K_{OA}: 8.84	log K_{OC}: 4.1		
Endrin - 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-=exo-1,4-exo-5,8-dimethanonaphthalene						
	[72-20-8]	$C_{12}H_8Cl_6O$	MW: 380.91	MP: 245*	Sw: 1.143	VP: 0.003
	H: 1.100	log K_{OW}: 4.94	log K_{OA}: 8.32	log K_{OC}: 4.0		
Chlordane - 1,2,4,5,7,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindane						
***Cis*-chlordane**	[57-74-9]	$C_{10}H_6Cl_8$	MW: 409.78	MP: 106	Sw: 0.533	VP: 0.007
	H: 5.700	log K_{OW}: 6.20	log K_{OA}: 8.83	log K_{OC}: 5.5	Half-Life. A:4 ; W:8 ; S:9	
***Trans*-chlordane**	[5103-74-2]	$C_{10}H_6Cl_8$	MW: 409.78		Sw: 0.615	VP: 0.010
	H: 6.800	log K_{OW}: 6.27	log K_{OA}: 8.83	log K_{OC}: 5.5	Half-Life. A:4 ; W:8 ; S:9	
Endosulfan [115-29-7] - 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a–hexahydro-6,9-methano-2,4,3-=benzodioxathiepin-3-oxide						
α isomer	[959–98–8]	$C_9H_6Cl_6O_3S$	MW: 406.93	MP: 106	Sw: 2.564	VP: 0.004
	H: 0.700	log K_{OW}: 4.94	log K_{OA}: 8.49	log K_{OC}: 3.4		
β isomer	[33213–65–9]	$C_9H_6Cl_6O_3S$	MW: 406.93	MP: 207-209	Sw: 36.216	VP: 0.004
	H: 0.045	log K_{OW}: 4.78	log K_{OA}: 9.53	log K_{OC}: 3.5		
Endosulfan sulfate - 6,7,8,9,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3,3-dioxide						
	[1031–07–8]	$C_9H_6Cl_6O_4S$	MW: 422.93	MP: 181		
Heptachlor - 1,4,5,6,7,8,8-heptachloro-3a-4,7-7a-tetrahydro-4,7-endo-methanoindene						
	[76-44-8]	$C_{10}H_5Cl_7$	MW: 373.32	MP: 95.5	Sw: 1.307	VP: 0.130
	H: 38.000	log K_{OW}: 5.94	log K_{OA}: 7.76	log K_{OC}: 4.4	Half-Life. A:3 ; W:5 ; S:7	

Table 1. (Continued)

Heptachlor epoxide - 1,4,5,6,7,8-heptachloro-2,3-epoxy-2,3,3a-4,7,7atetrahydro-4,7-methanoindene						
	[1024–57–3]	$C_{10}H_5Cl_7O$	MW: 389.32	MP: 160	Sw: 5.061	VP: 0.022
	H: 1.700	log K_{OW}: 5.42	log K_{OA}: 8.59	log K_{OC}: 4.0		
Mirex - 1,1a,2,2,3a,4,5,5,5a,5b,6-dodeca-chlorooctahydro-1,3,4-metheno-1H-cyclobuta(cd)pentalene						
	[2385-85-5]	$C_{10}Cl_{12}$	MW: 545.54	MP: 485	Sw: 0.000065	VP: 0.0001
	H: 839.400	log K_{OW}: 6.90		log K_{OC}: 6.0	Half-Life. A:4 ; W:6 ; S:9	
Toxaphene - 1,4,5,6,7,7-hexachloro-2,2-bis(chloromethyl)-3-methylidenebicyclo[2.2.1]heptane (Mixture of chlorinated camphene with 67–69% Cl content)						
	[8001-35-2]	$C_{10}H_{10}Cl_8$	MW: 413.81	MP: 65-90	Sw: 0.500	VP: 0.0009
	H: 0.745	log K_{OW}: 5.50		log K_{OC}: 5.0		
Hexachlorobenzene (HCB) – hexachlorobenzene						
	[118–74-1]	C_6Cl_6	MW: 284.78	MP: 228.83	Sw: 0.399	VP: 0.094
	H: 65.000	log K_{OW}: 5.64	log K_{OA}: 7.21	log K_{OC}: 4.9	Half-Life. A:8 ; W: 9 ; S: 9	

*Water solubility (mg/L at 25ªC), Vapor pressure (Pa at 25ªC), Henry's Law Constant (H/Pa.m^3.mol^{-1}), log K_{OW}, and log K_{OA}, for hexachlorocyclohexanes, methoxychlor, mirex, HCB, and toxaphene were selected from Mackay et al. (2006), and for the other compounds from Shen and Wania (2005). Log K_{OC} and half-life in the air (A), water (W), and sediment (S) for all compounds were selected from Mackay et al. (2006).

Factor: Environmental Characteristics and Conditions

As mentioned above, knowing the physicochemical properties of OCPs is not enough to understand and predict their behavior and environmental fate, it is also necessary to know the advective transport, the phase heterogeneity, and the prevailing environmental conditions (Ciffroy 2018). Advective transport is understood as the transport of the pollutant given by the movement of the phases in which it is dissolved or associated, for example, the movement of air masses and water in a river or ocean currents, and sedimentation or sediment resuspension (Figure 2). While the heterogeneity of the phases is understood as the presence of different fractions or domains within the same environmental compartment (atmosphere, water, soil, or sediment; Figure 2). The partition between the different fractions or domains of each compartment is a determining factor in the distribution, bioavailability, and toxicity of OCPs and, therefore, their study is essential to understand the environmental behavior of OCPs.

Water

Despite being hydrophobic, water is the main transport means for OCPs from their source to their sink, i.e., the atmosphere, sediment, or biota. Rains can wash particles or water from OCP-contaminated stockpiles or soils into rivers or storm/sewage drains by runoff and subsequently discharge them into the sea (Figure 2). At all stages (source, transport, and sink) several processes affect the phase distribution of the OCPs, mainly, the division between fractions of the same phase and other phases, and loss due to degradation or irreversible sorption.

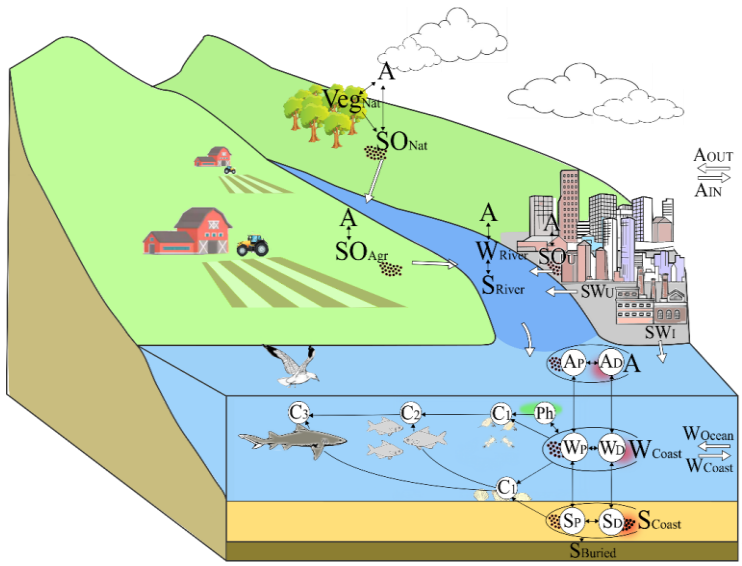


Figure 2. Schematic representation of the main organochlorine pesticide fate processes in coastal environments. A: air; A_P: air particulate fraction; A_D: air dissolved fraction; W: water; W_P: water particulate fraction; W_D: water dissolved fraction S: sediment; S_P: sediment particulate fraction; S_D: sediment dissolved fraction; C: consumer (C1, C2, C3: trophic levels); Ph: producers; SO_{AGR}: agricultural soil; SO_{NAT}: natural soil; SO_U: Urban soil; SW_U and SW_I: urban and industrial sewage; Veg: vegetation.

As can be seen in Table 1, the K_{OW} range is from x to x showing low water solubilities, hence, the OCPs tend to concentrate in the water particulate fraction. This trend is even greater in salty media because the solubility of OCPs can be up to 5.8 times lower than in distilled water due to the salty effect (Cetin et al. 2006).

The fraction of particles comprises solids that, according to their mass and the predominant local hydrodynamics, remain as suspended particles or yield to gravity, settling and becoming the sediment bed. Due to its high affinity for particles, the OCPs distribution both in sediments and in water in marine coasts is generally associated with the sedimentation patterns characteristic of inland discharges, in which coarser particles precipitate near the river mouth and finer and lighter particles and colloids (particles of ø <1 μm) precipitate further away or remain in suspension (Burgess 2012). However, other processes such as resuspension, bioturbation, turbulence, and changes in salinity and temperature play an important role in the fate of particles and OCPs in these environments (Ciffroy 2018).

Sediment

Dissolved, organism-accumulated, or particle-associated OCPs in the water column can become part of the sediment by advective transport, e.g., precipitation of particles or organic matter, and, lesser degree, by diffusive transport, through a concentration gradient. In the sediment, OCPs associated with particles/organic matter can bury under sediment layers or adsorb irreversibly and persist for decades (Doody 2002), metabolize by microbiological or chemical activity, or become secondary sources of contamination by resuspension or diffusion into the water column, either naturally, i.e., abiotic disturbances or bioturbation (action of benthic organisms), or anthropic, as occurred during dredging tasks.

In this compartment, as in the water column, OCPs are distributed between the dissolved and particulate fractions. This partitioning is largely regulated by the interaction of the compound with dissolved organic matter (DOM) or sedimentary organic matter (associated with sediment particles; SOM). Since the K_{OW} and K_{OC} of OCPs are in the high range, normally, under the same environmental conditions, their sedimentary levels are directly correlated to the content of SOM. However, sometimes, this relationship fails because the SOM is heterogeneous, varying the sorption capacity of OCPs from the sediment. Thus, two different types of SOM can be physically and chemically differentiated: the amorphous or "soft" domain and the condensed, "hard", or vitreous domain (Weber et al. 1992). The first is the dominant form of SOM and comprises mainly humic and fulvic substances (Xiao et al. 2004). Furthermore, it has a low sorption capacity of OCPs (and other chemicals) because it has linear sorption isotherms and relatively fast sorption and desorption rates, with little or no difference between both rates (e.g., Weber et al. 1992; Xiao et al. 2004). Instead, the condensed domain is a minor part of the SOM, which is formed as a consequence of the geological and thermal alteration of the amorphous domain, and comprises mainly kerogen, soot, charcoal, black carbon (BC), and humic acids in the vitreous form (Xiao et al. 2004). They are more structured forms, with higher H/O ratio, higher aromaticity, and greater sorption capacity due to presenting non-linear sorption isotherms, slow sorption and desorption rates, and sorption-desorption hysteresis, i.e., OCPs are trapped and retained (in some cases irreversibly) in micropores of condensed organic matter that can be deformed leading to different sorption and desorption isotherms (Weber et al. 1992, 1998; Xiao et al. 2004). This sorption/desorption kinetics plays an important role in the environment since they regulate the exchange of contaminants between the dissolved and particulate fractions

(Lasaga 2014), hence, influence their bioavailability. Consequently, according to the bioavailability of OCPs, three fractions can be established: immediately available in the dissolved fraction; potentially available, associated with DOM and the amorphous domain of the SOM; and the little or not available fraction, associated with the condensed domain of the SOM (Figure 3).

However, although the sediment sorption capacity is essential, other factors influence the OCPs bioavailability in sediments such as the nature and properties of the compounds, including K_{OW} and molecular size (Lyytikäinen et al. 2003), environmental conditions such as temperature, pH, salinity, and bulk density (Akkanen and Kukkonen 2001), and age of the OCPs in contact with the sediment, which increases the importance of the unavailable fractions (Reichenberg and Mayer 2006).

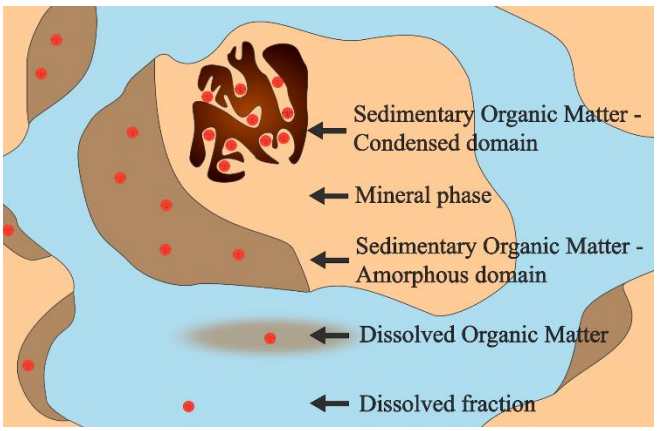


Figure 3. Schematic representation of the main phases and domains of geosorbents in sediments in the aquatic environment.

In addition to sorption processes, most of the OCPs degradation occurs in sediments, either by microbial or chemical action. Chemical degradation occurs by photolysis (in the photic zones of the sediment), hydrolysis, oxidation, and reduction (Andreu and Pico 2004). Meanwhile, the microbiological degradation of pesticides occurs by consumption, as a source of carbon and energy, by microorganisms generating structural modification, generally by dehydrochlorination (Vollhardt and Schore 2000), and by cometabolism (microbial consortium), which generates a series of consecutive transformations up to the total degradation of the pesticide (Borja et al. 2005; Alexander 1981). Usually, the degradation rate in the sediment is related to some key factors: the nature and properties of the chemical compound, the existence and abundance of relevant degradation agents, the bioavailability of the compounds (De Weert et al. 2008; Harwood et al. 2012), and the prevailing environmental conditions such as temperature, oxygen level, solar radiation, and pH (Kang and Kondo 2005; Ying and Kookana 2003).

Air

Atmospheric transport is one of the main ways of pollutant dispersion (Takeoka et al. 1991) and is the main pollution source in the polar regions and oceanic environments (Bacon et al. 1992; Ono et al. 1987). Due to the temperature gradient and consequently the K_{AW}

gradient, OCPs tend to volatilize in low latitude regions, enter atmospheric circulation cells, and transport to high latitudes, where they tend to condense and incorporate into soil and water bodies. This phenomenon is known as global distillation and may be responsible for the high concentrations of some OCPs in temperate and polar regions (Simonich and Hites 1995). OCPs with low molecular weight and high vapor pressure, such as hexachlorobenzene (HCB) and hexachlorocyclohexanes (HCH), are usually more effectively dispersed in cells, instead, those with low pressure and large molecular sizes, such as DDT or toxaphene, do not transport effectively to high latitudes (Iwata et al. 1994; Simonich and Hites 1995). However, the combination of the broad global use, environmental persistence, and relatively high Henry's law constant of these OCPs, promotes their presence, generally associated with biota or sediments, at high latitudes. In addition to global distillation, successive steps of volatilization/deposition and transport of OCPs occur throughout the world through so-called "grasshopping"; hence, they can affect all environments and ecosystems (Jurado and Dachs 2008). These processes influence the OCP levels in coastal waters, especially in remote areas such as Antarctica (Wu et a. 2020), Patagonia Argentina (Commendatore et al. 2015), and Arctic (Jin et al. 2017; Ma et al. 2015), since the chemicals can be incorporated by water-air exchange in these areas or in the soil, vegetation, and water of the influence drainage basins (Dachs and Méjanelle 2010; Figure 2).

Biota

Marine organisms can be exposed to OCPs through different routes. OCPs dissolved in the air can enter organisms through lung respiration. Meanwhile, OCPs dissolved in water, a fraction considered bioavailable, can enter organisms from ingested water or through external surfaces such as gills or skin tissues. Moreover, potentially available particle-associated OCPs (i.e., those associated with the amorphous domain of organic matter) and organism-incorporated OCPs can enter the consuming organisms through food intake and be incorporated from the intestine (Knezovich et al. 1987). Generally, OCP levels in small organisms are regulated by the equitable partition between body lipids and water (K_{OW}). Instead, OCP levels are better related to diet than to K_{OW} and lipid content in consuming organisms. The effect of diet is particularly important due to OCPs, being lipophilic and persistent compounds, increase their concentration through the trophic web; therefore, the concentration in top predators is usually higher than in those organisms with low trophic levels (biomagnification), reaching differences of up to 10,000 times greater (Kajiwara et al. 2004; Kleivane et al. 2004; Tanabe et al. 1994). Likewise, OCP levels show great interspecific and intraspecific variability, determined by the complexity of chemical, biological, and ecological factors that influence diets, such as habitat, feeding behavior, and digestion mechanisms, as well as the metabolizing capacity, chemical lipophilicity, and molecular size (Belfroid et al. 1996; Lyytikäinen et al. 2003; Weston et al. 2000).

After organisms assimilate them, the fate of OCPs in the body can be complex: they can store, metabolize, or excrete them. Mainly, OCPs tend to migrate towards lipid reserves due to their high K_{OW}, and it is common to find a concentration gradient gastrointestinal tract> liver or brain> kidney> muscle (Bryan et al. 1987; Dang et al. 2016; Zhao et al. 2014). While excretion through the urine is considered to be negligible, due to its low solubility in water. In contrast, the removal of OCPs by lipid mobilization in processes such as gonadal maturation, ovulation, pregnancy, or lactation is remarkable (Aguilar 1987). In lipid mobilization, some OCPs remain in the tissue, but others leave the tissue with the lipids (Aguilar 1987). This

explains the low residue concentrations in adult females of marine mammals about adult males (Girones et al. 2020; Kajiwara et al. 2004). Another way for removal OCPs is degradation, in which the organisms themselves transform the chemical compound into metabolites, which are sometimes the same or more harmful and persistent than the parent pesticide. This process occurs in most organisms and the main metabolic pathway is through the cytochrome P-450 system (CYP-450), which contains hemiproteins with iron used in the oxidation of hydrophobic xenobiotic compounds. Through a cytochrome P-450 reductase enzyme, the reduction of Fe3 + to Fe2 + occurs, which attracts an oxygen molecule and oxidizes the pollutant forming a hydroxyl group (hydroxylation), which makes the molecule more hydrosoluble and allows is excreted through the urine (Parkki et al. 1977). However, not all organisms have the same ability to degrade and remove OCPs, this ability is based on biochemical detox adaptations that would be mainly related to diet, for example, the detoxification ability of herbivores is greater than that of carnivores because they are frequently exposed to a greater number of natural toxins in their diet (Focardi et al. 1988; Fossi et al. 1988).

TOXICITY

Wild Animal Toxicity

Organochlorine pesticides were designed to eliminate arthropods by neurotoxic action, i.e., acting directly on the nervous system and causing sensory disturbances to seizures (ATSDR 2002). The magnitude and nature of this effect are determined partly by the way and concentration in which the OCPs reaches the receptor sensitive to it. If the OCP concentrations in the active site are too low, or if the pesticide has been metabolized to an inactive form, no impact will be observed.

Exposure to high environmental concentrations of OCPs generates harmful effects on the biota in the short and long term. The most notable effect was the decrease in the population of some wild bird species associated with agricultural sites with intensive use of DDT, where this compound was associated with the thinning of the eggshells, and consequently to their breakage and reproductive failure (Bitman et al. 1969; Burnett et al. 2013).

However, what generated the most interest in the scientific community were the effects that low OCP levels can produce on the reproduction and development of non-target organisms. These chemicals can act as endocrine disruptors (Petrovic et al. 2013; Sosa-Ferrera et al. 2013) because they can interfere with endogenous chemical messengers such as hormones, neurotransmitters, growth factors, and inhibitory substances, affecting direct development, control of homeostatic regulation and the function of these systems (Colborn et al. 1993). This hormonal system alteration has been linked to the increase in some types of neoplasms, malformations, and reproductive system dysfunctions, and immune response failures (Diamanti-Kandarakis et al. 2009). Its effects are more noticeable in embryos, fetuses, and newborns, producing in many cases irreversible effects as a product of delayed development and organization of the organism (Guillette et al. 1995; Mori and Nagasawa 1988).

Due to the large number of chemical indicators present among the cells of developing systems, there are numerous targets on which OCPs can act as chemical messengers. Therefore, detecting the small effects they cause and predicting developmental injuries or modifications is very difficult. Moreover, there may be a long delay between exposure and effects, hence the latter may not be expressed until the maturity of the individual. These individuals may appear normal but often experience early death or infertility as a result of exposure (Colborn and Smolen 1996). If the problem were to spread, these alterations in individuals could have serious consequences on population levels.

Human Toxicity

Where OCPs are still in use, dermal and aerial exposure is important, for example in cases where DDT is used to control malaria (Van Dyk et al. 2010). Furthermore, in some places, the consumption of contaminated fish and shellfish could be a source of human exposure to OCPs (Beard 2006). Both sources of OCPs exposure mean that significant levels can still be measured in serum, adipose tissue, or breast milk samples from populations not occupationally exposed (Gajski 2012).

Exposure to OCPs, even at very low levels, is associated with short gestational periods, low weight in newborns, and below-average head circumference measurements (Jacobson and Jacobson 1993). Furthermore, it can affect the fertility of the individual, especially if this exposure occurs during prenatal periods (Sharpe and Skakkebaek 1993), and generate liver, neurological, pulmonary, and cardiac problems (Furuya et al. 2005; Nakanishi et al. 2005). Besides, some pesticides would have a genotoxic (Canales-Aguirre et al. 2011) and/or carcinogenic capacity in humans, e.g., lindane is carcinogenic, DDT and dieldrin are probably carcinogenic, and others such as HCHs, HCB, and chlordane are possibly carcinogenic, according to IARC (IARC 2020).

CURRENT OCP LEVELS IN COASTAL SEDIMENTS

Literature Review and Information Processing

Scientific articles on OCPs in coastal sediments worldwide published in recent years (2015-2020) were reviewed. A comprehensive search was performed on the most common search engines using different word combinations. The compounds reviewed were: DDTs (o,p′-DDE, p,p′-DDE, o,p′-DDD, p,p′-DDD, o,p′-DDT, and p,p′-DDT); HCHs (α-HCH, β-HCH, γ-HCH, and δ-HCH); endosulfans (α-endosulfan, β-endosulfan, and endosulfan sulfate); heptachlor (heptachlor and heptachlor epoxide); chlordanes (*trans*-chlordane, *cis*-chlordane, *trans*-nonachlor, and *cis*-nonachlor); drins (aldrin, dieldrin, endrin, and endrin aldehyde); HCB; mirex; and methoxychlor. Levels below the detection limit were replaced by half the detection limit when necessary. Data analysis of OCP levels was performed with ArcGIS 10.3 (Esri, 380 New York Street, CA 92373, USA) and Microsoft Excel (ver. 2013, Microsoft, Redmond, WA, USA), and concentrations were expressed as $ng.g^{-1}$ dry weight (dw). Moreover, to determine the ecotoxicological risk associated with OCPs in the

sediments, the mean levels of each site were compared with the Norwegian Environmental Quality Classification System (NEQCS; Bakke et al. 2010; Molvær et al. 1997) and the guidelines proposed by Long et al. in 1995 and Macdonald et al. in 1996 (Table 2). NEQCS establishes five risk classes for five ranges of OCP concentrations in coastal marine environments: background (CI); no toxic effects (CII); toxic effects following chronic exposure (CIII); toxic effects following short term exposure (CIV); severe acute toxic effects (CV). Meanwhile, the guidelines proposed by Long and Macdonald are similar, they establish two limit values that define three ranges according to the frequency of adverse effects occurring in biota: rarely, occasionally, and frequently. Long et al. defined these limit values as "effects range low" (ERL) and "effects range median" (ERM), while Macdonald et al. defined them as "threshold effect level" (TEL) and "probable effects level" (PEL; Table 2).

Results and Discussion

DDTs

The current environmental concentrations of DDTs were higher than those of the other OCPs, between 3 and 20 times depending on the group of compounds compared. The general mean for 70 reviewed coastal sites in the world was 8.43 $ng.g^{-1}$ (SD = 30.84) with a global median of 1.71 $ng.g^{-1}$. The global spatial distribution of DDTs in coastal sediments was highly variable and was related to the proximity to the sources and the hydrodynamic characteristics of each environment, with Africa and Asia being the continents most affected by DDT pollution, which are regions where its use has not been totally discontinued (Van den Berg et al. 2017; Figure 4). When comparing the levels of DDT in the sediments with the international guidelines on ecotoxicological risk, it was observed that in many places these compounds would cause some damage to the biota (Table 3). In Jiulong Jiang, China (Lv et al. 2020) and iSimangaliso Wetland Park, South Africa (Buah-Kwofie and Humphries 2017) these levels were 209.23 $ng.g^{-1}$ and 138.14 $ng.g^{-1}$, respectively, and they could cause severe and frequent adverse effects on the biota according to the guidelines proposed by Long et al. (1995), Macdonald et al. (1996) and NEQCS. Moreover, the levels of DDTs from other sites could generate damage to the biota occasionally according to these guidelines (Table 3). Regarding the distribution of DDT congeners, a general predominance of DDT metabolites was observed over the original DDT, suggesting a historical input to the systems (Figure 4). Meanwhile, the DDE/DDD ratio varied mainly according to the oxygen conditions and the solar exposure of the evaluated sediments. In general, the subtidal sediments showed a predominance of DDD and the intertidal sediments showed a predominance of DDE. For example, the DDE/DDD ratios found in mangrove sediments studied by Chen et al. (2020) and Mitra et al. (2019) were much greater than 1. Instead, the DDE/DDD ratios found in subtidal sediments studied by Akhil and Sujatha (2014), Oliva et al. (unpublished), and Jin et al. (2017) were much lower than 1. However, in some places such as the Brazilian continental shelf (Santos et al. 2020) and Guanghai Bay (Haijun et al. 2019), where the sediments are permanently submerged, the DDE/DDD ratios were close to 1. This demonstrates that the prevalence of anaerobic or aerobic degradation depends not only on the waterlogging condition, but also on other factors, such as the diffusion of oxygen from the water column to the sediments, influenced by the content of organic matter, particle size, and the predominant hydrodynamics.

Table 2. Guidelines on ecotoxicological risk for organochlorine pesticides in marine sediments. Values expressed as ng.g^{-1}

Compounds	ERL[a]	ERM[a]	TEL[b]	PEL[b]	CII[d]	CIII[d]	CIV[d]	CV[d]
p,p,-DDT	1	7	1.19	4.77				
p,p'-DDE	2.2	27	2.07	374				
p,p'-DDD	2	20	1.22	7.81				
DDTs	1.58	46.1	3.89	51.7	0-15	15-165	165-1647	> 1647
γ-HCH			0.32	0.99				
HCHs					< 0.074	0.074-0.74	0.74-9.8	> 9.80
Endosulfans					< 0.073	0.073-0.6	0.6-6	> 6
Endrin			2.67c	62.4c				
Dieldrin	0.02	8	0.72	4.3				
Chlordane	0.5	6	2.26	4.79				
Heptachlor epoxide			0.6c	2.74c				
HCB					0-17	17-61	61-610	> 610

a. Long et al. 1995; b. Macdonald et al. 1996; c. CCME 2020 (www.ccme.ca); d. NEQCS

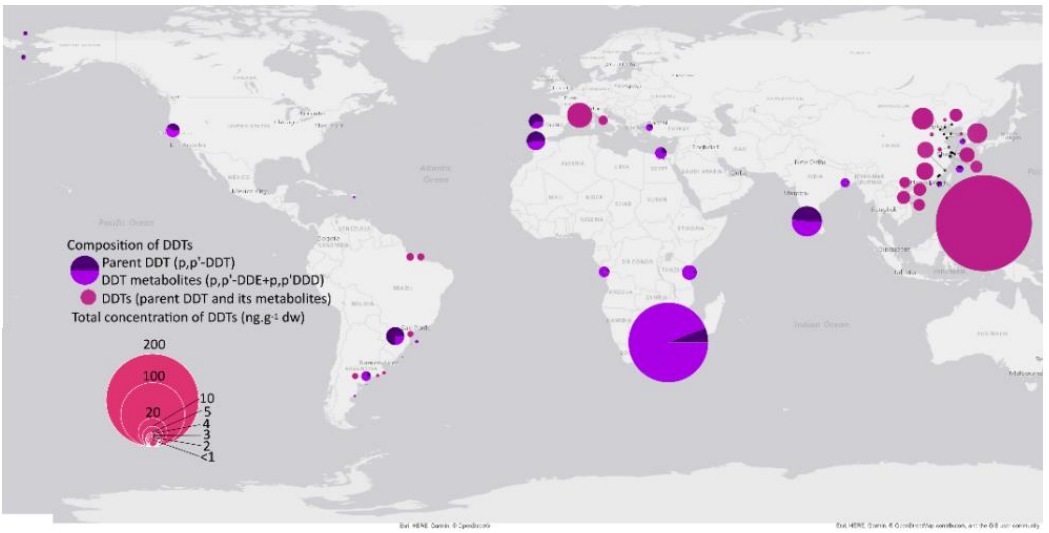


Figure 4. Spatial distribution and composition of the mean levels of DDTs in coastal sediments.

HCHs

HCH levels in sediment were on average 3 times lower than those of DDTs, with a general mean of 2.85 ng.g^{-1} (SD = 12.03) and a median of 0.41 ng.g^{-1}. Even so, high concentrations were found in some places around the world (Figure 5). The highest levels were found in iSimangaliso Wetland Park, South Africa (Buah-Kwofie and Humphries 2017), followed by Sado Estuary (Carvalho et al. 2009), Unguja Island, Tanzania (Mwevura et al. 2020), Guajará Bay, Brazil (Neves et al. 2018), the Yangtze Estuary, China (Adeleye et al. 2016), and the Nile River Estuary, Egypt (Abbassy et al. 2018) with an average of 95.8, 15.8, 4.65, 4.05, 3.93, and 3.61 ng.g^{-1}, respectively.

Due to its high toxicity, HCHs in sediment could cause damage to the biota in the short or long term in most of the sites analyzed in recent years (Table 3) according to the Norwegian

Environmental Quality Classification System (NEQCS; Bakke et al. 2010; Molvær et al. 1997). The sites most affected by HCHs were the iSimangaliso Wetland Park, South Africa (Buah-Kwofie and Humphries 2017), and the Sado Estuary, Portugal (Carvalho et al. 2009), where these compounds could generate severe damage to the biota in the short term.

Figure 5. Spatial distribution and composition of the mean levels of HCHs in coastal sediments.

In turn, the ecotoxicological risk associated with the current levels of the γ-HCH isomer (lindane) in sediments according to the guideline proposed by MacDonald et al. (1996) coincided with that of the HCHs and is also shown in Table 3.

Meanwhile, the α-HCH/γ-HCH ratio, which determines the predominant use of technical HCH when it is in the 3 and 7 range and of lindane (γ-HCH) when it is <1, indicated a predominant use of lindane in the most places (Figure 5). However, some sites in Africa, China, and the Arctic (Buah-Kwofie and Humphries 2017; Jin et al. 2017; Lv et al. 2020; Ma et al. 2015) showed indices higher than 3, indicating a predominant use of technical HCH or at least fast and efficient atmospheric transport from distant sources in progress (Ma et al. 2015).

Drins

The study of the other OCPs is much less extensive than that of HCHs and DDTs. Aldrin, dieldrin, and endrin were analyzed in 62%, 72%, and 62% of the reviewed studies, respectively. These compounds, usually grouped under the name of "drins", were found in considerably lower concentrations than DDTs and HCHs (Figure 6). The global mean and median concentrations were 1.44 $ng.g^{-1}$ and 0.05 $ng.g^{-1}$ for aldrin, 3.13 $ng.g^{-1}$ and 0.02 $ng.g^{-1}$ for dieldrin, and 3.65 $ng.g^{-1}$ and 0.13 $ng.g^{-1}$ for endrin, respectively. However, some places presented levels that could pose a risk to the associated biota according to the guidelines suggested by Long et al. (1995) and MacDonald et al. (1996) (Table 3). The mean levels of dieldrin in Cochin Estuary, India (Akhil and Sujatha 2014), iSimangaliso Wetland Park, South Africa (Buah-Kwofie and Humphries 2017), and Sado Estuary, Portugal (Carvalho et al. 2009) could cause frequent adverse effects on the biota according to both guidelines. The concentration of dieldrin in other sites around the world could cause occasional adverse effects on biota (Table 3). Meanwhile, endrin levels would not cause adverse effects at the studied sites, except in Cochin Estuary, India (Akhil and Sujatha 2014), Isimangaliso Wetland

Park, South Africa (Buah-Kwofie and Humphries 2017), and Island Unguja, Tanzania (Mwevura et al. 2020), where they could cause occasional damage to the associated biota, according to the guideline proposed by MacDonald et al. (1996).

Endosulfans

The two isomers of technical endosulfan (α and β) were poorly studied, they were analyzed only in 55% of the reviewed studies, and endosulfan sulfate, its main metabolite, was analyzed in less than 40% of the studies. The global mean concentration of endosulfans was 3.75 $ng.g^{-1}$, although with widely dispersed values (SD = 15.8; median = 0.11 $ng.g^{-1}$). The high standard deviation was mainly generated by high endosulfan concentrations at two sites: iSimangaliso Wetland Park, South Africa (Buah-Kwofie and Humphries 2017), and the Cochin Estuary, India (Akhil and Sujatha 2014) with averages of 93.5 $ng.g^{-1}$ and 21.01 $ng.g^{-1}$, respectively (Figure 7). These levels could generate acute toxic effects on the associated biota according to the NEQCS (Table 3). Furthermore, according to this guideline, the average levels found in some estuaries in Egypt, Vietnam, China, Argentina, and Portugal (Abbassy 2018; Carvalho et al. 2009; Chen et al. 2020; Lv et al. 2020; Oliva et al. unpublished; Tham et al. 2019) could cause damage to the associated biota in the short or long term (Table 3).

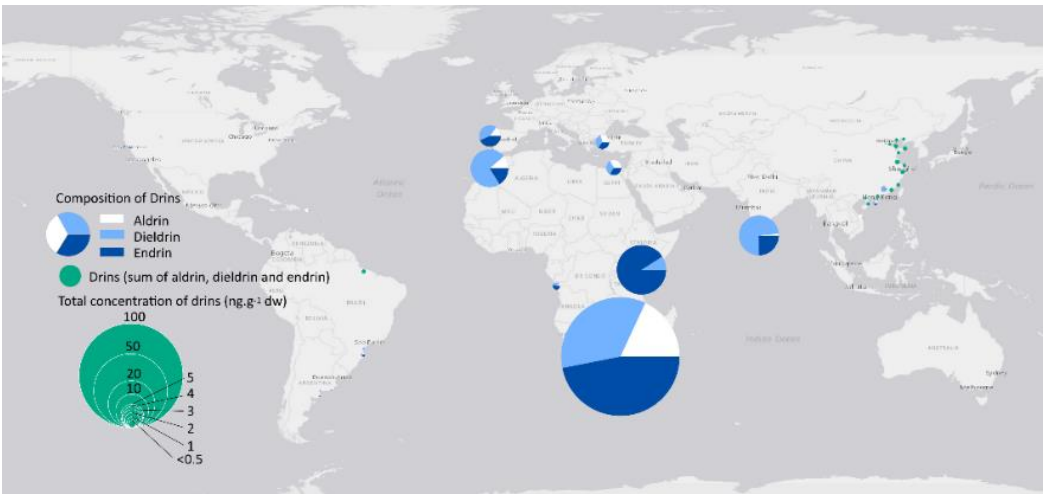


Figure 6. Spatial distribution and composition of the mean levels of drins in coastal sediments.

Regarding the distribution of congeners, a clear pattern was observed. In intertidal sediments, endosulfan sulfate was predominated over parent endosulfans (e.g., Chen et al. 2020; Lv et al. 2020) while in subtidal sediments the parent endosulfans were predominated (e.g., Abbassy 2018; Akhil and Sujatha 2014; Commendatore et al. 2018; Ma et al. 2015; Oliva et al. unpublished). This would be explained by the oxygen level in the sediments: under aerobic conditions, the parent endosulfan is easily oxidized to endosulfan sulfate, while in anaerobic or low-oxygen conditions, small amounts of this analyte are produced (Ghadiri and Rose 2001; Guerin 1999). Besides, in coastal sediments, under unfavorable environmental conditions for oxidative degradation, the recent use of technical endosulfan would promote the prevalence of the parent endosulfan over endosulfan sulfate. Meanwhile, the predominance of α or β isomers of endosulfan depended on both the environmental

conditions and the physicochemical properties of the isomers and the composition of the technical endosulfan (i.e., 70% α-isomer and 30% β-isomer). The β isomer is more resistant to environmental degradation and is less volatile than the α isomer (Breysse et al., 2015; Rand et al. 2010), which is why it is usually predominant in water, fish, and aerobic sediments (Granados- Galván et al. 2015; Rand et al. 2010). This pattern was observed in intertidal sediments in China (Chen et al. 2020; Lv et al. 2020), but also in subtidal sediments of the Nile River estuary, Egypt (Abbassy et al. 2018). The prevalence of α-isomer was observed in subtidal sediments of Svalbard, Norwegian Arctic (Ma et al. 2015), Bahia Blanca Estuary, Argentina (Oliva et al. unpublished), Cochin Estuary, India (Akhil and Sujatha 2014), and San Jorge Gulf, Argentina (Commendatore et al. 2015). This could be due to a recent entry of technical endosulfan into the system or to the low capacity to degrade both isomers in the absence or shortage of oxygen and/or low temperatures (Ghadiri and Rose 2001).

Other Organochlorine Pesticides

Heptachlor was analyzed in 57% of the studies. The current global mean concentration was 2.25 $ng.g^{-1}$ (SD = 7.61) and the median was 0.04 $ng.g^{-1}$, which were low values about the previously analyzed OCPs. However, the mean concentrations of heptachlor epoxide found in iSimangaliso Wetland Park, South Africa (28.60 $ng.g^{-1}$) and Cochin Estuary, India (2.77 $ng.g^{-1}$) would cause frequent damage to the associated biota according to the guideline proposed by Macdonald et al. (1996; Table 3). Regarding the distribution of heptachlor congeners, it was reported in the literature that heptachlor would transform into heptachlor epoxide in aerobic conditions through enzymatic epoxidation (Pokethitiyook and Toemthip 2012). However, a prevalence of this metabolite over parental heptachlor was not observed in intertidal sediments, where the oxygen level would be higher than in subtidal sediments. On the other hand, it should be taken into account that under oxygen-deficient conditions heptachlor would be metabolized to chlordane and 1-hydroxychlordene-2,3-epochloride chloride (Pokethitiyook and Toemthip 2012), and these metabolites were generally not analyzed. Consequently, the study of the parent heptachlor/metabolites ratio and its relationship to environmental conditions was very limited.

Chlordanes were evaluated in only 43% of the reviewed studies. The general mean levels were very low, with a mean of 0.18 $ng.g^{-1}$ (SD = 0.58) and a median of 0.07 $ng.g^{-1}$. Among the sites for which values were reported, the levels of this pesticide would only cause occasional damage to the biota in Anadim, Guajara Bay, Brazil (Neves et al. 2018), according to the guidelines proposed by Long et al. (1995) and Macdonald et al. (1996; Table 3). Regarding the distribution of congeners, *cis*-chlordane/*trans*-chlordane ratio ranged between 0.7 and 1.6 (Commendatore et al. 2015; Jin et al. 2017; Lv et al. 2020; Ma et al. 2015; SFEI 2014) and their main metabolite, oxychlordane, was hardly studied.

HCB was analyzed only in 27% of the studies reviewed. The mean levels (mean of 2.12 $ng.g^{-1}$, SD = 4.18, and median of 0.18 $ng.g^{-1}$) were similar to those of endosulfans and heptachlors. The sites with the highest HCB concentrations were Congo River estuary, Congo DR (Suami et al. 2020), Bahía Blanca Estuary, Argentina (Tombesi et al. 2018), and Rhone River delta, France (Salvadó 2013), with means of 14.06, 6.36, and 2.12 $ng.g^{-1}$, respectively. Even so and according to NEQCS, HCB levels in sediment would be biota-safe at all sites reviewed.

Table 3. Ecotoxicological risk associated with organochlorine pesticides in sediments in marine environments around the world according to guidelines proposed by Long et al. (L; 1995), Macdonald et al. (M; 1996), CCME (C), and NEQCs (N). OCPs contemplated were: p,p'-DDT (DDT); p,p'-DDE (DDE); p,p'-DDD (DDD); Sum of p,p'-DDT, p,p'-DDE, and p,p'-DDD (DDTs); lindane (γ-HCH); Sum of α, β, γ, and δ HCH (HCHs); Sum of α and β endosulfans and endosulfan sulfate (Endos); Sum of cis and trans chlordane (Chlord); dieldrin; heptachlor epoxide (Hepx); and HCB

	DDT		DDE		DDD		DDTs		γ-HCH		HCHs	Endos	Chlord		Dieldrin		Endrin	Hepx	HCB	Authors
	L	M	L	M	L	M	L	M	N	M	N	N	L	M	L	M	C	C	N	
South America																				
Bahía Blanca Estuary, Argentina	0	0	0	0	0	0	0	0	0	2	2	1	-	-	0	0	0	1	-	Oliva et al. unpublished
Bahía Blanca Estuary, Argentina	0	0	0	0	0	0	1	0	0	0	1	-	-	-	-	-	-	-	0	Tombesi et al. 2018
Samborombón Bay, Argentina	-	-	-	-	-	-	0'	0'	0'	-	1	1	0	0	0	0	-	-	-	Díaz-Jaramillo et al. 2018
Quequen River Estuary, Argentina	-	-	-	-	-	-	0'	0'	0'	-	0	1	0	0	1	0	-	-	-	Díaz-Jaramillo et al. 2018
Bahía Blanca Estuary, Argentina	-	-	-	-	-	-	0'	0'	0'	-	0	1	0	0	1	0	-	-	-	Díaz-Jaramillo et al. 2018
Mar Chiquita CoastalLagoon, Argentina	-	-	-	-	-	-	0'	0'	0'	-	0	1	0	0	0	0	-	-	-	Díaz-Jaramillo et al. 2018
San Blas Bay, Argentina	0	0	0	0	-	-	0	0	0	0	1	1	0	0	0	0	0	0	-	Commendatore et al. 2018
San Jorge Gulf, Argentina	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Commendatore et al. 2015
Santa Catarina (N), continental shelf, Brazil	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-	Santos et al. 2020
Santa Catarina (S), continental shelf, Brazil	0	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-	Santos et al. 2020
Rio Grande do Sul, continental shelf, Brazil	-	-	0	0	-	-	0	0	0	-	-	-	-	-	-	-	-	-	-	Santos et al. 2020
Anadim, Guajará Bay, Brazil	-	-	-	-	-	-	0'	0'	0'	-	2	-	1	1	-	-	-	-	-	Neves et al. 2018

	DDT		DDE		DDD		DDTs		γ-HCH		HCHs	Endos	Chlord		Dieldrin		Endrin	Hepx	HCB	Authors
	L	M	L	M	L	M	L	M	N	M	N	N	L	M	L	M	C	C	N	
Outerio, Guajará Bay, Brazil	-	-	-	-	-	-	0’	0’	0’	-	1	-	0	0	-	-	-	-	-	Neves et al. 2018
Babitonga Bay, Brazil	1	2	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	Rizzi et al. 2017a
Paranaguá Estuary, Brazil	-	-	-	-	-	-	0	0	0	0	2	-	0	0	0	0	-	-	-	Rizzi et al. 2017b
Central and North America and the Arctic Ocean																				
Bering Sea, Arctic	0	0	0	0	0	0	0	0	0	0	1	-	0	0	-	-	-	-	-	Jin et al. 2017
Chuckchi Sea, Arctic	0	0	0	0	0	0	0	0	0	0	1	-	0	0	-	-	-	-	-	Jin et al. 2017
Jobos Bay, Puerto Rico	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	-	Huber 2018
Kongsfjorden, Svalbard	-	-	-	-	-	-	-	-	-	0	0	0	0	0	-	-	-	-	-	Ma et al. 2015
San Francisco Bay, US	1	1	0	0	0	0	1	1	0	0	0	-	0	0	1	0	0	0	0	SFEI 2014
Asia																				
Jiulong Jiang, China	-	-	-	-	-	-	2’	2’	2’	-	2	1	0	0	-	-	-	-	-	Lv et al. 2020
Hangu, China	-	-	-	-	-	-	1’	1’	0’	-	1	1	0	0	-	-	-	-	-	Lv et al. 2020
Dagu He, China	-	-	-	-	-	-	1’	1’	0’	-	1	1	0	0	-	-	-	-	-	Lv et al. 2020
Zhu Jiang, China	-	-	-	-	-	-	1’	1’	0’	-	1	1	0	0	-	-	-	-	-	Lv et al. 2020
Min Jiang, China	-	-	-	-	-	-	1’	1’	0’	-	1	1	0	0	-	-	-	-	-	Lv et al. 2020
Chang Jiang, China	-	-	-	-	-	-	1’	1’	0’	-	1	1	0	0	-	-	-	-	-	Lv et al. 2020
Daliao He, China	-	-	-	-	-	-	1’	0’	0’	-	1	0	0	0	-	-	-	-	-	Lv et al. 2020
Dongzhai Gang, China	-	-	-	-	-	-	1’	0’	0’	-	1	0	0	0	-	-	-	-	-	Lv et al. 2020
Hangzhou Wan, China	-	-	-	-	-	-	1’	0’	0’	-	1	0	0	0	-	-	-	-	-	Lv et al. 2020
Yingluo Wan, China	-	-	-	-	-	-	1’	0’	0’	-	1	0	0	0	-	-	-	-	-	Lv et al. 2020

Table 3. (Continued)

	DDT		DDE		DDD		DDTs		γ-HCH		HCHs	Endos	Chlord		Dieldrin		Endrin	Hepx	HCB	Authors
	L	M	L	M	L	M	L	M	N	M	N	N	L	M	L	M	C	C	N	
Dongying, China	-	-	-	-	-	-	0’	0’	0’	-	1	0	0	0	-	-	-	-	-	Lv et al. 2020
Yancheng, China	-	-	-	-	-	-	0’	0’	0’	-	1	0	0	0	-	-	-	-	-	Lv et al. 2020
Beidaihe, China	-	-	-	-	-	-	0’	0’	0’	-	1	0	0	0	-	-	-	-	-	Lv et al. 2020
Sishili Wan, China	-	-	-	-	-	-	0’	0’	0’	-	1	0	0	0	-	-	-	-	-	Lv et al. 2020
Intertidal sediments, China	2	2	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	-	Lv et al. 2020
Zhang River Estuary, China	0	0	0	0	0	0	0’	0’	0’	0	1	2	-	-	1	0	0	0	-	Chen et al. 2020
Guanghai Bay, China	0	0	0	0	0	0	0	0	0	0	1	-	-	-	1	0	0	0	0	Haijun et al. 2019
Yangtze Estuary, China	0	0	0	0	-	-	0’	0’	0’	2	2	-	-	-	-	-	-	-	-	Adeleye et al. 2016
Hangzhou Bay, China	0	0	0	0	-	-	0’	0’	0’	2	2	-	-	-	-	-	-	-	-	Adeleye et al. 2016
Continental Margin East China Sea	0	0	0	0	-	-	0’	0’	0’	2	2	-	-	-	-	-	-	-	-	Adeleye et al. 2016
Hooghly estuary, India	0	0	0	0	0	0	1	0	0	0	1	-	-	-	-	-	-	-	0	Mitra et al. 2019
Cochin Estuary, India	2	2	1	1	1	1	1	1	1	-	2	3	-	-	2	2	1	2	-	Akhil and Sujatha 2014
Nhat Le Estuary, Vietnam	-	-	-	-	-	-	1	1	0	-	2	2	-	-	-	-	-	-	-	Tham et al. 2019
Cua Hoi Estuary, Vietnam	-	-	-	-	-	-	1	1	0	-	2	2	-	-	-	-	-	-	-	Tham et al. 2019
Han River Estuary, Vietnam	-	-	-	-	-	-	1	0	0	-	2	2	-	-	-	-	-	-	-	Tham et al. 2019
Lang Co Lagoon, Vietnam	-	-	-	-	-	-	1	0	0	-	2	2	-	-	-	-	-	-	-	Tham et al. 2019
Cua Dai Estuary, Vietnam	-	-	-	-	-	-	1	0	0	-	2	2	-	-	-	-	-	-	-	Tham et al. 2019
Cua Viet Estuary, Vietnam	-	-	-	-	-	-	1	0	0	-	2	2	-	-	-	-	-	-	-	Tham et al. 2019
Vientam estuaries, Vietnam	-	-	-	-	-	-	1	1	0	-	2	2	-	-	-	-	-	-	-	Tham et al. 2019

	DDT		DDE		DDD		DDTs		γ-HCH		HCHs	Endos	Chlord		Dieldrin		Endrin	Hepx	HCB	Authors
	L	M	L	M	L	M	L	M	N	M	N	N	L	M	L	M	C	C	N	
Cua Dai estuary, Vietnam	-	-	-	-	-	-	1	0	0	-	2	-	-	-	1	0	-	-	-	Nguyen et al. 2019
Mediterranea Sea and Europe																				
Nile River Estuary, Egypt	0	0	0	0	0	0	1’	0’	0’	1	2	2	-	-	0	0	0	1	-	Abbassy 2018
Rhone delta, France	-	-	-	-	-	-	1’	1’	0’	-	2	-	-	-	-	-	-	-	0	Salvadó 2013
Tiber River Estuary, Italy	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	Montuori et al. 2016
Southern portugal, Portugal	1	1	1	1	0	1	1	1	0	2	3	2	-	-	2	2	0	1	-	Carvalho et al. 2009
Northern portugal, Portugal	1	1	0	0	0	0	1	1	0	2	2	2	-	-	1	1	0	0	-	Carvalho et al. 2009
Edremit Bay, Aegean Sea, Turkey	0	0	0	0	0	0	0	0	0	0	0	-	0	0	0	0	0	0	-	Darilmaz et al. 2019
Sub-saharan Africa																				
Congo River estuary, Congo DR	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	Suami et al. 2020
iSimangaliso Wetland Park, South Africa	2	2	2	1	2	2	2	2	1	2	3	3	-	-	2	2	1	2	-	Buah-Kwofie and Humphries 2017
Unguja Island, Tanzania	0	0	1	1	0	0	1	1	0	2	2	-	-	-	1	1	1	-	-	Mwevura et al. 2020

Long et al. 1995 (L): Conc.(concentration) < ERL (0; rarely adverse effects in biota), ERL< Conc.< ERM (1; occasionally adverse effects in biota), Conc. > ERM (2; frequently adverse effects in biota); Macdonald et al. 1996 (M) and CCME (C): Conc. < TEL (0; rarely adverse effects in biota), TEL < Conc. < PEL (1; occasionally adverse effects in biota), Conc. > PEL (2; frequently adverse effects in biota); NECQS (N): CII (0; no toxic effects), CIII (1; toxic effects following chronic exposure), CIV (2; toxic effects following short term exposure), CV (3; severe acute toxic effects).

Mirex was determined in only 17% of the studies reviewed, in which it was presented at very low levels. The general mean concentration was 0.03 $ng.g^{-1}$ (SD = 0.04) and the median was 0.01 $ng.g^{-1}$. Meanwhile, for methoxychlor, the frequency of analysis was 23% and the global mean was 1.43 $ng.g^{-1}$ with high SD (1.92) and a median of 0.4 $ng.g^{-1}$. Neither of these two pesticides is included in any ecotoxicological classification systems used in this chapter, hence, we could not determine if the levels found would pose any risk to the associated biota.

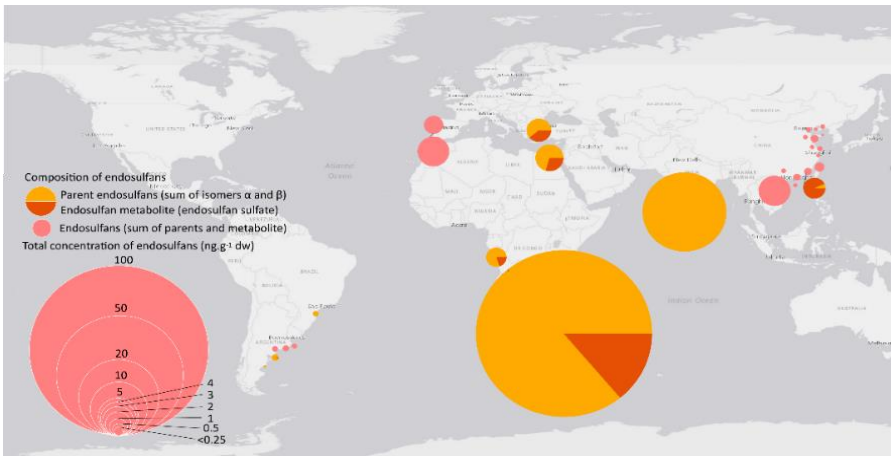


Figure 7. Spatial distribution and composition of the mean levels of endosulfans in coastal sediments.

CONCLUSION

Although it seems that the problem associated with the production and use of organochlorine pesticides has disappeared due to the worldwide restrictions, they remain as one of the most important pollutants for both the environment and humans. At almost all the coastal sites where were recently evaluated, OCP levels showed detectable concentrations of at least one congener. In some cases, mainly in sub-Saharan Africa and Southeast Asia, these concentrations were high and would pose a risk to the biota associated with these environments.

DDTs were the most abundant legacy POP in marine coastal sediments, 3 times more abundant than HCHs and much more abundant than the rest of the OCP. However, from an environmental point of view, other pesticides were shown to be more dangerous due to their greater ecotoxicity.

Regarding the environmental fate and behavior of OCPs, the factors influencing them were exhaustively described from the literature analysis. This information was then used to assess current levels of OCPs in the world's coastal sediments in various studies. We observe that the environmental behavior of these compounds is very complex and depends fundamentally on the intrinsic characteristics of each compound and the environment.

Parent pesticides were found at almost all the sites studied, which could represent a recent input into the system or poor system capacity to degrade them. This was observed mainly in subtidal sediments, which would have low oxygen content and little or no light exposure, i.e., unfavorable conditions for the aerobic degradation of the OCPs, which is the

main route of degradation in the environment. Moreover, this phenomenon was more noticeable in cold or temperate environments.

The predominance of each metabolite and parent isomer varied according to the prevailing environmental conditions. In intertidal sediments, the metabolites and isomers typical of oxygenated environments predominated, i.e., DDE, endosulfan sulfate, heptachlor epoxide, β-HCH, and β-endosulfan. Instead, metabolites typical of anaerobic environments such as DDD and the majority isomers of the technical mixtures (α-endosulfan, lindane, and α-HCH) predominated in subtidal sediments.

Finally, we must highlight the need to sustain permanent monitoring networks for these legacy pollutants, with a focus on non-surveyed regions and potentially polluted areas such as African and Asian coastal wetlands.

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Wu, X., Chen, A., Yuan, Z., Kang, H. & Xie, Z. (2020). Atmospheric organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) in the Antarctic marginal seas: Distribution, sources and transportation. *Chemosphere*, *127359*. https://doi.org/10.1016/ j.chemosphere.2020.127359.

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BIOGRAPHICAL SKETCHES

Lautaro Girones

Affiliation: Instituto Argentino de Oceanografía (IADO) - Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional del Sur (UNS), Bahía Blanca, Provincia de Buenos Aires, Argentina.

Education: Bachelor's Degree in Environmental Science, Universidad Nacional de Avellaneda (UNDAV), Avellaneda, Argentina.

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Research and Professional Experience:

Since April 2017 I have been a CONICET doctoral fellow developing my research activities at the Persistent Organic Pollutants Laboratory of the Instituto Argentino de Oceanografía (IADO), in Bahía Blanca, Argentina. My directors are Dr. Andres Hugo Arias and Dr. Jorge Eduardo Marcovecchio. I specialize in the study of Persistent Organic Pollutants such as organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), chlorinated paraffins (SCCPs and MCCPs), and polycyclic aromatic hydrocarbons (PAHs) in marine and continental aquatic environments. I have participated in several research projects, including research stays abroad, some of which have already been published in international scientific journals or will be published in the coming years. Among them, the study of pollution by POPs and their behavior and environmental fate in the Southwest of Buenos Aires stands out, analyzing both rivers and the Bahía Blanca Estuary, through the use of abiotic (e.g., sediments and water) and biotic matrices (e.g., snails, mussels, and fish). Besides, I also participate in the study of the river discharges impacts on the marine benthic community of the Province of Buenos Aires; the role of coastal wetland plants in the biogeochemical cycle of POPs; and the occurrence and environmental behavior of POPs in the Argentine continental shelf, among others.

Publications from the Last 3 Years (2018-2020):

Girones L., A.H. Arias, A.L. Oliva, T. Recabarren-Villalon, J.E. Marcovecchio. 2020. Occurrence and spatial distribution of organochlorine pesticides in the southwest Buenos Aires using the freshwater snail Chilina parchappii as environmental biomonitor. *Regional Studies in Marine Science*. Volume 33: 10089. https://doi.org/10.1016/j.rsma.2019.100898.

Girones L., A.H. Arias, J.E. Marcovecchio. 2019. Distribution of organochlorine pesticides (OCs) in coastal sediments of Latin America. *JAINA coasts and seas in the face of climate change*. Volume 1:2.

Girones L., A.L. Oliva, J.E. Marcovecchio, A.H. Arias. Spatial distribution and ecological risk assessment of residual organochlorine pesticides (OCPs) in South American marine environments. 2020. *Current Environmental Health Reports*. Volume 7: 147-160. https://doi.org/10.1007/s40572-020-00272-7.

Negrin V.L., L. Girones, A.V. Serra. 2019. Eco-friendly strategies of remediation in the marine system: bioremediation and phytoremediation. In: *Coastal and Deep Ocean Pollution* 1st Edition. Eds: Andres H. Arias and Sandra E. Botte. CRC Press/ Taylor & Francis Group. ISBN-10: 1138569399; ISBN-13: 978-1138569393. 370 pp.

Oliva A.L., A.C. Ronda, L. Girones, M.M. Orazi, T. Recabarren-Villalón, J.E. Marcovecchio, A.H. Arias. 2019. Polycyclic Aromatic Hydrocarbons: sources, occurrence, levels, distribution and ecotoxicological fate at coastal and Deep Ocean. In: *Coastal and Deep Ocean Pollution* 1st Edition. Eds: Andres H. Arias and Sandra E. Botte. CRC Press/ Taylor & Francis Group. ISBN-10: 1138569399; ISBN-13: 978-1138569393. 370 pp.

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Research and Professional Experience:

- PhD Scholarship: CONICET, Argentina. April 2020 – April 2025. "Organophosphorus pesticides in freshwater courses and coastal systems in the southwest of Buenos Aires Province, Argentina: evaluation of possible bioindicators." Instituto Argentino de Oceanografía, CONICET, Argentina.
- Research Scholarship for graduates of the Universidad Nacional del Sur: Secretaría General de Ciencia y Tecnología de la Universidad Nacional del Sur, Argentina. April 2019- April 2020. "Organophosphorus Pesticide Dynamics in a trophic chain of freshwater courses in southwestern Buenos Aires, Argentina." Instituto Argentino de Oceanografía, CONICET, Argentina.
- Research Scholarship for students of the Universidad Nacional del Sur: Secretaría General de Ciencia y Tecnología de la Universidad Nacional del Sur, Argentina. April 2018- April 2019. "Evaluation of the Corbicula fluminea clam as a bioindicator of contamination by persistent organic compounds intributaries of the Bahía Blanca Estuary, Argentina." Instituto Argentino de Oceanografía, CONICET, Argentina.

Professional Appointments: Chemistry Doctoral fellow

Honors: Argentine Chemical Society Prize 2019 for the best average in a Degree University Career, 11/22/2019.

Dr. Andres H Arias

Affiliation: Instituto Argentino de Oceanografía (IADO) - Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional del Sur (UNS), Bahía Blanca, Provincia de Buenos Aires, Argentina. Analytical Chemistry Area of the Chemistry Department of the National South University (Argentina)

Education: *PhD Biological Sciences* Marine Environmental Chemistry: water, soil and organism. Biogeochemistry. Environment Pollution and Monitoring. National South University, Argentina. Awarded with honour (10/10). *Biochemistry Sciences* (Analytical

Chemistry and Environmental Sciences Degree) at National South University, Argentina. Awarded with Honours. First award in qualifications.

Business Address: Instituto Argentino de Oceanografía. Florida 8000 (Camino La Carrindanga km 7,5), Complejo CCT CONICET Bahía Blanca | Edificio E1, B8000FWB Bahía Blanca | Argentina

Research and Professional Experience: My area of expertise falls between marine biogeochemistry and ocean monitoring, focusing in the pollution of the marine and coastal environments: Persistent Organic Compounds/Environmental Pollution Monitoring and Assessment. Plastics and Microplastics in the aquatic environment. Atmospheric transport of POPs to the coastal environments. Dry/Wet Deposition. Bioaccumulation and Biomagnification of POPs. Top predators. My main interests are to explore Persistent Organic Pollutants (POPs) and microplastic dynamics occurrence and bioaccumulation in marine and freshwater environments, the understanding of POPs movement through the trophic web, exploring POPs sources, origin and age of inputs PAHs, PBDEs, PCBs, OCs, OPs, emerging pollutants and microplastics in the marine/freshwater environment.

Professional Appointments: At present, researcher at the Argentinean Institute of Oceanography -IADO- (National Council of Scientific and Technological Research, CONICET, Argentina) at the Marine Chemistry Division. He is also Head of Laboratory (Teaching Position, permanent) at the Analytical Chemistry Area of the Chemistry Department of the National South University (Argentina). In addition, UNEP representative at Scientific Advisory Comitee Microplastics and Marine Litter and representative of Argentina at the GESAMP WG40. Member of the Directive Council Board of the Argentinean Institute of Oceanography (CONICET), he has performed posdoc at the University of Lille-1, Lille, France (CNRS) and UNAM, Mexico (CONACyT).

Honors: Awarded with the WIENER LAB Foundation prize (academic award), to the best overall qualification average of the year (2003) of the Biochemistry University Degree. First award for a conference paper at the VII Jornadas Chilenas de Física y Química Ambiental (Concepción; Chile, 2011). First award on conference paper in marine environments (Environment and Argentina, 2015). Has been awarded with several grants (PICES, Erasmus Mundus, Matsumae, IMBER ClimEco, etc)

Publications from the Last 3 Years (2020-2018):

(1) Alfonso, MB; Scordo, F; Seitz, C; Manstretta, GMM; Ronda, AC; Arias, AH; Piccolo, MC. (2020). First evidence of microplastics in nine lakes across Patagonia (South America). *Science of The Total Environment*, 139385.

(2) Girones, L; Oliva, AL; Marcovecchio, JE; & Arias, AH. (2020). Spatial Distribution and Ecological Risk Assessment of Residual Organochlorine Pesticides (OCPs) in South American Marine Environments. *Current Environmental Health Reports*, 1-14.

(3) Pozo, Karla; Urbina, Williams; Gómez, Victoria; Torres, Mariett; Nuñez, Dariela; PIbylová, Petra;Audy, OndeJ; Clarke, Bradley; Arias, Andrés; Tombesi, Norma; Guida, Yago; Klánová, Jana. Persistent organic pollutants sorbed in plastic resin pellet

"Nurdles"; from coastal areas of Central Chile. *Marine Pollution Bulletin*: Pergamon-Elsevier Science Ltd., 2020 vol. 151, n°. p - . issn 0025- 326X.

(4) Das, Shagnika; Arias, Andres HUGO; Cheng, Jing-O; Souissi, Sami; Hwang, Jiang-Shiou; KO, Fung-Chi. Occurrence and distribution of anthropogenic persistent organic pollutants in coastal sediments and mud shrimps from the wetland of central Taiwan. *Plos One.:* Public Library Science., 2020 vol.15 n°1. p - . issn 1932-6203.

(5) Alfonso, MB; Arias, HA; Piccolo, MC. Microplastics integrating the zooplanktonic fraction in a saline lake of Argentina: influence of water management. *Environmental Monitoring and Assessment*.: Springer. 2020, vol. 192, n°. p - . issn 0167-6369.

(6) Andrés Hugo Arias; Ronda, Ana Carolina; Oliva, Ana Laura; Jorge E. Marcovecchio. Evidence of Microplastic Ingestion by Fish from the Bahía Blanca Estuary in Argentina, South America. *Bulletin OF Environmental Contamination and Toxicology*.: Springer., 2019, vol. n°. p - . issn 0007-4861.

(7) Quintas, Pamela Y; Alvarez, Mónica B; Andrés H. Arias; Garrido, Mariano; Jorge E. Marcovecchio. Spatiotemporal distribution of organotin compounds in the coastal water of the Bahía Blanca estuary (Argentina). *Environmental Science and Pollution Research.* Heidelberg: Springer Heidelberg., 2019, vol. n°. p - .issn 0944-1344.

(8) Quintas, Pamela Y; Fernandez Eleonora; Carla V. Spetter; Andres H Arias; Garrido, Mariano; Jorge E. Marcovecchio. Preliminary studies about the role of physicochemical parameters on the organotincompounds dynamic in a South American Estuary (Bahía Blanca, Argentina). *Environmental Monitoring and Assessment.* Berlin: Springer. 2019 vol. n°. p - . issn 0167-6369.

(9) Oliva, Ana L; La Colla, Noelia S; Arias, Andrés H; Botté, Sandra E; Perillo, Gerardo ME; Piccolo, M. Cintia. First records of polycyclic aromatic hydrocarbons and metals in sediments from a shallow lake in the Pampean– Patagonian region (Argentina). *Marine and Freshwater Research*: Csiro Publishing, 2019, vol. n°. p - . issn 1323-1650.

(10) Recabarrén-Villalón, Tatiana; Ronda, Ana C; Arias, Andrés H. Polycyclic aromatic hydrocarbons levels and potential biomarkers in a native South American marine fish. *Regional Studies in Marine Science*: Elsevier BV., 2019, vol. n°. p - . issn 2352-4855.

(11) Orazi, Melina M; Arias, Andrés H; Oliva, Ana L; Ronda, Ana C; Marcovecchio, Jorge E. Characterization of atmospheric and soil polycyclic aromatic hydrocarbons and evaluation of air-soil relationship in the Southwest of Buenos Aires province (Argentina). *Chemosphere*: Pergamon-Elsevier Science Ltd. 2019, vol. n°. p - . issn 0045-6535.

(12) Ronda, Ana C; Arias, Andrés.; Oliva, Ana L; Marcovecchio, Jorge E. Synthetic microfibers in marine sediments and surface seawater from the Argentinean continental shelf and a Marine Protected Area. *Marine Pollution Bulletin*: Pergamon-Elsevier Science Ltd., 2019 vol. 149, n°. p - . issn 0025-326X.

(13) Girones, Lautaro; Arias, Andrés H; Oliva, Ana L; Recabarren-Villalon, Tatiana; Marcovecchio, Jorge E. Occurrence and spatial distribution of organochlorine pesticides in the southwest Buenos Aires using the freshwater snail Chilina parchappii as environmental biomonitor. *Regional Studies in Marine Science*: Elsevier., 2019, vol. n°. p - . issn 2352-4855.

(14) Quintas, Pamela Y; Oliva, Ana L; Alvarez, Mónica B; Arias, Andres H; Domini, Claudia E; Garrido, Mariano; Marcovecchio, Jorge E. Fast and Feasible Ultrasound-

Assisted Pretreatment for the Determination of Organotin Compounds in Environmental Samples. *Archives of Environmental Contamination and Toxicology*: Springer., 2018, vol. n°. p - . issn 0090-4341.

(15) Tombesi, Norma; Pozo, Karla; Arias, Andrés; Alvarez, Mónica; Pribylova, Petra; Audy, Ondrej; Klánová, Jana. Records of organochlorine pesticides in soils and sediments on the southwest of Buenos Aires Province, Argentina. *Environmental Earth Sciences*. Heidelberg: Springer Berlin Heidelberg, 2018, vol. 77, n°11. p - . issn 1866-6280. eissn 1866-6299.

(16) Vitale, Alejandro J; Perillo, Gerardo ME; Genchi, Sibila A; Arias, Andrés H; Piccolo, María Cintia. Low-cost monitoring buoys network tracking biogeochemical changes in lakes and marine environments; a regional case study. *Pure and Applied Chemistry.: Int Union Pure Applied Chemistry*, 2018, vol. 0, n° 0. p - . issn 0033-4545.

(17) Ronda, Ana Carolina; Oliva, Ana Laura; Andres H Arias; Orazi, Melina Mirta; Jorge E. Marcovecchio. Biomarker responses to polycyclic aromatic hydrocarbons in the native fish Ramnogaster arcuata, South America. *International Journal of Environmental Research: Univ Tehran.*, 2018, vol. n°. p - . issn 1735-6865.

Books:

(1) "Coastal and Deep Ocean Pollution". Editors: Andrés H. Arias, Sandra E. Botte-Taylor & Francis-/CRC Press, 2020. https://www.crcpress.com/Coastal-and-Deep-Ocean-Pollution/Arias-Botte/p/book/9781138569393.

Book Chapters

(1) The Northern Argentine Sea. Marcovechio JE, De Marco S, Gavio, Narvarte M, Fiori S, Gerpe M, Rodriguez D, Abbate C., La Colla N, Oliva AL, Zalva S., Bazterrica MC, Guinder V, Spetter V, Fernandes M, Arias AH, Botte S. En: *World Seas: An environmental Evaluation*. Volume I Europe America and West Africa, Charles Sheppard Ed., ISBN 978-0-12-805068-2.2019.

(2) Vulnerabilidad de ecosistemas marino costeros Sudamericanos a Contaminantes Orgánicos Persistentes: panorama actual e histórico. Arias AH, Oliva AL, Ronda AC, OraziMM, Marcovecchio JM. En: *Vulnerabilidad de las Zonas Costeras ante el Cambio Climático*, III Ed. ISBN 9786077887300. UNAM-ICMYT-UCampeche.2019.

(3) "The Coastal Zone and climate change: vulnerability and trends", Marcovecchio JE, La Colla NS, Vallina N, De Marco S., Hidalgo F, Arias AH, Spetter CV. In: *LAC coastal zones vulnerabilities under climate change*." ISBN 9786077887300. UNAM-ICMYT-UCampeche. Mexico.2018.

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Chapter 131

ATMOSPHERIC POPS THREAT THE MARINE ENVIRONMENTS

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ABSTRACT

Atmospheric input of POPs (e.g., organochlorine pesticides [OCPs], halogenated aromatic hydrocarbons -polychlorinated biphenyls (PCBs), chlorinated or brominated dibenzofuran and dibenzo-p-dioxins [PCDDs/DFs, PBDDs/DFs]) and polycyclic aromatic hydrocarbons [PAHs]) mainly comes from anthropic activities, including as primary emission sources those from agricultural production and industrial processes. Although the commercial use and application of some POPs have been totally prohibited or severely restricted (Stockholm Convention), they continue to be found in the air and other environmental compartments around the world.

Global atmospheric long-range transport has been widely demonstrated for these compounds and represents a dominant process by which POPs reach the oceans. This is far more relevant for POPs, such as OCPs and PCBs than for PAHs, as the first ones are only generated in the continental sector. On the contrary, PAHs can be generated in the marine environment, as in oil spills from ships. In addition to the atmospheric transport, atmosphere-sea interactions and the inter-compartmental transfer play an essential role in terms of deposition and subsequent entry of POPs into marine ecosystems. The transfer of POPs from the atmosphere to the ocean can take place by dry deposition, wet deposition and by diffusive gaseous flows. Fundamentally, in open marine systems,

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atmospheric deposition determines that the oceans could act as reservoirs for POPs. Also, aquatic planktonic species are believed to be impacted by atmospheric deposition, supporting the accumulation of POPs through the food chain. Once in the ocean, the fate of POPs will ultimately depend on several events such as movement flows, chemical transformations, physical and biological processes.

In this context, highlighting the important links between the atmospheric and oceanic systems, the objective of the present chapter is to consider the implication of atmospheric transport in the circulation of POPs and to present the relevance of atmospheric deposition to the oceans as a key process that affects the marine environments.

Keywords: POPs, atmospheric deposition, open ocean

INTRODUCTION

Among the thousands of chemical products that impact marine environments from anthropogenic activities, persistent organic compounds (POPs) present a scenario of special relevance due to their ability to remain intact in the environment for long periods of time, showing bioaccumulative characteristics in the fatty tissue and high toxic effects to both humans and biota. Polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs), halogenated aromatic hydrocarbons, polychlorinated biphenyls (PCBs), chlorinated or brominated dibenzofuran and dibenzo-p-dioxins (PCDDs/DFs, PBDDs/DFs) are semivolatile at atmospheric conditions (vapour pressures at 298 K in the range 10^{-6}-10^{-2} Pa) (Mulder et al. 2010) and subject to long-range atmospheric transport (LRAT), even when reaching remote zones such as open oceans. Except for PAHs, which can be generated in the marine environment by oil spills from ships, long-range transport enables terrestrial POPs to reach the remote oceanic environment (Castro-Jiménez et al. 2015).

The atmosphere plays an important role in the distribution of POPs at a global scale. The Convention on Long-Range Transboundary Air Pollution (CLRTAP Convention, 1979) is one of the first treaties on the potential of POPs to reach remote environments, including oceans, high mountains and the Arctic and Antarctic areas, where they have never been produced or used. Thus, POPs have been studied and incorporated into different atmospheric monitoring programmes around the globe (Global Monitoring Plan [GMP], Global Atmospheric Passive Sampling [GAPS] Network, and Arctic Monitoring and Assessment Programme [AMAP]). Furthermore, given the importance of persistence and LRAT, the Stockholm Convention (UNEP, 2017) recognises the need to eliminate, restrict or reduce the use and release of POPs. Despite the implementation of these treaties and regulations, POPs continue to cycle through the atmosphere and in aquatic and terrestrial environments. Due to the continuing threat that they pose to human health and the environment, it is necessary to identify and understand the distribution of POPs among environmental compartments. Different investigations and monitoring networks have aimed at better characterising the global fate of these toxic substances. Among them, the Integrated Atmospheric Deposition Network (IADN), in operation since 1990, evaluates the extent of atmospheric deposition in the Great Lakes region in North America (Castro-Jiménez et al. 2015).

Atmospheric deposition is the main mechanism by which POPs are removed from the atmosphere and deposited in other compartments. The atmospheric input of POPs into continental aquatic systems, coastal sites, large lakes and open oceans occurs through rain-

scavenging, falling particles and gas absorption. Also, some water bodies receive the contribution of POPs from local sources, run-off and discharge by tributaries. The coastal waters of the Mediterranean, Black, Aegean, and North Seas and of northeastern USA (Castro-Jiménez et al. 2012; Lammel et al. 2015; Lohmann et al. 2011; Mai et al. 2016; O'Driscoll, 2014), in the vicinity of primary emissions, reflected the influence of local sources, such as riverine input. Conversely, in the remote ocean, the main source is atmospheric deposition, and depositional processes are more relevant (Castro-Jiménez et al. 2015). Global oceans cover 3/4 of the planet's surface, the Pacific being the greatest of all, and they represent a key compartment during the global cycling of POPs. Nevertheless, few simultaneous measurements have been made in air and surface seawater in the open ocean, and POPs occurrence in the ocean-atmosphere system requires more research attention.

This chapter focuses on the relevance of the atmosphere as the main transport route for POPs to the open ocean. It describes long-distance transport and atmospheric deposition, considering the relevance of the depositional processes and gas exchange that allow POPs to reach oceanic waters. Moreover, it considers the implication of ocean biogeochemistry in the control of depositional flows, through the sinking fluxes (physical and biological) that accumulate POPs in the deep ocean. Finally, the chapter presents evidence that suggests the volatilisation of POPs in the open ocean.

CONSIDERATIONS ON THE ATMOSPHERIC TRANSPORT OF POPS

As for POPs to be distributed by LRAT, first, they must enter the atmosphere because they are directly emitted into it or by means of volatilisation from the medium where they were previously applied. Then, given their persistence in the air (half-life> 2 days), they can be transported through the atmosphere by air circulation. Furthermore, their behaviour and the probability that POPs undergo LRAT depend on the interaction of air-surface partitioning coefficients, the advective transport, and transformation and degradation processes (Hageman et al. 2015).

$$hops = \frac{\tau}{\frac{Inventory}{OutputFlux}} \quad (1)$$

Wania and Mackay (1996) described the migration of POPs using the "grasshopper effect", defined as successive cycles of volatilisation and deposition undergone by these compounds at normal ambient temperatures because of their sufficient volatility. This travel through the atmosphere in multiple hops, as opposed to a single emission-deposition event, is the key process that enables long-distance transport (Gouin et al. 2004).

The LRAT potential of each POP is associated with both its physical-chemical properties and the environmental characteristics where it is found. Therefore, the global fractionation of POPs will take place as a consequence of their volatility and the latitudinal variation in temperature. Thus, within the mixture of POPs, those more volatile will remain in the air longer than those less volatile, which will undergo partition faster on some surfaces. In turn, in tropical and subtropical regions, warm temperatures will enhance volatilisation from the surfaces. In areas of higher latitudes, colder temperatures favour deposition on surfaces and,

in mid-latitudes, seasonal temperature changes will regulate these cycles (Wania and Mackay, 1996).

In the case of an emitted molecule, a hop consists of a complete deposition-volatilisation cycle, which means that in air, the number of evaporation events defines the number of hops. By contrast, if the molecule is initially on a surface, the number of hops will be the number of depositions it undergoes. That number is an indicator of POP recycling in the atmosphere, and it is expressed as the residence time (τ) divided by the time a certain molecule of POP flushes out of the atmospheric compartment (Jurado and Dachs, 2008):

Equation 1 indicates how many times, on average, the molecules complete a deposition-volatilisation cycle while residing in the atmosphere (average time).

During the LRAT, it is important that transport and fractionation do not occur in a single hop. Multiple hops ensure that POPs will persist, and even if their emissions cease, they could be transported for years (Gouin et al. 2004).

Jurado and Dachs (2008) evaluated the grasshopping potential of POPs in the global oceanic atmosphere and observed that, during LRAT over the oceans, the number of hops had considerable spatial and seasonal variability. Although the air-surface partition coefficients are much more affected by temperature than the partitions involving other compartments, such as water-sediment or water-soil (Wania and Mackay,1996), the atmosphere-ocean exchange fluxes are fundamentally influenced by processes associated with the biogeochemistry of the water column. Thus, the global fractionation of POPs, as a complex interaction of many processes that maximise or minimise the number of hops, affects the transport potential of LRAT.

OCEANIC ATMOSPHERIC DEPOSITION: RELEVANCE, CONTROL AND IMPLICATIONS

Transfer of POPs from the air to the ocean surface occurs fundamentally through atmospheric deposition. POPs originate in the continental area from point or diffusive sources (agricultural, industrial and numerous combustion emissions), and like all semivolatile compounds, they are distributed in the atmosphere between the particulate and gas phases. The degree of association with the particulate phase will be determined by the vapour pressure of each compound, ambient temperature and the amount and nature of particles (aerosol) present. As shown in Figure 1, POPs arrive in the oceans after undergoing long-distance transport and are removed from the atmosphere by deposition processes involving dry particle deposition, wet deposition and gaseous deposition.

Atmospheric dry deposition occurs when the compounds are removed by gravitational settling associated with particle flux (Bidleman, 1988). Thus, in the open ocean, POPs adsorbed onto marine aerosols follow a downward way to the water surface.

Experimental measures of dry deposition fluxes (F_{DD}, pg m^{-2} d^{-1}) are estimated by Eq. (2) (Jurado et al. 2004):

$$F_{DD} = v_D C_P \quad (2)$$

where v_D is the overall particle dry deposition velocity (m d^{-1}) and C_P is the POP particulate-phase concentration (pg m^{-3}). Dry deposition velocity shows a high dependency on particle diameter distribution and wind speed (turbulence or stability atmospheric).

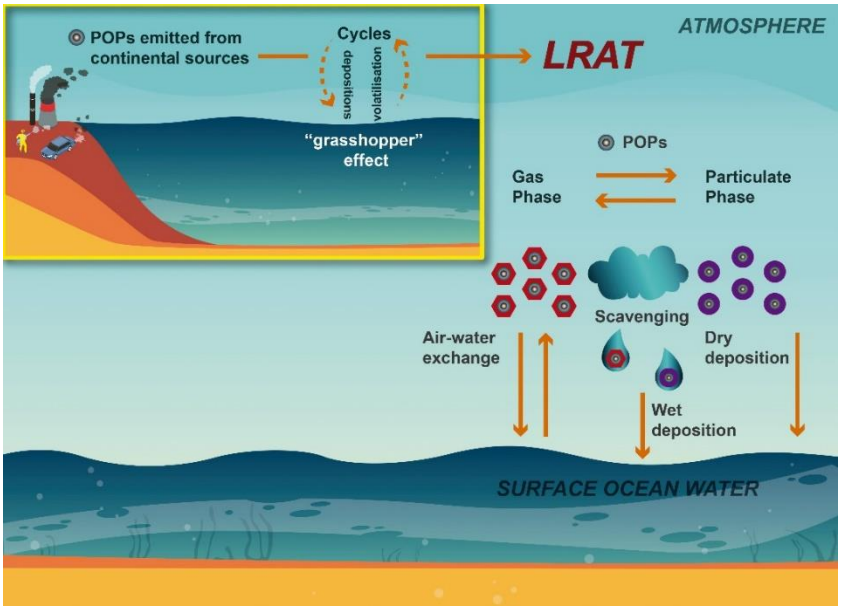

Figure 1. Key atmospheric processes that allow the arrival of POPs at the surface water of the open oceans. Adapted from Castro- Jiménez et al. (2015) and Lohmann and Dachs (2019).

Atmospheric wet deposition involves the removal of POPs through rain, fog, clouds and snow. Among these forms, rain is the most relevant washing mechanism (Simcik, 2004). Precipitation will deposit POPs in dissolved and particulate form. Wet atmospheric removal is very different for particle-associated compounds than for gas-phase compounds. POPs that are in the gas phase will be eliminated according to Henry's Law ($H' = H/RT$) equilibrium between the vapour and aqueous phases and will partition in the water droplets to be washed, falling on the ocean surface, while those that are in the atmospheric particulate matter will be eliminated by precipitation. Particle scavenging is a more complex process involving cloud weather conditions and the physical-chemical properties of the aerosol (Castro-Jiménez et al. 2015; Ligocki et al. 1985).

Wet deposition fluxes (F_{WD}, pg m^{-2} d^{-1}) can be calculated using Eq. (3) (Jurado et al. 2005):

$$F_{WD} = p_0 C_R \tag{3}$$

where p_0 is the precipitation rate per day (m^3 m^{-2} d^{-1}) and C_R is the concentration (atmospheric gas and particulate phases) of POP in rain (pg m^{-3}). Considering that the gas phase POP concentrations have been estimated using Henry's law constant (H', Pa m^3 mol^{-1}) and that particle scavenging results from processes controlled by properties of the aerosol, F_{WD} can be estimated by Eq. (4) (González-Gaya et al. 2015):

$$F_{WD} = p_0 \left(\frac{C_G}{H'} + W_P C_P \right) \quad (4)$$

where p_0 is the precipitation rate per day ($m^3\ m^{-2}\ d^{-1}$), C_G the POP concentration in the gas phase, H', Henry's Law constant and particle scavenging, based on the washout ratio (W_P) and C_P the POP concentration in the particulate phase.

The relative importance of each depositional process will depend on a combination of factors, such as the levels of atmospheric particulate matter, rain intensity, POP affinity to aerosols, etc.).

Generally, dry particle deposition is important for POPs with low-medium volatility and strong affinity to particle (PAHs and dioxins and furans of higher molecular weight). By contrast, more volatile POPs highly associated with the atmospheric gas phase (PCBs and HCHs) would be more affected by diffusive processes. Besides, particulate matter deposition depends on the aerosol diameter, wind speed, and atmospheric stability, including the effects of spray formation under high wind speed conditions and particle growth due to high relative humidities. POP partitioning to aerosols can occur through absorption into the organic phase of aerosols and adsorption onto soot carbon (Jurado et al. 2004).

Given the hydrophobic nature of POPs, removal from the atmosphere by wet deposition would be expected to be much less effective than removal by dry deposition of particles. Also, within clouds, interstitial aerosols are excluded from the aqueous phase and the particle scavenging is very inefficient. In addition, wet deposition is an episodic and intense process that strongly removes POPs when it first occurs and then loses capacity as a transport vector since the atmosphere has become depleted of POPs. However, during periods of abundant rainfall, the washout of POPs from the atmosphere is significant and wet deposition plays an important role in the mobility and environmental fate of POPs (Cetin et al. 2016, González-Gaya et al. 2015; Skrdlíková et al. 2011).

Diffusive air-water exchange (or dry gaseous deposition) consists in the transfer of POPs from the atmospheric gas phase to the water dissolved phase (Castro Jiménez et al. 2015)

In order to understand the diffusive processes, the fugacity approach is used. Fugacity is "a measure of chemical potential or partial pressure of a chemical in a particular medium that controls the transfer of chemicals between media" (Wang et al. 2011). The fugacities of POPs in the air (f_A) and water (f_W) were calculated using Eqs. (5) and (6) (González-Gaya et al. 2015), as follows:

$$f_A = C_G RT \quad (5)$$

$$f_W = C_W H' RT \quad (6)$$

where C_G and C_W are the POP concentrations in the gas phase ($pg\ m^{-3}$) and dissolved phase ($pg\ m^{-3}$), respectively; R, the gas constant (8.314 $Pa\ m^3\ mol^{-1}\ K^{-1}$), H', Henry's law constant (dimensionless) and T (K).

The fugacity ratio f_A/f_W between the air and water for POPs can be calculated using Eq. (7) (Nizzetto et al. 2010), and K_{AW} is the temperature corrected dimensionless air-water equilibrium partition coefficient (Henry's Law constant).

$$\frac{f_A}{f_W} = \frac{C_G}{C_W K_{AW}} \tag{7}$$

A fugacity ratio $f_A/f_W = 1$ indicates a condition of thermodynamic equilibrium of POPs between the air and water phases and no net transfer in either direction. Net deposition or volatilisation is indicated by values of f_A/f_W significantly smaller or greater than unit, respectively (Bigot et al. 2016). Thus, values of f_A/f_W >1 indicate deposition and f_A/f_W <1 indicate the potential for re-emission of compounds from surface water (Nizzetto et al. 2010).

The net air-water diffusive flux is always from high to low fugacity (González-Gaya et al. 2015). Thus, the concentration gradient is driven across the air-water interface and the tendency depends on environmental conditions (wind speed, temperature) and specific compound physical-chemical properties, all of which influence the mass transfer coefficient (k_{AW}, m d^{-1}). Absorption (F_{AW}, pg m^{-2} d^{-1}) and volatilisation fluxes ($F_{AW_{vol}}$, pg m^{-2} d^{-1}) are obtained using Eqs. (8) and (9) (Dachs et al. 2002):

$$F_{AW} = k_{AW}\frac{C_G}{H'} \tag{8}$$

$$F_{AW_{vol}} = k_{AW}C_W \tag{9}$$

Fluxes of POPs across the air-water interface will be controlled by the rate of gaseous exchange and is given by Eq. (10) (Dachs et al. 2002):

$$F_{AW} = k_{AW}\left(\frac{C_G}{H'} - C_W\right) \tag{10}$$

k_{AW} may be estimated by implementing the two-layer surface resistance model as the result of mass transfer coefficients in each phase ($k_A$and k_W), using Eq. (11) (Dachs et al. 2002):

$$\frac{1}{k_{AW}} = \frac{1}{k_A H'} = \frac{1}{k_W} \tag{11}$$

where k_A and k_W are the POP mass transfer coefficients (m d^{-1}) in the air and water layers, respectively. For their calculations, empirical standard coefficients are used: H_2O in the air ($k_{A,H2O}$) and CO_2 in the water ($k_{W,CO2}$) (González-Gaya et al. 2015).

On the air side, k_A may be calculated from the mass transfer coefficient of H_2O ($k_{A,H2O}$, m d^{-1}) by Eq. (12) (Dachs et al. 2002)

$$k_A = k_{A,H2O} + \left(\frac{D_{POP,a}}{D_{H2O,a}}\right)^{0.61} \tag{12}$$

where $D_{POP,a}$ and $D_{H2O,a}$ are the diffusivity coefficients of the POP and H_2O in air, respectively.

In addition, $k_{A,H2O}$ is given by Eq. (13)

$$k_{A,H2O} = 0.2U_{10} + 0.3 \tag{13}$$

where U_{10} is the wind speed at 10 m height from the atmosphere-ocean interphase.

Similarly, in the water, k_W may be calculated from the mass transfer coefficient of CO_2 on the water side ($k_{W,CO2}$, m d^{-1}) (Eq. 14)

$$k_W = k_{W,CO2} + \left(\frac{Sc_{POP}}{600}\right)^{-0.5} \tag{14}$$

where Sc_{POP} is the Schmidt number of the POP and 600 accounts for the Schmidt number of CO_2 at 298 K, respectively.

And $k_{W,CO2}$ is given by Eq. (15)

$$k_{W,CO2} = 0.24U_{10}^2 + 0.061U_{10} \tag{15}$$

Therefore, k_{AW} is strongly dependent on wind speed and also on temperature, Schmidt numbers, and H' (Dachs et al. 2002).

The dynamics of the atmosphere-ocean system is critical to global cycling and the fate and behaviour of POPs. From the surface ocean waters, they can enter the atmosphere and, by coupling to other mechanisms, they become susceptible to physical or biological alterations, such as degradation, incorporation into the food chain and deposition in the deep ocean.

The control on POPs fate includes the following processes:

- Geochemical processes: air-water exchange, degradation in air, degradation in water ("degradative pump") and vertical fluxes with settling particles to deep waters (sinking fluxes).
- Biological pump

In the atmosphere, POPs can undergo chemical reactions with oxidants, such as hydroxyl radical (·OH), the nitrate radical (NO3) and ozone (Keyte et al. 2013). The main degradation of POPs occurs by reaction with ·OH and depends on the concentration of ·OH in the atmospheric boundary layer and on the half-life (t1/2) of POPs. However, within-ocean biogeochemical processing of POPs is the most relevant control in the global dynamics and the role of oceans as sink of POPs.

As Figure 2 shows, in the ocean-surface mixed layer, POPs partition between dissolved and organic particulate phases (Jaward et al. 2004). Phytoplankton uptake and vertical fluxes of particles are the key to controlling the surface recycling, impact and sinks of POPs (Dachs et al. 2002). Although there are multiple and complex interactions in the water column that would greatly affect the atmospheric depositional processes (Jaward et al. 2004), under certain environmental conditions, the removal of POPs concentrations from the water column would deplete their concentrations in surface waters and modify the equilibrium conditions, thus enhancing the air-water diffusive flux.

Once POPs enter the ocean-surface mixed layer, POPs fluxes are established between water and plankton (F_{WP}, ng m^{-2} d^{-1}), as described in Eq. 16 (Dachs et al. 2002):

$$F_{WP} = k_{WP}\left(C_W - \frac{k_d}{k_u} C_P\right) \tag{16}$$

where C_P is the POP concentration in phytoplankton (ng kg^{-1}), k_u (m^3 kg^{-1} d^{-1}) and k_d (d^{-1}) are the uptake and depuration rate constants, respectively. The mass transfer coefficient between water and plankton (k_{WP}, m d^{-1}) depends on the mixed layer depth (MLD, m), the phytoplankton biomass (B_p, kg m^{-3}), and the uptake constant, as described in Eq. 17:

$$k_{WP} = MLD \cdot B_p \cdot k_u \tag{17}$$

Therefore, the dynamics of POPs in the marine environment is linked to the phytoplankton dynamics. POPs sorb to phytoplankton and a fraction will sink associated to cells, fecal pellets, and other aggregates. The biological carbon pump is the sinking of phytoplankton from the surface layers to the seabed. This mechanism involves a "sequestration" of atmospheric carbon (captured by phytoplankton) and its quick and efficient transfer to great depths. Thus, carbon captured at the surface is retained in the deep ocean for hundreds of years (Agusti et al. 2015). The biological pump induces depletion of POP concentrations in the photic water column by sequestering them in the particulate organic carbon that sediments, and therefore, the biological pump favours the diffusive air-water exchange.

Considering the role of the ocean as a final sink for POPs, the vertical sinking of particulate matter is a key process in removing organic carbon and POPs from the sea surface to the deep ocean. Particle sinking is a net unidirectional and non-fugacity driven process. According to Dachs et al. (2002), vertical fluxes of POPs out of the mixed-layer are given by the product of POP concentrations in sinking particles to the vertical flux of sinking organic matter (F_{OM}, ng m^{-2} d^{-1})

$$F_{sink} = k_{sink}\frac{k_d}{k_u} C_P \tag{18}$$

where k_{sink} (m d^{-1}) is the mass transfer rate coefficient for sinking POPs.

$$k_{sink} = F_{OM}\frac{k_u}{k_d} \tag{19}$$

Despite the limited number of studies conducted empirically in the open ocean, atmospheric deposition has been empirically verified in oceanographic campaigns along ocean transects. Diffusive air-water exchange is the mechanism that dominates the removal of atmospheric POPs and their entry into oceanic surface water. However, the occurrence of dry and wet deposition has been evaluated in some oceanic regions.

On the one hand, Jurado et al. (2004) evaluated the relative importance of dry particle deposition and air-water exchange for the Atlantic Ocean and at the global scale and determined that for all PCBs congeners and lower PCDD/Fs, air-water exchange dominates

the dry deposition mechanism. However, these authors were able to show a consistent spatial variability in depositional fluxes. The dry deposition fluxes increase at mid-high latitudes in association with the marked presence of marine aerosols, lower temperatures that improve the partition of pollutants in the aerosols and high wind speeds that favour turbulence and aerosol accumulation. The zone influenced by the Saharan-Sahel dust (between 10° N and 25° N) and the zone around 30° S registered the highest values of dry deposition fluxes in association with high concentrations of marine aerosol. On the other hand, Jurado et al. (2005) observed that wet deposition was highest in the Intertropical Convergence Zone (ITCZ), where convective precipitation rates are elevated.

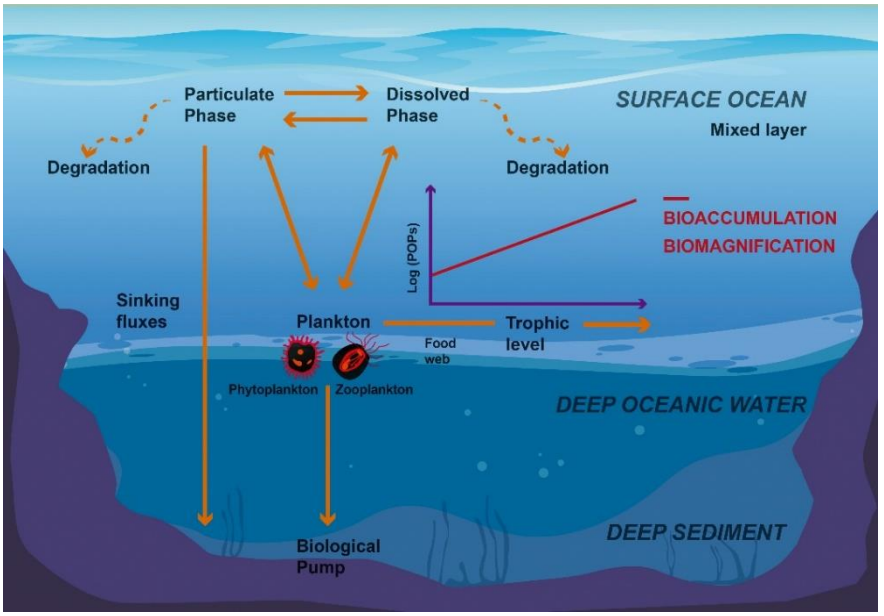

Figure 2. Main ocean biogeochemical processes that affect the distribution and fate of POPs. Adapted from Dachs et al. (2002), Jaward et al. (2004) and Lohmann and Dachs (2019).

These works characterize the atmospheric deposition of PCBs and PCDD/Fs to the Atlantic Ocean, reporting average values of dry aerosol deposition flux in the order of 66 $ng.m^{-2}\ yr^{-1}$ and 9 $ng.m^{-2}\ yr^{-1}$ respectively, and, average values of wet deposition fluxes in the order of 110 and 45 $ng.m^{-2}\ yr^{-1}$. Furthermore, the total dry deposition results in 2200 $kg.yr^{-1}$ (PCBs) and 500 $kg.yr^{-1}$ (PCDD/Fs); the total wet deposition results in 4100 $kg.yr^{-1}$ (PCBs) and 2500 $kg\ yr^{-1}$(PCDD/Fs); the net air-water exchange is higher, 22000 $kg.yr^{-1}$ for PCBs and 1300 $kg.yr^{-1}$ for PCDD/Fs.

In addition, other works presented evidence of atmospheric deposition in the open ocean. For instance, PAHs deposition over tropical oceans has been reported (Del Vento and Dachs, 2007). Atmospheric dry deposition velocities ranged between 0.1 and 0.3 $cm\ s^{-1}$. The important atmospheric input of Saharan dust in the Northeast Subtropical/Tropical Atlantic Ocean was reflected as dry deposition in the region between 26° and 21° N. In this region, for the more volatile PAHs outbreaks of Saharan dust there is a significant increase in the deposition velocities, reinforcing what was observed by Jurado et al. (2005). The South Atlantic and the Southern Ocean showed a net flux for α-HCH, γ-HCH and for Endosulfan

from air to seawater, indicating that these oceans remain net sinks for these pesticides (Lueck et al. 2017). The fugacity ratio calculations for hexachlorobenzene (HCB) and γ-HCH suggested that they were deposited from air to water in the North Atlantic (Zhang et al. 2012). The observed air–sea gas exchange gradients in the Arctic Ocean mainly favoured net deposition of OCPs (Cai et al. 2012). In the Southern Ocean, Bigot et al. (2016) determined that the Indian-Pacific sector acts as an environmental sink for OCPs and the air-water fugacity ratios and fluxes indicated net deposition for γ-HCH, dieldrin and chlorpyrifos.

Furthermore, trophic levels were coupled to air-water exchange through phytoplankton. Air-water fugacity ratios demonstrated the potential for lindane (γ- HCH) gas deposition to water and γ-HCH concentrations found in krill samples (*Euphausia superba*) and have been correlated with seawater concentrations in the Southern Ocean. Thus, it shows bioconcentration of HCHs from seawater (Cincinelli et al. 2009). High concentrations of PCBs in crustaceans from the deep Pacific Ocean illustrated their penetration into even the most remote regions of the ocean from atmospheric deposition (Jamieson et al. 2017). In addition to this, the strong bioaccumulation and magnification of PCBs, particularly in apical predator marine species, account for the impact of atmospheric deposition on the ecosystem. Thus, the presence of POPs in biota reflects the influence of atmospheric deposition from the base of the trophic pyramid of the marine ecosystem to apical predators. While phytoplankton exposure to POPs occurs only through water, so that bioaccumulation could respond to the dynamics of the equilibrium between cells and the surrounding water, zooplankton accumulates POPs from the water and from its diet. This suggests that zooplankton biomagnifies contaminants, such as PCBs and OCPs (Hallanger et al. 2011). As a consequence, top predators, such as large pelagic fish, of ecological importance in open oceans, have presented predominance of OCPs, especially DDTs, versus PCBs in the Indian Ocean (Munschy et al. 2020).

Phytoplankton plays a key role not only in acting as the first link in transferring these pollutants to higher trophic levels, but also as a carbon transporter from the atmosphere to deep waters and sediments (biological bomb). The latter role favours the sinking of POPs into the ocean. Morales et al. (2015) correlated PCDD/Fs and PCBs concentration with plankton biomass in the Atlantic, Pacific, and Indian Oceans, suggesting that atmosphere deposition-water column- plankton are coupled. The settling fluxes of organic matter bound PCDD/Fs and PCBs are of 400 and 10,500 kg y^{-1}, respectively. The atmospheric inputs are nearly 3 and 10 times larger for PCDD/Fs and PCBs. Thus, they support the role of the biological pump in the accumulation of POPs in deep ocean. With regard to the biological pump, it is expected to be a much more efficient process for higher hydrophobicity POPs than for the less hydrophobic ones. However, in areas of high productivity such as the North Atlantic region adjacent to the Arctic Ocean, the transport of HCB and HCHs (two OCPs of less hydrophobicity) was evidenced by the action of the biological pump (Galbán- Malagón et al. 2013).

More recently, Buesseler et al. (2020) have applied a metric approach to the ocean depths and the euphotic zone, determining the existence of regions where the action of the biological pump would significantly increase its carbon sequestration efficiency. Therefore, its relevance would be even greater, promoting the accumulation of POPs in the deep ocean and hence the atmospheric deposition (Nizzetto et al. 2012).

OCEANIC POPS OUTGASSING

Considering the global cycling of POPs, Meijer et al. (2003) suggested two possible scenarios based on primary emissions.

i) Primary emission levels are high and dominate the atmospheric concentrations. Once the chemical compound is released from its "primary source", it is deposited and remains retained, which prevents its volatilisation. Thus, the reservoirs (soil or sediments buried in deep ocean waters) would act as sinks.
ii) Emissions from the environment capacitors (secondary sources, such as soils and oceans) dominate the atmospheric level concentration. Environmental reservoirs control POPs atmospheric levels.

Historically, the ocean has acted as an ultimate sink for POPs. During the peak usage of POPs, primary sources dominated their atmospheric levels and important biogeochemical drivers, causing POPs burial in sediments. As expected, atmospheric levels of POPs gradually decreased after the application of regulatory actions and strict controls. In this new global environmental scenario, the sinks could play a role in the air input of POPs by re-emitting these compounds from the major environmental repositories.

Although POPs re-emission from ocean waters has rarely been documented, some research on modelling suggests the feasibility of occurrence of these compounds. Stemmler and Lammel (2009), for instance, employed global simulation and historical data on the usage of DDT. They observed that, in ocean models, some marine regions acted as the most significant secondary DDT source, coinciding with a decline in the large number of applications (1970s–1990s). The global multi-compartment chemistry-transport model with coupled atmosphere and ocean determined that the Western North Atlantic region was the most significant secondary DDT source. In large parts of the Tropical Ocean and in the mid-latitude meridional areas, there has been evidence for net volatilisation, showing a reversal of the usual net flux in the marine air system. For instance, Nizzetto et al. (2008) found PAHs along a North-South Atlantic transect (Σ_{10} PAHs ≈ 1.4- 2.5 ng m^{-3} air, and 0.7-1 ng L^{-1} seawater) and calculated f_A/f_W for selected compounds. For fluoranthene and pyrene f_A/f_W ≈ 1, suggesting air–water partitioning near equilibrium in remote, open ocean areas. In the remote tropical Atlantic Ocean, for anthracene and phenanthrene, f_A/f_W were < 0.3, suggesting net volatilization.

A relevant study on the evidence of degassing in the open ocean was carried out by Zhang and Lohmann (2010). These authors reported, for the first time, that PCBs could volatilise in the oligotrophic open ocean during a decline in land-based emissions. The fugacity ratio indicated that PCBs were volatilising from surface waters to the atmosphere, and air-water exchange fluxes were ~0.5 to ~30.4 ng m^{-2} d^{-1}.

These authors attributed the volatilisation of PCBs in the Pacific mainly to the following two causes:

A reduction in POPs in the environmental concentrations. During periods of high PCBs levels of emissions, the oceans act as PCBs accumulators. After the cessation of their use and production, due to the decrease in emissions, the atmosphere responds more quickly than large bodies of water (buffer function), resulting in low air-water fugacity ratios.

The extremely low rate of particle sinking in the South Pacific (one of the most oligotrophic oceanic regions). The South Pacific Gyre, part of the remote Pacific, presents low sedimentation rates because of the low surface concentrations of chlorophyll and the low surface biological production. It would cause an increase in the PCBs concentrations in the dissolved phase. This factor, together with the high temperatures of the water, would move the fugacity gradient towards net volatilisation. In this way, the South Pacific would be a secondary source of PCBs.

Although the cause of net volatilisation has not yet been elucidated, the removal of PCBs from the Open Pacific surface into the lower atmosphere has been clearly demonstrated. After the elimination of primary emission sources, the ocean subsurface can provide the ocean surface and the atmosphere with POPs. Whether due to rapid changes in the atmospheric patterns or to a longer-term trend in ocean occurrence, the ocean could act as a source rather than a sink for some POPs.

Nizzetto et al. (2010) provided experimental evidence across the Atlantic Ocean to illustrate that the ocean has a buffering capacity to moderate the rate at which the system would respond to an underlying change in continental emissions. This buffering capacity showed dependence on the type of POP.

While several polychlorinated dioxins and furans (PCDD/Fs) have experienced a marked decrease (even greater than that experienced in continental areas), the levels of PCBs have not decreased significantly over the remote oligotrophic Atlantic Ocean.

The analysis of loss/sink velocities for a range of PCBs and PCDD/Fs congeners evidenced that the main difference between these compounds was the reaction in surface water, with PCDD/Fs being faster (1-2 orders of magnitude) than PCBs. Given the oligotrophic characteristics of these waters, the mechanism by which POPs are degraded is likely the indirect photochemical pathway and not the biodegradation. During the day, the irradiation of chromophores found in the dissolved organic matter (DOM) of the surface water originates hydroxyl radical, which more reactively attacks the structure of PCDD/Fs (the four C atoms neighbour of O atom). Thus, PCDD/Fs decline faster ($t_{1/2}$) than PCBs. Although this mechanism requires further investigation, it suggests a remarkable role for the ocean DOM in controlling the global fate of POPs.

More recently, Li et al. (2020) presented the first field study describing the open Pacific Ocean as a secondary source of OCPs, reporting fugacity ratios that suggest volatilization of HCH from surface water to the atmosphere, with air-water exchange fluxes in the range 0.3-11.1 ng m^{-2} day^{-1}.

CONCLUSION

The transport dynamic of POPs to the open ocean is particularly associated with another dynamic: that of ocean-atmosphere system interactions. Not only has the Pacific Ocean atmosphere been affected by the arrival of these semivolatile organic compounds, travelling long distances from their source of origin, but also that of the Atlantic, Indian and polar oceans. The atmosphere deposition is the undisputed mechanism through which POPs may be introduced into the oceans. One of the most important depositional processes involves the air-water exchange.

The evaluation of these fluxes between the atmosphere and the ocean surface water provides the key to monitoring POPs in open oceans. Although oceans are proved to be compartments of great relevance to POPs environmental fate, studies considering the simultaneous measurements of these compounds in air and water are scarce.

Even though many ocean biogeochemical mechanisms have not been clearly studied, the highly hydrophobic properties of POPs allow them to be coupled to organic particles, and this assembled combination will then be present at greater depths. Apart from sedimentation, biological pumping processes encourage atmosphere deposition. As a consequence of their volume, oceans are the greatest final sinks for POPs. However, there have been historical changes in these compounds' emissions, and this fact is associated with the demonstration of surface waters acting as buffers between the atmosphere and the deepest ocean layers. This produces the re-emission of some POPs to the atmosphere, reversing the net deposition fluxes, with the oceans playing a degassing role.

In spite of the difficulties inherent in oceanographic campaigns, such as the costs involved in the use of ships, the required sample volume, the complication of comparing the data due to the influence of air masses, and ocean gyres, it is extremely important to increase the database of air-water exchange directions and the number of flux calculations in order to gain a better understanding of the dynamic and global fate of the main POPs in the open ocean. Moreover, as the atmospheric deposition of POPs has an impact on phytoplankton and thus on POPs accumulation at different trophic levels, it would be essential to evaluate the implications of depositional processes because they can pose a risk to the ocean value as a resource, thus affecting the health of the ecosystems.

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Publications from the Last 3 Years:

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Chapter 132

MICROPLASTICS POLLUTION: FROM CONTINENTAL SOURCES TO MARINE SYSTEMS

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ABSTRACT

Microplastics are amongst the contaminants of emerging concern for aquatic systems. These ubiquitous environmental contaminants began to be reported since the 1970s, with increasing studies about their distribution and impacts from 2004. Although marine microplastics research remains at the forefront, studies on freshwater and terrestrial environments have begun to occur in this field as a matter of priority in recent years. This chapter addresses the influence of plastic pollution on marine environments through continental water bodies. Rivers and effluents have been identified as significant pathways from terrestrial sources releasing between 4.8 and 12.7 million metric tons of microplastics per year that reach the ocean. Microplastics are present in different sizes, colors, shapes and textures influencing their behavior in the environment. Also, their interaction with the biotic and abiotic systems influence the final fate and transport of them. The varied hydrological characteristics (water flow velocity, water flow seasonal variation, water depth) in lakes and rivers significantly affect plastics transport within freshwater systems towards the ocean. In estuarine environments, the combined effect of turbulence and salinity can interact with particle density, size, and charge. Added to this, storms, floods and runoff contributes to microplastics incorporation in the watersheds

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from soil and atmosphere. Therefore, the study of continental microplastics sources and pathways are critical to understanding them as emerging global contaminants.

Keywords: microplastics, marine systems, continental sources, microplastics pathways

INTRODUCTION

The large scale of plastic production, the massive consumption and plastic waste production has been increasing since 1950 (Rochman, 2018). The global production of plastic reached approximately 360 million tons in 2018 and is expected to continue increasing in the next years (Plastics Europe, 2019). In a global approach, plastic production and consumption are not the same between developed and developing countries. Nowadays, China is considered the world's largest producer and consumer of plastic materials (Zhang et al., 2018) while pollution in non-developed countries is significantly lower (Xia et al., 2020); therefore, plastic impact is expected not to be uniform around the world.

From this plastic production, at the end of its life, more than 80% ends in the marine systems (Rochman, 2018), positioning plastic litter as a significant problem for the environment, particularly for aquatic systems. Plastics disposal mismanagement causes their spread in environments where they are fragmented, forming microplastics (MPs) (Cole et al., 2011). MPs are plastic particles less than 5 mm in diameter (Thompson et al., 2004) and can be divided into primary (directly produced MPs) or secondary MPs (resulting from plastics fragmentation) being the latter those most registered in worldwide aquatic systems. Among the identified MPs, polystyrene (PS), polyethylene (PE), polyamide (PA), polyethylene terephthalate (PET) and polypropylene (PP) were the most detected polymers in aquatic systems. Polystyrene (PS) is used in packagings such as foam cups, trays, food containers and eating utensils. Meanwhile, PET and PA are widely used in textile industries for synthetic clothes. PE is used in food packaging, films, plastics bottles and beauty care products.

According to Rochman (2018), rivers and effluents have been identified as significant pathways from terrestrial sources releasing between 4.8 and 12.7 million metric tons of microplastics per year that ultimately reach the ocean. Evertheless, the continental inputs' dynamics into the marine environments are poorly documented, as the information on the sources, fate and pathways of continental MPs is limited. Nowadays, MPs are ubiquitous in a broad type of environments from marine (Arias et al., 2019), freshwater (Besseling et al., 2017; Piehl et al., 2019; G. Wong et al., 2020), terrestrial (van den Berg et al., 2020) to the atmosphere (Dris et al., 2016; Enyoh et al., 2019). They are present in different sizes, colors, shapes and textures influencing their behavior in the environment. Also, the interaction with the biotic and abiotic systems influence their final fate and transport among these different environments. MPs in soil could be suspended due to wind action, then deposited by dry/wet atmospheric deposition processes and transferred by runoff to freshwater environments to finally end in the sea. MPs cycle is similar to C, N and other elements cycles where all compartments interact, making a complicated situation to determine a single pathway for MPs from continental to marine environments. Moreover, the varied hydrological characteristics significantly affect the plastic transport within freshwater systems towards the ocean. These comprise water flow velocity, water depth, bottom topography and water flow seasonal variation in lakes and rivers (Campanale et al., 2020). In estuarine environments, the

combined effect of turbulence and salinity can interact with particle density, size and charge (Díez-Minguito et al., 2020). Added to this, storms, floods and runoff contribute to MPs incorporation in the watersheds from the soil and atmosphere (G. Wong et al., 2020). Therefore, the study of continental MPs' sources and pathways is crucial to understanding them as emerging global contaminants.

The Relevance of Microplastics Contribution from Continental Sources

Since scientists began to study MPs, they were found in water, soil, air and biota registering a long-range transport between these compartments (Dris et al., 2016; Eckert et al., 2018; Arias et al., 2019; Ronda et al., 2019). Determination and quantification of MPs transport pathways from continental sources to the marine environment have been significant challenges. The results from numerous monitoring programs indicate that environmental plastic pollution problems can only be solved by attacking the pollution sources. In contrast, MPs removal from the environments is hardly possible (Bitter and Lackner, 2020). Long-range atmospheric transport from urban centers has been registered in remote mountain catchments (Allen et al., 2019) and Arctic snow (Bergmann et al., 2019) demonstrating that MPs are transported atmospherically to both local and faraway places. Thus, identifying the most relevant point sources of plastics release into the environment is a crucial factor.

Rivers and effluents have been identified as significant pathways from terrestrial sources (Rochman, 2018). From these releases, wastewater treatment plants (WWTP) and landfills are responsible for a considerable proportion (Kazour et al., 2019), with several factors influencing their discharge such as treated water volume, flow rate, filtration processes and sewer systems (Mason et al., 2016; Ziajahromi et al., 2017). MPs removal efficiencies in WWTPs generally range between 72 and 99.4% (Sun et al., 2019); nevertheless, a large amount of these residues still reach lakes and river catchments (Mathalon and Hill, 2014; Murphy et al., 2016). Moreover, sludges from WWTP are usually used in agricultural fields, returning a significant MPs proportion to the environment (van den Berg et al., 2020).

Continental MPs Sources

Although marine microplastics research remains at the forefront, studies on freshwater and terrestrial environments have begun to occur in this field as a matter of priority in recent years (Table 1). Therefore, it is possible to determine the different inputs and influence of plastic pollution from terrestrial environments through continental water bodies that reach the ocean (Fig. 1). In the last years, more studies quantifying MPs presence in soils (van den Berg et al., 2020), dust (Yukioka et al., 2020), atmosphere (Dris et al., 2016, 2018; Stanton et al., 2019), lakes (Wang et al., 2018; Zhang et al., 2018; Alfonso et al., 2020a, 2020b) and rivers (Lebreton et al., 2017; Piehl et al., 2019) were worldwide developed. Nevertheless, the connected pathways among all these compartments still need for more research. Moreover, all MPs do not end in the ocean without first having passed through terrestrial and freshwater environments. Therefore, according to Rochman (2018), MPs' study in these ecosystems will give us more information about their source as the particles will be less weathered, fouled and fragmented than in the ocean.

Table 1. Overview of microplastic sources, sample type, lower particle size limit (mm), concentration, main category and affected sea/ocean

Study site/Country	Sample	Lower size limit	Concentration	Main category	Affected Ocean (sea)	Reference
Lakes Superior, Erie, and Huron, United States	SW, SE	0.355	43,157 MPs km^{-2}	NA	Atlantic ocean	Eriksen et al., 2013
Lake Garda Italy	SE	1 µm	108-1,108 MPs km^{-2}	NA	Adriatic Sea	Imhof et al., 2013
Lake Bolsena, Italy	SW, SE	0.3 mm	0.82-4.42 MPs m^{-3}, 112 MPs kg^{-1}	Fibers	Mediterranean Sea	Fischer et al., 2016
20 Wuhan lakes, China	SW	50 µm	1,660-8,925 MPs m^{-3}	Fibers	Pacific Ocean (China Sea)	Wang et al., 2017
Lake Winnipeg, Canada	SW	0.33 mm	193,240 MPs km^{-2}	NA	Atlantic Ocean (Artic Sea)	Anderson et al., 2017
Lake Ontario, Canada	SW	125 µm	0.8 ± 0.7 MPs L^{-1}	Fibers, blue	Atlantic Ocean	Grbić et al., 2020
Poyang Lake, China	SW	< 0.1 mm	5–34 MPs L^{-1}	Fibers, coloured	Pacific Ocean (East China Sea)	Yuan et al.. 2019
Lake Vembanad, India	SE	NA	252,800 MPs km^{-2}	NA	Indian Ocean (Arabic Sea)	Sruthy and Ramasamy, 2017
Yangtze River, China	SW	50 µm	2,516.7 MPs m^{-3}	NA	Pacific Ocean (China Sea)	Wang et al., 2017
Hanjiang River, China	SW	50 µm	2,933 MPs m^{-3}	NA	Pacific Ocean (China Sea)	Wang et al., 2017
River Thames, United Kingdom	SE	1 mm	185-660 MPs kg^{-1}	Fragment, Fibers	Atlantic Ocean (Northern Sea)	Horton et al., 2017
Mame River, France	SW	0.8 mm	100.6 MPs m^{-3}	Fibers	Atlantic Ocean	Dris et al., 2018
Bloukrans River, South Africa	SE	63 µm	6.3-160.1 MPs kg^{-1}	NA	Indian Ocean	Nel et al., 2018
Valencia, Spain	S	50 µm	930-3,060 MPs kg^{-1}	Fragments	Mediterranean Sea	van den Berg et al., 2020
Anacostia River, United States	S	250 mm	1,270 MPs kg^{-1}	Fibers, Fragments	Atlantic Ocean	Helcoski et al., 2020
Greater Paris, France	A, WWTP	100 µm	29-280 MPs m^{-2} day^{-1} 14,000-320,000 MPs m^{-3}	Fibers	Atlantic Ocean (Celtic Sea)	Dris et al., 2015
Seine and Mame Rivers, Paris	SW	50 µm	4-108 MPs m^{-3}	Fibers	Atlantic Ocean (Celtic Sea)	Dris et al., 2016
Pearl River Estuary, China	SW	0.355 mm	2.376 MPs m^{-3}	Fragments, transparent and white	Pacific Ocean (South China Sea)	Lam et al., 2020
London, U.K	A	20 µm	575-1,008 MPs m^{-2} day^{-1}	Fibers	Atlantic Ocean	Wright et al. 2020
Kusatsu (Japan), Da Nang (Vietnam)	RD	100 µm	1970-2,000 MPs km^{-2}	Fragments	Pacific Ocean (China Sea and Japan Sea)	Yukioka et al. 2020

Abbreviations: SW: surface water, SE: sediment, S: soil, RD: road dust, A: atmospheric fallout, WWTP: wastewater treatment plant, NA; no available.

Soil

The presence of MPs in soil dust is becoming more apparent. As research advances, we know that soils can act as a long-term sink for microplastics (Rochman, 2018; Wang et al., 2020). Plasticulture application on agricultural fields (e.g., plastic sheeting in greenhouses or plastic film mulching covering) for production, contributes to MPs presence from its continuous weathering and fragmentation in situ (Wang et al., 2019). Other sources of MPs in soils are open waste dumping (Wang et al., 2020), road littering with tire abrasion (Yukioka et al., 2020) and landfills (Bläsing and Amelung, 2018). However, the application of sewage sludge from WWTP in worldwide agricultural lands was recognized as the leading cause of MPs presence in soils (van den Berg et al., 2020). According to Nizzetto et al. (2016), the annual MPs input into agricultural lands from sewage sludge/biosolids application could exceed the total amount of MPs in oceans.

Most previous studies concerning soil MPs were conducted in China, one of the greatest producers and consumers of plastic (Zhang et al., 2018). A study in coastal soils adjacent to the Bohai Sea and Yellow Sea in China showed that MPs concentrations ranged from 1.3 to 14712.5 MPs kg^{-1} (Zhou et al., 2018) and were associated with local anthropogenic activities as aquaculture, port construction and tourism. Also, soils from farmlands and forest zone around Dian Lake, registered MPs concentration between 7100 and 42960 MPs kg^{-1} (mean value 18760 MPs kg^{-1}) (Zhang and Liu, 2018). Furthermore, it was estimated that sewage sludge application in farmlands from Europe and North America, releases about 63000 to 430000 and 44000 to 300000 tons of MPs per year, respectively (Nizzetto et al., 2016b). Furthermore, a study in four WWTP and 16 agricultural fields from Spain found that on average, soils' plastic loads increased by 280 light density MPs kg^{-1} and 430 heavy density MPs kg^{-1} with each successive application sewage sludge (van den Berg et al., 2020). For South America, a study case in agricultural fields from Chile found soil MPs contents ranging from 600 to 10400 MPs kg^{-1}, depending on sludge rates applied (Corradini et al., 2019).

Atmosphere

The ubiquitous presence of MPs in the environment from soil, biota and aquatic systems leads us to think about their presence in the atmosphere. Nevertheless, the number of studies about the occurrence and distribution of suspended atmospheric microplastics (hereafter SAMPs) is recently beginning to increase in comparison with other environmental compartments (Dris et al., 2015, 2016; Liu et al., 2019), considering the atmospheric transport as a potential pathway for continental MPs to the ocean. First studies were carried out in urban areas, as the developed-in Greater Paris, located on the River Seine basin (that drains in the North Atlantic Ocean) and where concentrations from 29 to 280 SAMPs m^{-2} day^{-1} were registered (Dris et al., 2015). The higher concentrations were associated with rainfall events, suggesting that this could be an important environmental factor for atmospheric deposition. Another study developed in London found deposition rates ranging from 575 to 1008 SAMPs m^{-2} day^{-1}, where fibers dominate the shape category in 92% of the samples (Wright et al., 2020). Therefore, more studies should focus on environ-mental variables' effects on atmospheric MPs presence, emphasizing the plastic sources in the catchments exposed to severe anthropogenic impacts, mainly urban areas.

A recent study sampled sea air during a cruise in the western Pacific Ocean, founding SAMPs concentrations from 0 to 1.37 MPs m^{-3} (Liu et al., 2019), and fibers dominated the samples (60%). Higher SAMPs concentrations were registered in the coastal area (0.13 ± 0.24

SAMPs m^{-3}), than in the pelagic area (0.01 ± 0.01 SAMPs m^{-3}), and during the daytime (0.45 ± 0.46 SAMPs m^{-3}) than the nighttime (0.22 ± 0.19 SAMPs m^{-3}) (Liu et al., 2019). These are the first results providing consistent evidence that SAMPs are critical MPs sources pollution in the oceans and emphasize textile microfibers' presence. Another recent study using high-resolution spatial and temporal data revealed that near 132 SAMPs m^{-2} day^{-1} are deposited in remote U.S. conservational areas trough atmospheric deposition (Brahney et al., 2020). The authors remark that microfibers dominated the sample composition, suggesting that laundry drying could be the primary source in which microfibers are released several times more than through wastewater during the washing (Pirc et al., 2016). They also estimated averaged deposition rates of >1000 metric tons per year in these protected lands (Brahney et al., 2020). According to these results, urban centers and resuspension from soils or water are the primary sources of plastics wet-deposited; meanwhile, under dry conditions, SAMPs smaller in size were associated with longer-range or global transport (Brahney et al., 2020).

Lakes, Rivers and Estuaries

Lakes, rivers and estuarine systems are among the most studied MPs sources to the date, linking the continental systems to marine environments (Sighicelli et al., 2018; Arias et al., 2019; Meng et al., 2020; Xia et al., 2020). The studies developed in these systems focused on quantifying water and sediment MPs as the most relevant plastic sources. Most of these studies were focused on pollution monitoring alone, rather than discussing the possible pathways or their interaction with the environmental factors in their way into the ocean (Ajith et al., 2020).

The vast majority of these studies were developed in North America, Europe and Asia and still increasing in South America and Africa during the last years (Blettler et al., 2017; Alfonso et al., 2020a). These studies are particularly relevant, considering that freshwater and estuarine ecosystems are highly diverse. MPs found in these systems give us relevant information about their sources on their way to the oceans. Based on the literature, MPs concentrations in continental surface waters are comparable and even higher than marine systems in some cases (Table 1) (Grbić et al., 2020). Besides those MPs studies developed in endorheic lakes, most of them were developed in lakes and rivers that directly or indirectly connect with estuaries and the ocean, contributing to their final deterioration.

Microplastics were first reported in freshwater lakes in 2013 when a study developed in the Great Laurentian Lakes recorded MPs abundances up to 466,000 MPs km^{-2} downstream from two major cities, with a mean value of 43,000 MPs km^{-2} (Eriksen et al., 2013). A recent study in Lake Ontario, Canada, analyzed the MPs' presence in surface waters (800 MPs m^{-3}), stormwater runoff (15400 MPs m^{-3}), agricultural runoff (900 MPs m^{-3}) and treated wastewater effluent (13300 MPs m^{-3}) (Grbić et al., 2020). Studies in larger shallow lakes from China registered similar or higher MPs concentrations ranging from 900 to 4650 MPs m^{-3} in Dongting and Hong lakes, respectively (Wang et al., 2018); between 1660 and 8925 MPs m^{-3} in 20 urban lakes from Wuhan (Wang et al., 2017); and up to 34,000 MPs m^{-3} in Poyang Lake, the largest freshwater lake from China (Yuan et al., 2019). In European lakes, MPs concentrations in the Bolsena and Chiusi lakes in Italy were 2.51 and 3.02 MPs m^{-3}, respectively (Fischer et al., 2016). In South America, there are few studies in lakes registering concentrations from 143.3 ± 40.4 MPs m^{-3} in a shallow lake (Alfonso et al., 2020a) and 0.9 MPs m^{-3} in nine deep lakes from Patagonia (Alfonso et al., 2020b); nevertheless, all of them correspond to closed basins, with no connection to the ocean. The only study in a lake

discharging the Atlantic Ocean through the Paraná River is in a floodplain lake (Setúbal Lake) where concentrations of 704 MPs m^{-2} in sediment samples were found (Blettler et al., 2017).

MPs concentrations from rivers worldwide resulted in similar or higher than lakes depending on the contamination level. For example, a study in two rivers from China registered 2516.7 ± 911.7 MPs m^{-3} and 2933 ± 305.5 MPs m^{-3} in the Yangtze River and Hanjiang River, respectively (Wang et al., 2017). Another study in the Seine River and Marne River in France found lower concentrations between 4 and 108 MPs m^{-3} with a plankton net, and between 0.28 to 0.47 MPs m^{-3} with a manta net (Dris et al., 2015); nevertheless, in a more recent study in the Marne River, higher concentrations (100.6 ± 99.9 MPs m^{-3}) were registered (Dris et al., 2018). In South America, a study in an Andean tropical river from Ecuador (Guayllabamba River Basin) registered concentrations up to 1,186,339 MPs m^{-3} (Donoso and Rios-Touma, 2020). It is estimated that 1.15 and 2.41 million tons of plastic waste, currently enters the ocean every year from rivers, with over 74% of emissions occurring between May and October. It has been reported that the 20 most polluted rivers reported in the world, mainly located in Asia, account for 67% of the global total plastic debris that enters the ocean every year (Lebreton et al., 2017). In Europe, studies estimated that the Danube River releases 530–1,500 tonnes of plastic into the Black Sea annually (Lechner et al., 2014) and that 20–31 tons flow into the North Sea every year from the Rhine River (van der Wal et al., 2015). Reported range in raw and treated wastewater in WWTPs from Europe, USA and Australia were between 1–3160 MPs L^{-1} and 0.0007–125 MPs L^{-1}, respectively, with PS and PE as the most detected polymers (Sun et al., 2019).

Finally, estuarine systems are the final step of MPs from continental sources into the ocean where urban discharges trough river could be significant sources of these particles to coastal ecosystems (Figueiredo and Vianna, 2018) and the ocean (Andrady, 2011). Populated catchments estuaries have been documented as hotspots of plastic pollution (Lam et al., 2020). A study developed in the Mississippi Sound, located along the coasts of Mississippi and Alabama in the northern Gulf of Mexico, registered concentration between 1200 to 381000 MPs m^{-3}, founding higher concentrations in the estuaries than in their riverine inputs (Scircle et al., 2020). The authors' explanation is based on that estuary acts as a sink for these plastics from the river where mechanical and photolytic degradation to smaller particles increases their concentrations (Scircle et al., 2020). A study in the Pearl River Estuary, China, registered concentrations of 2.376 ± 0.700 MPs m^{-3}, where the highest values were found in the river mouth (Lam et al., 2020). In a previous study, the Pearl River was tagged as the third-largest source of continental-based plastics in oceans, with an annual discharge of 0.1 million tonnes of plastic debris into the South China Sea (Lebreton et al., 2017). These results reveal the critical role of rivers and estuaries to transport MPs from continental sources to the oceans. In Spain, a study developed in the estuary from Ría de Vigo registered concentrations of 0.64 ± 0.10 MPs m^{-3} and 0.48 ± 0.11 MPs m^{-3} during upwelling and downwelling conditions, respectively (Díez-Minguito et al., 2020). Another estuary from the Elbe River (Germany) found 600,000-fold higher MPs concentrations in sediments (3,350,000 MPs m^{-3}) than in water (5.57 MPs m^{-3}) (Scherer et al., 2020). Finally, a study in Brazil, including the Juagaribe and the Timonha-Ubatuba estuaries, found concentrations between null and 0.31 MPs m^{-3} (Garcia et al., 2020).

Pathways of Continental Microplastics: Environmental Variables and Particles Characteristics

There are complex and multiple relations between the environmental variables and human activities influencing MPs' presence and distribution in the environment. Therefore, determine their pathways and transport from continental sources to the ocean has been a significant challenge. In a first approach, the scientific community has considered lakes, rivers and coastal inputs as the principal sources of marine plastic pollution (Rochman, 2018). However, the latest studies also recognized that atmospheric MPs deposition could also be a significant source for marine MPs pollution (Brahney et al., 2020).

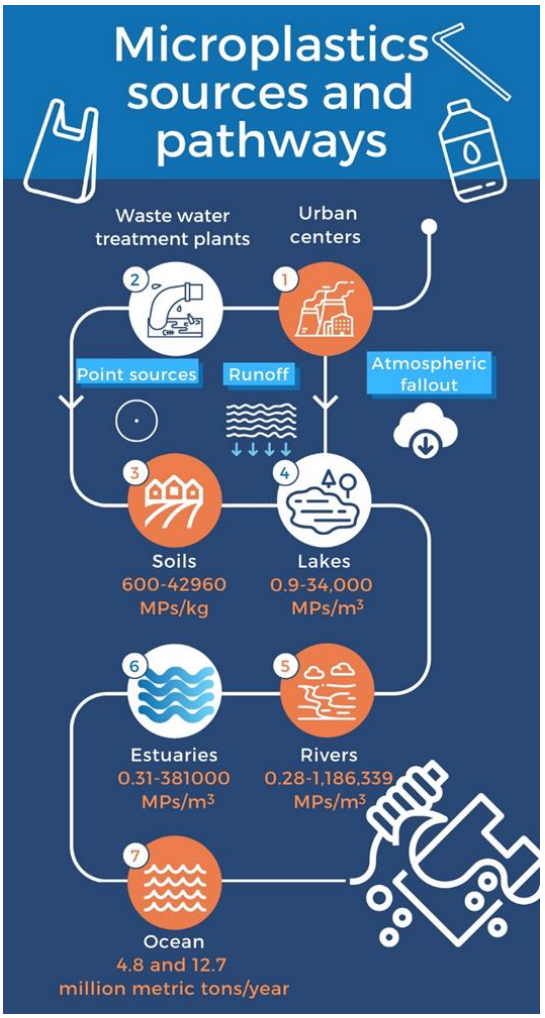

Figure 1. Microplastics sources and pathways from the continent to the ocean.

Nowadays, it is senseless to think about marine plastic pollution remediation actions without assessing and performing practical solutions focusing on the understanding and managing emissions and pathway processes on continental sources. Scientists should elucidate the "microplastics cycle" to understand MPs' global fate in oceans (Rochman and

Hoellein 2020). Here we proposed a conceptualization of how different MPs pathways interact in an agro-urban global context based on the consulted bibliography (Figure 1). As we establish in previous sections, the anthroposphere (1), which includes urban and agricultural environments, is the leading continental source of plastic pollution to the oceans. From it, primary and secondary MPs enter and cycles between the four environment compartments: the lithosphere (soil) (2), the atmosphere (3), the hydrosphere (lakes, rivers, estuaries and oceans) (4) and the biosphere (5). As the interactions between plants and animals are broad and complicated because of trophic interactions, in this section, we will focus on the sources and pathways between compartments from 1 to 4.

In the anthroposphere, we can cite many MPs pathways across the complex gradient between urban and rural environments. Among them are industrial emissions, resuspension from lithosphere (soils), building and traffic dust to the atmosphere. Plastics may also reach the hydrosphere through direct MPs inputs in aquatic systems due to irresponsible in situ disposal wastes, urban WWTP in rivers, or runoff on catchments. Simultaneously, WWTP sludge on agricultural soils and atmospheric plastic particles fallout contributes to the lithosphere contamination. Finally, trough wind resuspension or runoff, MPs can reach aquatic systems again. Little is known about the MPs behavior inside and between these spheres and their interaction with the environmental variables as flow velocity, turbulence, seasonal flow, water depth variation, catchments topography, salinity and tidal activity in estuarine environments, precipitations and runoff, atmosphere radiation and oxidative processes, among others.

Dry and Wet Atmospheric Deposition

Atmospheric transport, which has been recently considered one of the pathways by which continental MPs reach the ocean, has been poorly investigated and understood (Liu et al., 2019). A recent study quantified the wet and dry fallout of MPs, suggesting that as in the case of soil transport (Mahowald et al., 2014), those MPs with a size < 25 µm can be transported in a long-range or global scale distance (Brahney et al., 2020). Meanwhile, more prominent MPs as fiber can be transported on a regional scale (10 to 1000 km) (Lawrence and Neff, 2009). Moreover, if we consider that MPs densities are lower than those of soil particles (0.65 - 1.8 g cm^{-3} vs. ~2.65 g cm^{-3}), it can be expected that MPs will be more transportable. Also, microfibers with higher surface area/volume ratios will increase dragging forces and reduce settling velocities (Brahney et al., 2020). Therefore, according to these results, it is expected that large MPs and wet deposition will contribute less than dry deposition, highlighting the role of regional storms in the local fallout of MPs and a broader scale of atmospheric pattern to a global dispersion (Brahney et al., 2020).

According to SAMPs studies, rain, snow and wind events are responsible for the atmospheric deposition (Dris et al., 2016; Liu et al., 2019; Brahney et al., 2020). It has been reported by Allen et al. (2019) that rainfall and snowfall events duration was negatively correlated with SAMPs fallout, suggesting that frequency and intensity of these events are more relevant than duration. According to climate models, these results are relevant, considering that more frequent and intense rainfalls are expected in the future (Westra et al., 2013).

Microfibers are the most cited MPs type in worldwide studies, and those related to atmospheric deposition are not the exception (Dris et al., 2018; Liu et al., 2019). A recent study found that fibers (60%) dominate the SAMPs in the Pacific Ocean, also accompanied

by fragments (31%), granule (8%) and microbeads (5%) (Liu et al., 2019). The authors found that SAMPs decreased in abundance from the coastal area towards the ocean by ten times and that during the daytime was twice than nighttime, as a result of the settlement caused by the high relative humidity at night, especially on the sea (Liu et al., 2019). According to Liu et al. (2019), the nighttime could potentially contribute to SAMPs settlement since relative humidity is higher, being crucial this period for the SAMPs entering to oceans from the atmosphere. They speculated that 52% of SAMPs could deposit and dispersed in the ocean during the daytime (Liu et al., 2019). These results confirm that atmospheric deposition from the continent is an essential pathway of MPs pollution in the ocean, especially that caused by textile microfibers. Besides considering the distance from continental sources, other variables as time sampling hour, wind speed and relative humidity should be considered when atmospheric MPs pollution pathways are assessed.

Respect to the shape composition of SAMPs, it was observed that samples become less diverse as the distance from the coastline increases (Liu et al., 2019). Also, it was observed that the number of SAMPs decreased at night, being fibers the shape that most decreased, which are prone to the settlement due to their density and size. Agreeing with previous studies the authors finally state that small-sized SAMPs could be easily spread to more remote areas by the wind (Allen et al., 2019; Brahney et al., 2020). Respect to polymer composition, the study found a high percentage of PET in SAMPs samples, and that the proportion PET and PE−PP decreased at night compared with the daytime. These results could be explained by their physicochemical properties (density and hygroscopicity) and relative humidity conditions. In the case of Allen et al. (2019), the authors registered that SAMPs with different composition varies with climate, registering higher amounts of PS when rain or snow events were lower, and higher amounts of PE when these events increased.

In conclusion, the concentration of SAMPs in the atmosphere will vary with changing atmospheric pressure, wind, temperature, rainfall and snowfall conditions (Allen et al., 2019; Enyoh et al., 2019). The dispersion of SAMPs increases with higher wind speed and turbulence conditions. On the other hand, low atmospheric pressures generate strong air turbulence and, thus, increasing SAMPs dispersion; finally, high pressures systems generate air stability developing episodes of plastic pollution (Enyoh et al., 2019). A vertical temperature gradient could also improve SAMPs ascending movement. In the case of temperature inversion, plastic particles may be blocked in the atmosphere's low layers, which prone pollution episodes. Finally, it should be considered that SAMPs are under the effect of humidity and solar radiation, which may contribute to smaller particles formation that could be easily transported to long distances (Allen et al., 2019; Enyoh et al., 2019; Liu et al., 2019; Brahney et al., 2020).

Precipitation Patterns and Runoff

Rainfall events affect MPs' presence and dispersal: inland lakes and rivers are how pollutants are transported by runoff from their terrestrial anthropogenic sources (urban and agricultural areas) by rivers towards estuaries and finally reaching the ocean (Xia et al., 2020). Recent studies remarked the importance of rainfall over MPs and other associated contaminants due to the first flush effect (Piñon-Colin et al., 2020). This process consists that during a precipitation event with the surface runoff increase, high concentrations of pollutants are recorded at the beginning of the event to decrease gradually. Finally, when MPs are present in lakes and rivers, they can enter to the lower trophic chain levels by

adsorption/absorption or ingestion, to subsequently move upwards by bioaccumulation, which presents a considerable risk to the ecosystem and people that make use of these systems (Bradney et al., 2019). Nevertheless, the accuracy of MPs' emission estimates is currently hindered by the lack of data about MPs transport efficiency in runoff and streams.

A study developed in the Keelung River and the Xindian River found positive correlations between precipitations and MPs concentrations (G. Wong et al., 2020). This positive relation was also registered in rivers such as Los Angeles River in the USA (Moore et al., 2011), River Seine in France (Dris et al., 2015) and Venoge River in Switzerland (Faure et al., 2015). According to G. Wong et al. (2020), this could be explained by deposited MPs' resuspension in the river banks. The authors also suggest that when storm sewers cities network is not connected to WWTP, all kinds of pollutants directly flow into the rivers through the runoff (G. Wong et al., 2020). They also reported that an increasing amount of MPs indicating a land-based origin was observed after heavy rainfall events. Furthermore, authors suggest a lag period of 24 hours between the rainfall and the MPs concentrations increase corresponding to the time that the rainwater takes to drag the particles from land into the storm sewers to finally deposit them in the river (G. Wong et al., 2020).

Depending on precipitation patterns (G. Wong et al., 2020), catchment soil characteristics (Nizzetto et al., 2016a), and river hydrodynamics (Besseling et al., 2017), MPs retention as well as vertical distribution may vary significantly along with rivers (Lebreton et al., 2017). Studies in rivers registered temporal differences in MPs concentrations associated with seasonal rainfall differences and runoff. A study in three rivers of California found differences of three orders of magnitude (Moore et al., 2011). Likewise, a study in four estuarine rivers around the Chesapeake Bay showed that the highest MPs concentrations occurred after significant rain events (Yonkos et al., 2014). These results emphasize the role of runoff and seasonality of freshwater contamination by MPs. Respect to rainfall and global runoff patterns, a study found two distinct river plastic input peaks: one for African, North and Central American rivers occurring between June and October, and the other for European, South American and Australia-Pacific rivers occurring from November to May (Lebreton et al., 2017). According to the authors, rainfall and global runoff patterns for Asia are not as pronounced as for other continents since there is a balance between inputs from East Asia plus the Indian Subcontinent and Southeast Asia during the northern hemisphere and southern hemisphere summer, respectively (Lebreton et al., 2017).

Hydrodynamics

Another factor to consider when MPs are studied in lakes and rivers is that the hydrodynamics determine the speed in which MPs arrive to the ocean. We can assume that for a singular plastic particle, the buoyancy will be different in the case of estuaries where fresh and saline water effect are combined added to turbulence, which will interact with particle size, density and charge, respect to freshwater environments as rivers (Eerkes-Medrano et al., 2015; Leslie et al., 2017; J. K. H. Wong et al., 2020). Therefore, a greater particle flocculation and deposition were observed in estuaries in comparison with other aquatic environments. The advance in knowledge about modeling in aquatic systems and the increase in MPs field data, starts to clarify the mechanisms influencing these particles' presence and distribution pathways in diverse aquatic environments. A study based on a literature review developed a hydrological model to determine spherical MPs fate and transport (100 nm to 10 mm). The authors assessed the advective transport effect, homo- and

hetero-aggregation, sedimentation-resuspension, polymer degradation, biofilm presence and burial (Besseling et al., 2017). They found that river hydrodynamics affect MPs size distributions with consequences in their emission to the ocean. Spatial characteristics such as width, depth and flow rate result in net sedimentation and resuspension areas (Quik et al., 2015). Also, MPs size has a relevant effect on their fate and accumulation in hot spots along with river sediments (Besseling et al., 2017). Polymer density and biofilm formation effects were not significant since heteroaggregates formation was observed between suspended solids and MPs. The authors conclude that intermediate-sized particles of about 5 mm will be less retained (18-25%) than the smaller submicron size range particles as well as micro- and millimeter-sized MPs (Besseling et al., 2017).

On the other hand, a study developed in the Thames River upgraded a mathematical model to assess catchment hydrology, soil erosion and sediment budgets effects on rivers MPs fate (Nizzetto et al., 2016a). The model assumed that physical controls on soil erosion and sediment transport are the same that control MPs' transport. According to their results, those particles with a size < 0.2 mm will not be retained in streams; meanwhile, those larger particles with higher densities than water will be retained in sediments, which could be remobilized under high flow periods (Nizzetto et al., 2016a). Furthermore, those river sections experiencing low stream power are likely hotspots for MPs deposition on sediments. Therefore, MPs storage in soils and river sediments should be considered temporary or permanent sinks of relevance, contributing to the delay or prevention of the MPs to reach the marine environment.

Internal currents of water bodies are responsible for plastic litter accumulation. It has been demonstrated that in the ocean, currents are responsible for accumulating these particles in different sizes in the subtropical convergence zones (Van Sebille et al., 2015). Previous studies in large lakes as the Laurentian Great lakes or Qinghai Lake, found that plastic debris distribution is affected by the currents lake (Hoffman and Hittinger, 2017; Xiong et al., 2018). Moreover, source inputs and wind direction are responsible for MPs distribution in some small lakes (Imhof et al., 2013; Free et al., 2014; Zhang et al., 2016).

In estuarine systems, a recent study assessed the MPs distribution patterns in a coastal upwelling environment, Ría de Vigo estuary (Spain). Using water and sediment MPs field data during upwelling and downwelling conditions and modeling (2D-vertical model), the authors investigated the relative importance of river discharge, wind-driven and density-driven circulation (Díez-Minguito et al., 2020). According to their results, wind force was the main driver of MPs mobilization/sedimentation. Its domain on the water circulation is more significant than those of the density-driven and river flows circulation. Also, they observed that MPs' presence was higher in surface waters at the outer half of the estuary during upwelling. The flush out of MPs to the sea was affected by wind-induced circulation in the water surface; meanwhile, near the bottom, the effect of wind landward and gravitational circulation produces the presence of a maximum MPs concentration inside the estuary (Díez-Minguito et al., 2020). Therefore, according to their modeling results, MPs pathways circulation in estuarine environments are dominated by winds in the estuary's outer part, and near the head are dominated by the gravitational circulation. Nevertheless, according to Wolanski and Elliott (2015), MPs transport in lower rivers and estuaries could differ among studies because their differences in river morphology and tidal activity, tidal currents, circulation and geometry of each estuary.

Worldwide Continental Influence on Ocean Pollution by MPs

To solve marine plastic pollution, we should quantify and understand the plastic debris from inland sources to seas and the actual plastic pollution damage to human and environmental health. According to modeling studies, MPs exports differ spatially among European rivers, according to their socio-economic status and technological development of WWTP (Siegfried et al., 2017). Therefore it is wise to assume that MPs exports will differ in the same manner a global scale, founding significant differences among countries and continents as we can observe in the different studies reviewed here (Table 1).

In a first attempt to determine the global contribution of plastic from continental sources trough rivers, a modeling study calibrated with available data in the literature, found that up to 2.41 million tons year^{-1} of plastic waste end in the ocean only from rivers (Lebreton et al., 2017). According to their results, more than 90% of plastic inputs corresponds to the top 122 polluting rivers (4% of total surface area and 36% of the global population), from which, 103 are located in Asia, eight in Africa, eight in South and Central America and one in Europe (Lebreton et al., 2017). These results agree with the values found in the literature (Table 1) and the fact that most MPs studies are developed in China. Nevertheless, the authors point out that this input estimate is conservative, as it only comprises plastic litter from 0.3 mm to 0.5 m, and neglect the contribution outside this size range. According to their modeling, 1.21 million tons per year (range: 1.00–2.06) are only contributed from Asia, with China and Indonesia as significant contributors. The remaining 14% of river plastic mass input is distributed as follows: 7.8% coming from Africa (109,200 tons per year), 4.8% from South America (67,400 tons per year), 0.95% from Central and North America (13,400 tons per year), 0.28% from Europe (3,900 tons per year) and 0.02% from the Australia-Pacific region (300 tons per year) (Lebreton et al., 2017). A study suggests that MPs atmospheric transport into the ocean seems weak compared to rivers (Liu et al., 2020). This study estimated that between 7.64 and 33.76 tons of atmospheric microplastic fibers were generated in 2018 in the world. Compared to MPs input from the Yangtze River and The Pearl River, they only represent 3% and 31% of riverine input, respectively (Liu et al., 2020).

Finally, quantifying MPs abundance in the ocean environment for actual and future conditions is a crucial aspect to understand and sort out marine plastic pollution. Using numerical model approaches allows us to map the abundance of MPs in the past, present and future (Isobe et al., 2019). Understand how plastic debris is transported from continental and marine sources is essential to quantify the global inventory of marine plastics, which is relevant for mitigation or policy strategies (Van Sebille et al., 2020). A study for the Pacific Ocean determined that pelagic MPs concentrations will increase approximately twofold by 2030 and therefore, fourfold by 2060, from condition registered in 2016 (Isobe et al., 2019). Furthermore, it was projected that plastic debris weight concentration would exceed 1000 mg m^{-3} in summer in parts of the East Asian seas by the 2060s onward (Isobe et al., 2019). According to the authors, these values will vastly exceed the suspended particular matter in the North Pacific Ocean, which will place MPs as the predominant non-organic suspended matter in the Ocean before the 2060s (Isobe et al., 2019).

Figure 2. Percentages per continent of inland microplastics contributions and ocean plastic pollution studies based on literature research.

It is worth mentioning that, from literature searching, most of the plastic pollution research efforts were developed in China, North America and Europe; nevertheless, in Africa and South America, two of the most critical contributors have a lesser study (Table 1, Fig. 2). Therefore, investigation efforts should increase in these continents. A review of marine MPs studies suggests that only 22.9% (44 from 192) of the world's countries have developed research on this topic, mainly focused on the Pacific and Atlantic Oceans. Meanwhile, very few have included the Arctic and Antarctic oceans and minimal studies in the Indian Ocean, where plastic pollution is probably high (Ajith et al., 2020). From studies developed between 2010 and 2019, 86% of them were distributed in Europe (38%), Asia (36%) and North America (12%) being the remaining 14% distributed in South America (7%), Africa (2%), Australia (4%) and Antarctica (1%) (Ajith et al., 2020).

CONCLUSION

Microplastics are among the contaminants of emerging concern for aquatic systems. Nowadays, MPs are ubiquitous in broad environment types from marine, freshwater, terrestrial to the atmosphere. Plasticulture application on agriculture, the massive use of plastic disposals, WWTP and road dust are among the anthropogenic activities that contribute to ocean plastic pollution in the world. Lakes, rivers and estuarine systems are among the most studied MPs sources, linking the continental systems to marine environments. Rivers and effluents were identified as significant pathways from inland sources. Microplastics are present in different sizes, colors, shapes and textures, and their final fate and transport among the different compartments of natural environments are influenced by their interaction with the biotic and abiotic variables. More studies should be focused on discuss MPs pathways or their interaction with the environmental factors in their way into the ocean, besides their pollution monitoring alone. There are complex and multiple relations between the environmental variables and human activities influencing MPs' presence and distribution in the environment. As with global biogeochemical cycles, the elucidation of the "microplastics cycle" helps us understand their global fate for future remediation. MPs reach aquatic systems

through direct MPs inputs, urban WWTP in rivers, or runoff on catchments. Simultaneously, the use of WWTP sludge on agricultural soils (containing MPs) and atmospheric plastic particles fallout, contributes to the soil's contamination, which trough wind resuspension or runoff, leads MPs to aquatic systems again. The varied hydrological characteristics as water flow velocity, water flows seasonal variation, water depth and the combined effect of turbulence and salinity in aquatic systems, affect the plastics transport within freshwater systems towards the ocean. It is worth to mention that most plastic pollution research efforts were developed in China, North America and Europe. Nevertheless, Africa and South America have lesser studies; therefore, investigation efforts should increase in these continents in the next years.

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BIOGRAPHICAL SKETCHES

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Education:

2018 – PhD in Biology, Universidad Nacional del Sur, Bahía Blanca, Argentina

2013 – Biology, Universidad Nacional del Sur, Bahía Blanca, Argentina

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Research and Professional Experience:

2020 – 2021. Posdoctoral fellow in the Kyushu University, Fukuoka, Japan. Directors: Ph.D. Atsuhiko Isobe. Research tasks activities related to the modelling of microplastics transport.

2018 – 2020. Posdoctoral fellow in the Instituto Argentino de Oceanografía (IADO – CONICET/UNS), Bahía Blanca, Argentina. Directors: Ph.D. María Cintia Piccolo and Ph.D. Andrés H. Arias. Research tasks activities related to the effect of climate variability in the presence and dispersion of microplastics, organochlorinated pesticides and polycyclic aromatic hydrocarbons in lakes.

2013 – 2018. Doctoral fellow in the Instituto Argentino de Oceanografía (IADO – CONICET/UNS), Bahía Blanca, Argentina. Directors: Ph.D. María Cintia Piccolo and Ph.D. Horacio E. Zagarese. Research tasks activities related to the effect of climate variability and water management on zooplankton ecology and water quality of lakes.

2011 – 2012. Student Fellow BENTR11 from the Centro de Investigaciones Científicas (CIC, Pcia. Buenos Aires) in the Instituto Argentino de Oceanografía (IADO – CONICET/UNS), Bahía Blanca, Argentina. Research tasks activities related to the study of zooplankton communities in lakes. Director: Ph.D. María Clara Menéndez and Ph.D. María Cintia Piccolo.

Professional Appointments:

2020 – 2023. Member of the Collaborative Climate Committee in the Global Lake Ecological Observatory Network

Publications from the Last 3 Years:

(1) Alfonso, M.B., Scordo, F., Seitz, C., Mavo Manstretta, G.M., Ronda, A.C., Arias, A.H., Tomba, J. P., Silva, L. I., Perillo, G.M. E. & Piccolo, M.C. (2020). First evidence of microplastics in nine lakes across Patagonia (South America*). Science of the Total Environment*, 139385. https://doi.org/10.1016/j.scitotenv.2020.139385.

(2) Alfonso, M.B., Arias, A.H. & Piccolo, M.C. (2020). Microplastics integrating the zooplanktonic fraction in a saline lake of Argentina: influence of water management. *Environmental Monitoring and Assessment*, 192: 117. https://doi.org/10.1007/s10661-020-8080-1.

(3) Alfonso, M.B., Brendel, A.S., Vitale, A. & Piccolo, M.C. (2020). Impact of heatwaves events on latent and sensible surface heat flux and future perspectives in shallow lakes based on climate change models. *Geography Research Letters*, 47. https://doi.org/ 10.18172/cig.4456.

(4) Alfonso, M.B., Baleani C., Fornerón F., & Piccolo M.C. (2020). The mesozooplankton (Crustacea: Cladocera, Copepoda, and Decapoda) spatial and temporal dynamics in a Pampean shallow hyposaline lake during drought conditions. *Biología Acuática*, 34. https://doi.org/10.24215/16684869e008.

(5) Alfonso, M.B., Vitale, A., Piccolo, M.C. & Perillo, G.M.E. (2019). Usefulness of lagoon environmental monitoring with multiparametric buoys in the Pampean region: La Barrancosa Lake. In: *Destination La Barrancosa. An invitation to know the Pampean lakes*. Eds: Fabián Grosman, Pablo Sanzano y Bertora Andrea. Ecosystems and Sustainable Development Multidisciplinary Institute. National University of the center of Buenos Aires Province, Tandil, Buenos Aires. 293 pp. https://drive.google.com/file/d/ 1lXo88FjzRzwW6Q5AOkyIpgVNbeY7sm2R/view.

(6) Fontanarrosa, S., Rojas Molina, F., Alfonso, M.B., García Souza, J, & Diovisalvi, N. (2019). Abundant but not seen animals in the lake, the zooplankton of La Barrancosa Lake. In: *Destination La Barrancosa. An invitation to know the Pampean lakes*. Eds: Fabián Grosman, Pablo Sanzano y Bertora Andrea. Ecosystems and Sustainable Development Multidisciplinary Institute. National University of the center of Buenos Aires Province, Tandil, Buenos Aires. 293 pp. https://drive.google.com/file/d/ 1lXo88FjzRzwW6Q5AOkyIpgVNbeY7sm2R/view.

(7) Alfonso, M.B., Brendel, A.S., Vitale, A., Seitz, C., Piccolo, M.C. & Perillo, G.M.E. (2018). Drivers of ecosystem metabolism in two managed shallow lakes with different salinity and trophic conditions: Sauce Grande and La Salada lakes (Argentina). *Water*, 10: 1136. https://doi.org/10.3390/w10091136.

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Research and Professional Experience: Climate variability in Wetlands

Professional Appointments:
- Emeritus Professor UNS
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Honors:
- 2005. Prize "Geographical Merit 2005" for his participation in the work "Urban Climate of Bahía Blanca". GAEA Argentine Society of Geographical Studies.
- 2011. On International Women's Day, Diploma of Honor and an award, in recognition of her professional career, commitment to research and national development. Command of the Third Division of the Argentine National Army “Tte. Gral. Julio A. Roca ”
- 2011. INNOVAR 2011 2nd Prize for the project 9402 Buoy for Environmental Monitoring in Hydrology (Lakes, Rivers and Ocean). In conjunction with Drs. G. M. E. Perillo and A. Vitale.
- 2013. Gold medal awarded by the Senate of the Province of Buenos Aires to Outstanding Women on International Women's Day in recognition of their scientific career (Women who have excelled in any of the disciplines into which Science is divided).
- 2013. Distinction awarded by the Bahía Blanca Industrial Chemical Association (AIQBB) for scientific career and community support.
- 2016. ESRI ESPAÑA 2016 Award granted by Papeles de Geografía Journal to the work: Downscaling of climatic variables from the NCEP / NCAR Reanalysis in the southwest of the province of Buenos Aires (Argentina) Coauthors F. Ferrelli, ML Bustos, MC Piccolo, MA Huamantinco Cisneros and G. M. E. Perillo.

Publications from the Last 3 Years:

(1) Ferrelli, F., Huamantinco Cisneros, M. A., Delgado, A. L., Piccolo, M. C., 2018. Spatial and temporal analysis of the LST-NDVI relationship for the study of land cover changes and their contribution to urban planning in Monte Hermoso, Argentina. *Documents d’Análisi Geográfica*, vol. 64,1, 25-47. https://doi.org/10.5565/rev/dag.355.

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(3) Ferrelli, F., Vitale, A. F. y Piccolo, M. C., 2018. Urban microclimate : Thermo-Hygrometric Variations of Bahía Blanca, Argentina. *Anuário do Instituto de Geociências – UFRJ*, 41 (1) 283-295.

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(6) Scordo, F., Bohn, V. Y., Piccolo, M. C. and Perillo, G. M. E., 2018. Mapping and Monitoring Lakes Intra-Annual Variability in Semi-Arid Regions: A Case of Study in Patagonian Plains (Argentina). *Water*, 10, 889 doi:10.3390/w10070889.

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(20) Fernández Severini, M. E., Menéndez, M. C., Buzzi, N. C.; Delgado, A. L., Piccolo, M. C., Marcovecchio, J. E., 2019. Metals in the particulate matter from surf-zone waters of a southwestern Atlantic sandy beach (Monte Hermoso, Argentina). *Regional Studies in Marine Sciences*, 29. (https://doi.org/10.1016/j.rsma.2019.100646).

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Publications from the Last 3 Years:

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(1) Ronda, A. C., Oliva, A. L., Arias, A. H., Orazi, M. M., & Marcovecchio, J. E. (2019). Biomarker Responses to Polycyclic Aromatic Hydrocarbons in the Native Fish Ramnogaster arcuata, South America. *International Journal of Environmental Research*, 13(1), 77-89.

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Chapter 133

BIOINDICATORS OF POLLUTION IN MARINE ENVIRONMENTS

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ABSTRACT

World marine ecosystems are constantly impacted by anthropogenic activities, accumulating pollutants from the tropics to the poles. Physical, chemical, and biological stressors produced by industrial processes, intensive agriculture and urban development, have the potential to alter the balance of ecosystems by triggering changes in the biotic communities, including the marine environment. Traditionally, the pollution assessment at these ecosystems has been based on the analysis of specific xenobiotics levels in selected matrices. However, the present challenges require an integrated assessment of contamination levels along with their possible adverse effects on organisms, even if they are found in concentrations below to the permitted limits. Then, it is essential to develop consistent tools for biomonitoring, while also assessing their potential impacts on human health. Essentially, bioindicators are organisms, populations, or communities that undergo through a certain change when exposed to contamination; these changes are based on the complexities of the ecosystem and provide representative responses which allow the assessment of the environmental health from a dynamic perspective. A suitable bioindicator should have a wide range of properties; however, the selection in marine environments should be based on specific criteria considering the different habitats (from coastal areas to offshore, from benthic environments to pelagic waters) and the sensitivity degree of organisms to diverse pollutants (or pollutants complex) to which they are

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exposed. This chapter summarizes numerous marine species from different taxonomic groups that have been tagged in the last 20 years to be used as bioindicators of diverse physicochemical pollutants in marine environments; it will address their suitability according to the different habitats and life habits, ranging from microorganisms to marine mammals. Besides, this chapter presents the most important challenges and advances in the field of marine bioindicators in recent years, which could be relevant for integrative monitoring purposes and the development of new approaches and technologies in marine pollution monitoring.

Keywords: bioindicators, pollution, molluscs, metals, plastics, emerging contaminants

INTRODUCTION

Our oceans health is essential for maintaining the planet. However, it is one of the ecosystems most severely affected by anthropic impact (Moura 2012; Potters 2013). Marine ecosystems are constantly being impacted by pollution caused by human activities, from the tropics to the poles. Physical, chemical, and biological stressors produced by industrial processes, intensive agriculture, and urban development, have the potential to alter ecosystems balance by triggering changes in the biotic communities that constitute them (Bellas et al. 2020; Moura 2012). A wide variety of effects produced by marine pollution have been reported, such as the promotion of algal blooms, reduction of biodiversity, stress, mortality and failure of organisms development and reproduction; and diseases that can also affect humans (Potters 2013). The alarming loss of biological diversity in the last decades represents a major challenge to the scientific community. Additionally, together with the ecological consequences, important economic processes can be affected. Consequently, the development of proper management strategies and monitoring tools are essential to maintain oceans health (Zuarth and Vallarino 2014). Traditionally, marine ecosystems assessment has been based on the analysis of specific pollutant levels in selected matrices. However, these studies only offer a partial approach to the question of how marine organisms and ecosystem functioning are affected by pollutants. The current challenges require an integrated assessment of contamination levels and their possible adverse effects on organisms (Bellas et al. 2020). In the intense search for precise, inexpensive, easy-to-implement methods that allow the early detection of environmental disturbances, the use of bioindicators is a method that has gained popularity over the years (Zuarth and Vallarino 2014).

Bioindicators are organisms, populations or communities used to indicate the pollution adverse effects on an ecosystem, taking into account changes in their presence/absence, abundance, morphology, physiology or behaviour (Gerhardt 2002). These are based on the complicated intricacies of ecosystems, providing representative responses that allow assessing the health of the environments from a dynamic perspective (Holt and Miller 2011). The terms “bioindicators”, “biomonitors” and “sentinels” are used and defined in different ways by the different authors. In strict terms, bioindicators assess qualitative responses while biomonitors assess quantitative responses. The term sentinel refers to a special kind of indicator organism that accumulates and concentrates pollutants in its tissues. However, the concept of “bioindicator” is generally used for most of the authors as a collective term including all terms related to biotic responses detection to environmental stress (Gerhardt 2002; Holt and

Miller 2011; Parmar et al. 2016; Zuarth and Vallarino 2014). In this chapter, the term "bioindicator" encompasses all these concepts (Figure 1).

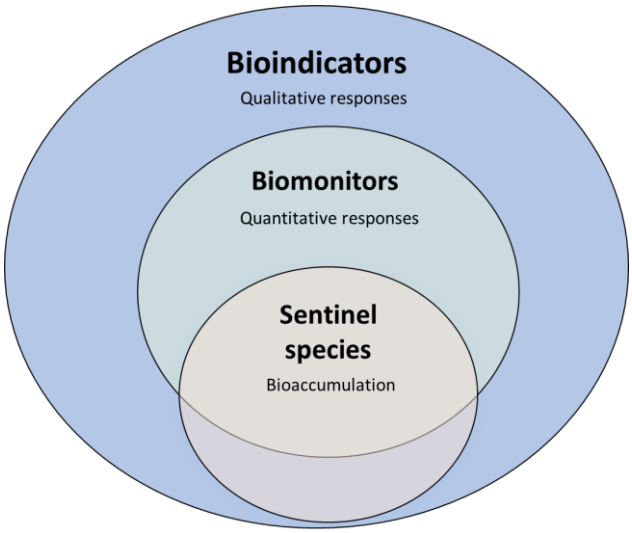

Figure 1. Bioindicator concept used in this chapter.

A suitable bioindicator should have several properties such as a wide distribution, sensitivity, environmental representativeness, practicability (easy for sampling, taxonomy, recognition and grow in the laboratory and low cost), societal importance, and ecological characteristics (abundance, specificity, and good knowledge about the position in the trophic system, feeding strategy, position in ecosystem compartment; Gerhardt 2002; Zuarth and Vallarino 2014). The ideal bioindicator should fulfil all these criteria; however, until now no species, community or population has been found that can comply with all of them at the same time. Marine ecosystems are complex, multivariate, and simultaneously exposed to a wide variety of stressors with poorly understood cumulative effects (Zuarth and Vallarino 2014). Because of this, bioindicator selection in marine environments is based on specific criteria considering the different habitats (from coastal to offshore areas, from benthic environments to pelagic waters) and the degree of organism sensitivity to the diverse pollutants to which they are exposed (Fossi et al. 2017). Also, every monitoring program or search will have its objectives, in terms of spatio-temporal scale and areas investigated, founding the bioindicator selection on these criteria (Fossi et al. 2017).

This chapter is a review of numerous marine species belonging to different taxonomic groups that have been used as bioindicators over the past 20 years (total of revised works: 182), including the new challenges in marine pollution monitoring taking into consideration pollutants of emerging concern and new techniques and technologies (Bellas et al. 2020). For this chapter, the works focused on the evaluation or use of bioindicators were selected, considering their suitability according to the different habitats and life habits. Bioindicators of diverse pollutants, such as persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), plastics, metals, nutrients, emerging pollutants (EPs) and noise pollution were reviewed (Figure 2), ranging from microorganisms to marine mammals. Many works were found for pollutants such as metals (74 works), POPs (38 works), plastic (35

works), and nutrients (22 works); compared to EPs (12 works) and noise pollution (one work; Figure 2). This revision could be relevant for future comprehensive monitoring development with new approaches and technologies that result in representative data to support environmental decision-making and regulations.

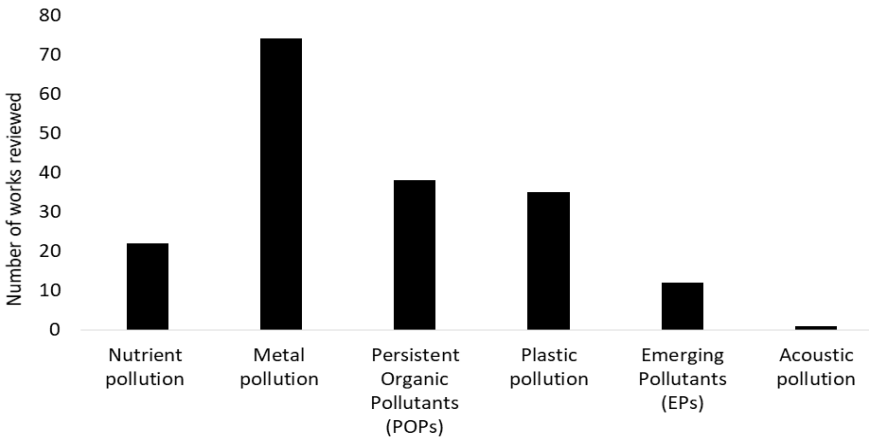

Figure 2. Number of works reviewed for each pollutant.

NUTRIENT POLLUTION BIOINDICATORS

Pollution from nutrients, also called over-enrichment, represents one of the largest sources of water quality degradation at coastal waters (Howarth et al. 2000). Nutrient loading that leads to eutrophication arrive in the marine environments due to human activities, such as fossil fuels combustion, use of agricultural fertilizers and mainly from untreated and treated sewage effluent emissions present in many coastlines in the world (Benson et al. 2008; Dudley and Shima, 2010). Nutrient over-enrichment has a range of effects on coastal systems, such as fish mortalities, coral reef destruction and ecological changes that decrease the biological diversity. Eutrophication changes the ecological communities' structures by two mechanisms: indirectly through oxygen depletion and directly by increasing nutrient concentrations. The main nutrients causing eutrophication are nitrogen (N) and phosphorous (P); although silica (Si) may also play a role in regulating algal blooms in coastal waters and, in determining some eutrophication consequences (Howarth et al. 2000). Therefore, it is important to detect and monitor the nutrients flow in coastal areas to predict and prevent harmful effects of eutrophication. A potentially informative way of monitoring an increase in environmental nutrients is using bioindicators (Lilliesköld and Mork 2009; Todd et al. 2006).

Traditional monitoring of dissolved nutrients in the water column is complicated by short term fluctuations, especially when their levels are low and have a fast turnover. As nutrient bioindicators, algae seem to be preferred (Figure 3). Macroalgae integrate nutrients overtime in their tissues from those that are bioavailable in the environment, the use of these organisms as nutrient bioindicators may be less expensive and labour-intensive than traditional water quality sampling (Dudley and Shima 2010; Lilliesköld and Mork 2009; Lin and Fong 2008). Additionally, algae as bioindicators since changes in the composition of their population,

abundance and dominance relationships of species proved to be an effective method to monitor nutrient contamination (Sliskovic et al. 2011). Experiments under controlled conditions using the alga *Gracilaria edulis* have been shown that amino acids, tissue nitrogen and chlorophyll content response to nutrient pulses indicating that this alga species could be a suitable bioindicator for investigating the source and geographical extent of nutrients entering oligotrophic coastal waters (Costanzo et al. 2000). *In situ*, *Ulva* spp. was able to reflect N gradients, with up to 90% higher tissue N levels in the vicinity of major cities compared to adjacent areas (Lillieskold and Mork 2009). The brown macroalgae, *Sargassum flavicans*, was investigated as a potential bioindicator for monitoring time-integrated uptake of nitrogen from nutrient-enriched industrial effluent since increased 3-4-fold in $\delta^{15}N$ (nitrogen stable isotope) compared to reference sites (Alquezar et al. 2013). Moreover, the red macroalgae *Acanthophora spicifera,* has been used as a bioindicator to monitor the enrichment of nutrients from the shrimp culture (Lin and Fong 2008). Other herbaceous species such as *Zostera marina* was also described as a proper bioindicator of eutrophication, measuring $\delta^{15}N$ in sewage areas (Schubert et al. 2013).

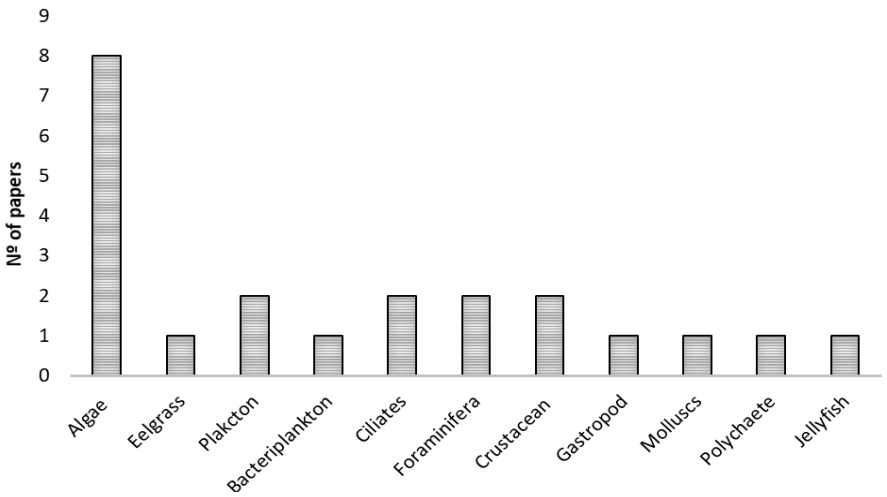

Figure 3. Bioindicators for nutrient pollution. Number of papers reviewed.

Algae have also been used as bioindicators along with other organisms to propose them jointly as bioindicators of nutrient contamination. Thus, the macroalgae *Gracilaria* sp. was evaluated together with the oyster species *Crassostrea virginica* to determine spatial and temporal patterns of nitrogen sources through $\delta^{15}N$ (Ferting et al. 2009). While the macroalgae responded quickly without fine-scale spatial patterns, the oyster responded slowly over time and exhibited a spatial scale at a slight gradient. Also, Babaranti et al. (2018) confirmed the use of the *Ulva lactuca* algae and the mussel *Mytilus galloprovincialis* to monitor the impact of sewage-derived organic matter. Dudley and Shima (2010) used three different trophic levels species: a kelp (*Carpophyllum maschalocarpum*), an isopod (*Amphoroidea media*) and a filter-feeding crab (*Petrolisthes elongatus*) as bioindicators along a sewage exposure gradient. $\delta^{15}N$ measuring was useful in *C. maschalocarpum* and in *A. media* while $\delta^{13}C$ evaluation was useful in *P. elongatus*, suggesting multiple-isotope and multi-species bioindicator approaches for detecting both patterns to understand the nutrients incorporation into food webs (Dudley and Shima 2010).

Zooplankton has also been evaluated as a bioindicator of nutrient contamination by some authors elsewhere (Neto et al. 2014; Xu et al. 2016). Both works evaluated changes in their structure, number and/or biomass of the population, however, these studies were non-conclusive and more studies were suggested to postulate them as appropriate bioindicators.

Microbial communities respond rapidly to environmental disturbances, therefore, bacteriplankton was also described as a sensitive bioindicator of eutrophication, with changes significantly correlated with the abundance of 27 families (Dai et al. 2017). Abundance and pattern of species richness of ciliate, such as tintinnids and aloricate (Xu et al. 2011; Sivasankar et al. 2018) and benthic foraminifera (Mojtahid et al. 2006; Vidovic et al. 2014) have been proposed as excellent bioindicators of eutrophication.

Taking into account crustaceans as nutrient bioindicators, density of the amphipod crustacean *Metaprotella sandalensis* was effective nitrogen and phosphate bioindicator in coral reefs, showing the highest densities in more stressed sites (Guerra-García and Koonjul 2005) while prawns (family *Penaeidae*) from different sites along Asia-Pacific coastlines were suitable for nitrogen monitoring through isotope measurement (Fry et al. 2016).

Although less studied, some other organisms were suggested as bioindicators for nutrients. The gastropod *Amphibola Crenata* proved to be a proper bioindicator of nutrient changes levels in estuaries through their growth and survival measurements (Marsden and Baharuddin 2014). Also, studies on mollusc community structure (35 taxa identified) (Atalah and Crowe 2012) and polychaete assemblages (Surugiu 2005) were useful to indicate marine eutrophication. Finally, the jellyfish *Cassiopea xamachana* proved to be an effective bioindicator for integrating relevant information about phosphate availability in low nutrient environments (Todd et al. 2006).

METAL POLLUTION BIOINDICATORS

Heavy metals are natural constituents widely distributed in the environment. Their sources include industrial and agricultural activities and flow into the sea through effluents and sewage or are directly discharged affecting the physicochemical and biological parameters of the water (Chiarelli et al. 2014). In coastal areas, heavy metals involve a serious concern in terms of environmental and ecological degradation (Conti et al. 2008; Chakraborty et al. 2014). Some of them have essential functions and are toxic only in an overdose, others are extremely toxic, and others are essential for some organisms but toxic for others (borderline). Thus, metals can be divided into three class: essentials, nonessentials, and borderline classes (Chiarelli et al. 2014). When they are highly concentrated are harmful to animals and plants, can bioaccumulate in different organisms and through the different trophic levels affecting finally to humans (Conti et al. 2008; Chakraborty et al. 2014). Heavy metal overload can cause serious ecotoxicological effects having inhibitory effects on the development or could disrupt oxygen levels, reproductive, physiological and metabolic processes from organisms (Gheorghe et al. 2017). Thus, bioindicator organisms are commonly used to predict the toxicity of metals in biota (Chakraborty et al. 2014).

Among the papers reviewed in this chapter (74 works in the last 20 years), bivalves were the metal bioindicator most widely used (20%, Figure 4). They have the ideal characteristics to be bioindicators such us being cosmopolitan, sedentary, and filter-feeding organisms that

bioaccumulate pollutants dissolved and present in the particulate matter due to their low metabolic transformation, providing time-integrated information on contaminants levels in coastal zones. Additionally, numerous laboratory and experimental studies have shown that these organisms accumulate metals in proportion to the bioavailable metals in the environment (Chandurvelan et al. 2015; Ozden et al. 2010). From a multiple biomarker approach, the mussel *Perna canuliculus* proved to be a useful bioindicator in New Zealand with significant metal accumulation correlations with metallothionein-like protein content, catalase activity and lipid peroxidation (Chandurvelan et al. 2015). Studies on indigenous *Mytilus galloprovincialis* had also demonstrated that it can provide a measure of observable cellular and physiological responses, such as oxidative stress due to cellular oxy-radical generation. However, these responses may also be affected by seasonality (Vlahogianni et al. 2007). Similarly, *Mytilus galloprovincialis* from aquaculture farms in an estuarine ecosystem, have been used as bioindicators of copper (Cu), chromium (Cr), nickel (Ni), zinc (Zn), iron (Fe) and manganese (Mn). However, they presented high seasonal variability, strongly influenced by the abiotic and biological parameters of the marine environment (Catsiki and Florour 2006). Bivalves *Perna perna*, *Brachidontes variabilis*, and the clam *Anadara granosa* proved to be proper heavy metal bioindicators, but seasonal variations were also described (Belabed et al. 2013; El-Moselhy et al. 2016; Januar et al. 2019). Therefore, environmental parameters and seasonal variations must be considered when using bivalves as heavy metal pollution bioindicators. Mussels *Mytilus galloprovincialis* and *Perna perna* were also used as bioindicators by other authors and including more heavy metals such us cadmium (Cd), mercury (Hg), lead (Pb), cobalt (Co), tin (Sn), vanadium (V), silicon (Si) and arsenic (As) (Jovic et al. 2011; Spada et al. 2013; Ozden et al. 2010). For *P. perna*, the use of shells as bioindicators for metal pollution has been validated. Significant correlations between concentrations in soft tissue and shells were demonstrated under controlled conditions of laboratory (Belloto and Miekeley 2007). Heavy metals and trace elements (As, Cd, Cr, Cu, Pb, selenium-Se, and Zn) were also analyzed in bivalve blood (Yusof et al. 2004). Different clam species were proved to be proper heavy metal bioindicators such us *A. granosa*, *Polymesoda expansa*, *Tivela mactroides*, *Gafrarium tumidum (*Dabwan and Taufiq 2016; Acosta and Lodeiros 2003; Hédouin et al. 2011). Moreover, it has been demonstrated that the rock oyster *Saccostrea glomerata* could be a precise bioindicator for heavy metals in port areas (Jahan and Strezov 2019).

Algae were also widely used as bioindicators for heavy metals (15%; Figure 4) being recognized as useful bioindicators for metal pollution in seawater due to their sedentary lifestyle. Also, they are representative of the study area, are abundant throughout most of the world's rocky coasts, accumulate contaminants without being severely affected, have a long life span and are easy to sampling and identify (Burger et al. 2007; Chakraborty et al. 2014; El-Din et al. 2014). In Australia, twelve species of estuarine macrophytes, including red, green, brown algae and seagrasses were evaluated as potential metal bioindicators (As, Cd, Cu, Pb, Se and Zn). The authors suggested that the analysis of multiple species may be necessary for a comprehensive understanding of estuary-wide metal pollution and concluded that *Ulva australis*, *Zostera muelleri* and *Ruppia megacarpa* have potential as heavy metal pollution bioindicators, based on their availability and bioaccumulation pattern (Farias et al. 2018). In the Gulf of Kutch (India), *Ulva lactuca* has been shown to accumulate the highest levels of Cu, Fe and Zn; *Padina gymnospora* the highest levels of Cd, Mn and Ni; *Enteromorpha sp.* accumulate the maximum Pb concentrations, while *Dictyota D.*

bartayresiana accumulate the maximum Cr levels, demonstrating that different algae specie can be used as bioindicators depending on the metal in question (Chakraborty et al. 2014).

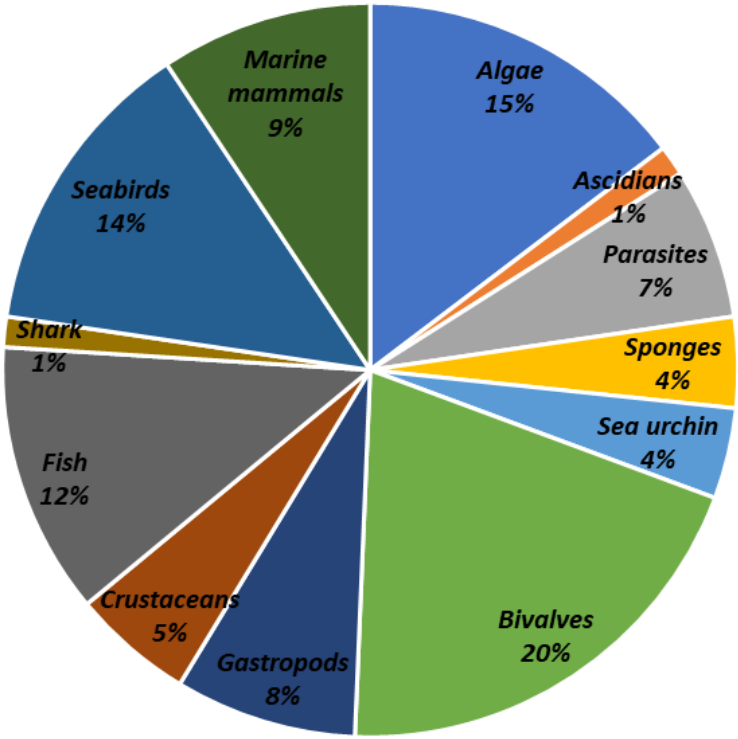

Figure 4. Bioindicators for metal pollution. Percentage of papers reviewed.

Also, in the Egyptian Mediterranean Coast, demonstrated that others three algae species were useful metals bioindicators: the green alga *Enteromorpha compressa*, the brown alga *Padina boryana* and the red alga *Jania rubens* (El-Din et al. 2014). This study was based on the maximum metal pollution index (MPI), the bioconcentration factor (BCF) and the Tomlinson pollution load index (PLI). Four other brown algae (*Sargassum angustifolium*, *Sargassum boveanum*, *Sargassum latifolium*, and *Padina gymnospora*) were described as effective bioindicators for Zn in Saudi Arabia due to consistency of the concentrations in all the algae evaluated (Alkhalifa et al. 2012). The brown alga *Lobophora variegata* has been shown to take up Cd, Co, Cr, Ni and Zn in direct proportion to their ambient dissolved concentrations describing it as a useful metal bioindicator with a fast response not only in laboratory experiments (Metian et al. 2008) but also *in situ* (Hedouin et al. 2008). In Poland, seven macrophytobenthonic algae (*Polysiphonia fucoides*, *Furcellaria lumbricalis*, *Coccotylus truncates*, *Stuckenia pectinate*, *Zanichellia palustris*, *Cladophora spp*., and *Chara baltica*) proved to be suitable metal bioindicators (Pb, Cd, Hg and Ni), based on the efficiency of heavy metal accumulation, frequency of occurrence and biomass (Zalewska and Danowska 2017). *Ulva lactuca* alga was described to be a proper bioindicator for Cu, Co, Ni, Cr, Mn, Zn, Cd, Pb, and Fe (Mourad and El-Azim, 2019; Parus and Karboska, 2020). However, Kalesh and Nair (2005) determined low levels of Ni, Cr, strontium (Sr) and Ag bioaccumulation in this species, concluding that it may not be a good bioindicator for such

metals. Other reports have been shown that some metals are differentially accumulated but not others in several algae specie (*Centroceras clavulatum*, *Grateloupia filicina*, *Chaetomorpha antennina*, *Enteromorpha intestinalis*, *Gracilaria corticate*, *Alaria nana*) demonstrating that depending on their physiology and place these herbacea organisms could be suitable bioindicator of a specific metal. Moreover, considering that some algae can be more than 25 m long, authors warn about different concentration patterns in particular parts of the algae, in addition to seasonal variations, recommending harmonized protocols.

Seabirds have been used as useful bioindicators of metals for years, especially to monitor Hg concentrations in biological systems at different spatial and temporal scales (Sadat and Hosseini 2018). Among the reviewed papers, seabirds were the third most widely used bioindicator for metals (14%, Figure 4). *Anas crecca* (migratory) and *Ringa solitaria* (resident) were used as bioindicators of Hg and methyl mercury (MMHg) pollution and variations among species and tissues were found, confirming that mercury concentrations were strongly affected by habitat and feeding habit (Sadat and Hosseini 2018). Other species of seabirds (*Pterodroma baraui*, *Puffinus lherminieri bailloni*, and *Phaethon lepturus*) have also been used as bioindicators of Hg, as well as Cd, Cu, Fe, Mn, Se and Zn finding differences between tissues (liver, kidney, pectoral muscles, and feathers) (Kojadinovic et al. 2007). Among 17 species studied, it has been concluded that common eider (*Somateria mollissima*), Leach's stormpetrel (*Oceanodroma leucorhoa*), double-crested cormorant (*Phalacrocorax auritus*) and black guillemot (*Cepphus grille)* are the most effective bioindicators of Hg (Goodale et al. 2008). These authors have also demonstrated that seabird blood could be an effective method for using seabirds as bioindicators of Hg in non-destructive ways and suggest that eggs are preferred for long-term Hg monitoring since they are easy to collect. Seabird eggs were also used as bioindicators by other authors; thus Cienfuentes et al. (2003) found that *Sterna trudeaui* eggs are useful bioindicators for Hg, but they were rare and inaccessible in the sampling area; and Burger and Gochfeld (2004) explained that *Sterna hirundo* eggs can serve as effective bioindicators of temporal trends in exposure to metals (Cd, Cr, Pb, Mn, Hg, Se, and As). Penguin species were widely used as metal bioindicators. Feathers from different penguin species (*Aptenodytes patagonicus*, *Eudyptes chrysolophus*, *Eudyptes chrysocome filholi* and *Pygoscelis papua*, *Pygoscelis antarctica*, *Spheniscus humboldti*, *Eudyptula minor)* proved to be useful to demonstrate metal accumulation, including Hg (Carravieri et al. 2013; Metcheva et al. 2006; Squadrone et al. 2019). Little Penguin blood (*Eudyptula minor*) was also used to analyze trace metals and metalloids (Finger et al. 2015) describing this species as an efficient bioindicator with concentrations linked to industrialisation levels from adjacent to the foraging zones, and a clear correlation between blood and feathers for mercury, lead, and iron levels. Thus, there is extensive use of feathers as bioindicators; however, a work that analysed feathers with synchrotron radiation X-ray fluorescence (SR-XRF) microanalysis in snow petrel (*Pagodroma Nivea*) showed external contamination of Fe, barium (Ba), Pb, Zn and Hg suggesting that feathers could be suitable bioindicator only of external contamination (Xie et al. 2008).

Twelve percent of the reviewed papers used fish as metal bioindicators (Figure 4). Thus, because many fish species have the required criteria as bioindicators for aquatic ecosystems assessment and they are considered one of the best organisms to study bioaccumulation characteristics and pollutants effects on ecosystems (Tsygankov et al. 2017). Additionally, metal bioaccumulation has been described in their tissues, particularly in the liver where they

are bio-transformed and excreted or passed over to consumers through the food chain (Mehana et al. 2020). In estuarial environments, two species (*Micropogonias furnieri* and *Mugil liza)* were described as proper bioindicators for Hg, Cd and Zn by Marcovecchio (2004), while six species (*Brevoortia aurea, Odontesthes argentinensis, Micropogonias furnieri, Cynoscion guatucupa, Mustelus schmitti* and *Paralichthys orbignyanus*) were described as useful bioindicators of metal pollution by La Colla et al. (2017; 2018). Benthic fish are also suitable bioindicators because they have relatively low mobility and are present in the sediment where the bioavailability of contaminants is typically higher (Azevedo et al. 2012), such as *Salaria basilisca* that was described for Cd accumulation (Barhoumi et al. 2009), *Cathorops spixii* and *Genidens genidens* were described as effective metal bioindicators including for trace levels (Azevedo et al. 2009; 2012). Other benthic and pelagic fish species (*Arius heudelotii, Mullus barbatus, Merlangius merlangus*) were used as metal bioindicators, finding significant differences between species probably due to variances in diet or trophic level (Fonge et al. 2011; Fındık and Cicek 2011; Storelli and Marcotrigiano 2005).

Marine mammals were used in 9% of the reviewed works (Figure 4). Marine mammals have demonstrated high potential as bioindicators since they accumulate metals due to their position at the top of the marine food web and long life-span (Lemos et al. 2013). The skin of Commerson's dolphins (*Cephalorhynchus commersonii*) was proposed as a useful bioindicator for Hg taking into account its correlation with internal tissues concentrations such as lung, liver, kidney and muscle (Cáceres-Saez et al. 2015). The Guiana dolphin (*Sotalia guianensis*) was also used as a bioindicator for Hg, and even positive correlations with body length were found (de Moura et al. 2012). High concentrations of total-mercury, organic mercury, and Cd were found in the melon-headed whale (*Peponocephala electra*), and levels of other 10 elements (V, Cr, Mn, Co, Cu, Zn, Sr, molybdenum-Mo, and cesium-Cs) presented seasonal variations (Hirata et al. 2010). As part of a program monitoring the cetacean contamination by heavy metals, striped dolphins (*Stenella coeruleoalba*) stranded dead on the beach between 1996 and 1997 were compared to those between 1990 and 1991. As a result, a significant decrease in Cu, Hg, Ni and Pb concentrations was shown, but a slight decrease in the concentrations of Cd, and Zn levels remained high. This was in response to clean up efforts the zone coast, which corroborates the use of cetaceans as reliable bioindicators (Augier et al. 2001). Similarly, the sperm whale (*Physeter macrocephalus*) proved to be a proper metal bioindicator, but high variability between individuals and tissues was found. High metal concentrations were found in pregnant oldest females, warning the necessity to assess the impact of these pollutants on vulnerable individuals since pregnant females and younger individuals have low or altered detoxification capacity (Squadrone et al. 2015). Multiple cetaceans were used as bioindicators by Chen et al. (2017) *Grampus griseus, Kogia simus, Lagenodelphis hosei* and *Stenella attenuate* demonstrating marked Ag and Cd pollution in the western Pacific Ocean in the last two decades. Moreover, Lemos et al. (2013) analysed essential (Cu, Mn, Se and Zn) and non-essential (Cd and Hg) elements in *Feresa attenuate*, *Orcinus orca*, *Pontoporia blainvillei, Sotalia guianensis, Stenella frontalis, Steno bredanensis*, *Tursiops truncates*, observing bioaccumulation variations between species probably due to the preference for certain preys and their bioavailability in the environment. All these studies demonstrate cetaceans as effective bioindicators, however metals bioavailability must be also considered to fully understand the accumulation mechanisms.

Gastropods have been widely used as metal bioindicators due to their biological characteristics of sedentary and thus representativity of the study area, they are hardy and tolerant to high levels of metals, abundant in coastal environments, easy to identify and collect and provide sufficient tissue for analysis of metal concentrations (Ramirez 2013). Eight percent of the studies reviewed in this chapter used gastropods as effective bioindicators (Figure 4). *Monodonta turbinata* was used as a bioindicator of Fe, Zn, Cd, Cu, Co, Ni, Al, Mn, Pb and Cr; and although accumulations of all elements were affected from seasonal changes, this gastropod was a very efficient heavy metal accumulator and, therefore, a valuable bioindicator (Duysak and Ersoy 2014). It has been shown that variations of antioxidant parameters such as reduced glutathione (GSH), malondialdehyde (MDA), glutathione S- transferase (GST) and catalase (CAT) in *Stramonita haemastoma* could be used as prospective biomarkers of metals toxicity; demonstrating that this specie could be a useful bioindicator although seasonal variations were also found (Bouzahouane et al. 2018). Besides, *Osilinus atrata* presented great potential as a bioindicator of heavy metals (Cd, Cu, Pb and Zn), however, intraspecific variations were observed (Ramirez 2013). Gastropod gills (*Cerithium scabridum*) were also successfully used as metals bioindicators with seasonal variations, with significant correlations between metal concentrations in gills and sampling sites (Bu-Olayan and Thomas 2001). Finally, *Patella caerulea* was used by two authors who observed that shells and soft tissue were useful bioindicators of Cd, Pb, Cu, Fe, Ni, Mn, Zn, Hg, and Cr (El-Damhogy et al. 2019; Storelli and Marcotrigiano 2005).

Parasites have been shown to be proper bioindicators of essential and nonessential metals (7%, Figure 4) due to the number of parasitic species that exist compared to free-living species. Also, with their complex life cycles, the numbers of potential indicators increase with the number of different developmental stages, and some parasites are highly sensitive to heavy metal pollution (Malek et al. 2007; Mehana et al. 2020). The cestode/seabird system (*Tetrabothrius bassani*/*Morus bassanus*) was considered a promising bioindicator to monitor environmental Cd and Pb pollution in marine ecosystems (Mendes et al. 2013). Also, shark parasites proved to be suitable PB and Cd bioindicators, such as *Anthobothrium sp*. and *Paraorigmatobothrium sp*. cestodes in the shark *Carcharhinus dussumieri*; while *Gyrocotyle plana* infecting the spiral intestine of *Callorhinchus capensis* shark was a useful bioindicator for As, Mn, Pb, Ti and Zn. These organisms were extremely sensitive as early warning bioindicators, particularly in environments under threat but where pollution levels are presently low (Malek et al. 2007; Morris et al. 2016). Finally, for fish parasites, *Siganus rivulatus*/*Sclerocollum rubrimaris* system, and nematodes in the fish *Dicentrarchus labrax* demonstrated their sustainability as bioindicators of metal pollution (Al-Hasawi 2019; Morsy et al. 2012).

Crustaceans are generally excluded as possible bioindicators due to their ability to metabolically regulate essential metals (Simonetti et al. 2013). Nevertheless, the bioaccumulation of non-essential metals has been described and different crustaceans showed to be suitable bioindicators (5%, Figure 4). The crab, *Neohelice granulate*, was an appropriate candidate for bioindication of Ni and Pb pollution. However, seasonal variations and gender differences were found, therefore these factors must be considered (Simonetti et al. 2013). Besides, the benthic crustacean *Melicertus plebejus* was an effective bioindicator of some trace elements such as Pb and Cu (Munroe et al. 2018) and the isopod crustacea *Ligia italica* described as a reliable and representative bioindicator of Hg pollution (Longo et al. 2013) where ultrastructural alterations in the hepatopancreas epithelium were found. Finally, a study

compared the metal bioaccumulation of barnacles, ghost shrimps, polychaetes, and bivalves. The barnacle, *Amphibalanus amphitrite*, was the most suitable bioindicator. Also, barnacles are sessile or at least show restricted mobility, are available in all seasons, and can be easily sampled (Amoozadeh et al. 2013).

Sea urchins are suitable bioindicator organisms for their wide distribution, easy collection, filter-feeding habits, and good tolerance to pollutants (Salvo et al. 2014) and were used in 4% of the reviewed works (Figure 4). For Pb and Cd metals, *Diadema antillarum* proved to be an excellent bioindicator, and concentrations of metals in tissue and shell were positively correlated with the sample size (Dolores Hernández et al. 2009). *Paracentrotus lividus* was a suitable organism to be used as a bioindicator for different metals in situ and in bioassays (Salvo et al. 2014, Soualili et al. 2008).

Sponges are promising bioindicators because they process large amounts of water, show a wide distribution and year-round availability, are abundant in sublittoral areas, and can accumulate a wide range of metals from both the suspensions and dissolved phases. Additionally, sponges submitted to metal contamination experience morphological, biological, physiological, and biochemical responses, which can be easily monitored (Cebrian et al. 2007; Venkateswara et al. 2009). These organisms have been used as metal bioindicators in the 4% of the revised works (Figure 4). A comparative study on metal bioaccumulation by four widespread sponge species (*Crambe crambe*, *Chondrosia reniformis*, *Phorbas tenacior*, and *Dysidea avara*) showed that *Crambe crambe* provide accurate information on the background levels of metals in the area, and it reflected fluctuations of the bioavailable metals (Cebrian et al. 2007). *Haliclona tenuiramosa* was also a suitable bioindicator for As, Cd, Co, Cu, Fe, Mn, and Ni with positive correlations between concentration levels in water and bioaccumulation in tissues (Venkateswara et al. 2009). It has been demonstrated that the sponge *Hymeniacidon perlevis* closely reflected the heterogeneous distribution of metals in the environment (Mahaut et al. 2013) and that it can accumulate *in situ* more contaminants than the blue mussel *M. edulis*, meeting all the requirements of a proper bioindicator for integrated monitoring programs (Mahaut et al. 2013).

Sharks and ascidians were scarcely used as metal bioindicators among the reviewed works, with only 1% each. The blue shark, *Prionace glauca*, showed great potential as a bioindicator of Al, Cr, Mn, Fe, Ni, Cu, Zn, As, Se, Ag, Cd, Pb, and Hg (Alves et al. 2016). On the other hand, two invasive ascidian species, *Phallusia nigra* and *Microcosmus exasperates*, could be effective as heavy metal bioindicators due to high levels of bioaccumulation coupled with changes in the hepatosomatic index (Tzafriri-Milo et al. 2019).

BIOINDICATORS OF PERSISTENT ORGANIC POLLUTANTS (POPS)

In marine environments, persistent organic pollutants (POPs) are of great environmental and health concern**.** POPs are toxic chemicals resistant to chemical and biological degradation, characteristics that make them have a global distribution and be persistent in the environment. Furthermore, due to their fat solubility, POPs ingestion by organisms leads to bioaccumulation throughout their lives, generally in fatty tissues, and to biomagnification in the food chain (Luzardo et al. 2014; Noventa et al. 2011). Among POPs, organochlorine

pesticides (OCs), polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), dioxins, polybrominated diphenyl ethers (PBDEs), brominated flame retardants (BFRs), and perfluorinated compounds (PFCs) have been found in marine organisms and environments (Alves et al. 2016; Luzardo et al. 2014). Strictly speaking, polycyclic aromatic hydrocarbons (PAHs) cannot be considered POPs since they are easily metabolized by some organisms, however, due to their high prevalence in the environment and lipophilicity, these compounds are usually considered as POPs being the most frequently found in marine coastal areas (Luzardo et al. 2014). All POPs arrive in the marine environment from anthropogenic sources and have significant detrimental effects on wildlife and humans, including carcinogenesis, mutagenesis, endocrine disruption, and immunotoxicity (Batista et al. 2013; Noventa et al. 2011).

To monitor the contamination status of POPs in marine ecosystems, sophisticated and highly sensitive techniques of chemical analyses are required. Marine organisms have been commonly used as bioindicators to assess regional/global contamination by POPs as possible integrators of these contaminants (Ueno et al. 2003b). According to reviewed works in this chapter and considering POP pollution, invertebrates were the preferred organisms for biomonitoring these compounds (63.2 %; Figure 5A), probably due to the higher capability to bioaccumulate contaminants than vertebrates. Within the invertebrate group, mollusc bivalves were the most widely used (58.3%; Figure 5B). This is because, in addition to the characteristics mentioned above, bivalves have been used as marine indicator organisms in national and international monitoring studies, such as Mussel Watch Program, U.S. National Oceanic and Atmospheric Administration (NOAA), Status and Trends Program (NS&T), Exxon Valdez contamination monitoring and Gulf of Mexico Produced Water Bioaccumulation Study (Francioni et al. 2005; Kimbrough et al. 2008). In Brazil, the mussel *Perna perna* is the main species used as a bioindicator for PAHs due to its economic importance and wide geographical distribution (Batista et al. 2013) being a reliable bioindicator for monitoring coasts and estuaries (Francioni et al. 2005). This species is also able to bioaccumulate PAHs, and physiological and biochemical biomarkers were subsequently demonstrated to be sensible (Pereira et al. 2007; 2010). Other mussel species were described as suitable bioindicators of PAHs such us *Brachidontes rodriguezii* and *Tagelus* sp. in Argentina (Arias et al. 2009; Oliva et al. 2015); and *Sanguilonaria (Soletellina) acuminate* in India (Zuloaga et al. 2009).

On the other hand, for organochlorine pesticides (OCs) *Mytilus galloprovincialis* was used as bioindicator in Spain and the Adriatic Sea (Campillo et al. 2017; Carro et al. 2014; Giandomenico et al. 2013). A correlation with effects on physiological parameters, such as lipid content and shell length were demonstrated (Carro et al. 2014). The brown mussel (*Perna perna*) was used as a sentinel species for OCs and dioxins, and bioaccumulation of these compounds was demonstrated without seasonal variation (Galvao et al. 2015); *Perna viridis, Mytilus gallorovincialis* and *Crenomytilus grayamus* from Asia waters were shown to be suitable bioindicators of OCs (Monirith et al. 2003); and *Perna viridis* proved to be a useful bioindicator of PCBs and PBDEs in Singapore's marine environment (Bayen et al. 2003). Wang et al. 2008 compared seven bivalves and gastropods species for using as OCs, PBDEs and PCBs bioindicators proving that *Crassostrea talienwhanensis* and *Mytilus edulis* bivalves were the most sensitive bioindicators among those selected in the Chinese Bohai Sea. *Mytilus edulis* was also described as a very sensitive bioindicator for PAHs and PCBs during a bioremediation process (Walker et al. 2013), and for PBDEs, HBCDs, PCBs and

OCs in Korea coastal waters (Ramu et al. 2007). Bivalves from other families such us *Polymesoda arctata, Anadara tuberculosa and Larkinia grandis* were also suitable POPs bioindicators used in Nicaraguan coasts to detected PAHs, PCBs, PBDEs, OCs, and organophosphorus pesticides (Aguirre-Rubí et al. 2019).

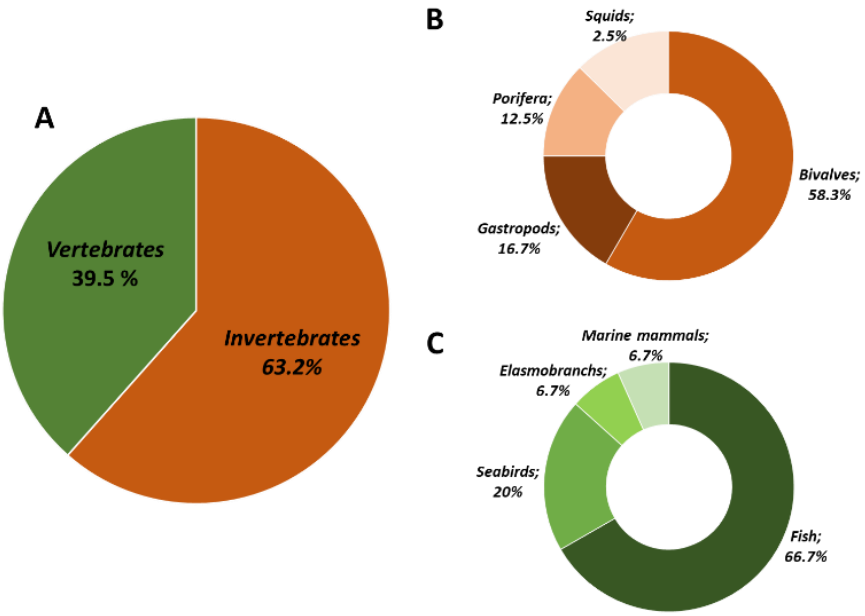

Figure 5. Bioindicators used for Persistent Organic Pollutants (POPs). A: Proportion of vertebrate and invertebrate organisms, B: Invertebrate bioindicators (%), C: Vertebrate bioindicators (%).

Gastropods were the second group of invertebrates most used as bioindicators (16.7%; Figure 5B). These have been used for years as effective bioindicators of tributyltin (TBT) due to their propensity to develop the imposex and intersex phenomena in which superimposition of male sexual organs onto females concentration-dependent occurs, resulting in infertility and population declines in extreme cases. TBT is an organotin compound (OTCs) and is not strictly considered as POP but has been banned and regulated along with them (Noventa et al. 2011). It has been demonstrated in West Africa that *Gemophos viverratus* is sensitive to TBT since imposex could be artificially induced by injecting very small doses of TBT into females (Lopes-Dos-Santos et al. 2014). In Venezuela three species of gastropods, *Chicoreus brevifrons*, *Chicoreus (Phylonotus) margaritensis* and *Leucozonia nasa* were used as TBT bioindicators, but their concentrations were not determined (Miloslavich et al. 2007). In Canada, *Nucella lapillus* proved to be a useful TBT bioindicator with high correlations between imposex and their concentrations (Titley-O'Neal et al. 2011). *Littorina littorea* was described as an effective PAHs and organotin compounds (OTCs) bioindicator along the southwestern English coast, verifying the bioaccumulation of both compounds and strong correlations between PAHs concentrations and DNA damage, as well as OTC levels and Intersex development (Noventa et al. 2011).

Sponges (Porifera Phylum) have been proposed as deep waters bioindicators where mussels are not found. They are also among the most abundant benthic animals colonizing consolidated substrates in tropical zones, coral reefs, rocky shores and many artificial

structures. They are widely distributed in marine systems and have high filtration rates (Batista et al. 2013; Mahaut et al. 2013; Perez et al. 2003). *Hymeniacidon heliophile* was described as a suitable deep-water PAHs bioindicator since having desirable characteristics such as wide distribution, easily identified in the field, encrusting massive sponge with a large amount of soft tissue and petrogenic and pyrolytic PAHs effective accumulation (Batista et al. 2013). *Spongia officinalis* and *Hymeniacidon perlevis* proved also to be useful PCBs and PAHs bioindicators, respectively, being able to concentrate these contaminants in higher proportions than bivalves (Mahaut et al. 2013; Perez et al. 2003).

Another invertebrate, the squid *Todarodes pacificus* has been described as an effective POPs bioindicator in offshore waters. This species has a wide distribution with a minimal migration and it has been considered as an important commercial food item which ecology and biology have been extensively studied. These organisms have been partly used in ‘‘Squid Watch’’ Program as a pollution bioindicator for monitoring (Ueno et al. 2003a). *T. pacificus* proved to be a proper OCs bioindicator (Ueno et al. 2003A; Wong et al. 2009), PBDEs and HBCDs (Kim and Stapleton 2010).

The characteristics that make fish suitable bioindicators have been mentioned in the previous section (Metal pollution bioindicators). Among vertebrates, fish were preferred with a 66.7% (Figure 5C). Four species of Pacific salmon (*Oncorhynchus gorbuscha, O. keta, O. nerka, and O. tshawytscha*) were successfully used as OCs bioindicators in the Sea of Okhotsk and the Western Bering Sea (Tsygankov et al. 2017). On the other hand, a fish species of widespread distribution and high abundance in near-shore seawaters (*Trematomus bernacchii*) was considered a useful bioindicator of PCBs, PBDEs, HCBs compounds and changes in the Antarctic ecosystem quality and temporal trends (Cincinelli et al. 2016). The piscivorous young of year bluefish (*Pomatomus saltatrix*) has a high lipid content and has been described as an effective bioindicator of PCBs, PBDEs and OCs in estuaries since it has been shown greater sensitivity to contaminants than other estuary-resident fish, being their residence in this environment during the critical life stages (Smalling et al. 2016). The fish *Boops boops* have been successfully used as a bioindicator of PCBs, OCs, BDEs, and PAHs since significant pollutant concentrations differences between wild fish compared to farmed fish were found (Heriquez-Hernandez et al. 2018). On the other hand, as an offshore waters bioindicator, skipjack tuna fish (*Katsuwonus pelamis*) was shown to have desirable qualities such as it is an important commercial fish, its ecology and biology have been well studied, it is widely distributed and OCs accumulation suggests that their concentrations rapidly reflect pollution levels of this compound in seawaters (Ueno et al. 2003B). The same fish species has been used as a bioindicator of dibenzo-p-dioxins (PCDDs), PCDFs, PCBs (Ueno et al. 2005) and hexabromocyclododecane (HBCD; Ueno et al. 2006). In estuarial environments, *Ramnogaster arcuata* has been suggested as a bioindicator of PAHs due to its short life cycle and its completion within estuaries. Additionally, *R. arcuata* showed significant correlations of PAH concentrations with metabolic enzymes, proteins and lipid peroxidation (Ronda et al. 2018). Similarly, *Cynoscion guatucupa* juveniles, residents of estuarine environments during their first year of life, proved to be suitable bioindicators of PAHs, showing changes in the metabolic enzyme activities, lipid peroxidation and condition factor in response to PAH concentrations (Recabarren-Villalón et al. 2019). Finally, the seahorse (*Hippocampusreidi reidi*) was described as an excellent bioindicator for PAHs. This neo-tropical fish has low mobility and does not migrate from contaminated areas being a proper bioindicator for oil spills (Delunardo et al. 2015).

At higher trophic levels, organisms are more exposed to pollutants by bioaccumulation and biomagnification processes through the food web. Elasmobranchs, such as sharks are ideal marine pollution bioindicators because they are also widely distributed and are important in the ecosystem. The blue shark (*Prionace glauca*) is one of the most frequently caught shark species all over the world, particularly in Portugal where it was demonstrated to be an effective bioindicator for BFRs and PFCs with high correlations between chemical analysis and biochemical responses such as DNA damage and lipid peroxidation (Alves et al. 2016).

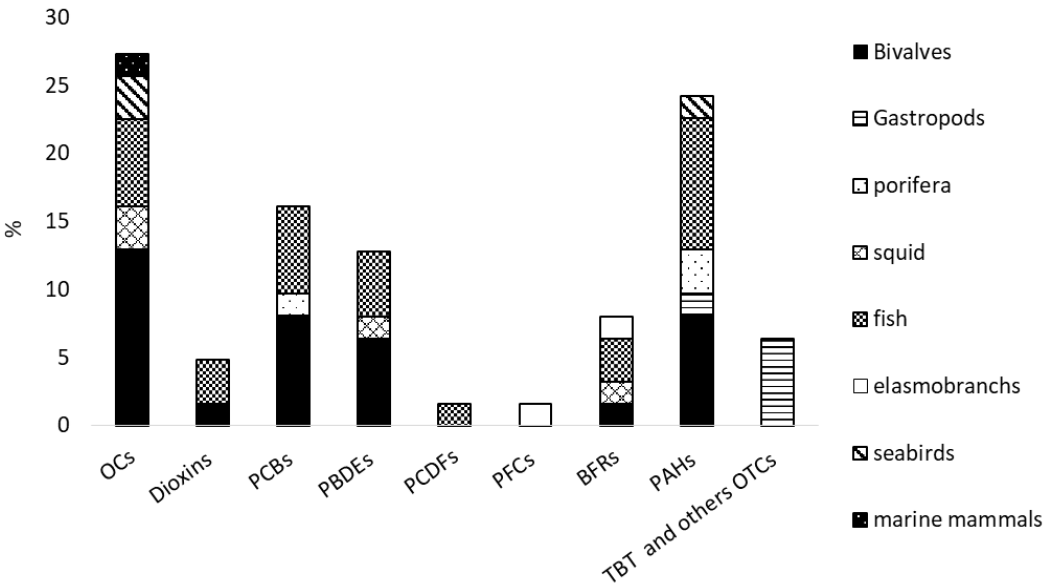

Figure 6. Bioindicators for each POP (in percentage of reviewed works).

Seabirds are especially prone to POP accumulation due to their high position on the food chain, food consumption high rates and rapid responses to contamination events. Nonmigrating birds can reflect the background contamination of their habitat and if there are no local pollution sources, birds reflect the global pollution resulting from the pollutants transboundary transport (Cienfuentes et al. 2003; Tsygankov et al. 2017). Five species of seabirds (*Larus schistisagus, Aethia cristatella, Aethia pusilla, Fulmarus glacialis, and Oceanodroma furcate*) were used as useful bioindicators of OCs in Okhotsk and Western Bering seas (Tsygankov et al. 2017). Bird eggs have also been demonstrated to be excellent bioindicators because during the breeding season energy reserves are mobilized and females consume large quantities of food. The assimilated proteins and fat, as well as the pollutants bound to them, are transferred into the eggs (Cienfuentes et al. 2003). In Cienfuentes et al. 2003 seabird eggs of five species (*Larus maculipennis, Larus dominicanus Sterna trudeaui, Phalacrocorax brasilianus, and Puffinus creatopus*) were used as bioindicators of OCs pollution. However, based on their abundance and sampling ease, only *Larus dominicanus* and *Larus maculipennis* eggs proved to be suitable bioindicators of OCs. Petroleum products are toxic to seabirds and their characteristics make them particularly vulnerable to oil pollution. They spend much of their lives on the ocean's surface and their populations

concentrate in habitats prone to high oil exposure. Nevertheless, because of their great ability to metabolize PAHs, seabirds are not commonly used as PAHs bioindicators, being necessary to develop alternative techniques for the use of these organisms as bioindicators. The analysis of PAHs in the *Larus michahellis* blood turned out to be a promising method for the use of seabirds as bioindicators of PAHs in a non-mortal way (Pérez et al. 2008).

Marine mammals are considered key species for monitoring of POPs in the marine environment all over the world. Thus, they can be used as bioindicators of global pollution. The grey whales (*Eschrichtius robustus*) and Pacific walrus (*Odobenus rosmarus divergens*) have been successfully used as bioindicators of OCs (Tsygankov et al. 2017).

Finally, from the reviewed works, it was observed that among the different POPs, the greatest attention has been focused on OCs, with a wide range of bioindicators described, followed by PAHs, PCBs, PBDEs and BFRs summarized in the Figure 6. Other pollutants such as dioxins, PCDFs and PFCs are still receiving little attention regarding bioindicators, so they still represent a challenge for research and environmental monitoring.

PLASTIC POLLUTION BIOINDICATORS

Marine pollution due to plastic is a global threat widely recognized by policymakers, the scientific community and citizens (Bonanno and Orlando-Bonaca 2018). Plastics are persistent pollutants, lasting hundreds to thousands of years; therefore, they accumulate over time and the fate and consequences are just beginning to be understood (Santana et al. 2016). Depending on their size, plastic debris can be classified as macro-, meso-, micro and nanoplastics. Its widespread use and persistent nature have made that especially the microplastics (MPs) were ubiquitous in marine waters, sediments, organisms and even sea salts (Li et al. 2016). MPs are particles less than 5 mm of diameter that could be intentionally produced in this small size (primary microplastics used in products such as abrasive products and exfoliating creams) or originated from the fragmentation of larger plastic (secondary microplastics). Additionally, they represent a special threat to marine ecosystems due to their high bioavailability, persistence and capacity to be toxic by themselves or adsorb other pollutants in their surfaces (Santana et al. 2016). In the marine environment, plastic debris is exposed to a combination of chemical and physical agents that lead to a degradation process producing potentially harmful and toxic chemicals, such as organic pollutants and chemical additives (Khoironi et al. 2018). Therefore, the biomonitoring of plastic pollution is necessary to generate appropriate and tailored actions of prevention and mitigation, for better sea ecosystems protection. Although the ingestion of plastic has been documented in more than 300 species, the use of plastic bioindicators is still at a preliminary stage, with few documented monitoring campaigns in the marine environment. The need to identify potential useful bioindicators is thus urgent given the ever-increasing impact of plastic debris on the sea (Bonanno and Orlando-Bonaca 2018; Lim et al. 2020). The species that have been evaluated, used, and/or recommended as bioindicators of plastic contamination have been summarized in Table 1, with bivalves, fish, and seabirds as the preferred organisms.

Table 1. Plastic pollution bioindicators

Bioindicators	Species	Location	Sampling method	Organs investigated	Technique	References
Bivalves	*Mytilus edulis* and *Crassostrea virginica*	United States	Bioassays	Whole body	Endoscopic examination in vivo	Ward et al. 2019
	Mytilus edulis	China	Collection of natural habitats	Whole body	Observation under microscope and verification with a micro-Fourier Transformed Infrared Spectroscope and Scanning Electron Microscope Energy Dispersive Spectrometer.	Li et al. 2016
	Perna perna	Brazil	Collection of natural habitats	Whole body	Observation under polarized light microscope (PLM)	Santana et al. 2016
	Mytilus edulis	France	Collection of natural habitats and transplanted	Whole body	Observation under Stereomicroscope and identification by Micro-Raman Xplora Plus	Kazour and Amara 2019
	Mytilus edulis	United Kingdom	Bioassays	Digestive gland and intestine	Observation under microscope	Lim et al. 2020
	Mytilus edulis, M. trossulus, M. galloprovincialis and *hybrids*	Norway	Collection of natural habitats	Whole body except byssus and foot	Observation under Stereomicroscope and identification by μ-transmission FT-IR	Brate et al. 2018
	Perna viridis	Indonesia	Collection of natural habitats	Whole body	Observation under microscope and verification by Scanning Electron Microscopy Electron Dispersive X-Ray (EDX)	Khoironi et al. 2018
	Mytilus galloprovincialis	Greece	Collection of natural habitats	Gills and digestive glands	Observation under a Stereomicroscope and verification by infrared spectroscopy (FT-IR)	Digka et al. 2018
Seabirds	*Calonectris diomedea, Puffinus yelkouan* and *Puffinus mauretanicus*	Spain	Accidentally caught	Stomach and gizzard	-	Codina-García et al. 2013
	Fulmarus glacialis	Canada	Local Inuit hunters	Proventriculus, gizzard and faecal precursor	Observation under Stereomicroscope	Provencher et al. 2018

Bioindicators	Species	Location	Sampling method	Organs investigated	Technique	References
Seabirds	*Diomedea exulans, Thalassarche melanophris, T. chrysostoma, Macronectes halli* and *M. giganteus*	United Kingdom	Chicks targeted for diet studies	Induced regurgitation	-	Phillips and Waluda 2020
	Puffinus lherminieri, Ardenna pacifica, Pterodroma baraui, Pseudobulweria aterrima, Phaethon lepturus, Anous stolidus pileatus, Anous tenuirostris, Ornithoprion fuscatus and *Morus capensis*	France	Necropsy of dead birds	Gizzard and proventriculus	Observation under microscope	Cartraud et al. 2019
	Fulmars	North Sea	Comparative analysis of fulmar data	Stomach	Observation under microscope	van Franeker and Lavender 2015
Fish	*Boops boops*	Spain	Caught by local fishermen	Gastrointestinal tract	Observation under a Stereomicroscope and verification by infrared spectroscopy (FT-IR)	Garcia- Garin et al. 2019
	Engraulis encrasicolus, Boops boops, Hygophum benoiti, Myctophum punctatum, Electrona risso, Mullus barbatus barbartus and *Chelidonichthys lucerna*	Mediterranean Sea	Academic database	Stomach contents and reports of necropsies	Bioindicator Index	Bray et al. 2019
	Sardina pilchardus, Pagellus erythrinus and *Mullus barbatus*	Greece	Trawling	Stomach and intestine	Observation under a Stereomicroscope and verification by infrared spectroscopy (FT-IR)	Digka et al. 2018
	Mullus barbatus and *Merluccius merluccius*	Italy	Collected by local professional fishermen	Gastrointestinal tract	Observation under a Stereomicroscope and verification by infrared spectroscopy (FT-IR)	Giani et al. 2019

Table 1. (Continued)

Bioindicators	Species	Location	Sampling method	Organs investigated	Technique	References
Fish	*Boops boops*	Mediterranean Sea Spain, France, Italy and Greece.	Caught by local fishermen	Gastrointestinal tract	Observation under a Stereomicroscope and verification by infrared spectroscopy (FT-IR)	Tsangaris et al. 2020
	Sardina pilchardus, Trachurus trachurus, Engraulis encrasicolus, Scomber colias, Scomber scombrus, and *Boops boops*	Portugal	Collection of natural habitats	Stomachs	Observation under Stereomicroscope and verification by infrared spectroscopy (FT-IR)	Lopes et al. 2020
	Boops boops	Italy	Collected both by local artisanal fisheries and by professional fisheries	Gastrointestinal tract	Observation under a Stereomicroscope and verification by infrared spectroscopy (FT-IR)	Sbrana et al. 2020
	Cathorops spixii, Cathorops agassizii and *Sciades herzbergii*	Brazil	Collection of natural habitats	Stomachs	Observation under binoculars (45x)	Possatto et al. 2011
	Micropogonias furnieri	Argentina	Collection of natural habitats	Gastrointestinal tract	Observation under a Stereomicroscope	Arias et al. 2019
Crustaceans	*Neohelice granulata*	Argentina	Collection of natural habitats	Gills and digestive tract	Observation under a Stereomicroscope	Villagran et al. 2019
	Nephrops norvegicus and *Aristeus antennatus*	Italy	Collected from framework of the MEDiterranean International Trawl Survey (MEDITS)	Stomachs	Observation under microscope and identification by μ-transmission FT-IR	Cau et al. 2019
	Nephrops norvegicus	Italy	Collected during trawl surveys	Stomach and intestine	Observation under Stereomicroscope and verification by infrared spectroscopy (FT-IR)	Cau et al. 2020
	Ocypode quadrata	Brazil	Captured by hand and euthanized on ice	Guts	Observation under Stereomicroscope	Lopes Costa et al. 2019
	Amphibalanus amphitrite	China	Collection of natural habitats	Whole body	Observation under Stereomicroscope and identification by μ-transmission FT-IR	Xu et al. 2020

Bioindicators	Species	Location	Sampling method	Organs investigated	Technique	References
Marine mammals	*Otaria byronia, Arctocephalus phillippii, Arctocephalus australis* and *A. australis un-named*	Peru and Chile	Collection of natural habitats	Scats	Observation under microscope and identification by μ-transmission FT-IR	Perez-Venegas et al. 2020
	Pontoporia blainvillei and *Sotalia guianensis*	Brazil	Accidentally captured by gillnet fisherie	Stomachs	-	Di Beneditto and Arruda 2014
	Balaenoptera physalus	Italy	Stranded fin whales	Blubber and muscle	Detection of phthalate content	Fossi et al. 2014
Shark	*Cetorhinus maximus*	Italy	Accidentally caught	Muscle	Detection of phthalate content	Fossi et al. 2014
Turtles	*Caretta caretta*	Italy	Streanded and hand-picked by fishermen and yachtsmen	Gastrointestinal tract and residual faecal pellet	Observation under Stereomicroscope	Camedda et al. 2014
Sponges	*Carteriospongia sp., Ircinia spp.* and *Tethyid spp.*	Indonesia	Non-lethal samples of natural habitats	Whole body subsample	Raman spectra were taken on a confocal Raman microscope	Girard et al. 2020
Corals	*Astrangia poculata*	United States	Bioassays	Polyps	Identification by Smiths IlluminatIR II, attenuated reflectance (ATR) Fourier transform infrared spectroscopy (FTIR) with an attached Olympus scope	Rotjan et al. 2019
Algae	*Pyropia yezoensis, Ulva prolifera, Sargassum horneri, Cladophora sp., Undaria pinnatifida,* and *Ulva pertusa*	China	Collected in culture period and non-culture period	-	Observation under Stereomicroscope and identification by μ-transmission FT-IR	Feng et al. 2020

Regarding bivalves, multiple works point to their suitability as MPs bioindicators (Digka et al. 2018; Khoironi et al. 2018; Li et al. 2016; Lim et al. 2020; Santana et al. 2016). Brate et al. (2018), described the ingestion of small particles suggesting that *Mytilus* spp. is a suitable bioindicator of small MPs (<1mm). An experiment with transplanted mussels proved that a balance between intake and defecation/egestion reached in approximately 6 weeks being this information adequate to reflect the native mussel's ingestion. However, a study evaluated the use of two bivalve's specie (*Mytilus edulis* and *Crassostrea virginica*) demonstrating that these organisms are not robust indicators of MPs pollution because they only ingest the smallest spheres and microfibers. The authors strongly recommend searching for new marine bioindicators for MPs (Ward et al. 2019). Consequently, bivalves could be proper MPs bioindicators, but it is necessary to continue their study because microplastic monitoring is complex and it is in its initial phase (Kazour and Amara 2019).

Most of the reviewed works evaluated the use of fish as bioindicators through MPs ingestion (Bray et al. 2019; Digka et al. 2018; Garcia-Garin et al. 2019; Giani et al. 2019; Lopes et al. 2020; Possatto et al. 2011; Tsangaris et al. 2020). Of relevance, a significant correlation was described between the frequency of MPs ingestion with fish condition factor (Sbrana et al. 2020) and hepatosomatic index (Arias et al. 2019).

Various species of seabirds were described as effective MPs bioindicators (Codina-García et al. 2013; Phillips and Waluda 2020; Cartraud et al. 2019; van Franeker and Lavender 2015); besides, Provencher et al. (2018) suggested that seabirds could also act as microplastic vectors, bioaccumulating MPs in their colonies through the faeces.

For crustaceans, five species are suitable bioindicators of MPs pollution (Table 1; Cau et al. 2019; Cau et al. 2020; Lopes Costa et al. 2019; Villagran et al. 2019). It has been demonstrated that the Atlantic ghost crab was a indicator but only qualitatively since MPs prevalence in the gut content of this organism did not follow the constant MP density in the sediment at spatial-temporal scales. Therefore, the authors warn about the need of searching for other beach bioindicators and agree with the assumption that plastics biomonitoring in marine ecosystems is complex, and should rely on the combination of several indicator species with distinct characteristics, particularly feeding habitats and/or mobility (Lopes Costa et al. 2019). Also, it is important to highlight that Cau et al. (2020) documented the occurrence of MPs biological fragmentation in the crustacean *Nephrops norvegicus*, but future studies are necessary to better understand this process.

Other organisms have been related to MPs pollution studies. Cultivation of Nori algae releases large amounts of microplastics that are trapped in the algae for human consumption (Feng et al. 2020). Possible adverse effects of MPs were also described for sponges (Girard et al. 2020); and corals (Rotjan et al. 2019), demonstrating capability of these organisms to be MPs bioindicators, although more studies are necessary to elucidate this.

BIOINDICATORS OF EMERGING POLLUTANTS (EPS)

Emerging pollutants (EPs) or contaminants of emerging concern (CECs) encompass a wide range of chemicals that have been released into the environment and only recently detected in the environment, or they are present in the environment and have become of recent concern, or well-recognized contaminants for which new information on

environmental risk has become available. For most of these, there are no current regulations (Kroon et al. 2019; Tang et al. 2019). EPs can be categorized in multiple ways depending on their chemical structure and/or mode of action. The US EPA lists cover pharmaceuticals and personal care products (PCPs), veterinary medicines, endocrine-disrupting chemicals (EDCs) and some persistent organic pollutants (POPs). Other studies also contemplate lifestyle compounds, such as caffeine and nicotine, drugs and more recently MPs (Kroon et al. 2019). POPs and MPs have already been mentioned above in this chapter and are widely studied. However, the rest of the EPs are not commonly monitored in the environment and have the potential to enter to the diverse environmental compartments and cause adverse ecological and human health effects (Cunha et al. 2017). They are persistent, bioactive, bioaccumulative, and endocrine-disrupting being their effects not fully elucidated (Horricks et al., 2019; Rzymski et al., 2017). Sources of these compounds are diverse and include urban and industrial wastewater and diffuse runoff from agricultural land uses (Kroon et al. 2019).

Emerging pollutants are currently the newest and most serious challenges to natural resources, ecosystems and human health. Scientifically validated processes and tools are necessary to address this problem leading to future regulations (Gavrilescu et al. 2015). To overcome these challenges, mussels (*Mytilus galloprovincialis and Mytilus edulis)* and macroalgae (*Laminaria digitata*) were used for diclofenac (a nonsteroidal anti-inflammatory drug) monitoring. Levels of this compound in mussels were closely related to the environmental contamination turning them as potential bioindicators of diclofenac contamination in coastal environments (Cunha et al. 2017). For other pharmaceutical products such as carbamazepine, ibuprofen, fluoxetine, 17α-ethynylestradiol and propranolol, the marine bioindicator *Hediste diversicolor* (polychaete) has been used in bioassays and *in situ*. Analyses of antioxidant system (glutathione peroxidase-GPX and glutathione reductase-GR), neurotoxicity (acetylcholinesterase-AChE) and oxidative effects (lipidperoxidation-LPO and DNA damage *strand breaks*) comprised the battery of biomarkers recommended to evaluate in this bioindicator the environmental evaluation of pharmaceutical contamination (Maranhoet al. 2014; Pires et al., 2016). Marine macroalgaes *Pterocladia capillacea*, *Ulva lactuca* and *Sargassum hornschuchii* turned out to be suitable bioindicators of pharmaceutical contamination through measurements of fatty acid contents (Mohy El-Din 2017). Moreover, antibiotic resistant bacteria were used as bioindicators by several authors either, in effluents (Al-Bahry et al. 2012), as in organisms such as sea turtles (*Chelonia mydas*) through a non-invasive procedure (Al-Bahry et al. 2011) or the fish *Sparus aurata* (Barros et al. 2011).

Regarding personal care products (PCPs), those using organic ultraviolet (UV) filters such as oxybenzone, 4-methylbenzylidene camphor, Padimate-O or octyl methoxycinnamate were evaluated on muscle and stomach content from the invasive Pacific lionfish (*Pterois volitans*) showing that this organism may be bioaccumulating their residues and could be a useful bioindicator for such compounds (Horricks et al. 2019). Seaweeds specially *Asparagopsis taxiformis*, were proper bioindicators of benzotriazole ultraviolet stabilisers (BUVSs; Pacheco-Suárez et al. 2019) and *Boops boops* an aquaculture-associated fish, proved to be an effective bioindicator of chemical sunscreens (UVFs; Henriquez-Hernandez 2016). Also, *Artemia salina* and *Allium cepa* were used as bioindicators of cosmetics with hormonal composition (Viega et al. 2019).

Finally, rare earth elements (REEs) of anthropogenic origin are also contaminants of emerging concern. Feathers of the Humboldt penguin (*Spheniscus humboldti*) showed to be suitable as bioindicators of 16 REEs (Squadrone et al. 2019).

ACOUSTIC POLLUTION BIOINDICATORS

Marine noise pollution is a relatively recent development and is now recognized as a worldwide problem (Williams et al. 2015). It is introduced intentionally and incidentally into the oceans through human activities. Sound waves can differ in frequency (measured in hertz [Hz] or cycles per second), in wavelength and amplitude (generally measured in decibels [dB]) (Harm and Oude 2000). Anthropogenic noises vary significantly in terms of frequencies and intensities and can be categorized into two main types: high-intensity impulsive noise and low-frequency stationary noise. High-intensity noise can be produced by pile driving, underwater blasting, seismic exploration and active sonar application. Low-frequency stationary noise can be generated by various ships and vessels (Peng et al. 2015).

Assessing the impacts of noise pollution is complex and expensive. There is limited information on sound processing in marine organisms or the role that plays in populations balance and development, and up to what sensory and systemic levels the impact can reach is still unknown, being an effect not immediately observed but in the medium or long term. Also, there are no well-defined protocols to measure the noise pollution impacts, which lead to heterogeneous and fragmented results, useless to guide management actions (Andre et al. 2009).

As top predators with vital dependence and an almost exclusive relationship with sound, cetaceans are the best bioindicators of marine acoustic pollution. They depend on acoustic exchange for a great number of activities and vital behaviours such as communication, geographical orientation, habitat use, feeding and a wide range of endeavours within the broader social group (cohesive action, warnings and maternal relationships), which makes them especially sensitive to acoustic pollution (Andre et al., 2009). The effects can be physical, physiological and behavioural and therefore, different actions have been recommended to determine acoustic contamination impact such as the detection of cetacean strandings and mortalities along with noise events worldwide that should be disclosed and documented. Moreover, post-mortem inspection to determine injuries into the acoustic reception channels and lesions in "non-acoustic" organs have been carried out as well as studies of noise effects on ecological processes, population dynamics, patterns of distribution and behaviour, stress hormones levels (e.g., in faeces) in noisy and quiet areas, and hearing studies in high-noise areas compared with suitable controls (Andre et al. 2009; Weilgart 2007).

Currently, there is still uncertainty and many questions regarding noise pollution since many species have not been studied, such as fish that seem quite vulnerable, and bivalve "can sessile marine species adapt to non-lethal noise environments?" (Peng et al. 2015; Weilgart 2007).

CONCLUSION

A wide range of pollutants that affect marine environments have been reviewed in this chapter. All these pollutants can severely alter both the marine world stability and biodiversity. Therefore, constant monitoring and identification of impacts in time are essential to avoid irreparable damage. The use of bioindicators has proven to be reliable tools for

constant monitoring of marine environments through the evaluation of qualitative and quantitative responses. Organisms of different taxonomic groups, from microorganisms to marine mammals have been described. Bivalve molluscs were the preferred bioindicators while fish were widely used for POPs, metals, and plastic pollution. The use of algae specie as bioindicators were useful for metals and nutrients, and seabirds were frequently used for metal and plastic pollution. Many other promising bioindicators were described for particular environments or pollutants, such as marine mammals, marine sponges, gastropods, crustaceans, parasites, plankton, bacteria, among others, although these were less frequent.

Among the newest challenges, the use of bioindicators for some pollutants such us plastic, emerging pollutants, acoustic pollution, and some POPs, is still in the preliminary stage, and the need to identify useful bioindicators is urgent, considering the increasing impacts that these entails. Furthermore, it is important to note that some authors suggest a multi-species approach as a more effective monitoring method; and non-invasive methods have been explored, such as the use of faeces or blood in seabirds, the use of shells or blood in bivalves, non-invasive methods in sea turtles, and behavioural studies in cetaceans. Finally, there are still many considerations that must be evaluated in the use of bioindicators, advantages, disadvantages and techniques according to the objectives sought. However, its use is currently essential for obtaining representative data that can support decision-making and regulations for environmental preservation and human health.

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Chapter 134

OCCURRENCE, BEHAVIOR AND ECOTOXICITY OF ORGANOPHOSPHORUS PESTICIDES (OPPS) IN MARINE ENVIRONMENTS: A REVIEW

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ABSTRACT

Since the banning of popular organochlorine pesticides in 1970s, organophosphorus pesticides (OPPs) started to be widely used throughout the world for agricultural purposes. With an increasing popularity since the 1980s, they were simultaneously applied with organochlorine pesticides for several years. OPPs represent the most used pesticides around the world, to protect agricultural crops against pests, private houses, gardens and in veterinary practices.

Chemically, although they have a lower half-life in the environment than organochlorine pesticides, a moderate persistence and ecotoxicological effects on non-target species like invertebrates, fish, birds, and even humans have been proved. OPPs resistance to degradation leads to a half-life ranging from hours-at elevated temperature, extreme pH, or high radiation to more than 6 months in the marine environment.

They commonly enter to the marine environments transported by river runoff from the continent in dissolved phase or sorbed to particulate matter. Once in the water, they can enter the trophic network causing damage to the biota; in simultaneous, they can undergo chemical, photochemical and biological degradative processes which could result in more toxic metabolites than the parental compounds.

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This review-chapter will describe the development history of the organophosphorus pesticides class, their environmental fate, behavior, and currents concentrations in marine environments and the state of the art of their effects to the marine biota.

Keywords: organophosphorus pesticides, marine pollution, organic pesticides, ecotoxicity,·coastal-marine environment, organophosphorus metabolites, diazinon, chlorpyrifos, parathion, malathion

1. INTRODUCTION: HISTORY, CHEMISTRY AND USES OF ORGANOPHOSPHORUS PESTICIDES

At the present time, many chemical compounds are widely dispersed in the environment. Frequently, the sea is the destination of the contaminants such as sewage, oils, metals, radioactive isotopes, plastics, and synthetic organic compounds after their use in industrial, agricultural, and urban activities (Serrano et al. 1995). Coastal marine environments are some of the most anthropogenically-stressed environments in the world and are known to receive copious amounts of hazardous organic chemicals. These organic chemicals include carcinogenic and toxic polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, and among others. These chemicals can not only adversely benthic and pelagic organisms but also accumulate to higher trophic levels, ultimately affecting humans (Smith 1987).

Pesticides, because of their widespread distribution, physicochemical properties, and toxic nature, have become an important class of aquatic pollutants, and so have been the focus of intense research (Jun et al. 2001; Serrano et al. 1995). In many countries, restrictions have been imposed on the use of organochlorine pesticides; and this has shifted the use patterns away from organochlorine toward organophosphorus and carbamate pesticides (Jun et al. 2001).

Organophosphorus are the most widely used pesticides in most countries around the world to protect agricultural crops against pests and also elimination of ectoparasites of animal husbandry (Kazemi et al. 2012). OPPs have become increasingly popular for both agricultural and home use because their unstable chemical structure leads to rapid hydrolysis and little long-term accumulation in the environment (Kumar et al. 2010).

Because of the extensive use of organophosphorus pesticides, the risk of contamination affects different types of aquatic environments, namely rivers, estuaries, lagoons, shallow waters, and wetlands (Barceló et al. 1990). Although they have lower persistence in the environment than organochlorine pesticides, their moderate persistence, toxicity, and bioaccumulation in different species have been reported in many scientific articles (Mercado-Borrayo et al. 2015, Giesy et al. 2014, Klosterhaus et al. 2003, Varó et al. 2002, Escartín and Porte 1996, Keizer et al. 1991).

1.1. History and Evolution of Organophosphorus Compounds and Opps

In order to describe the history of organophosphorus pesticides (OPPs), it is necessary to mention the events that made the development of this type of compound possible and also the

difference between organophosphorus compounds and organophosphorus pesticides. Organophosphorus compounds are used as insecticides, nematicides, acaricides, fungicides, herbicides, defoliants, fire retardants, solvents, plasticizers, drugs, and chemical warfare nerve agents (Bey et al. 2001). Thus, OPPs (insecticides, nematicides, acaricides, molluscicides, fungicides, and herbicides) are a particular type of organophosphorus compounds, however in this review article herbicides would not be considered. The synthesis of the first organophosphorus compound and even the development of chemical warfare agents were very important developments for evolution in the synthesis of OPPs and these made possible large-scale production for agricultural purposes in the following years.

The systematic study of organophosphorus compounds began in the 19th century, by Jean Louis Lassaigne –a French scientist- in 1820, with the esterification of acid phosphoric that resulted in the obtaining of triethyl phosphate (TEP), (Dos Santos et al. 2007, Chambers 1992). However, the first organophosphorus compound acetylcholine esterase inhibitor (anti-AChE) was produced by Philippe de Clermont, another French scientist, and it was the tetraethyl pyrophosphate (TEPP) in 1854. Then in 1873, Von Hofmann synthesized an organophosphorus compound called methyl phosphoryl chloride (Balali-Mood and Saber 2012). Methyl phosphoryl dichloride is the first example of the C-P linkage and is one of the important steps in the synthesis of modern C-P compounds (Holmstedt 1963).

By the end of the nineteenth century, Carl Michaelis – a german chemist, professor at the University of Rostock- reported a compound with a P-CN bond, diethylamidoethoxy phosphoryl cyanide. This was an important contribution that later led to the synthesis of very important insecticides and the nerve gas, Tabun (Holmstedt 1963).

On the other hand, a few decades later, in 1932, Willy Lange and Gerda Von Krueger, at the University of Berlin, synthesized some compounds containing the P-F linkage -dimethyl fluorophosphates and diethyl fluorophosphates- and noticed that these compounds have strong odor and after inhaling for a few minutes cause difficulties in breathing, loss of consciousness and painful sensitivity to sunlight (Popov and Popov 2009).

Two years later, Dr. Gerhard Schrader, a German chemist at I. G. Farbenindustrie, was given the task to develop a pesticide (Soltaninejad 2014). However, it was not until 1936, that he turned his interest to the phosphorus compounds (Holmstedt 1963). The organophosphorus compounds are highly toxic substances with effect on the nervous system (Popov and Popov 2009). Indeed, in 1937 Schrader synthesized secret compounds bearing the code names Sarin, Tabun and Gelan. Their high volatility and toxicity made them potentially suitable as chemical warfare agents (CWA). These compounds have been called “nerve gases,” because their effects are predominantly on the nervous system (Costa 1988). These three nerve gases were known as “G agents,” the G letter means German. Seven years later Schrader synthesized Soman and thionophosphorus esters including parathion and its oxygen analog paraxon, two well-known pesticides. Till the end of the war, Schrader and his coworkers developed about 2000 organophosphorus compounds (Soltaninejad 2014). The formulas of Tabun and Sarin were published in 1948 by Valade and Sallé who also reported some preliminary pharmacological experiments in the same year (Holmstedt 1963).

After the WWII, American companies gained access to information from Schrader’s laboratory, and began to develop OPPs in copious quantities. Hence, in 1950 the American Cyanamid Company began to produce malathion, an extremely low human toxicity pesticide that become the most popular pesticide worldwide for more than half a century (Soltaninejad 2014; Gupta et al. 2011).

Table 1. Chronology of the synthesis and development of organophosphorus compounds

Date	Event
1820	Synthesis of TEP by Lassaigne
1854	First OP anti-AChE by Clermont
1873	Von Hofmann synthesized methyl phosphor chloride
1890s	Michaelis reported a compound with a P-CN linkage, the Diethyl amido ethoxy phosphoryl cyanide
1932	Lange and Krueger noticed the toxic effects in humans of dimethyl and diethyl fluorophosphates
1936	Schrader started working on phosphorus compounds
1937	Schrader synthesized sarin, tabun and gelan as CWA
1944	Soman, parathion and paraoxon were synthesized by Schrader
1948	Formulas of tabun and sarin published by Valade and Sallé
1950	Malathion was produced by the American Cyanamid Company in 1950
1951	Introduction of Systox (demeton or mercatophos) by Schrader
1952	British scientists produced the VX Russian scientists produce the RX Synthesis of dichlorvos and trichlorfon by Perkow reaction
1957-1959	Chlortion, fenthion and fenitrothion were synthesized
1970s	The use of OPPs increased due to ban on organochlorine compounds

In 1951 Schrader and Lorenz developed a new type of sulfur- having OPPs, including Systox (demeton or mercaptophos, a mixture of the thiono- and thioloisomers of O, O-diethyl-2-ethylmercaptoethyl phosphorothioate).

In 1952 occurred three notable events for the evolution and development of organophosphorus compounds and so of OPPs too. First, British scientists in the United Kingdom produced CWA known as "V agents" – "V" for "Victory" after WWII- which were sulfur-containing organophosphorus compounds (Balali-Mood and Balali-Mood 2008). These compounds are ten-fold more toxic than sarin (Soltaninejad 2014). Secondly, Russian chemists developed a similar nerve agent, variably referred to as VR or "Russian VX" (N, N-diethy-2-methyl-2-methylpropoxy phosphorylsulfanylethanamine, Popov and Popov 2009; Mikler et al. 2011). Finally, the Perkow reaction was discovered, and many important vinyl phosphate esters –as dichlorvos and trichlorfon- begun to be introduced as practical insecticides (Eto 1979). In the latest years of the 1950s, chlorthion, fenthion and fenitrothion were also synthesized (Eto 1979).

Therefore, the use of OPPs peaked in the 1970s when most widely used organochlorine insecticides where phased out or banned (Costa 2018). While there has been a considerable increase in the annual use of organophosphorus insecticides for crop protection since 1970, the overall increase has been less since the early 1980s. However, new uses and formulations have been introduced and the use of OPPs is still high in most developing countries, particularly because of the low cost of these chemicals compared to newer insecticides (Costa 2018; WHO 1986).

1.2. Chemistry

Organophosphorus pesticides are normally esters, amides, or thiol derivatives of phosphoric, phosphonic, phosphorothioic, or phosphonothioic acids and are currently used in agriculture or animal husbandry for crop protection and/or elimination of ectoparasites (Sud

and Kaur Sidhu 2012). The general formula of organophosphorus is presented in Figure 1, where R_1 and R_2 are usually simple alkyl or aryl groups, both of which may be bonded directly to phosphorus (in phosphinates), or linked via -O-, or -S- (in phosphates and phosphorothiolates, respectively), or R_1 may be bonded directly and R_2, bonded via one of the above groups (phosphonates, WHO 1986).

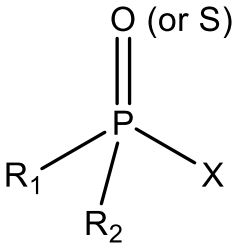
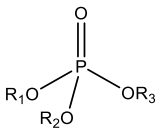
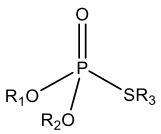
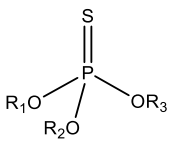
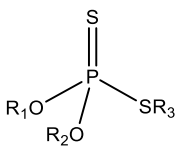


Figure 1. General formula of organophosphorus pesticides.

Table 2. Chemical structures of main groups of OPPs

Main groups of OPPs	Chemical structure	Examples
Phospate		Dichlorvos, Diazoxon, Dicrotophos, Mevinphos, Monocrotophos, Naled, Phosphamidon, Tetrachlorvinphos
Phosphorothiolate		Demeton-S, Oxydemeton-Me
Phosphorothioate		Chlorpyrifos, Chlorpyrifos-Me, Demeton-O, Diazinon, Fenitrothion, Fensulfothion, Fenthion, Isazofos, Methyl- Parathion, Parathion, Pirimiphos-Me, Pirimifos-Ethyl, Temephos, Tolclofos-Me
Phosphorodithioate		Azinfos-Me, Carbophenothion, Dimethoate, Disulfoton, Malathion, Phorate, Phosmet, Terbufos, Thiometon

The double-bonded atom may be oxygen or sulfur and related compounds would, for example, be called phosphates or phosphorothioates (WHO, 1986). In phosphoramidates, carbon is linked to phosphorus through an -NH group. The X group can be diverse and may belong to a wide range of aliphatic, aromatic or heterocyclic groups linked to phosphorus via a bond of some lability (usually -O- or –S-). The X group is also known as a leaving group because on hydrolysis of the ester bond it is released from phosphorus (Singh and Walker 2006).

Organophosphorous pesticides vary in chemical structures and toxicities. For instance, a phosphorthioate compound such as Parathion is much more toxic than a phosphorodithioate compound like Malathion (Balali-Mood and Saber 2012).

Based on the molecular structures, OPPs are typically categorized into four subgroups: phosphates, phosphorothioates, phosphorodithioates, and phosphorothiolates (Pehkonen and Zhang 2002).

1.3. Uses of OPPs

Organophosphorus pesticides include not only insecticides, but also fungicides, herbicides, molluscicides, nematicides and others. They are used to protect crops and improve agricultural production by controlling insects, diseases, fungi, and other pests. OPPs are wide spectrum pesticides, not only used for field crops, fruits, vegetables, ornamental plants, trees, horticulture, cattle parasites and, pet ectoparasites, but also in public health for disease vector control. For instance, azinphos methyl is frequently used for cotton, apples, pears, citrus crops, and blueberries, whereas chlorfenvinphos is used as pesticide to control root flies, root worms and other soil insects in vegetables; foliar application on potatoes; scale insects and tick eggs on citrus fruit; stem borers and leafhoppers on rice, corn and sugar cane; and white flies on cotton; aside from control of mosquito larvae, acaricide and animal ectoparasiticide (Mackay et al. 2006).

1.4. Currently Used OPPs, Quantities and Restricted Compounds

The population explosion and resulting increased demand for agro-products have resulted in such an increase in the consumption of pesticides worldwide. The U.S. Environmental Protection Agency (USEPA) had registered about 23.400 different pesticide products by the year 1991 (Kumar et al. 2018). The top five highest pesticide-consuming countries globally (tonnes per year) are Italy (63.305), Turkey (60.792), Colombia (48.618), India (40.379), and Japan (36.557, Verma et al. 2014).

Currently, organophosphorus pesticides are the most commercially favored group of pesticides, with large application areas all over the world (Kumar et al. 2018). Out of 2.300.000 tonnes of pesticides which are used worldwide every year, organophosphosphorus pesticides (mostly insecticides) constitute 20-38%, and the main candidates are chlorpyrifos, dichlorvos, diazinon, dimethoate, fenitrothion, methyl parathion, monocrotophos, malathion, and profenophos (Mahajan et al. 2019).

According to Pesticides Industry Sales and Usage 2008 – 2012 USEPA report, the amount of OPPs used in the United States OPPs usage as a percentage of total insecticide use has decreased from 71% in 2000 to 33% in 2012 and the most usage include acephate, chlorpyrifos, malathion, naled, phorate, dicrotophos, phosmet, dimethoate, terbufos, ethoprophos, and tetrachlorvinphos. The decrease in OPPs usage reflects a shift in usage to other classes of pesticides (i.e., pyrethroids, neonicotinoids, and other new formulations) because of the phasing out and use restrictions placed on OPPs.

In the case of the European Union, over the period 2011-2018 (EU agri-environmental indicator consumption of pesticides 2020), the sales of pesticides remained stable at around 360.000 tonnes per year. Particularly, in 2018 total pesticides sales were of 428.800 tonnes, of which 12.52% were insecticides and acaricides. From that insecticides and acaricides 11.7% were insecticides based on organophosphorus, constituting 6.286 tonnes. However,

organophosphorus pesticides also include acaricides, fungicides, and molluscicides, therefore the number of total organophosphorus pesticides should be higher.

As for other continents, there is only few data on South and Central America (Argentina, Belize, Panama, Paraguay and Suriname) of total organophosphorus pesticides use of 2.908 tonnes in year 2017 (FAOSTAT, Pesticides use 2020) and Asia (Bangladesh, Cyprus, Japan, Maldives, Myanmar and Sri Lanka) of 5.591 tonnes in the same year. No data of Africa, and Oceania were found in the FAOSTAT Pesticide use registers.

Regarding international conventions on organophosphorus pesticides, Annex III of the Rotterdam Convention include pesticides and industrial chemicals that have been banned or severely restricted for health or environmental reasons by two or more parties. The Annex III includes: azinphos-methyl, metamidophos (severely hazardous pesticide formulations), monocrotophos, parathion, phorate, trichlorfon, methyl-parathion and phosphamidon. Moreover, fenthion is recommended for list in Annex III.

On the other hand, the European Union for instance recently banned chlorpyrifos, because it is linked to health problems in children, but the U.S. has allowed its use because the USEPA did not agree with the assessment in Europe (Topping et al. 2020).

2. Chemical Properties of OPPs and Environmental Behavior

2.1. Physical- Chemical Properties of OPPs: Structures and Related Parameters of 28 Compounds

After organophosphorus pesticides are deposited on or incorporated into the soil matrix, they distribute between the air, soil air, soil water and soil solid phases (Pedersen and Suffet 2002). The major differences between behavior profiles of organic chemicals in the environment are attributable to their physical-chemical properties (Mackay et al. 2006).

A comparison of the octanol-water partition coefficients, K_{ow}, reveals the role the leaving group (X) plays in determining compound hydrophobicity and provides a direct estimate of partitioning tendency from water to organic media such as lipids, waxes and natural organic matter such as humin or humic acid (Mackay et al. 2006; Pedersen and Suffet 2002). Thus, it is used in environmental fate studies and large values (+4 or higher) are regarded as an indicator that a substance will bio-accumulate according to the Pesticide Properties DataBase website from University of Hertfordshire (https://sitem.herts.ac.uk/aeru/ppdb/).

Furthermore, the differences between the K_{ow} coefficients are mostly due to the leaving group of the molecule. For instance, the high K_{ow} value of chlorpyrifos (log K_{ow} = 4.70) is due primarily to the hydrophobic 3,5,6-trichloro-2-pyridinyl X group, while the low K_{ow} value of dimethoate (log Kow = 0.50) is due to the polar 2-mercapto-N-methyl acetamide X group (Pedersen and Suffet 2002).

Another important property is the water solubility, which is the mass of a given substance (the solute) that can dissolve in each volume of water (Pesticide Properties DataBase website from University of Hertfordshire). In addition to this, water solubility values above 500 mg/L means compounds with high solubility and below 50 mg/L low solubility. Acephate (818000 mg/L at 25ºC), Dicrotophos (1000000 mg/L at 25ºC), and Monocrotophos (1000000 mg/L at

25ºC) have high solubility in water, while Ethion (1 mg/L at 25ºC), and Chlorpyrifos (2 mg/L at 25ºC) have extremely low solubility in water.

Instead, to analyze the pesticide volatilization process, the vapor pressure property of each compound must be reported. This is the pressure at which a liquid is in equilibrium with its vapor at 20ºC (Pesticide Properties Data Base website from University of Hertfordshire). Besides, it is a measure of the tendency of a material to vaporize, hence can be viewed as a "solubility in air" (Mackay et al. 2006). According to the interpretation of parameters provided by the Pesticide Properties Data Base website from University of Hertfordshire; vapor pressure values above $1x10^{-2}$ Pa means highly volatile compounds; and values below $5x10^{-3}$ means low volatility compounds. Therefore, Dichlorvos (1.60 Pa at 20ºC) is one of the most volatile OPPs and by contrast Temephos (9.5 x 10-6 at 20ºC) is one of the less volatile compounds. Volatilization of soil and foliar applied pesticides reduces the amount available for transport with runoff water (Pedersen and Suffet 2002). Volatilization depends on the vapor pressure and water solubility of the compound. Partitioning between the water and vapor phases is expressed by the Henry's law constant, $K_H = C_a/C_w$, where C_a and C_w are the air, and water phase concentrations, both expressed in mol/L (Pedersen and Suffet 2002). Henry's law constant supplies a sign of the preference of a chemical for air compared to water i.e. its volatility (Mackay et al. 2006). Table 3 displays physicochemical properties of representative organophosphorus pesticides.

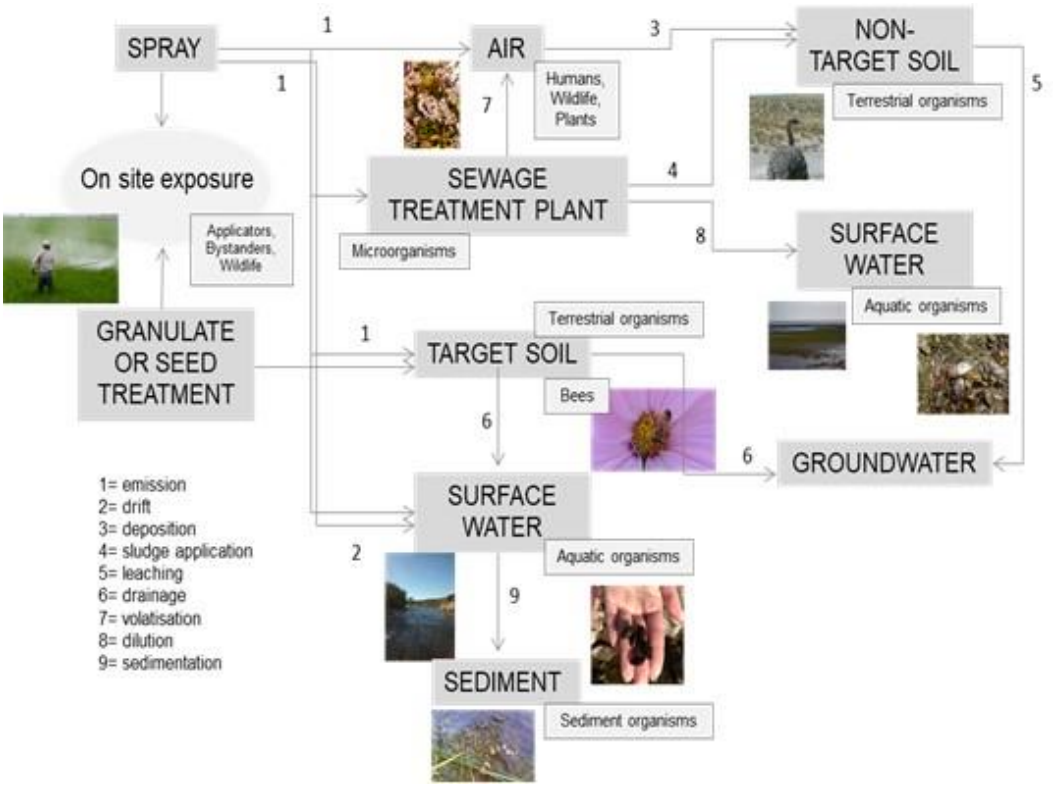


Figure 2. Distribution pathways of OPPs in the environment. Adapted from WHO 2001.

Table 3. Physical- chemical properties and related parameters of 28 organophosphorus pesticides

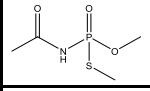
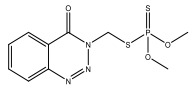
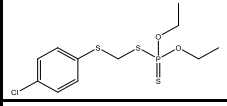
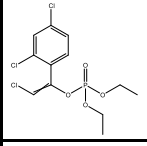
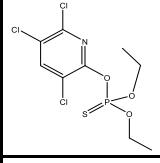
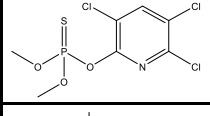
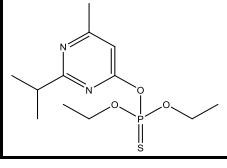
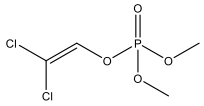
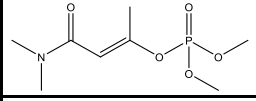
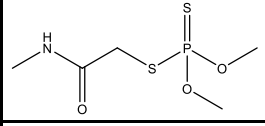
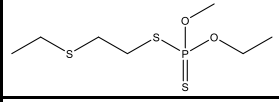
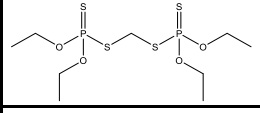
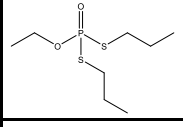
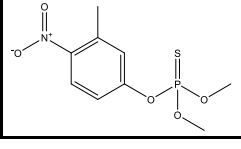
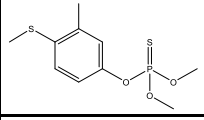
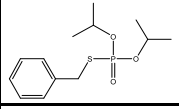
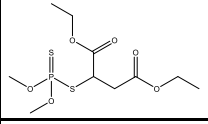
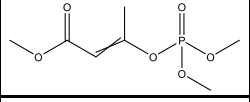
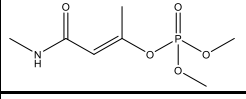
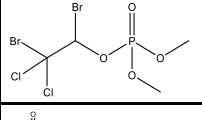
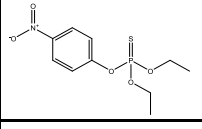
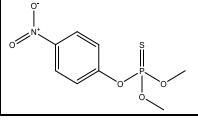
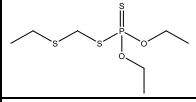
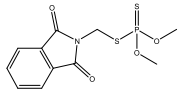
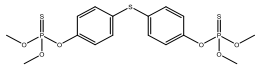
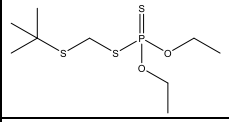
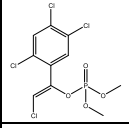
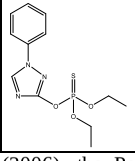
Name	Structure	Type	MW*	Vapor pressure (Pa at 25°C or as indicated) *	Water Solubility (g/m^3 or mg/L at 25°C or as indicated) *	Log KOW*	Henry's Law Constant ($Pa \cdot m^3/mol$ at 25°C) *
Acephate acetylphosphoramidothioic acid O, S-dimethyl ester		Insecticide	183.16	2.26 × 10–4 at 20°C	81.18 × 105	–0.85	5.27 × 10–8
Azinphos methyl O, O-dimethyl-S-[-4-oxo-1,2,3-benzotriazin-3(4H)-yl)methyl)] phosphorodithioate		Insecticide and acaricide	317.324	2.93 × 10–5 at 20°C	30 at 20ºC	2.69	0.0032 at 20°C
Carbofenthion S-chlorophenylthio methyl O, O-diethyl phosphorothioate		Insecticide and acaricide	342.866	1.11 × 10–3	0.34	5.50	0.0046 at 20°C
Chlorfenvinphos 2-chloro-1-(2,4-dichlorophenyl) vinyl diethyl phosphate		Insecticide and acaricide	359.569	1.0 x 10-3	145	3.10	0.324 at 20°C
Chlorpyrifos O, O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate		Insecticide	350.586	2.5 x 10-3	2.0	4.70	0.421
Chlorpyrifos-methyl *O, O*-dimethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate		Insecticide and acaricide	322.534	5.60 × 10–3	4.0	4.31	0.235
Diazinon O, O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate		Insecticide and acaricide	304.345	1.87 x 10-2	40 at 20°C	3.81	0.0114 at 20°C

Table 3. (Continued)

Name	Structure	Type	MW*	Vapor pressure (Pa at 25°C or as indicated) *	Water Solubility (g/m^3 or mg/L at 25°C or as indicated) *	Log KOW*	Henry's Law Constant ($Pa \cdot m^3/mol$ at 25°C) *
Dichlorvos 2,2-dichlorovinyl-O, O-dimethyl phosphate		Insecticide and acaricide	220.976	1.60 at 20°C	1× 104	1.43	0.194
Dicrotophos (E)-2-dimethylcarbamoyl-1-methylvinyl dimethyl phosphate		Insecticide and acaricide	237.191	9.3 x 10-3 at 20°C	1 × 106	–0.49	5.05 × 10–6
Dimethoate O, O-dimethyl S-methylcarbamoyl-methyl phosphorodithioate		Insecticide	229.258	1.1 × 10–3	2.5× 104	0.50	1.15 × 10–4
Disulfoton O, O-diethyl S-[2-(ethylthio) ethyl] phosphorodithioate		Insecticide and acaricide	274.405	2.4 x 10-3 at 20°C	25 at 22°C	4.02	0.090
Ethion bis(S-(dimethoxyphosphinothioyl)mercapto)methane		Insecticide and acaricide	384.476	1.50 × 10–4 at 20°C	1.0	5.07	0.0384
Ethoprophos O-ethyl S, S-dipropylphosphorodithioate		Insecticide and nematicide	242.340	4.65 x 10-2 at 26°C	700 at 20°C	3.59	0.0161
Fenitrothion O, O-dimethyl O-(3-methyl- O, O-dimethyl O-4-nitro-m-tolyl phosphorothioate		Insecticide	277.234	1.5 × 10–4 at 20°C	21 at 20°C	3.43	0.0962

Name	Structure	Type	MW*	Vapor pressure (Pa at 25°C or as indicated) *	Water Solubility (g/m^3 or mg/L at 25°C or as indicated) *	Log KOW*	Henry's Law Constant ($Pa \cdot m^3/mol$ at 25°C) *
Fenthion O, O-dimethyl O-(3-methyl-4-(methylthio)phenyl) phosphorothioate		Insecticide and acaricide	278.328	4.0 × 10–3 at 20°C	7.51 at 20°C	4.09	0.547
Iprobenfos di(propan-2-yloxy)phosphorylsulfanylmethylbenzene		Fungicide	288.34	1.22 × 10–2 at 20ºC	540 at 20ºC	3.37	-
Malathion S-[1,2-bis(ethoxycarbonyl)ethyl] O, O-dimethyl phosphorodithioate		Insecticide and acaricide	330.358	1.30 × 10–3 at 20ºC	145	2.36	2.03 × 10–3
Mevinphos 2-carbomethoxy-1-methylvinyl dimethyl phosphate		Insecticide and acaricide	224.148	1.7 x 10-2 at 20ºC	6x105	1.20	6.35 × 10–6
Monocrotophos dimethyl (E)-1-methyl-2-(2-methylcarbamoyl) vinyl phosphate		Insecticide and acaricide	223.164	9.00 × 10–3	1x106	-0.22	2.08 × 10–6
Naled 1,2-dibromo-2,2-dichloroethyl dimethyl phosphate		Insecticide	380.784	2.66 x 10-1 at 20ºC	2x103	1.38	6.60
Parathion O, O-diethyl O-4-(nitrophenyl) phosphorothioate		Insecticide and acaricide	291.261	5.0 × 10–3 at 20°C	24	3.83	0.0087
Parathion methyl O, O-dimethyl O-4-(nitrophenyl) phosphorothioate		Insecticide and acaricide	263.208	2 × 10–3	57	2.86	0.0101

Name	Structure	Type	MW*	Vapor pressure (Pa at 25°C or as indicated) *	Water Solubility (g/m^3 or mg/L at 25°C or as indicated) *	Log KOW*	Henry's Law Constant ($Pa \cdot m^3/mol$ at 25°C) *
Phorate O, O-diethyl-S-(ethylthio)methyl phosphorodithioate		Insecticide, acaricide and nematicide	260.378	0.11 at 20°C	50	2.92	0.648
Phosmet O, O-dimethyl S-phthalimidomethyl phosphorodithioate		Insecticide and acaricide	317.321	6.5 × 10–5	25	2.78	9.53 × 10–4
Temephos *O, O, O'*, O'-tetramethyl *O, O'*-thiodi-p-phenylene bis(phosphorothioate)		Insecticide	466.47	9.5 x 10-6 at 20ºC	0.01 at 20ºC	4.95	1.99 x 10-4
Terbufos S-(tert-butylthio)methyl O, O-diethyl phosphorodithioate		Insecticide and nematicide	288.431	3.46 x 10-2	5.07	4.48	2.463
Tetrachlorvinphos (Z)-2-Chloro-1-(2,4,5-trichlorophenyl) vinyl dimethyl phosphate		Insecticide	365.896	5.59 x 10-6	11 at 20ºC	3.53	1.86 x 10-4
Triazophos diethoxy-[(1-phenyl-1,2,4-triazol-3-yl)oxy]-sulfanylidene-λ5-phosphane		Insecticide, nematicide and acaricide	313.3	1.33 x 10-3	35 at 20ºC	3.55	4.90 x 10-3

* Parameters were selected from Mackay et al. (2006), the Pesticide Properties Database (https://sitem.herts.ac.uk/aeru/ppdb/) and from Pehkonen and Zhang (2002).

2.2 Environmental behavior. Distribution pathways. Persistence. Half-lives.

Table 4. t ½ for air, surface water and sediments of organophosphorus pesticides

Organophosphorus pesticides	t ½ Air*	t ½ Surface water*	t ½ Sediments*
Acephate	-	50 d when incubated at 9°C in creek water	-
Azinphos methyl	1.3 h	11 d under sunlight conditions for seawater at pH 8.1	-
Carbofenthion	-	-	-
Chlorfenvinphos	-	> 400 h at pH 9.1	-
Chlorpyrifos	6.34 h	24 d in an estuary	24 d in untreated sediment of a pesticide-seawater solution
Chlorpyrifos-methyl	-	11.1 d in estuarine water, pH 7.8	-
Diazinon	4.1 h	47 d under sunlight conditions for seawater, pH 8.1 10.6 d in estuarine water, pH 7.8	14.4 d under aerobic conditions
Dichlorvos	320 d	4 d in lakes and rivers DA 81 d under sunlight conditions for seawater at pH 8.1	-
Dicrotophos	-	-	-
Dimethoate	0.469–4.69 h	74 d under sunlight conditions for seawater, pH 8.1	-
Disulfoton	0.50–4.80 h	8–28 d for summer in river water	-
Ethion	6.95 h	4 wk in river water	-
Ethoprophos	-	-	-
Fenitrothion	-	3 d under sunlight conditions for seawater, pH 8.1	-
Fenthion	-	5 d under sunlight conditions for seawater at pH 8.1 4.6 d in estuarine water, pH 7.8	-
Malathion	1.0–9.8 h	14 d under sunlight conditions for seawater at pH 8.1 4.4 d in estuarine water, pH 7.8	1.4 d under aerobic conditions
Mevinphos	-	-	-
Monocrotophos	--	-	-
Naled	-	Completely hydrolyzed within 2 d	-
Parathion	< 1 d	18 d under sunlight conditions for seawater at pH 8.1	-
Phorate	-	-	-
Phosmet	-	33 d at 6°C VFD under sunlight conditions for seawater at pH 8.1	-
Temephos	-	5 d in estuarine water, pH 7.8	-
Terbufos	-	6.5 d in surface water	-
Tetrachlorvinphos	-	-	-

* Parameters were selected from Mackay et al. (2006), Lacorte et al. (1995) and from Lartigues y Garrigues (1995).

DA: Disappearance after, VFD: Very fast degradation.

The OPPs enter the environment (target soil or air or surface water) through different pathways after their application onto crops (by spraying) or into the soil (as seed treatment). These routes include emissions, leakage, drainage, and volatilization (Mahajan et al. 2019). These kinds of pesticides are applied to crops via a variety of methods. Malathion is often applied from low-flying airplanes in agricultural as well as urban areas while fenamiphos is applied as a spray or in granular form to the soil surface and incorporated post application (Pedersen and Suffet 2002). On the other hand, phorate is an example of a soil pesticide that is applied as granules on the land where the protected crop is cultivated, while chlorpyrifos belongs to a group of OPPs that are applied directly to the fruit and/or leaves (Pehkonen and Zhang 2002).

Regarding transport through the air, except for dichlorvos, most organophosphorus pesticides show low volatility. Dispersion of spray dew by wind is possible, but, in general, only insignificant amounts are likely to be distributed in this way (WHO 1986). From the soil, the OPPs residues can enter either surface water or groundwater via drainage or leakage.

As a result of the movement of the OPP residues in different environmental components, several non-target species are adversely affected. These non-target species range from microorganisms in the soil to aquatic and terrestrial organisms (Mahajan et al. 2019).

Factors controlling the distribution of organophosphorus pesticides discharged to marine environment are the degree of natural degradation and the extent of adsorption onto particulate matter while moving toward the offshore area (Stoker and Seager 1976). In addition to this, temperature, pH, contributions from microorganisms, and levels of suspended solids in seawater are also important (Paris et al. 1975).

The half-life of a chemical in the environment depends not only on the intrinsic properties of the chemical, but also on the nature of the environmental compartments. Factors such as sunlight intensity, hydroxyl radical concentration and the nature of the bacterial community, as well as temperature, affect the chemical's half-life so it is impossible (and misleading) to document a single reliable half-life (Mackay et al. 2006). Several works concerning the persistence of OPPs in the aquatic environment have been carried out; however, the most of them were studied in freshwater environments. In 1995 Lartigues and Garrigues, studied the degradation kinetics of a mixture containing 19 organophosphorus (OP) and organonitrogen (ON) pesticides at ppb level was studied over a 6-month period in different water types (ultrapure water, natural seawater -from Arcachon Bay, France-, river water, filtered river water) and under various conditions (Lartigues and Garrigues 1995). The mixture was analyzed by gas chromatography coupled with a nitrogen phosphorus detector (GC/NPD) and the half-lives times of the OPPs were decided (Lartigues and Garrigues 1995).

In the same year, Lacorte et al. studied the degradation of 10 organophosphorus pesticides in natural estuarine waters. The estuarine water samples of Ebro River in Spain were placed in Pyrex flasks, spiked with OPPs 50 μg/L and exposed outdoor to ambient sunlight and temperature from January to march (Lacorte et al. 1995). The analytical determinations were performed by solid phase extraction (SPE) followed by GC-NPD and GC-MS (Lacorte et al. 1995). As a result, five OPPs were stable for less than 1 week (disulfoton, fenamiphos, fenthion, malathion, and temephos), others had a half-life of ca. 1 week (chlorpyrifos-methyl, methidathion, and diazinon), and the rest showed a half-life of ca.10 days (isofenphos and pyridafenthion, Lacorte et al. 1995). The half-life of three pesticide transformation products: disulfoton sulfoxide, disulfoton sulfone, and fenthion sulfoxide varied from 7 to 12 days (Lacorte et al. 1995).

2.2. OPPs Mechanisms of Degradation and Sorption in Estuaries and Oceans

In the aquatic environment, the survival time in water may be influenced by light intensity, oxygen concentration, salinity, and pH. Most OPPs are more stable in the pH range that may be met in the environment (pH: 3 - 6), than at neutral pH (WHO 1986). Consequently, in the marine environments –usually with alkaline pH- the OPPs tend to be degraded faster. In addition to this, the influence of microbiological factors in the degradation of these pesticides in water may be considerable. Different climatic conditions, especially temperature and humidity, before, during, and after application may influence the survival time of OPPs markedly before reaching to the marine ecosystem (WHO 1986).

Organophosphorus pesticides in water can suffer different process of degradation, such as oxidation –can occur in a biotically way by ozone, dissolved oxygen and radical mechanisms, and in biotically way by specific enzymes-, photolysis - by direct or indirect ways- and hydrolysis, which seems to be the principal route of degradation in the environment and the most studied. Abiotic and biotic transformation processes reduce the amount of parent compound available in the environment. Transformation products call for concern, however, when they show significant toxicity (Pedersen and Suffet 2002). Therefore, identification of metabolites after their release into the environment is necessary for the evaluation of the OPPs toxicity to the biota (Battala et al. 2012).

2.2.1. Hydrolysis

All organophosphorus pesticides are subject to degradation by hydrolysis yielding water-soluble products that are believed to be non-toxic at all practical concentrations. The toxic hazard is therefore essentially short-term, in contrast to that of the persistent organochlorine pesticides, though the half-life at neutral pH may vary from a few hours for dichlorvos to weeks for parathion (WHO 1986). In addition to this, half-lives studies are often carried out in "model" environments and conditions, making extremely complicated to predict the results in the natural marine environments and at natural conditions.

The mechanism of hydrolysis plays a critical role in the overall degradation process of OPPs, which accounts for the cleavage of P–S or P–O bonds (Mahajan et al. 2019). Despite of hydrolysis is the most common route of degradation of OPPs in natural waters, the rate of hydrolysis depends of the type of OPP, for instance phosphorothioate pesticide hydrolysis is typically less than that of the corresponding phosphate compounds because the greater electrophilicity of the oxon renders it more susceptible to nucleophilic attack (Schwarzenbach et al. 1993). Hydrolysis can occur by a concerted mechanism, where H2O and HO^- (H^+ catalysis is less common) act as nucleophiles in an SN_2 mechanism (Pehkonen and Zhang 2002). It is important to realize that nucleophiles can attack either the phosphorus atom (with an alcohol or thiol residue being the leaving group) and the carbon atom bound to the oxygen or sulfur of an alcohol or thiol moiety respectively (with one of the the diester being the leaving group, Hong and Pehkonen 1998). For organophosphorus pesticides, HO^- is a better nucleophile than H_20. The base-catalyzed reaction results in detachment of the best leaving group from the phosphorus atom (Pedersen and Suffet 2002). In most cases, acid-catalyzed hydrolysis is unimportant unless the leaving group holds a basic functional group that enhances reactivity when protonated (Schwarzenbach et al. 1993).

Moreover, constituents of soil, sediments of surface water may themselves catalyze degradation of OPPs (WHO 1986). Hydrolysis of organophosphorus pesticides can be

catalyzed by dissolved metal ions -particularly Cu^{2+} for Diazinon or Hg^{2+} for malathion, fenitrothion, fenthion and parathion methyl- and constituent or exchangeable metal ions in aluminosilicate minerals -e.g., montmorillonite-. (Pedersen and Suffet 2002; Smolen and Stone 1997; Wan et al. 1994; Pehkonen and Zhang 2002).

Hong an Pehkonen (1998) studied the hydrolysis of phorate under simulated environmental conditions and with several metal oxides –like aluminum hydroxide, hematite, goethite and ferrihydrite- as solid phase in natural aquatic systems, although this four metal hydroxide or oxides (the most common in natural environments) did not seem to have significant influence on the half-life of phorate, under the experimental conditions employed in the study, i.e. no statistically significant catalytic effect was observed (Hong and Pehkonen 1998). They also found that abiotic hydrolysis of phorate under alkaline conditions produces diethyl disulfide, hydrogen sulfide, and formaldehyde, all of which are toxic compounds (Hong and Pehkonen 1998). In addition to this, Battala and co-workers studied the degradation of phorate in different water types –well water, Penna river water and seawater of Nellore, Bay of Bengal, India- and found that this pesticide had degraded at faster rates in sea water compared to river and well water. This shows that the hydroxide-catalyzed hydrolysis is a major pathway for the degradation in marine systems (Battala et al. 2012).

Batalla and co-workers (2012) studied the degradation of malathion in seawater of Bay of Bengal in India. They figured out that malathion had a half-life of 2 days in the seawater/sediment system -pH 7.3 to 7.7- (Battala et al. 2012). This half-life time value is in agreement with what was studied earlier by Cotham and Bidleman in 1989 at the same pH ranges for the seawater obtained from North Inlet estuary, Georgetown County, USA (Cotham and Bidleman 1989).To conclude, for malathion hydroxide-catalyzed hydrolysis, as well as in the case of phorate, is a major pathway for their degradation in marine systems, and is regulated by the pH of water (increase with alkaline pH) and salinity (faster degradation with increase of salt content, Cotham and Bidleman 1989; Lartigués and Garrigués 1995). Speedup of the reaction in saltwater is attributed to the higher ionic strength which influences hydrolysis through primary and/or secondary salt effects (Weber 1976).

On the other hand, Lartigués and Garrigués (1995) studied the degradation kinetics of several OPPs for a 6-month period in different water types, included natural seawater, under various conditions. Quite different degradation behavior with respect to physicochemical conditions and molecular structures of the pesticides was seen (Lartigués and Garrigués 1995). Moreover, the authors determined half-life times ($t_{1/2}$) of the pesticides in seawater. For phosmet, a very fast degradation in seawater at (T = 6ºC, pH = 8.1) was observed due to a rapid hydrolysis , whereas $t_{1/2}$ for dimethoate were 219 day (T = 6ºC, pH = 8.1) and in the case of azinphos-ethyl (T = 6ºC, pH = 8.1) no degradation at all was observed (Lartigués and Garrigués 1995).These experiments confirm that half-lives of OPPs can be more than several months and consequently lead to lasting environmental pollution. It is worth mentioning that there are marine environments that may present conditions that do not favor degradation. In this case, it was evaluated for seawater, in the dissolved fraction and with controlled conditions –pH and temperature-. Thus, in natural marine environments, there are multiple factors that influence the behavior of OPPs and it is not possible to generalize what may occur in a natural environment from a laboratory study.

2.2.2. Oxidation

The oxidation of OPPs can occur by a direct absorption of light, by an attack of photoproduced radicals, or by aqueous oxidants such as dissolved oxygen, aqueous chlorine, or by enzymatic reagents such as oxygenases (Pehkonen and Zhang 2002). The possible mechanisms include oxidation of the P = S bonds or the isomerization (Mahajan et al. 2019). Oxidation of OPPs to the corresponding oxons, sulfones, and sulfoxides has been reported widely (Pehkonen and Zhang 2002). Oxidation of OPPs by ozone produces oxons -where a sulfur atom from the thioate group is substituted by an oxygen atom- and sulfates due thiophosphorile bonds were oxidized by ozone into phosphorile bonds (Ohashi et al. 1994).

Lacorte and co-workers (1997) studied the oxidation with N-bromosuccinimide (NBS) of fenthion and temephos in water. In the case of fenthion the products were fenthion oxon (from the replacement of the doubly bonded sulfur with oxygen), fenthion sulfoxide (from the oxidation of the sulfur in one of the ester side chains), fenthion oxon sulfoxide (from the combination of the above two), and finally the S-methyl isomer of fenthion (changes the sulfur of the dimethyl-phosphorotioate group with the oxygen of the methoxy group) (Lacorte et al. 1997). Moreover, another oxidation product whose formation is possible is sulfone –is formed when sulfoxide in the ester side chain is further oxidized to yield two S = O bonds on a single sulfur atom- (Lacorte et al. 1997). Likewise, in the case of temephos similar products than fenthion were founded. The main difference is that temephos has two P = S bonds, thus dioxon can also be formed (Pehkonen and Zhang 2002). In the next figure are shown all degradation products of oxidation of fenthion with NBS.

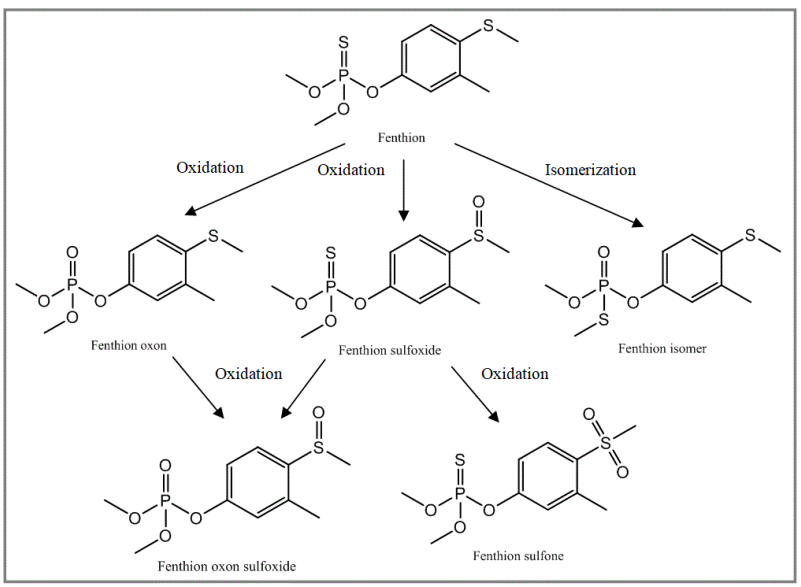


Figure 3. Structural formula of fenthion and its degradation products and schema of the oxidative pathways after oxidation of fenthion with NBS. Adapted of Lacorte et al. 1997.

2.2.3. Biodegradation

Pesticides are degraded in the environment principally by the action of indigenous microorganisms, a process termed as biodegradation, which is defined as the breakdown of a substance to small inert end products (Aislabie and Lloyd-Jones 1995). According to Kumar

et al. 2018 most organophosphate pesticides are degraded by microorganisms in the environment as a source of their limiting nutrients, carbon (C) and/or phosphorus (P). The principal reactions involved in the degradation process are oxidation, hydrolysis, alkylation, and dealkylation (Singh and Walker 2006).

As cited Kumar (2018) different species of *Pseudomonas* isolated from agricultural soils and contaminated effluents throughout different regions have proven to be very efficient in the biodegradation of chlorpyrifos.

Wang and Hoffman (1991) studied the persistence and degradation of parathion in the Indian River estuary, which is found along the east coast of Florida. Test water was put into a 1 L borosilicate glass bottle and was spiked with 2.5 mL of 200 ppm pesticide solution. As a result of the study, the most significant pathway for parathion degradation was biological interaction (Wang and Hoffman 1991). After 30 days in sterilized water, 64%, 57%, and 49% of initial parathion in pH 6, 7, and 8.16 respectively was still present (Wang and Hoffman 1991). By contrast, persistence in nonsterile water after 30 days revealed that only 21%, 14%, and 6% were present in pH 6, 7, and 8.16, respectively. Both alkaline hydrolysis and sunlight photolysis of parathion occurred, but they were not as competitive as the biological degradation pathway, i.e. both are secondary pathways of degradation (Wang and Hoffman 1991).

2.2.4. Photodegradation

Photodegradation can occur either by a direct photolysis of OPPs, which have an absorption spectrum overlap with the solar spectrum or by indirect photodegradation, whereby dissolved humic and fulvic acids can act as a sensitizer or when particles can lead to semiconductor promoted photodegradation (Pehkonen and Zhang 2002).In static water, in a simulated aquatic environment, there is evidence of the contributions of light, suspended particulates, and bacteria to degradation. Thus, the degradation of fenitrothion in lake water under illumination occurred with a half-life of about 2 days, compared with 50 days in the dark (Greenhalgh et al. 1980).

Lacorte and Barceló (1994) were the first ones who studied the degradation of fenitrothion at environmental conditions in estuarine water. Fenitrothion was applied in the irrigation ditches of the Ebro Delta in Spain at a concentration of 200 and 20 µg/L to eliminate the American crab (Lacorte and Barceló 1994). The transformation products (TPs) formed 4 days after its application were 3-methyl-4-nitrophenol, fenitrooxon, and S-methyl isomer of fenitrothion (Lacorte and Barceló 1994). The concentration of fenitrothion decayed sharply in 2 h to less than 10% of the initial amount and reached a steady state within 10 h, while TPs were at a very low 0.01 µg/L level. Half-life of fenitrothion was of 13 h, with a disappearance rate of 0.053 and photolysis being the main pathway (Lacorte and Barceló 1994).

2.2.5. Sorption

Adsorption to soil solids, sediments, and plant cuticular material represents an important process influencing the chemodynamic behavior of insecticides, including their transport in surface runoff. Sorption phenomena affect the volatilization, hydrolysis, photolysis, and microbial transformation of organophosphorus insecticides (Pedersen and Suffet 2002). The degree of adsorption and the rate and extent of ultimate degradation are influenced by many

factors, which include solubility, volatility, charge, polarity, molecular structure, and the size of the pesticides (Kumar et al. 2018).

As cited Pedersen and Suffet (2002) sorption of organophosphorus insecticides to soil particles depends primarily on compound hydrophobicity and the fraction of natural organic matter (NOM) in the soil. This is because the particles of organic matter or clay provide soils and sediments with an increased number of adsorptive sites onto which pesticides molecules can bind. In the case of chlorpyrifos, its strong association with suspended sediments presents a potential migration route unique to aquatic environments and may explain reported detections of chlorpyrifos in water wells and marine sediments (Gebremariam et al. 2012).

Readman and co-workers studied the presence of OPPs in sediments of tropical marine environments -coastal areas of Central America and Mexico-. In sediments from the areas chosen for study, chlorpyrifos was found to be the most widely distributed compound. Traces of parathion and methyl-chlorpyrifos were, however, also encountered (Readman et al. 1992). However, the physical chemistry of chlorpyrifos differs to that of most other organophosphorus pesticides. Not only is generally less soluble in water (2.0 mg/L compared to, for example 145 mg/L for malathion and 24 mg/L for parathion) but also the log K_{ow} of 4.70 contrasts those for malathion (2.36) and parathion (3.83) and approaches that of pp'-DDT (6.39) (Chiou et al. 1977, Mackay et al. 2006). Whilst this would enhance partitioning of chlorpyrifos onto sediments compared to most other organophosphorous compounds, it is likely that the identification of primarily chlorpyrifos in the sediments is also a function of rapid degradation as well as solubilization of the others (Schimmel et al. 1983; Readman et al. 1992).

3. CURRENT OPP LEVELS IN MARINE ENVIRONMENTS

Scientific articles of organophosphorus pesticides in marine environments – in water and sediments- around the world in the last decade (2010-2020) were reviewed. Despite of being harmful pesticides for aquatic biota very few information was found about research conducted in marine environments. The compounds reviewed were: azinphos methyl, chlorfenvinphos, chlorpyrifos, chlorpyrifos methyl, diazinon, dichlorvos, dimethoate, disulfoton, ethion, fenitrothion, iprobenfos, malathion, parathion, parathion methyl, Terbufos and triazophos.

Regarding the detection limits (LOD) of the reviewed works, a disparity was seen in them. It is hard to get a wide interpretation of a ND (Not detected) or below the detection limit (<LOD) value; if this is a high LOD, since is possible that there are organophosphorus pesticides but they are below this limit of detection. Therefore, the finding of pesticides may be because the LOD was low and in other cases where no compounds are found it is because the LOD is high. The above explained complicates the item QA/QC because for the comparison of works the harmonization of LODs is necessary; setting a limit as a minimum and standardizing a common protocol. LODs of OPPs in seawater ranged from 0.001 pg. L^{-1} to 3.3 ng. L^{-1} and in sediments from 0.067 pg g^{-1} dw to 10 ng g^{-1} dw. In the particular case of chlorpyrifos in seawater, as an example, its LOD were 0.4 ng.L^{-1} for Moreno-González and coworkers (Moreno-González et al. 2013) using capillary gas chromatography coupled to mass spectrometry (SBSE- GC- MS); (0.20 – 1 ng.L^{-1}) for Montuori and coworkers using gas chromatography coupled to nitrogen-phosphorus detector (GC-NPD) (Montuori et al. 2015;

Montuori et al. 2016); (0.001 pg.L^{-1} - 2 pg.L^{-1}) for Zhong and coworkers using GC-MS in the German Bight, North sea (Zhong et al. 2012); 0.06 pg.L^{-1} for the same author from East China to high Artic Ocean (Zhong et al. 2011) and 1 ng.L^{-1} for Harino and coworkers using GC-MS (Harino et al. 2013).

On the other hand, in order to determine the ecotoxicological risk associated with de presence of OPPs in sediments and water, the mean levels found of OPPs in the marine environments in the reviewed articles were compared with the benchmarks proposed by the SQuiRTs (Screening Quick Reference Tables) of the NOAA (National Oceanic and Atmospheric Administration) from USA (Buchman 2008); the Australian and New Zealand Guidelines for Fresh and Marine Water Quality, 2000 and the Environmental Quality Guidelines of Canadian Council of Ministers of the Environment (CCME 2002).

3.1. OPPs in Marine Sediments

In the case of sediments, the studies reviewed were from research in Europe, Asia and North America. No research studies of OPPs in marine environments from Central and South America, Oceania, or Africa for the last decade were found; fourteen scientific articles were reviewed. By contrast, several works for freshwater sediments were found, like determination of OPPs in the Zio River, Togo, África by Mawussi et al. 2014; in the Guan River, China by He et al. 2014; in the Lake Zumpango, Mexico by Dzul-Caamal et al. 2014; and in the Llobregat River, Spain by Masiá et al. 2015.

Furthermore, the only guidelines applicable to levels in sediments are those corresponding to SQuiRTs of NOAA. However, in these tables the only OPPs with benchmarks for sediments are azinphos methyl, diazinon, and parathion. For diazinon and parathion the benchmark is Ecotox Thresholds (ETs) -defined as media specific contaminant concentrations above which there is sufficient concern regarding adverse ecological effects to warrant further site investigation- whereas for azinphos methyl are target benchmark -meaning that analyte concentrations in the sediment matrix are set such that they should not contaminate another matrix above risk- and intervention benchmark –meaning risk that the analyte may migrate to other matrix-.

The current environmental mean concentration of OPPs in sediments range from ND to 311.81 ng. g^{-1} dw (Table 7). On one hand chlorpyrifos was the most studied and quantified pesticide in sediments, and its general mean for the reviewed sites in the world is 9.60 ng. g^{-1} dw. On the other hand, the highest concentration of pesticide found in sediments was for iprobenfos with a mean concentration of 331.81 ng. g^{-1} dw in the East China Sea, reported by Lan et al. 2019.

Table 5. Benchmarks for OPPs given in SQuiRTs tables from NOAA. Parameters were selected from (Buchman 2008)

Organophosphorus Pesticides	Target (ppb or ng. g-1)	Intervention (ppb or ng. g-1)	EcoTox (ppb or ng. g-1)
Azinphos Methyl	0.05	2000 S*	-
Diazinon	-	-	1.9
Malathion	-	-	0.67

*S: Serious contamination.

Table 6. Levels of OPPs in marine sediments (ng. g^{-1} dw)

Location- sample year	Azinphos -m	Chlorfenvinphos	Chlorpyrifos	Chlorpyrifos-m	Diazinon	Dichlorvos	Dimethoate	Disulfoton	Ethion	Fenitrothion	Iprobenfos	Malathion	Parathion	Parathion-m	Terbufos	Triazophos	References
Óbidos Lagoon, Portugal 2013		37 ± 3,7 (34,2-41,2)	5,0 ± 4,5 (2,0-14,3)														Pinto et al. 2016
Mar Menor coastal lagoon, Spain 2010			38,3 ± 28,3(S) 20,0 ± 15,8(A)		6,0± 9,2(S) 7,7± 17,4(A)									17,4 ± 32,1(S) 8,8 ± 19,5 (A)			Moreno-González et al. 2017
Cádiz Bay, Spain 2011			2,1														Pintado-Herrera et al. 2017
Sarno River Estuary, Italy 2008	ND		1 ± 0,78 (0,19-2,6)		ND	ND	ND			ND		ND					Montuori et al. 2015
Tiber river Estuary, Italy 2014-2015			0,26 ± 0,09 (0,26-14,32)		4,08 ± 4,39 (0,18-0,37)		ND			ND		ND					Montuori et al. 2016
Berre Lagoon, France 2008-2009		(0,02-0,03)	(ND- 0,02)														Kanzari et al. 2012
Berre Lagoon, France 2010		(0,02-0,08)	ND														Kanzari et al. 2015
Vistula Lagoon, Baltic Sea, Poland 2009		10	10							10		20 ± 10 (10-50)		10 ± 10 (10-30)			Staniszewska et al. 2013
East China Sea, China 2011											311,81±215,88 (79,14 - 1169,18)					9,77 ±8,35 (ND-32,10)	Lan et al. 2019
Bohai and Yellow seas, China 2009-2010			Max 0,14														Zhong et al. 2014

Table 6. (Continued)

Location- sample year	Azinphos -m	Chlorfenvinphos	Chlorpyrifos	Chlorpyrifos-m	Diazinon	Dichlorvos	Dimethoate	Disulfoton	Ethion	Fenitrothion	Iprobenfos	Malathion	Parathion	Parathion-m	Terbufos	Triazophos	References
Seto Inland, Japan 2012-2013										24 (ND-44); (2012) 26 (9-51); (2013)							Kaonga et al. 2015
Maizuru Bay, Japan 2012			<1 (<1-2)		21 (2-27)					5 (<1-11)	<1						Harino et al. 2013
Arabian Gulf, Saudi Arabia								2,03 ± 1,30 (0,6-4,31)					0,003 ± 0,001 (0,001-0,005)	0,002 ± 0,001 (0,002-0,006)			Alaamri 2013
Coastal Lagoon of the gulf of México, México 2016			0,201 ± 0,002	ND	ND		78,82 ± 0,246		0,46 ± 0,097			ND	ND	ND	ND		Ponce-Velez et al. 2019

ND: Not detected. Mean ± SD, or Mean if it is only one value; Range is expressed with a hyphen between and in some case in the study was only informed the maximum. S: Summer, A: Autumn.

Table 7. Benchmarks for OPPs in marine surface water given in SQuiRTs tables from NOAA (Buchman 2008), CCME (2002) and Australian and New Zealand Guidelines (2000)

Organophosphorus Pesticides	Australian and New Zealand Guidelines	NOAA Guidelines		Canadian Guidelines for the protection of aquatic life (CCME)	
Benchmarks	Trigger values for marine water (µg. L-1)	Acute effect (µg. L-1)	Chronic effect (µg. L-1)	Concentration short term effect (µg. L-1)	Concentration long term effect (µg. L-1)
Azinphos-methyl			0.01		
Chlorfenvinphos			0.1		
Chlorpyrifos	0.0005	0.011	0.0056		0.002
Diazinon		0.82	0.82		
Dimethoate					
Fenitrothion					
Malathion			0.1		
Parathion					

When comparing levels of OPPs in the sediments with the international guidelines on ecotoxicological risk proposed by SQuiRTs tables of NOAA, it was observed that in the case of diazinon in the three places – Mar Menor coastal Lagoon, Spain in summer and autumn studied by Moreno-González et al. 2017; Tiber river Estuary in Italy, studied by Montuori et al. 2016 and Maizuru Bay, Japan studied by Harino et al. 2013- detected the mean concentration levels found of this compound indicate there is sufficient concern regarding adverse ecological effects to warrant further investigation of the sites and it can cause damage to marine biota.

Meanwhile, malathion was only detected in Vistula lagoon, Baltic Sea, Poland (Staniszewska et al. 2013) where the mean concentration exceeds EcoTox threshold of SQuiRTs tables meaning, like diazinon, there is sufficient concern regarding adverse ecological effects to warrant further investigation of the sites.

3.2. OPPs in Seawater

To analyze the current levels of OPPs in seawater, eleven articles were reviewed, all from Europe, Asia, and North America. No available studies were found for other continents. It is remarkable that the number of studies available on OPPs in seawater is much lesser than in freshwater (e.g., Shaw et al. 2010; Polidoro et al. 2017; Bonansea et al. 2013; Phillips et al. 2012; Mamta et al. 2018).

Guidelines applicable to levels in marine water are those corresponding to SQuiRTs of NOAA, trigger values of Australian and New Zealand guidelines and Canadian guidelines for the protection of aquatic life. For the NOAA tables the benchmarks are marine surface water acute effects (health effects usually develop rapidly after a short-term exposure to hazardous chemicals) and chronic effects (health effects become apparent and/or continue for some time after exposure to hazardous chemicals). Meanwhile, Canadian water quality guidelines for the protection of aquatic life help to protect species that live oceans by establishing acceptable levels for substances that affect water quality. As long as conditions are within the levels

established by the guidelines, one would not expect to see negative effects in the environment. Finally, in the Australian and New Zealand guidelines the benchmark is a trigger value for marine biota, which represents either bioavailable concentrations or unacceptable levels of contamination.

The current environmental mean concentration of OPPs in seawater range from ND to 19.37 µg. L^{-1} (Table 8). The most studied pesticides in seawater were chlorpyrifos and diazinon. In the same way that happened in the sediments, iprobenfos was the pesticide with the highest mean concentration detected; 19.37 µg. L^{-1} in the East China Sea, reported by Lan et al. 2019.

OPPs levels in seawater where compared with the international guidelines on ecotoxicological risk proposed by the SQuiRTs tables from NOAA. From this, it was observed that azinphos-methyl mean concentrations did not exceed the benchmark proposed for marine surface waters, meaning there would not be adverse effects on biota in none of the three places where it was detected –in coastline of Catalonia in Spain, Sarno river Estuary in Italy and in German Bight in the North Sea (Köck- Schulmeyer et al. 2019, Montuori et al. 2015, Mai et al. 2013)-. Meanwhile, the mean levels of chlorfenvinphos, in seawater of the Catalan, German Bight and Portuguese coasts (Köck- Schulmeyer et al. 2019, Mai et al. 2013, Sousa et al. 2020) were below the SQuiRTs benchmark, however the maximum concentration in the Portuguese coast was extremely close to the benchmark, meaning it would be adverse effects to marine biota. By contrast, diazinon was detected in six places –coastline of Catalonia, Mar Menor coastal lagoon, Tiber river Estuary, Sarno river Estuary, German Bight and Maizuru Bay- but in none of them the mean concentration were higher than the benchmark for surface marine water (Köck- Schulmeyer et al. 2019, Moreno- González et al. 2013, Montuori et al. 2015, Montuori et al. 2016, Mai et al. 2013, Harino et al. 2013); whereas the same happen with Malathion concentrations in the Sarno river Estuary, Tiber river Estuary, and in the German Bight (Montuori et al. 2015, Montuori et al. 2016, Mai et al. 2013). From this, no adverse effects would be expected to marine biota of the places diazinon and Malathion were detected.

On the other hand, chlorpyrifos were detected in seven of eleven studied places. The maximum concentrations found along the Portuguese coast and in the Mar Menor coastal lagoon (Moreno- González et al. 2013) exceeded the benchmarks proposed by NOAA –both acute and chronic adverse effects-, the trigger value for marine water of Australian and New Zealand Guidelines and also the marine concentration long term of Canadian Guidelines for the protection of aquatic life. This means that in both places are unacceptable levels of contamination by this pesticide, and there would be acute and chronic adverse effects on marine biota. While, in the Sarno River Estuary and in the Maizuru Bay (Montuori et al. 2016, Harino et al. 2013) the mean concentrations of chlorpyrifos were higher than the benchmarks of Australian-New Zealand -and Canadian guidelines, whereas the mean concentration in the Tiber River Estuary (Montuori et al. 2015) only exceeded the Australian-New Zealand benchmark, meaning there were unacceptable levels of contamination and it would expect to see negative effects on the marine biota. Instead, the mean concentrations in the German Bight, North Sea and from the East China Sea to the high Artic (Zhong et al. 2012, Zhong et al. 2011), did not exceed any of the benchmarks mentioned before, meaning it would not pose an ecotoxicological risk to marine biota.

Table 8. Levels of OPPs in seawater (µg. L^{-1})

Location- sample year	Azinphos-m	Chlorfenvinphos	Chlorpyrifos	Chlorpyrifos-m	Diazinon	Dichlorvos	Dimethoate	Fenitrothion	Iprobenfos	Malathion	Triazophos	References
Portuguese coast, Portugal 2017		(ND - 0.0989)	(ND- 4,095)									Sousa et al. 2020
Coastline of Catalonia, Spain 2015	0,00229	0,00038			0,00006							Köck-Schulmeyer et al. 2019
Mar Menor coastal lagoon, Spain 2010			Max 0,0458	(ND- 0,0044)	(ND- 0,0054)							Moreno-González et al. 2013
Sarno River Estuary, Italy 2008	0,0006 ± 0,0001 (0,0005-0,001)		0,0032 ± 0,0023 (0,001-0,012)		0,0006 ± 0,0004 (0,0003-0,002)	0,00010 ± 0,0002 (0,0007-0,001)	0,0002 ± 0,001 (0,001-0,006)	0,0002 ± 0,0008 (0,001-0,004)		0,0023 ± 0,0008 (0,001-0,005)		Montuori et al. 2015
Tiber river Estuary, Italy 2014-2015			0,0018 ± 0,0024 (0,0002-0,0146)		0,0040 ± 0,0045 (0,0002-0,0203)		0,0068 ± 0,0074 (0,0003-0,0376)	0,0013 ± 0,0012 (0,0006-0,0063)		0,0033 ± 0,0033 (0,0010-0,0167)		Montuori et al. 2016
German Bight, North Sea, Germany 2010			2,7 x10-5 ± 8x10-6 (1,4 x 10-5-1. 42,7 x 10-4)									Zhong et al. 2012
German Bight, North Sea, Germany 2009-2010	(ND- 4,3x 10-5)	(ND- 0,0003)			(ND- 0,0004)		(ND -0,0020)			(ND- 0,0001)		Mai et al. 2013
East China Sea to high Artic Ocean, 2010			7,7x10-6 ± 2,6x 10-5 (1x10-7- 0.000111)									Zhong et al. 2011
East China Sea, China 2011									19,37		8,53	Lan et al. 2019
Seto Inland, Japan 2012-2013								0,025 (ND-0,042); (2012) 0,028 (0,011-0,052); (2013)				Kaonga et al. 2015
Maizuru Bay, Japan 2012			0,002 (<0,001-0,005)		0,006 (0,004-0,009)			0,019 (0,018-0,02)	< 0,001			Harino et al. 2013

ND: Not detected. Mean ± SD, or Mean if it is only one value; Range is expressed with a hyphen between and in some case in the study was only informed the maximum.

4. ORGANOPHOSPHORUS PESTICIDES TOXICITY ON NON-TARGET ORGANISMS

OPPs are one of the two major classes of cholinesterase-inhibiting pesticides. The main mode of action of the OPPs is inhibition of acetylcholinesterase, the enzyme that ends the action of acetylcholine neurotransmitter, which is released by nerve stimulation, on postsynaptic cholinergic receptors in the nervous system. OPPs produce an irreversible inhibition of acetylcholinesterase, in contrast to the carbamates (the second major class) that produce a reversible inhibition (WHO 2001).

4.1 Mode of Action of OPPs

The primary biochemical effect associated with toxicity caused by organophosphorus pesticides is inhibition of acetylcholine esterase (AChE, WHO 1986). AChE is an enzyme that catalyzes the breakdown reaction of Acetylcholine –a neurotransmitter which is required for the transmission of nerve impulses in the brain, skeletal muscles, and other areas (Toole and Toole 1995). OPPs inhibit the normal activity of the AChE by covalent bonding to the enzyme, thereby changing its structure and function. The leaving group is replaced –by nucleophilic substitution- by the oxygen of serine in the AChE active site (Elersek and Filipic 2011). Then, the leaving group binds to the positive hydrogen of His 447 and breaks off the phosphate, leaving the enzyme phosphorylate (Singh and Walker 2006).The rate of AChE inhibition depends on the leaving group; higher tendency of leaving results in higher affinity of the inhibitor to the enzyme (Elersek and Filipic 2011). Loss of AChE activity may lead to a range of effects resulting from excessive nervous stimulation, convulsion, paralysis and finally death for insects and mammals and culminating in respiratory failure and death (WHO 1986; Ragnarsdottir 2000).

4.2. Bioaccumulation and Bioconcentration of OPPs in Marine Biota

Aquatic organisms may absorb dissolved chemicals directly from water through respiratory organs (e.g., gills), through the body surface, or may ingest chemicals through intake of contaminated sediments or prey (Katagi 2010). Uptake of the OPPs from the water may occur across the gills, through the skin, and by intestinal adsorption. This process is known as bioaccumulation and is affected by different factors (Serrano et al. 1995). It is primarily controlled by the physico-chemical properties of the chemicals involved (K_{OW}, water solubility, molecular weight, and size), the physiological disposition of each organism (lipid content is considered the most important determinant for bioaccumulation), and the surrounding environmental conditions (Katagi 2010). This process includes uptake of dissolved pesticides from water, pesticides sorbed to particulate components in water or sediments, and also trough dietary. Bioaccumulation is the net result of competing processes of chemical uptake into the organism at the respiratory surface and from the diet and chemical elimination from the organism including respiratory exchange, fecal egestion, metabolic biotransformation of the parent compound and growth dilution (Arnot and Gobas 2006).

Serrano et al. studied the bioconcentration of selected OPPs in *Mytilus galloprovincialis and Venus gallina* at different concentrations and found that dimethoate presented low concentrations in mollusc tissues, while chlorpyrifos was bioconcentrated by molluscs at high levels (1995). Moreover, phosmet was not detected at any of the pesticide exposure concentrations. This is in accordance with Log K_{ow} partition coefficients of the OPPs studied; chlorpyrifos can be considered a fat-soluble pesticide due to its high Log K_{ow} of 4.70. Instead, dimethoate has a Log K_{ow} of 0.50, and perhaps it causes low concentration of this pesticide in the mollusk tissues.

In more recent research, bioaccumulation of fenitrothion was demonstrated in different marine species in Seto Inland Sea in Japan by Kaonga et al, 2015. In that study, the animals with the higher bioaccumulation factors were mollusks, followed by fish, plankton, crustaceans, and sea cucumber, with mean of 0.313, 0.250, 0.189, 0.149 and 0.089 µg. g^{-1} dw respectively.

Besides, bivalves are one of the most widely used animals to study the bioaccumulation of pesticides due to their high lipid content, feeding by water filtration, and presence worldwide. This is the case of the study carried out by Harino et al. (2013) in Maizuru Bay, Japan and by Dodder et al. (2014) in California coast, USA. On the one hand, Harino and coworkers studied bioconcentration of diazinon, iprobenfos, fenitrohion and chlorpyrifos in *Mytilus galloprovincialis;* and the range concentrations of diazinon, iprobenfos and fenitrothion in mussel samples were 4.3–25, 22–60 and 9.2–13 mg.kg^{-1} ww respectively, however chlorpyrifos was only detected at the concentration of 1.0 mg.kg^{-1} ww in one sample station (Harino et al. 2013). On the other hand, Dodder and coworkers studied the occurrence of contaminants of emerging concern in mussels (*Mytilus spp*.) along the California coast and found a maximum concentration of chlorpyrifos of 0.036 mg.kg^{-1} dw (Dodder et al. 2014). Hence, it can be stated that certain marine species, with a particular focus on bivalves, are capable of bioaccumulating OPPs and this can lead to a risk for these species, the ecosystem in general and even for human beings who usually include this type of mollusks in their diet.

4.3. Common Effects and Toxicity on Marine Biota

These chemicals are neurotoxic that act by inhibiting acetylcholine esterase in the central and peripheral nervous system, resulting in choline and acetate formation (Elersek and Filipic 2011). The inhibition of this enzyme results in a continuous binding of acetylcholine to the receptor, leading to an overstimulation of this cholinergic receptors, which may result in alterations in behavior, potentially reducing the organism's ability to move, eat, and reproduce (Azevedo-Pereira et al. 2011). This suppression leads to convulsion, paralysis, and lastly death for insects and mammals (Ragnarsdottir 2000). The degree of brain AChE inhibition has often been used to distinguish OPPs related adverse effects from exposure. For example, an inhibition of brain AChE activity greater than 20% in birds is generally accepted as an index of exposure, whereas an inhibition exceeding 50% is a sufficient cause of adverse effects (Ludke et al. 1975). In mammals, inhibition of brain AChE greater than 50% has been suggested for sublethal exposure and inhibition exceeding 80% as a cause of death (Walker 1998).

Additionally, OPPs also bear the potentiality to cause genotoxic and carcinogenic effects (Kaushik and Kaushik 2007).

Reductions in cholinesterase (ChE) activity have been widely used to indicate exposure to OPPs in vertebrate and invertebrate species (Cooper and Bidwell 2006). Silva and coworkers (2019) studied the results of sublethal concentrations of chlorpyrifos in cholinesterases (ChE) in the marine snail *Gibbula umbilicalis* and determined that this inhibition cause an alteration on its behavior, capacity to turn over, lead to increased drift and disruption in their escape reaction from predators, feed, or even reaction (Silva et al. 2019).

Ferrero and coworkers (2001) studied acute toxicity of malathion in *Chasmagnathus granulata*, a crab present in Bahía Blanca Estuary, Argentina (Ferrero et al. 2001). In that study, only adult male crabs, with mean carapace width of 20.9 ± 0.73 mm and 33.0 ± 2.94 mm, were used and the estimated LC50 (concentration of a substance that can be expected to cause death, during exposure or within a defined period after it, of 50% of the population exposed to that substance during a given period) values to malathion were 13 $\mu g.L^{-1}$ and 19 $\mu g.L^{-1}$ respectively. Marine crustaceans are particularly vulnerable, as they tend to be highly susceptible to the toxic effects of OPPs (Johnston and Corbett 1985) and estuaries serve as critical feeding and nursery grounds for many aquatic organisms, including commercially and recreationally important fish and shellfish species. These productive, diverse ecosystems are particularly vulnerable to pollution because they serve as repositories for pollutants from upland sources (De Lorenzo et al. 2001).

On the other hand, acute toxicity of several OPPs in two marine species (*Artemia sp* and *Brachionus plicatis)* was studied by Guzella et al, 1997. Comparisons of the EC50 (concentration of a compound in which 50% of its maximum effect is seen) values show that chlorpyrifos was the most toxic OPP to both species. In order of toxicity decreasing in both the marine organisms tested azinphos-ethyl was the second compound followed by fonofos while diazinon, parathion-methyl, azinphos-methyl showed a lower toxic response (Guzzella et al. 1997).

4.4. Effects on Human Health

These compounds can also cause a high toxicity to humans. In occupational and non-occupational poisonings, exposure to these pesticides may occur via oral, inhalation or dermal route. But the majority of studies available in the literature involve the oral route (Gupta et al. 2011). Therefore, not only the application of pesticides in agricultural activities but also the consumption of marine organisms inhabiting pesticides polluted areas are a serious threat to people (Serrano et al. 1995). In general, following oral exposure, these pesticides are rapidly absorbed, widely distributed, metabolized in the liver, and eliminated in the urine (Gupta et al. 2011).

OPPs phosphorylate acetylcholinesterase in an irreversible reaction that inhibits the activity of cholinesterase to hydrolyze the neurotransmitter at the nerve synapse. The accumulation of acetylcholine results in continuous nerve firing and eventual failure of nerve impulse propagation (Smith 1987). Bronchial constriction and increased bronchial secretions are characteristic signs of OPPs poisoning (Serrano et al. 1995). Consequently, respiratory paralysis is generally the immediate cause of death (Murphy 1975). Brain cholinesterase activity is used in the diagnosis of OPPs poisoning in humans, with a reduction of 20 % of normal activity indicating exposure while a cholinesterase inhibition of 50% or more is considered the diagnostic threshold for determining the cause of death (Smith 1987).

Once entering the body, OPPs can be enzymatically converted to their oxon form, which then inhibits the enzyme acetylcholinesterase (AChE) responsible for the breakdown of the neurotransmitter acetylcholine (Prapamontol et al. 2014). The oxon also can be enzymatically or spontaneously hydrolyzed to form a dialkyl phosphate (DAP) metabolite and an organic molecule specific to the pesticide. If the pesticide is not converted to its oxon form, it can hydrolyze similarly to form its specific and dialkylthionate metabolites (Barr et al. 2004). Thus, quantification of these metabolites in human urine offers information on cumulative exposure of this class of pesticides and the data are used in several epidemiological studies for the health outcome assessment (Prapamontol et al. 2014).

Absorption following inhalation is also rapid. Dermal absorption varies depending on the site of application and dose. Compared to many OPPs, dermal absorption of chlorpyrifos is low (Gupta et al. 2011). Chlorpyrifos is moderately toxic to human beings; however, there are reports of genotoxic and mutagenic effects of chlorpyrifos in human beings and rat (Mulla et al. 2019).

Moreover, OPPs occupational exposure was associated with an elevated risk of breast cancer (RR = 1.20, 95% CI 1.01–1.43); one of the most commonly used OPPs, malathion was associated with the elevated risk of thyroid cancer and whereas diazinon was positively associated with increased risk of ovarian cancer (RR=1.87, 95% CI 1.02–3.43) (Lerro et al. 2015). According to the IARC (International Agency for research on Cancer, WHO 2020); malathion and diazinon are assigned to Group 2A (*probably carcinogenic to humans*), and tetrachlorvinphos and parathion are assigned to Group 2B (*possibly carcinogenic to humans*) based on epidemiological and experimental evidence of carcinogenicity and mechanistic and other relevant data.

CONCLUSION

Currently, organophosphorus pesticides are used worldwide; however, these pesticides are being replaced by new formulations as carbamates, neonicotinoids and pyrethroids, most recently developed and less harmful for the environment.

Despite of their extensive use trough the last decades, there is a lack of data on the behavior of organophosphorus pesticides in the marine environment; hence it is probable that potential impacts on marine ecosystems have been substantially underestimated. On the contrary, in freshwater environments, toxicity, behavior and current levels in different matrices are well known and studied.

In what refereed to current levels in different countries in the reviewed period, most of studies have been performed in Europe and Asia; there are a few studies in North America; and no research in Central and South America, Africa or Oceania marine environments was found. It is notable that are just a few studies focused on developing countries, in which legal OPPs are often applied in large amounts. The most extensively studied matrix is sediments.

Although everything seems to indicate that chlorpyrifos is the most toxic pesticide (a bias can be posed since it is the most studied congener in the marine environments), iprobenphos and triazophos exhibited the highest mean concentrations in the studied matrices.

Another output to consider is that despite their rapid environmental degradation, there are no recent OPP degradation research studies tracking metabolites in marine environments.

This is not a minor aspect since some metabolites are more toxic than the parent compounds and may represent a higher ecotoxicological risk for the marine biota.

On the other hand, regarding the OPPs detection limits (LOD), a high disparity was seen which difficult comparisons and data interpretation. There is a general need for OPPs method´s harmonization.

The scarcity of studies on OPPs bioaccumulation and ecotoxicity in marine environments during the last ten years does not allow us to affirm whether the present OPPs levels represent a risk for humans; however, they do represent a risk for the marine biota, with ability to cause chronic and acute adverse effects on it.

Finally, considering that the oceans and seas are the ultimate sink for all kinds of pollutants, including organophosphorus pesticides, apart from the already internationally regulated, there is a need for global regulation for OPPs in sediments and waters of the oceans, estuaries, and seas.

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Chapter 135

REMOVAL OF EMERGING ORGANIC POLLUTANTS FROM SEAWATER USING PHOTOACTIVATED PERSULFATE

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ABSTRACT

Sulfate radical-based advanced oxidation processes (AOPs) have become emergent technologies for the destruction of emerging organic pollutants (EOPs) in water and soil. UV activation of persulfate generates both sulfate and hydroxyl radicals ($SO_4^{\bullet-}$, $^{\bullet}OH$) which are the most oxidizing species researched for water treatment. These radicals work together to make the destruction of EOPs very easy. The efficiency and, accordingly, the success of AOPs have generally been evaluated on the basis of degradation kinetics. In practice, chloride in saline wastewater is often found to inhibit degradation processes. Therefore, it is highly desirable to develop more effective processes which are not affected by chloride.

We herein show the potential application of UV/persulfate process for the degradation of EOPs in seawater, taking chlorazol black (CB) as a substrate model. CB is very persistent azo dye that is not only refractory to biodegradation but also toxic and mutagenic. This compound constitutes a factor of risk for the marine life. Firstly, the fast conversion of $SO_4^{\bullet-}$ and $^{\bullet}OH$ into dichlorine radical anion ($Cl_2^{\bullet-}$) has been proven experimentally through radical probes technique. $Cl_2^{\bullet-}$ was found to be the main oxidant implicated in the degradation of CB in seawater. Secondly, the impact of influencing

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factors such as initial persulfate and pollutant concentrations and solution pH was clarified. Besides, the influence of several surfactants on the process efficiency was elucidated. The process performance was found to be more efficient in acidic and neutral pH under relatively high concentration of persulfate (< 5 mM) and low concentration of CB. More interestingly, there was no significant difference between the degradation rate of CB in seawater and deionized water, meaning that $Cl_2^{\bullet-}$, although less reactive than $SO_4^{\bullet-}$ and $^{\bullet}OH$, may has other promising properties that make its performance comparable to those of $SO_4^{\bullet-}$ and $^{\bullet}OH$. In fact, $Cl_2^{\bullet-}$ is more selective and has much longer lifetime than $SO_4^{\bullet-}$ and $^{\bullet}OH$.

Keywords: seawater, emerging organic pollutants (EOPs), advanced oxidation processes (AOPs), UV/persulfate process, degradation, $Cl_2^{\bullet-}$ radical

INTRODUCTION

The sea is usually the receptor of discharges and dumping wastes containing high concentrations of persistent organic pollutants (POPs) produced from the diverse anthropogenic and human industrial activities [1, 2]. Polychlorinated biphenyls, polycyclic aromatic hydrocarbons, endocrine disrupting chemicals and synthetic dyes are among the various kinds of POPs detected in different marine environments [3–5]. Azo dyes are among the most notorious and widespread environmental pollutants associated with textile, food, cosmetic and leather industries [6]. They are characterized by an -N=N- azo bond in their molecular structure. Due to their high water solubility, their facile transportability and their high persistence [6], these compounds are of potential risks on human health and aquatic ecosystems. Dyes interfere with penetration of sunlight into seawater, retards photosynthesis, inhibits the growth of aquatic biota and interferes with gas solubility in water bodies [7]. Moreover, these contaminants are known or suspected as mutagenic and carcinogenic [8].

In situ chemical oxidation (ISCO) technologies utilizing persulfate have been increasingly applied for the remediation of contaminated soil and groundwater [9–11]. There are several techniques for persulfate activation into reactive $SO_4^{\bullet-}$ radicals including heat, Fe^{2+}, UV, basic conditions (pH > 11), metal oxides, zerovalent metals, sonolysis, radiolysis, microwaves and organic compounds (e.g., quinones and phenols), and all these techniques were extensively employed in both laboratory research and field applications [12, 13, 22–29, 14–21]. However, each activation method has its pros and cons. For example, heat is an effective method for persulfate activation, but this technique suffers from potential high costs as the treated soil and groundwater must be heated continuously [30]. Fe^{2+} activation suffers from pH limitation as the process could be operated at low pH to prevent iron precipitation as hydroxides [30]. Basic activation operates with high pH level, which required higher quantity of bases and subsequent neutralization step.

UV light with $\lambda < 400$ nm is one of the most effective activators of persulfate for the remediation of soil and groundwater heavily contaminated by organic pollutants [18]. This technique is relatively low-cost and environmentally friendly, specifically when solar irradiation is employed [31, 32]. UV activation of persulfate has been found to provide an alternative for the destruction of organic contaminants including phenols [33, 34], perfluorocarboxylic acids [35, 36], dyes [37, 38] and endocrine disruptor chemicals [39]. A summary of the persulfate activation chemistry is given below [30, 40, 41]:

$S_2O_8^{2-} \xrightarrow{h\nu} 2\ SO_4^{\bullet-}$ $k_1 = 1\times10^{-7}\ s^{-1}$ (1)

$SO_4^{\bullet-} + H_2O \rightarrow {}^{\bullet}OH + H^+ + SO_4^{2-}$ $k_2 = 660\ s^{-1}$ (2)

$SO_4^{\bullet-} + HO^- \rightarrow {}^{\bullet}OH + SO_4^{2-}$ $k_3 = 7\times10^7\ M^{-1}s^{-1}$ (3)

$SO_4^{\bullet-} + S_2O_8^{2-} \rightarrow S_2O_8^{\bullet-} + SO_4^{2-}$ $k_4 = 6.5\times10^5\ M^{-1}s^{-1}$ (4)

${}^{\bullet}OH + S_2O_8^{2-} \rightarrow S_2O_8^{\bullet-} + OH^-$ $k_5 = 1.2\times10^7\ M^{-1}s^{-1}$ (5)

$SO_4^{\bullet-} + SO_4^{\bullet-} \rightarrow S_2O_8^{2-}$ $k_6 = 7\times10^8\ M^{-1}s^{-1}$ (6)

${}^{\bullet}OH + {}^{\bullet}OH \rightarrow H_2O_2$ $k_7 = 5.5\times10^9\ M^{-1}s^{-1}$ (7)

The degradation process proceeds with production of the sulfate and hydroxyl radicals, which is demonstrated experimentally by ESR spin trapping technique [42]. Sulfate ion will be generated as the end-product, which is practically inert and not considered to be a pollutant.

Although persulfate photochemistry in pure water has been widely investigated and has been relatively well elucidated, there are, until now, scarce data on the applicability of this technique in seawater contaminated with POPs, where the high salinity (~35 g/L) may inhibit or suppress the reaction of free radicals with target contaminants [43]. Also, the seawater may completely change the radicals distribution due to its high content of chloride.

The present investigation deals with the application of UV/persulfate process for the degradation of organic pollutants in seawater, taking chlorazol black (CB) dye, as substrate model of POPs. The specific physicochemical properties of chlorazol black are available in refs. [5, 44]. Chlorazol black is a highly water soluble azo dye which is widely used for various applications like dyeing of fabric, leather, cotton, cellulose materials and plastic [45]. It is an eye irritant chemical and is moderately toxic by inhalation and ingestion [46]. The carcinogenic and mutagenic effects of the dye towards humans and animals have been experimentally confirmed [46]. Additionally, laboratory experiments showed that CB is very persistent to direct oxidation with H_2O_2, persulfate and periodate oxidants [5]. Therefore, any presence of this dye in seawater would have detrimental effects on aquatic life. Before proceeding, a succinct view on previous works conducted on the effect of chloride ions on the performance of the persulfate photoactivated process is given in the following section.

EFFICIENCY OF $SO_4^{\bullet-}$ -BASED AOPS IN SALINE WATERS

In sulfate radical-based AOPs, both ${}^{\bullet}OH$ and $SO_4^{\bullet-}$ coexist. These processes have been successfully applied for the degradation of recalcitrant contaminants in industrial wastewaters [30, 47]. However, as known, industrial effluents may contain large amount of mineral salts, specifically chloride ions (Cl^-), which are known as radical scavengers [48]. Chloride ions upon reactions with $SO_4^{\bullet-}$ and ${}^{\bullet}OH$ may induce a radical-based chain reactions (Eqs. 8-20)

allowing to form reactive chlorine species RCS, mainly $Cl^{\bullet}$, $HClO^{\bullet-}$ and $Cl_2^{\bullet-}$, which may have a significant influence on the overall treatment efficiency of AOPs [40, 49–53].

$$Cl^- + SO_4^{\bullet-} \rightleftharpoons SO_4^{2-} + Cl^{\bullet} \quad k_8 = 4.7\times10^8\ M^{-1}s^{-1},\ k_{-8} = 2.5\times10^8\ M^{-1}s^{-1} \tag{8}$$

$$Cl^- + {}^{\bullet}OH \rightleftharpoons HClO^{\bullet-} \quad k_9 = 4.7\times10^8\ M^{-1}s^{-1},\ k_{-9} = 2.5\times10^8\ M^{-1}s^{-1} \tag{9}$$

$$Cl^{\bullet} + Cl^- \rightleftharpoons Cl_2^{\bullet-} \quad k_{10} = 8.5\times10^9\ M^{-1}s^{-1},\ k_{-10} = 6\times10^4\ M^{-1}s^{-1} \tag{10}$$

$$Cl^{\bullet} + H_2O \rightarrow HClO^{\bullet-} + H^+ \quad k_{11} = 2.5\times10^5\ s^{-1} \tag{11}$$

$$Cl^{\bullet} + OH^- \rightarrow HClO^{\bullet-} \quad k_{12} = 1.8\times10^{10}\ s^{-1} \tag{12}$$

$$Cl_2^{\bullet-} + H_2O \rightarrow Cl^- + HClO^{\bullet-} + H^+ \quad k_{13} = 1\times10^5\ s^{-1} \tag{13}$$

$$HClO^{\bullet-} \rightleftharpoons {}^{\bullet}OH + Cl^- \quad k_{14} = 6.1\times10^9\ M^{-1}s^{-1},\ k_{-14} = 4.3\times10^9\ M^{-1}s^{-1} \tag{14}$$

$$HClO^{\bullet-} + H^+ \rightarrow Cl^{\bullet} + H_2O^{\bullet-} \quad k_{15} = 2.1\times10^{10}\ M^{-1}s^{-1} \tag{15}$$

$$HClO^{\bullet-} + Cl^- \rightarrow Cl_2^{\bullet-} + OH^- \quad k_{16} = 1\times10^5\ M^{-1}s^{-1} \tag{16}$$

$$Cl_2^{\bullet-} + Cl_2^{\bullet-} \rightarrow Cl_2 + 2Cl^- \quad k_{17} = 6.3\times10^8 M^{-1}s^{-1} \tag{17}$$

$$Cl_2^{\bullet-} + {}^{\bullet}OH \rightarrow HClO + Cl^- \quad k_{18} = 1\times10^9\ M^{-1}s^{-1} \tag{18}$$

$$Cl_2^{\bullet-} + Cl^{\bullet} \rightarrow Cl_2 + Cl^- \quad k_{19} = 2.1\times10^9\ M^{-1}s^{-1} \tag{19}$$

$$Cl^{\bullet} + Cl^{\bullet} \rightarrow Cl_2 \quad k_{20} = 8.8\times10^7\ M^{-1}s^{-1} \tag{20}$$

Previous investigations have focused on the influence of Cl^- on the performance of UV/persulfate process. In most cases, the applicability of the process was evaluated by assessing the degradation efficiency in saline wastewater. Since the resulted RCS from the reaction of chloride ions with $^{\bullet}OH$ or $SO_4^{\bullet-}$ are less reactive than the primary radicals, the involvement of Cl^- would mostly inhibit the radical reaction chains, thus significantly reducing the overall efficiency of the selected treatment process [30,54]. Chen and Chu [55] have studied the degradation of atrazine by the activation of peroxymonosulfate, and found that Cl^- exhibited inhibitory effects in the process; they deduced that the reason was likely due to the scavenging of $SO_4^{\bullet-}$ and the formation of weaker radical species such as $Cl^{\bullet}$ and $Cl_2^{\bullet-}$. Fang et al. [56] have reported insignificant effect of chloride addition for up to 300 mM on the degradation of four monochlorophenols by persulfate photoactivated, but several chlorinated aromatic intermediates were identified confirming the involvement of chlorine reactive radicals in the degradation pathway. Fang et al. [40] showed that the presence of chloride ions at 20 mM greatly inhibited the degradation of polychlorinated biphenyls; transformation by-products were monitored, confirming that Cl^- can react with $SO_4^{\bullet-}$ to produce chlorine radicals, which react with biphenyl to generate chlorinated compounds.

Additionally, a significant reduction in the degradation of 1,1,1-trichloroethane by heated activated persulfate was reported by chloride addition at 100 mM [54]. Merouani et al. [15] found that the effect of Cl^- on the degradation of safranin O in heated persulfate system was not significant when Cl^- concentration was below 50 mM.

In contrast, Anipsitaki et al. [23] observed faster degradation rate of phenol and 2,4-dichlorophenol due to the low amount of Cl^- in the cobalt/persulfate system. Yang et al. [37] reported that the destruction of an azo dye can be enhanced by Cl^- without catalyst, possibly resulting from the activation of persulfate with unsymmetrical structure.

Besides, a dual role of chloride ions has been recently reported. Wang et al. [57] found that the degradation of azo dyes by sulfate radical was significantly inhibited in the presence of Cl^- (0-10 mM), while the degradation of the dye was significantly enhanced for higher chloride ions concentration (>100 mM). The same trend has been reported by Yuan et al. [58,59] for the degradation of acid orange 7 by UV/persulfate and Co/persulfate processes.

Therefore, the different reported observations on the effect of Cl^- revealed that the importance of converting $SO_4^{\bullet-}$ and $^\bullet OH$ into $Cl_2^{\bullet-}$ depend on the concentration and reactivity of $Cl_2^{\bullet-}$ toward the target contaminant. If $Cl_2^{\bullet-}$ is not reactive toward the pollutant, the effect of Cl^- addition is detrimental toward the degradation process. If $Cl_2^{\bullet-}$ is reactive with the target substrate, the additions of low Cl^- concentration could reduce the degradation process via reducing the concentration of $SO_4^{\bullet-}$ and $^\bullet OH$ which are more reactive than $Cl_2^{\bullet-}$. However, using higher concentration of Cl^- could generate higher concentration of $Cl_2^{\bullet-}$ which may compensate of the loss in $SO_4^{\bullet-}$ and $^\bullet OH$ level in the solution.

RADICALS DISTRIBUTION IN SALINE WATERS: KINETICS MODELING DATA

To better understand the underlying mechanisms of radical generation and distribution in $SO_4^{\bullet-}$-based AOPs in the presence of chloride, many authors have developed many kinetic models as a tool for predicting the effect of chloride ion on the types of radical species and their distributions.

The results of Fang et al. [40], reported in Figure 1, showed that chloride ion could influence the selectivity of radical species and their distribution, and increase the concentration of the sum of radical species. In the absence of Cl^-, $SO_4^{\bullet-}$ was the main radical species for pH < 10; above which $^\bullet OH$ was predominated (Figure 1a). In the presence of Cl^- at 100 mM, $Cl_2^{\bullet-}$ was the predominate radical species for pH < 9, while both $^\bullet OH$ and $ClOH^{\bullet-}$ coexist at alkaline conditions, i.e., pH > 10 (Figure 1b). Besides, distribution percentage of $Cl_2^{\bullet-}$ increased rapidly as Cl^- concentration increased, while that of $SO_4^{\bullet-}$ decreased substantially (Figure 1c). When Cl^- concentration reached 100 mM, most of $SO_4^{\bullet-}$ was converted to $Cl_2^{\bullet-}$. The results suggested the rapid conversion of $SO_4^{\bullet-}$ to $Cl_2^{\bullet-}$. Even in the presence of 10 mM Cl^-, approximately 40% of $SO_4^{\bullet-}$ was converted to $Cl_2^{\bullet-}$.

The total radical concentrations (TRC) were calculated in the presence and absence of chloride ion. As shown in Figure 1d, the TRC in the presence of 100 mM Cl^- was significantly higher than that without Cl^- (Figure 1d). As pH < 6, the TRC in the presence of Cl^- was fifty times larger than that in the case of absence of Cl^-, and the TRC values with and without Cl^- ion were 8.8×10^{-12} and 1.6×10^{-13} M, respectively. This is ascribed to reactions

(8) and (10) which favored the conversion of $SO_4^{\bullet-}$ into $Cl_2^{\bullet-}$ in the presence of Cl^-, and increased the TRC.

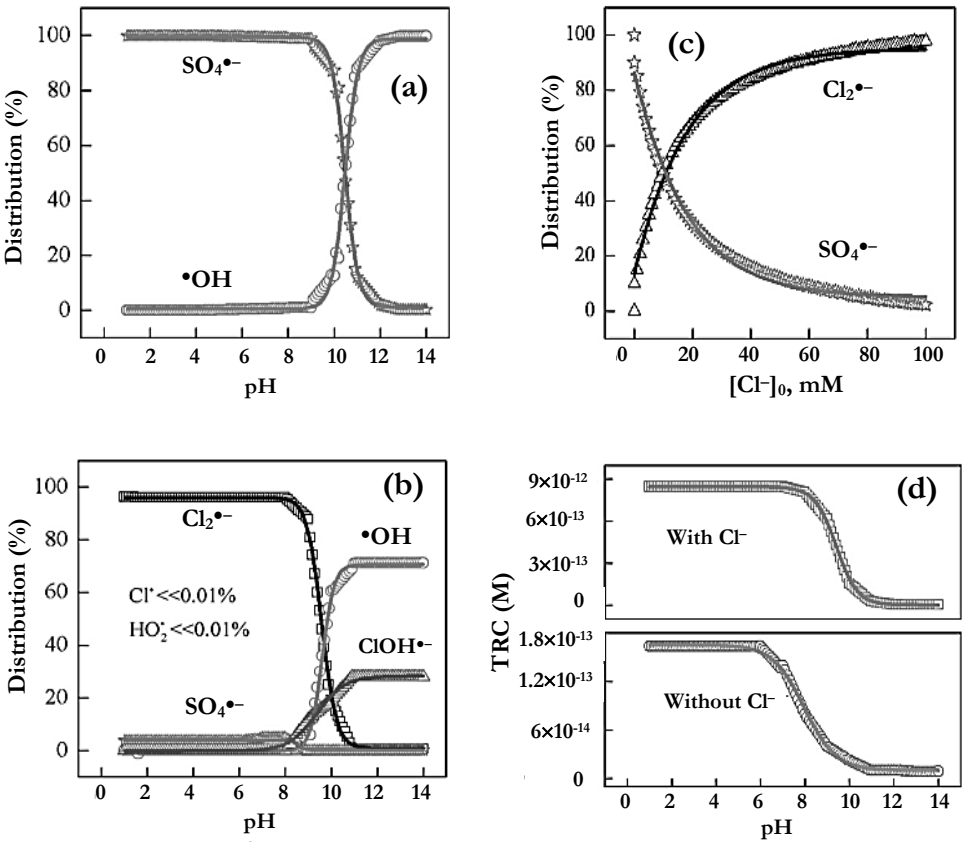

Figure 1. Effects of pH on the distribution of radical species in the absence (a)/presence (b) of Cl^- ($[S_2O_8^{2-}]_0$ = 16.8 M; $[Cl^-]_0$ = 100 mM; 25°C), effects of Cl^- concentration on the distribution of radical species (c) and calculated total radical concentration (TRC) in the absence/presence of $[Cl^-]_0$ = 100 mM) (d). Reprinted with permission from [40].

In contrast, most of $SO_4^{\bullet-}$ in the absence of Cl^- was scavenged by reactions (4) and (6), resulting in reducing the TRC. When pH increased from 6 to 8, the TRC decreased rapidly in the absence of Cl^-. As discussed in Figure 1a, the concentration of $SO_4^{\bullet-}$ decreased as pH increased to 8.0 due to reactions (4) and (6), while the TRC slightly changed in the presence of Cl^-. This suggests that reactions (6) and (8) are not only related to their reaction constants, but also to their concentration, although the reaction rate constant of $SO_4^{\bullet-}$ with $SO_4^{\bullet-}$ (Eq. 6) is slightly higher than that with Cl^- (Eq.8). The concentration of Cl^- was 100 mM, which was far greater than $SO_4^{\bullet-}$ concentration. Thus, the slight change in $SO_4^{\bullet-}$ had limited effects on reaction (8), and the TRC slightly changed as pH increased. At pH > 8, the TRC decreased rapidly in the presence of Cl^- due to reactions (12) and (17).

Similar radical distribution has been reported by Yuan et al. [58] whose used a kinetics model consisting in 76 reactions for simulating the degradation of acid orange 7 by UV/persulfate system in the presence of chloride ions for up to 500 mM.

The finding in Figure 1b indicated that the TRC was greatly increased in the presence of Cl^-, which can explain some experimental results reported in previous studies. For example, Wang et al. [57] found that the degradation of azo dyes was greatly enhanced in the presence of high concentration of Cl^- (> 50 mM), and attributed this to a dual effect of chlorine and sulfate radicals for azo dyes degradation. Theoretically, if $SO_4^{\bullet-}$ is completely converted to $Cl_2^{\bullet-}$, the degradation of organic pollutants should be inhibited due to the lower reactivity of $Cl_2^{\bullet-}$. However, in Wang's study, they observed that the degradation of azo dyes was enhanced in the presence of high Cl^- concentration (> 50 mM). This is due to the fact that the total radical concentrations increased in the presence of Cl^-. Although its reactivity is less than that of sulfate radical, its concentration was greatly increased under these conditions. Thus, the degradation rate of organic pollutants increases in the presence of high concentration of Cl^-. Similarly, the results presented in Figure 1 could also explain Yuan's experimental results [59].

EXPERIMENTAL

Seawater of the Mediterranean Sea (collected from the north-East of Algeria in the autumn of 2019) has been used as water matrice. Its salinity was of ~ 35.7 g L^{-1}, distributed as $Na^+ \approx 11$ g L^{-1}, $Mg^{2+} = 1.3$ g L^{-1}, $Ca^{2+} \approx 0.4$ g L^{-1}, $Cl^- \approx 20$ g L^{-1} and $SO_4^{2-} = 3$ g L^{-1}. Chlorazol black degradation experiments were conducted in the setup described early [60]. It consists in a cylindrical water-jacketed glass cell of 500 mL. The operating solution volume was 250 mL. A low-pressure mercury lamp (Oriel 6035, 15 mW cm^{-2}) emitting maximum irradiation at 253.7 nm was mounted in a quartz tube and submerged vertically in the center of the solution. A thermocryostat (RC6 Lauda) has been used to maintain the temperature of the treated solution at 25±1°C through flowing water at 23°C in the jacket surrounding the cell.

During irradiation, the temperature of the magnetically stirred solution, was displayed by a thermocouple submerged in the solution. A pH-meter Jenway 3505 was used to measure and adjust the solution pH. The dye concentration was determined spectrophotometrically at $\lambda_{max} = 578$ nm as described in refs. [5, 60]. Runs were repeated at least 3 times and averages values were plotted (error bars ensuring the maximum deviation of the means were inserted in plots).

EFFICIENCY OF UV/PERSULFATE PROCESS IN SEAWATER: CB DEGRADATION

CB degradation runs in seawater upon UV and UV/persulfate treatments have been conducted at pH 3 and ambient temperature of about 25 °C, for various initial persulfate dosages in the range of 0.1 to 5 mM. The degradation results, reported in Figure 2a, obviously demonstrated the super advantage of the combined process against the treatment with the UV sole. Complete removal of CB was achieved after 20 min with UV irradiation in the presence of 5 mM of persulfate; the time at which only 22% of CB was removed with the sole UV irradiation. The degradation rate increased with increasing initial persulfate loading in the

solution. At 20 min, the removal efficiency increased from 29% for 0.1 mM of persulfate to 55%, 76%, 90% and 100% for 0.5, 1, 2 and 5 mM of persulfate, respectively. Note that there was no CB removal with persulfate in the absence of UV over the same concentration range of persulfate, i.e., 0.1–5 mM. Therefore, the rise in the persulfate dosage could increase the RCS concentration, which makes the degradation faster.

Many studies conducted in deionized water have reported the existence of certain optimum persulfate dosage above which the degradation rate was detrimentally affected by persulfate due to the radicals-quench by the excess of persulfate (Eqs. 4 and 5) or by the radicals quench by themselves (Eqs. 6 and 7) at higher RCS concentration in the solution [15–17]. This scenario was not obtained for the case of seawater, which is may be attributed to the fact that (i) the maximum dosage of persulfate in Figure 2a did not yet achieving a higher level for becoming a radicals-quencher in the solution or (ii) the reaction of persulfate with RCS is not efficient as those of persulfate with $^{\bullet}OH$ and $SO_4^{\bullet-}$ which diminishes the impact of higher persulfate dosage on radicals consumption. On the other hand, the radical-radical recombination reactions are too fast for $^{\bullet}OH$ and $SO_4^{\bullet-}$ (Eqs. 6 and 7) than those of RCS (Eqs. 17 and 20). Therefore, the detrimental concentration of persulfate in deionized water could be much lesser than that required in seawater.

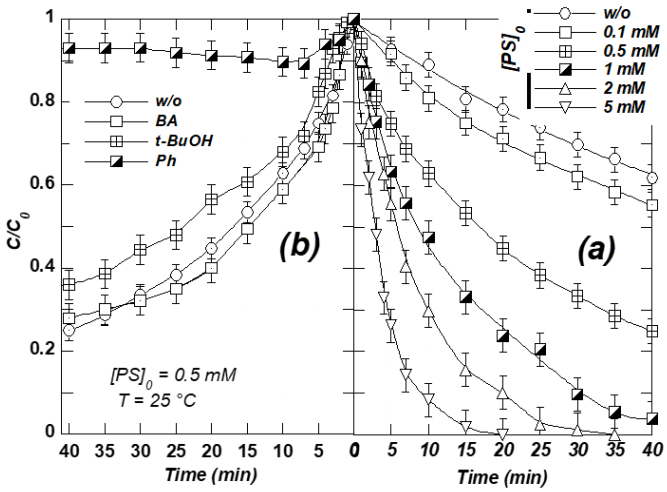

Figure 2. (a) CB degradation kinetics for various PS dosages and (b) radical scavenger tests for 0.5 mM persulfate using Benzoic acid (BA), tert-butanol (t-BuOH) and phenol (Ph) (conditions – C_0 = 25.5 µM, pH 3, $[t\text{-BuOH}]_0$ = 100 mM, $[BA]_0 = [Ph]_0$ = 1 mM, ~ 25±1°C).

EVIDENCE OF RCS IMPLICATION

As illustrated early, the reactive species $^{\bullet}OH$, $SO_4^{\bullet-}$ and RCS ($Cl^{\bullet}$, $HOCl^{\bullet-}$ and $Cl_2^{\bullet-}$) may all be formed in seawater irradiated by UV light in the presence of persulfate. The contribution of these radicals in the overall CB degradation rate was evaluated using some specific radical scavengers, namely, benzoic acid (BA), *tert*-butanol (*t*-BuOH) and phenol (Ph). Table 1 shows the reactivity of these three scavengers with $SO_4^{\bullet-}$, $^{\bullet}OH$, $Cl^{\bullet}$ and $Cl_2^{\bullet-}$. Ph can scavenge all radicals; it has been used for confirming the radical pathway for CB

degradation in seawater. *t*-BuOH can scavenge $^{\bullet}OH$ and $Cl^{\bullet}$ but its scavenging effect on $SO_4^{\bullet-}$ and $Cl_2^{\bullet-}$ is negligible. *t*-BuOH was used for appreciating the global role of $SO_4^{\bullet-}$ + $Cl_2^{\bullet-}$. BA can efficiently scavenge $^{\bullet}OH$, $SO_4^{\bullet-}$ and $Cl^{\bullet}$ but its scavenging effect on $Cl_2^{\bullet-}$ is negligible. It has been then used to determine the contribution of the sole $Cl_2^{\bullet-}$.

Table 1. Second-order rate constant between scavengers and radicals in the UV/persulfate system [30,61,62]

Radicals	Benzoic acid (BA)	*t*-butanol (t-BuOH)	Phenol (Ph)
$^{\bullet}OH$	1.8×10^9 M^{-1} s^{-1}	6×10^8 M^{-1} s^{-1}	6.6×10^9 M^{-1} s^{-1}
$SO_4^{\bullet-}$	1.2×10^9 M^{-1} s^{-1}	8.9×10^5 M^{-1} s^{-1}	8.8×10^9 M^{-1} s^{-1}
$Cl^{\bullet}$	1.8×10^{10} M^{-1} s^{-1}	3×10^8 M^{-1} s^{-1}	$(0.8\text{-}2.5)\times10^9$ M^{-1} s^{-1}
$Cl_2^{\bullet-}$	$(0.2\text{-}1.8)\times10^6$ M^{-1} s^{-1}	0-700 M^{-1} s^{-1}	$(2.5\text{-}5)\times10^8$ M^{-1} s^{-1}

Figure 2b shows the influence of the three radical scavengers on the removal kinetics of 25.5 µM of CB in seawater. The initial concentrations of scavengers were 100 mM for *t*-BuOH and 1 mM for BA and Ph. These doses were selected to ensure high excess of these scavengers against the dye pollutant, i.e., $[t\text{-BuOH}]_0/[CB]_0 \sim 3920$ and $[Ph]_0/[CB]_0 \sim 40$. Phenol addition drastically inhibits the CB removal; i.e., more than 90% of CB was remained in the solution for up to 40 min of reaction. Thus, CB degradation happens mainly through radical pathway. Besides, *t*-BuOH addition reduces the dye removal after 40 min by only about 10%, revealing that both $SO_4^{\bullet-}$ and $Cl_2^{\bullet-}$ can take place in the degradation of CB by the UV/persulfate in seawater. However, the dye removal was totally unaffected by BA addition, meaning that $Cl_2^{\bullet-}$ plays the dominant role in the degradation of CB by the UV/persulfate in seawater. This distribution of radicals consists with that furnished by the simulation results of Figure 1b where the concentration of $Cl_2^{\bullet-}$ is dominated over all other radicals in saline water containing 100 mM of chloride ions, i.e., the typical concentration in seawater is 565 mM.

For deionized water, Liang and Su [63] have researched active radical species formed by thermally activated persulfate in deionized water under various pH conditions using a chemical probe method. Their results revealed that $SO_4^{\bullet-}$ is the predominant radical oxidant at pH < 7. Both $SO_4^{\bullet-}$ and $^{\bullet}OH$ are present at neutral pH, and $HO^{\bullet}$ is the predominant radical at more basic pH (pH > 9). Alternative spectroscopic method (ESR spin trapping) [64] also demonstrated that the predominant radical species at different pH conditions in the persulfate activated system are similar to those reported by the chemical probe method. Therefore, when Cl^- is present at huge quantity as in seawater, i.e., 565 mM, the rate of $SO_4^{\bullet-}$ consumption by Cl^- to yield $Cl^{\bullet}$ (Eq. 8) could be several orders of magnitude higher than that of $SO_4^{\bullet-}$ reaction with the dye pollutant, specifically at pH 3. Thus, $SO_4^{\bullet-}$ could have a low chance to react with CB.

Besides, the resulted $Cl^{\bullet}$ radical may conduct four main reaction pathways: (i) fast reaction with Cl^- to produce $Cl_2^{\bullet-}$ (Eq. 10), (ii) rapidly hydrolyzed to generate the $HClO^{\bullet-}$ radical anion (Eq. 11), (iii) radical recombination with them self (Eq. 20) or (iv) reaction with CB to induce degradation. The rate constant of pathway (i), i.e., 8.5×10^9 M^{-1} s^{-1}, is much higher than those of all other pathways [2.5×10^5 M^{-1} s^{-1} for pathway (ii) and 8.8×10^7 M^{-1} s^{-1} for pathway (iii)], assuming that its rate constant with CB is of the order of 10^9 M^{-1} s^{-1} [50]. Therefore, $Cl^{\bullet}$ could principally be consumed via pathway (i) and, hence, its implication in the degradation process is insignificant. In fact, a lifetime of less than 5 µs was estimated for

$Cl^{\bullet}$, against fractions of milliseconds for $Cl_2^{\bullet-}$ [65]. Accordingly, the contribution of $HClO^{\bullet-}$ can also be neglected, as it is unreactive with synthetic dyes [58].

Consequently, the present radical scavengers studies revealed that CB degradation by UV/persulfate in seawater was mainly driven by $Cl_2^{\bullet-}$, generated from reactions associated to the photolysis of persulfate in seawater.

ROLE OF BROMIDE IONS

Seawater contains bromide ions at ~ 1 mM, wich means that bromide reaction chain may also be produced from reactions of bromide-containing seawater with $SO_4^{\bullet-}$/$^{\bullet}OH$ and their associate reactions (Eqs. 21-37), where many brominated reactive species, i.e., BRS: $Br^{\bullet}$, $Br_2^{\bullet-}$ and $HBrO^{\bullet-}$, and other Cl-Br mixed radicals have been suspected to be generated [41, 51, 66–68]. However, given that the Cl^- concentration in seawater is 565 folds much higher than that of Br^- (565 mM for Cl^- against 1 mM for Br^-), the Br-radical pathway is not suspected as all of $SO_4^{\bullet-}$/$^{\bullet}OH$ radicals would be scavenged by chloride, favoring the predominance of CRS instead BRS. Besides, reactions between RCS and Br^-, i.e., like those of Eqs. 26-28, are also possible but their happening in seawater is also unsuspected due the same previous reason; RCS could preferably react with the Cl^- rather than with Br^- due to the high excess of the former.

$$SO_4^{\bullet-} + Br^- \rightarrow Br^{\bullet} + SO_4^{2-} \qquad k_{21} = 3.5\times 10^9\ M^{-1}s^{-1} \tag{21}$$

$$^{\bullet}OH + Br^- \rightarrow BrOH^{\bullet-} \qquad k_{22} = 1.1\times 10^{10}\ M^{-1}s^{-1} \tag{22}$$

$$BrOH^{\bullet-} + H^+ \rightarrow Br^{\bullet} + H_2O \qquad k_{23} = 4.4\times 10^{10}\ M^{-1}s^{-1} \tag{23}$$

$$Br^{\bullet} + Br^- \rightleftharpoons Br_2^{\bullet-} \qquad k_{24} = 1.2\times 10^{10}\ M^{-1}s^{-1},\ k_{-24} = 1.9\times 10^4\ M^{-1}s^{-1} \tag{24}$$

$$BrOH^{\bullet-} + Br^- \rightarrow Br_2^{\bullet-} + OH^- \qquad k_{25} = 1.9\times 10^8\ M^{-1}s^{-1} \tag{25}$$

$$Cl^{\bullet} + Br^- \rightleftharpoons ClBr^{\bullet-} \qquad k_{26} = 1.2\times 10^{10}\ M^{-1}s^{-1},\ k_{-26} = 2\times 10^3\ s^{-1} \tag{26}$$

$$Cl_2^{\bullet-} + Br^- \rightleftharpoons ClBr^{\bullet-} + Cl^- \qquad k_{27} = 1.2\times 10^{10}\ M^{-1}s^{-1},\ k_{-27} = 1.9\times 10^3\ s^{-1} \tag{27}$$

$$ClOH^{\bullet-} + Br^- \rightleftharpoons ClBr^{\bullet-} + OH^- \qquad k_{28} = 4.0\times 10^9\ M^{-1}s^{-1},\ k_{-28} = 1.1\times 10^2\ s^{-1} \tag{28}$$

$$Br_2^{\bullet-} + Cl_2^{\bullet-} \rightarrow Br_2 + 2Cl^- \qquad k_{29} = 4\times 10^9\ M^{-1}s^{-1} \tag{29}$$

$$Br_2^{\bullet-} + {}^{\bullet}OH \rightarrow HOBr + Br^- \qquad k_{30} = 1\times 10^9\ M^{-1}s^{-1} \tag{30}$$

$$BrCl^{\bullet-} + {}^{\bullet}OH \rightarrow BrCl + OH^- \qquad k_{31} = 1\times 10^9\ M^{-1}s^{-1} \tag{31}$$

$$BrCl^{\bullet-} + Cl_2^{\bullet-} \rightarrow BrCl + 2Cl^- \qquad k_{32} = 2\times 10^9\ M^{-1}s^{-1} \tag{32}$$

$$BrCl^{\bullet-} + Br_2^{\bullet-} \rightarrow Br_2 + Cl^- + Br^- \quad k_{33} = 4\times10^9\ M^{-1}s^{-1} \tag{33}$$

$$Br_2^{\bullet-} + Br_2^{\bullet-} \rightarrow Br_2 + 2Br^- \quad k_{34} = 1.9\times10^9\ M^{-1}s^{-1} \tag{34}$$

$$Br_2^{\bullet-} + Br^{\bullet} \rightarrow Br_2 + Br^- \quad k_{35} = 2\times10^9\ M^{-1}s^{-1} \tag{35}$$

$$BrO^{\bullet} + BrO^{\bullet} + H_2O \rightarrow BrO_2^- + OBr^- + 2H^+ \quad k_{36} = 5\times10^9\ M^{-1}s^{-1} \tag{36}$$

$$BrCl^{\bullet-} + BrCl^{\bullet-} \rightarrow BrCl + Br^- + Cl^- \quad k_{37} = 1\times10^9\ M^{-1}s^{-1} \tag{37}$$

OVERALL EFFECT OF SEAWATER SALINITY

To appreciate the overall impact of the seawater salinity on the process performance, CB degradation runs upon UV and UV/persulfate processes was assessed in two matrices: deionized water and seawater, and the results are shown in Figure 3. The photochemical removal of the dye was appreciably reduced in seawater, as compared to the pure water; i.e., a 15% of reduction was remarked. This is in fact due to the salts-induced light attenuation, in which the seawater acts as light screens, thus reducing the photon receiving efficiency and, subsequently, shrinking the degradation rate of the pollutant [69]. However, for UV/persulfate process, the difference in the removal yields is much lower, i.e., ~ 7%, than that obtained by the photochemical treatment. This means that the generated RCS, although less reactive than $SO_4^{\bullet-}$, can compensate of the lower UV treatment in the seawater via the generation of high concentration of $Cl_2^{\bullet-}$, which has a longer lifetime than $SO_4^{\bullet-}$. Thanks to these benefic specifications of $Cl_2^{\bullet-}$, the seawater can be regarded as a strong oxidation environment of organic pollutants when applying UV/persulfate as a decontamination process. Consequently, the higher concentration of salts containing in seawater, i.e., mainly Cl^-, could be helpfully for oxidizing persistent organic pollutants in marine media.

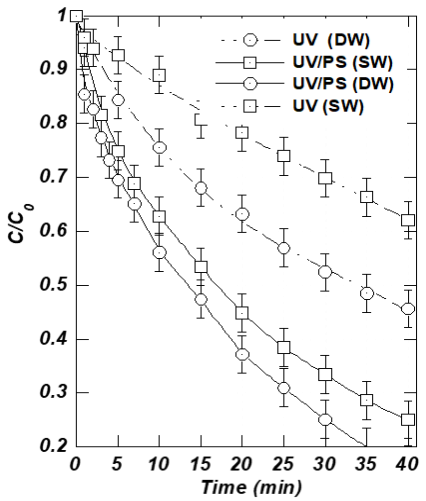

Figure 3. CB degradation kinetics in seawater (SW) and deionized water (DW) upon sole photolysis (UV) and UV/PS system (conditions – C_0 = 25.5 µM, $[S_2O_8^{2-}]_0$ = 0.5 mM, pH 3, ~ 25±1°C).

IMPACT OF SOLUTION pH AND POLLUTANT CONCENTRATION

A series of degradation experiments in seawater has been conducted by varying pH from 3 to 9 and initial CB concentration in the range of 3.37–63.75 µM. The application of UV/persulfate process for decontaminating seawater was not affected by pH elevation in the range of 3 to 7, while a reduction in the degradation rate is looked at pH 9, but only after an advanced reaction period of 10 min (Figure 4a). This latter reduction was due to an observed precipitation phenomenon, which was taken place at basic medium.

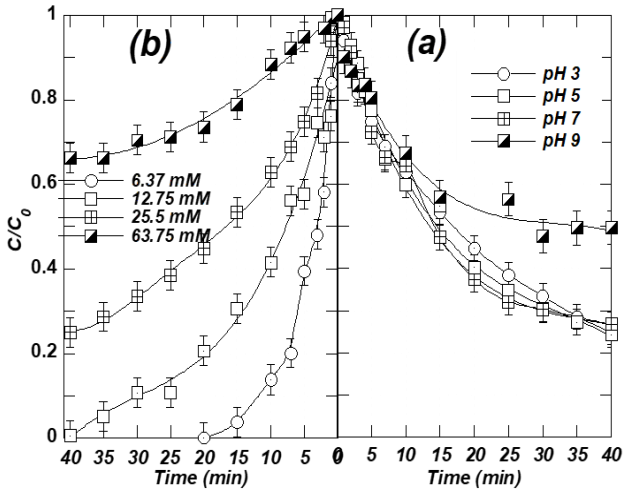

Figure 4. CB degradation kinetics for different pH (a) and various initial CB concentration (b) (conditions – C_0 = 25.5 µM for (a), pH 3 for (b), $[S_2O_8^{2-}]_0$ = 0.5 mM, ~ 25 ±1°C).

In deionized water, the solution pH affects the radicals distribution in the reacting medium [42,63]. ESR spin trapping studies during photolysis of persulfate showed that $SO_4^{\bullet-}$ is the main product species at pH < 8.5. For pH > 8.5, $SO_4^{\bullet-}$ decays rapidly into $^{\bullet}OH$ via reaction (2) and only $^{\bullet}OH$ was detected for pH > 10.8 [42]. Alternative chemical probes method in heat activated persulfate system revealed that $SO_4^{\bullet-}$ is the predominant radical oxidant at pH < 7; both $SO_4^{\bullet-}$ and $^{\bullet}OH$ are present at neutral pH, and $^{\bullet}OH$ is the predominant radical at more basic pH (pH > 9) [63]. However, the above distribution was completely modified in seawater as stated in Figure 1, where the $Cl_2^{\bullet-}$ becoming the dominant radical species in the solution for pH 1–9. Therefore, as $Cl_2^{\bullet-}$ was the sole radical oxidant implicated in the degradation of CB, the pollutant removal rate could insignificantly be changed with the solution pH in the interval of 3-8. Consequently, the photoactivated persulfate process could be efficiently operable for seawater treatment under acidic to neutral pH-conditions.

On the other hand, the efficiency of CB removal at 20 min decreased from 100% for 6.37 µM of initial CB concentration to 80% for 12.75 µM, 55% for 25.5 µM and 27% for 63.75 µM, as reported in Figure 4b. The process is then more efficient for low pollutant concentration in which a shorter treatment time is required. Meanwhile, the initial degradation rate increased from 0.77 µM min^{-1} for 6.37 µM of CB to 1.1 µM min^{-1} for 12.75 µM, 1.27 µM min^{-1} for 25.5 µM and 1.4 µM min^{-1} for 63.75 µM. These results are in good agreement with those reported in deionized water for several $SO_4^{\bullet-}$-based AOPs [15–17]. The rise of

initial degradation rate with initial CB concentration is not linear, as expected for a first order kinetics law. Therefore, the degradation of organic pollutants in seawater with the UV/persulfate could not be represented with a pseudo-first order kinetics law, as reported for many cases of $SO_4^{\bullet-}$-based AOPs conducted in deionized water [21, 54, 70, 71].

The augmentation of the degradation rate with augmenting the initial pollutant concentration could be associated to the following reasons: at fixed irradiation intensity and initial persulfate dosage, the overall production rate of reactive radicals, i.e., $Cl_2^{\bullet-}$, could be constant. Hence, with increasing the initial pollutant concentration, the portion of radicals that could be scavenged by the pollutant molecules increases, thereby accelerating the destruction rate of contaminants [5, 7, 15, 17, 60, 72, 73].

IMPACT OF SURFACTANTS

Surfactants are usually used as additives in dyeing processes and are widely discharged along with the dye effluents [15]. It is of practical point to test their impact on the efficiency of UV/persulfate process in seawater. The effect of four surfactants (SDS 'sodium dodecyl sulfate', Tween 20, Tween 80 and Triton X-100) on the degradation of CB by photoactivated persulfate in seawater was investigated at pH 3 by fixing persulfate dosage at 0.5 mM. SDS, Tween 20, and Tween 80 did not affect the CB degradation rate, whereas the process losses more than 50% of its efficiency in the presence of Triton X-100, as highlighted in Figure 5. The dye removal after 40 min of treatment decreased from 73% without surfactant to 55% and 38% in the presence of Triton X-100 at 20 and 80 mg L^{-1}, respectively. It seems that the reactivity of $Cl_2^{\bullet-}$ radical is strongly affected by this type of surfactant. This radical is very selective for olefins and aromatics containing hydroxyl or amino groups [61, 74, 75].

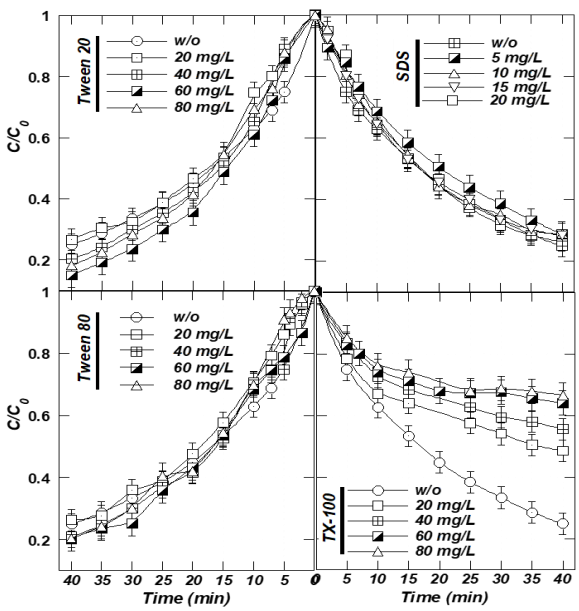

Figure 5. Effect of different surfactants on the removal kinetics of CB in seawater upon UV/persulfate treatment (conditions – C_0 = 25.5 µM, pH 3, $[S_2O_8^{2-}]_0$ = 0.5 mM, ~ 25 ±1°C).

Conversely to these results, Tween 20 and Tween 80 at 80 mg L^{-1} have reduced the dye elimination in deionized water by about 10-15%, while Triton X-100 accelerated the process performance by about 15%, both as compared to runs performed without surfactants. Thus, the water matrix affects sensitively the performance of the UV/persulfate process through changing the radicals distribution in the reacting system. In general, surfactants can scavenge both $SO_4^{\bullet-}$ and $^{\bullet}OH$, thereby decelerating the process efficiency in deionized water [17, 76–79]. However, the $Cl_2^{\bullet-}$ radical is strongly affected by the nature of the surface-active solute [5, 61, 75, 80].

CONCLUSION

Sea has historically been subject to high anthropogenic weights of direct and indirect discharges of persistent organic pollutants (POPs) from exhaustive industrial and agricultural activities. The findings of this study showed that UV/persulfate oxidation process can supply a good performance toward the degradation of POPs in seawater. The high quantity of chloride ions existing in seawater exerts a conversion of radicals, in which $SO_4^{\bullet-}$ and $^{\bullet}OH$ were totally converted into $Cl_2^{\bullet-}$, which has a longer life time than the primary radicals formed during persulfate photoactivation. The degradation rate of CB by UV/persulfate was drastically improved as compared to the sole UV irradiation, due to the involvement of free radical pathway. The degradation rate increased with the increase in persulfate dosage whereas low dye concentrations favor a quick removal rate. UV/persulfate process can be practicable in seawater for up to pH 7. Interestingly, many surfactants did not affect the degradation of the dye, making persulfate photoactivated treatment a promising method for treating seawater contaminated with emerging organic pollutants.

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Chapter 136

DIVERSITY AND CLASSIFICATION OF DINOFLAGELLATES

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ABSTRACT

This chapter summarizes the diversity of dinoflagellates divided into several groups: basal dinoflagellates (i.e., Syndineans and Noctilucales), unarmored dinokaryotes and thecate forms such as Prorocentrales and Dinophysales, Gonyaulacales, Peridiniales, thin-walled dinoflagellates and unclassified taxa. The morphology of the main representatives are illustrated, phylogenetic trees of each group and a scheme on the tentative evolution are provided. The trends on dinoflagellate biodiversity research, problematic and unresolved issues (i.e., biases in the availability of molecular markers for the less accessible taxa, recent proposals on nomenclature, etc.) are outlined. A classification scheme for dinoflagellate levels above genus is proposed within a context of unresolved relationships of higher ranks in the molecular phylogenies.

Keywords: Dinoflagellata biodiversity, Dinophyta classification, Dinophyceae systematics, alveolate evolution, protist diversity

INTRODUCTION

Dinoflagellates (Alveolata, Dinophyceae) are protists with a truly remarkable diversity in lifestyles (free-living, parasites and mutualistic symbionts), habitats (marine, freshwater, plankton, benthos), and trophic modes (heterotrophic, chloroplast-containing) (Gómez 2012a). Dinoflagellates and ciliates are the most diverse groups of alveolates. About ~2400 species of dinoflagellates are currently accepted (Gómez 2012b), several tens of new species are described every year, and the environmental molecular surveys reveal numerous lineages

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of basal dinoflagellates that has not been documented yet (Guillou et al. 2008). This chapter summarizes the main groups of dinoflagellates and its classification, with illustrations of the diversity, and some brief notes on ecological aspects and problematic issues.

A first issue is to delimitate what a dinoflagellate is. In a strict sense, a dinoflagellate is a dinokaryote which is characterized by a peculiar nucleus, the dinokaryon, devoid of histones and with permanently condensed chromosomes, and a huge genome with peculiar characteristics (Lin 2011). Most of these biflagellate cells have a wavy transversal flagellum, although this character is missing in the desmokont (prorocentroid) dinokaryotes. The cingulum or girdle, a groove that encircles the cell harboring the transversal flagellum, is not only missing in prorocentroids. It is also apparently absent in planktonic (*Podolampas*) or benthic species (*Adenoides*) that have the wavy transversal flagellum. Alternatively, if the cingular plates are not in a depressed or sunken groove, they can be mistaken for the precingular or postcingular plate series (Gómez et al. 2015c).

BASAL DINOFLAGELLATES

Unexpectedly, the earlier molecular studies reveal the phylogenetic relationship between the dinoflagellates and the apicomplexans that include agents of human diseases (*Plasmodium*, *Toxoplasma*; Gunderson et al. 1987). Since then, the molecular phylogeny has placed numerous lineages between the apicomplexans and the dinokaryotes (Figure 1). Among these groups of basal dinoflagellates are parasites such as the perkinsids (*Dinovorax*, *Parvilucifera*, *Perkinsus*, *Snorkelia*; Figure 2A; Goggin and Barker 1993, Nóren et al. 1999, Reñé et al. 2017), the ellobiopsids (*Thalassomyces*, *Ellobiopsis*, Figure 2B; Silberman et al. 2004, Gómez et al. 2009a) and the free-living heterotrophic genus *Oxyrrhis* (Figure 2C, Saldarriaga et al. 2003a). The nuclei of the syndineans have intermediate characteristic between the typical eukaryotic and dinokaryotic nucleus (Ris and Kubai 1974). The syndineans are divided into two groups: the eduboscquellids with *Ichthyodinium*, a parasite of fish eggs (Figure 2D–E), and *Eduboscquella*, a parasite of ciliates (Figure 2F–G; Yuasa et al. 2007, Harada et al. 2007, Coats *et al*. 2010, Gómez and Gast 2018) and the Syndiniales with the parasites of crustaceans *Hematodinium* and *Syndinium* (Figure 2H, Skovgaard et al. 2005) and the dinophagous parasite *Amoebophrya* (Figure 2I–K, Gunderson et al. 1999). The genera *Syndinium*, *Hematodinium* and *Amoebophrya* can be placed in the Syndiniales or Syndinea, and *Eduboscquella* and *Ichthyodinium* can be placed in the family Eduboscquelliaceae or alternatively in the order Coccidiniales following Cachon and Cachon (1987). These basal dinoflagellates remain unreported in epicontinental waters, and they have an important role in the marine ecosystems, including the control of the proliferations of harmful dinoflagellates (Chambouvet et al. 2008). The molecular data reveal a huge genetic diversity of basal dinoflagellates (Guillou et al. 2008). The lack of distinctive characters, even in the dinospores, make difficult the species characterization. In the past, the species names were proposed citing the host as the species epithet. This could be interpreted as a high host-specificity. Further studies have revealed that the relationship between host and parasite is not often species specific, and a single parasitic species may infect different species of hosts, and a host species may be infected by different syndinean parasites (Salomon et al. 2003).

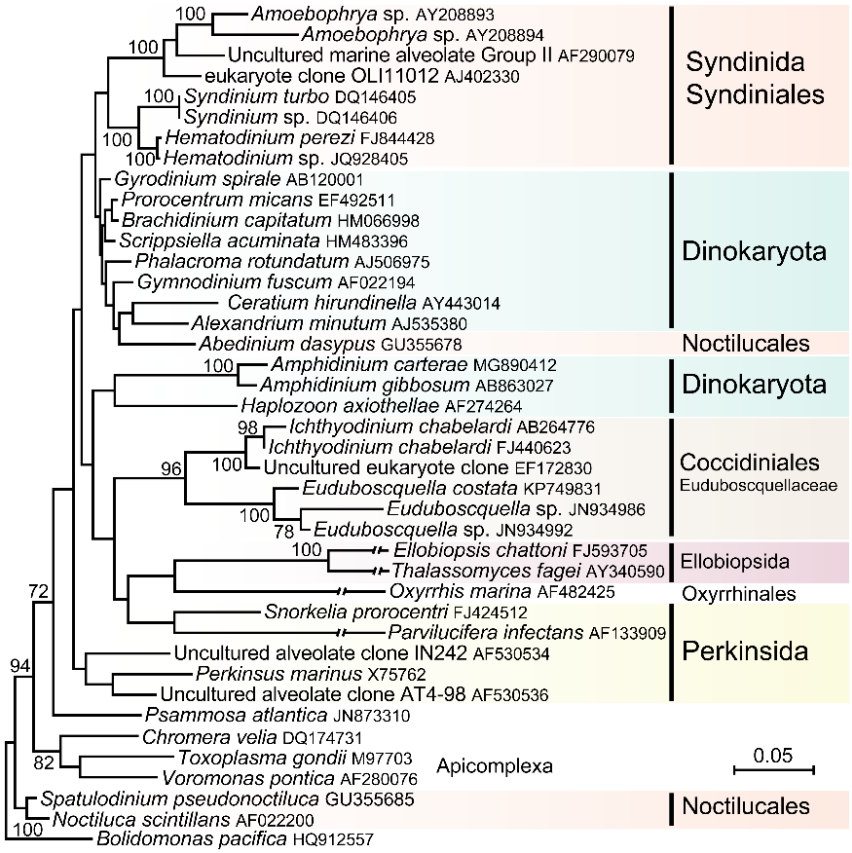

Figure 1. The phylogenetic position of dinoflagellates and other alveolate lineages based on Maximum Likelihood analysis inferred from SSU rRNA gene sequences. Bootstrap values ≥70 are noted to the left of internodes. The stramenopile *Bolidomonas pacifica* was used as outgroup. Scale bar = 0.05 substitutions site^{-1}.

The Noctilucales are the basal dinoflagellates closer to the dinokaryotes, and have dinokaryotic characteristics in some life stages. *Noctiluca scintillans* was the first observed dinoflagellate because it is the biggest species, bioluminescent and common in coastal temperate waters. The other noctilucaceans have received less attention and numerous species remain undescribed (Gómez 2010). *Noctiluca scintillans* (Figure 2L) and *Spatulodinium* spp. are closely related in the molecular phylogenies (Gómez et al. 2010a), and they share a tentacle-like extension used for feeding. In some tropical areas, *Noctiluca* may harbor a free-swimming chlorophyte, *Spatulodinium* possesses chloroplasts of an unknown nature (Figure 2O), and *Pomatodinium* possesses a photosynthetic endosymbiont (Figure 2P). A close relative is *Kofoidinium*, which possesses an extracellular dome as well as *Spatulodinium* used for prey capture (Figure 2M–N). The members of the Leptodiscaceae are even less known due to their fragility. These heterotrophic species have highly flattened cell bodies and able of fast changes of shape. *Leptodiscus* (Figure 2Q) and *Cymbodinium* (Figure 2R) swim like a medusa, and *Abedinium* and *Scaphodinium* (Figure 2S) are also able of sudden contractions. The molecular data on the Leptodiscaceae are restricted to partial ribosomal RNA gene sequences of *Abedinium* (Gómez et al. 2010a). The phylogenetic position is instable, being more distantly related to the other noctilucaceans (Figure 1). The Noctilucales are

characterized by sequences with long branches in the ribosomal RNA gene phylogenetic trees. This is associated with the long-branch attraction artefact that is a form of systematic error where distantly related lineages with long branches appear closely related in the phylogenetic trees (Philippe et al. 2005).

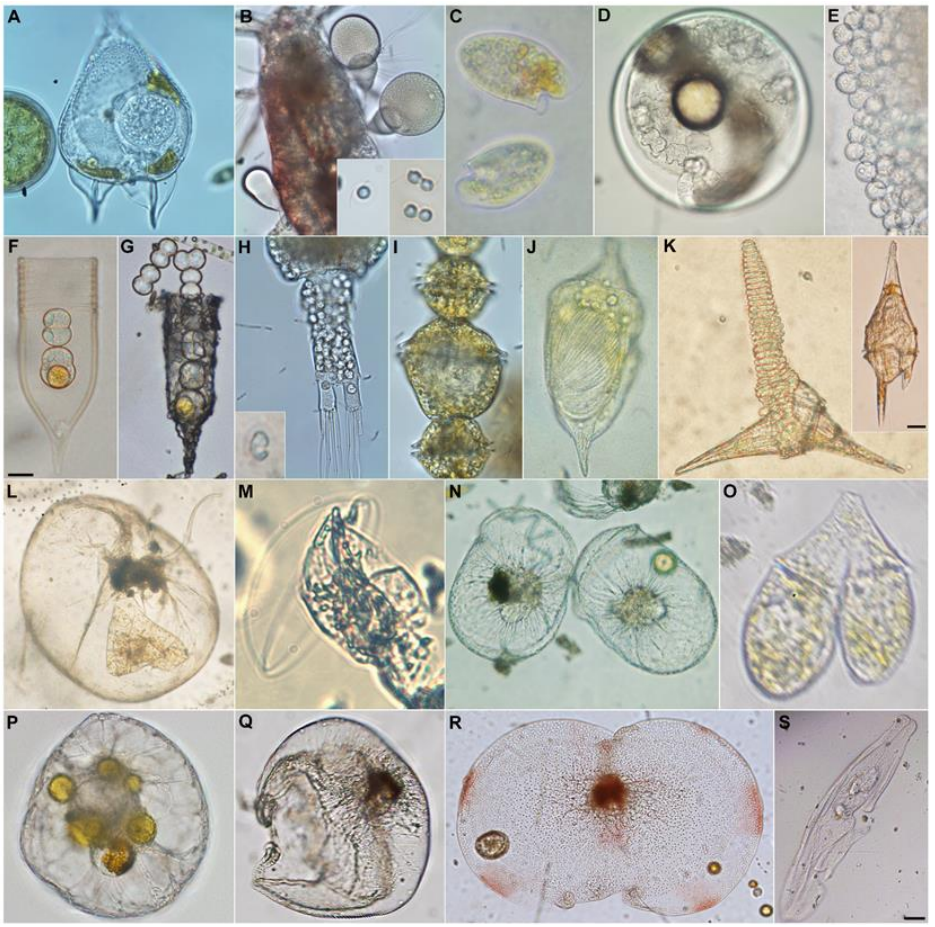

Figure 2. Light micrographs of basal dinoflagellates. A. *Parvilucifera* in *Podolampas*. B. *Ellobiopsis* on a copepod. The inset shows the infective spores. C. *Oxyrrhis*. D–E. *Ichthyodinium*. F–G. *Eudubosquella*. H. *Syndinium*. The inset shows the infective spore. I–K. *Amoebophrya* in *Protogonyaulax*, *Schuettiella* and *Tripos*. L. *Noctiluca*. M–N. *Kofoidinium*. O. *Spatulodinium.* P. *Pomatodinium*. Q. *Leptodiscus.* R. *Cymbodinium*. S. *Scaphodinium*. Scale bar = 20 μm.

UNARMORED DINOFLAGELLATES (GYMNODINIPHYCIDAE)

The dinoflagellates have been traditionally classified as armored (thecate) and unarmored dinoflagellates (athecate or naked dinoflagellates). As all the known basal dinoflagellates are unarmored, we could speculate that this is an ancestral trait and the thecate dinoflagellates constituted the most derived forms. Recent data based on dinoflagellate transcriptomes confirmed this view and placed the unarmored dinoflagellates as a basal group among the dinokaryotic dinoflagellates (Janouškovec et al. 2017).

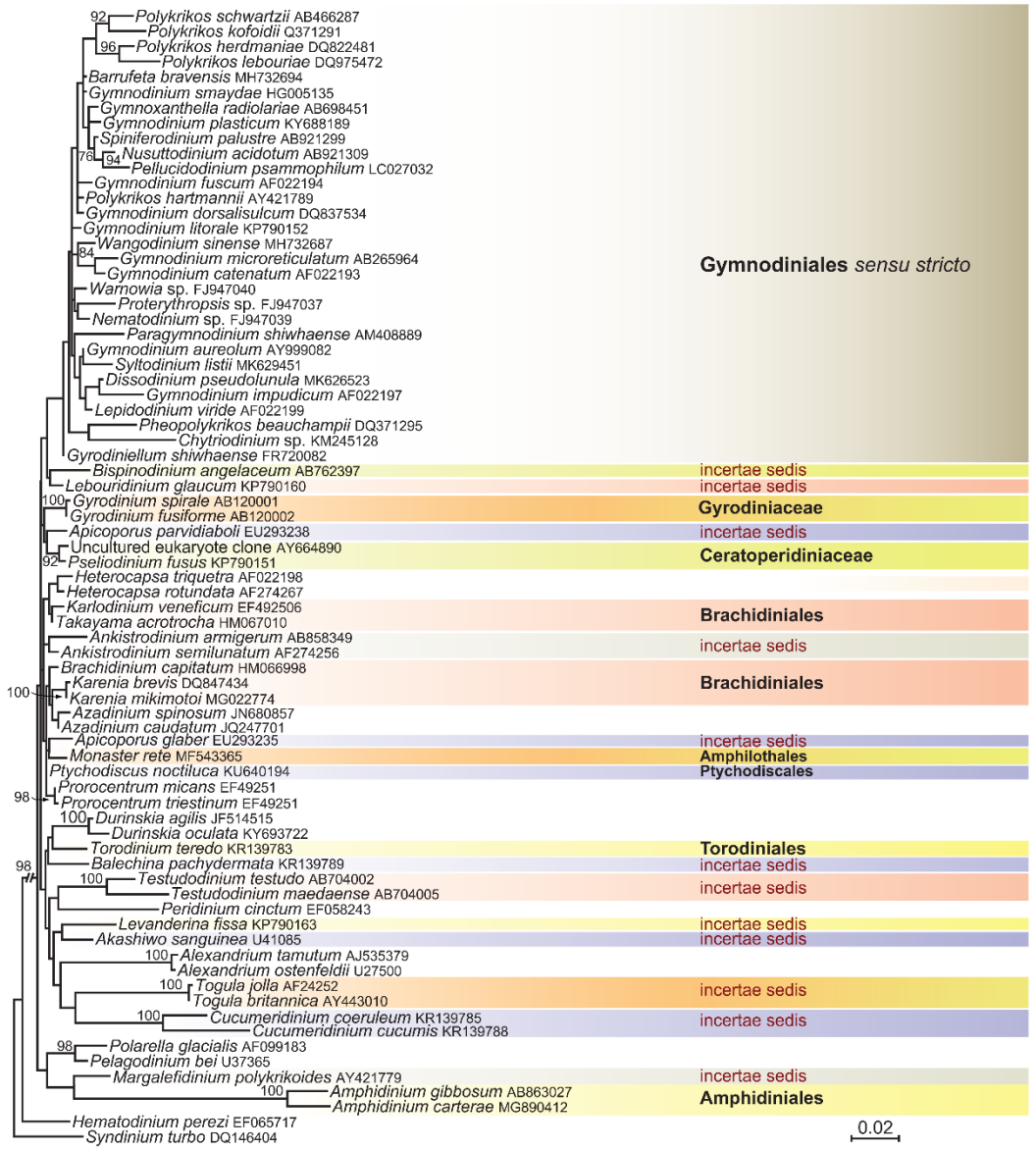

Figure 3. The phylogenetic position of dinoflagellates based on Maximum Likelihood analysis inferred from SSU rRNA gene sequences, with especial focus on the unarmored forms. Bootstrap values ≥70 are noted to the left of internodes. The syndinean *Syndinium turbo* was used as outgroup. Scale bar = 0.02 substitutions site^{-1}.

There is an important gap in the knowledge between unarmored and armored dinoflagellates due to practical reasons. When compared to the thecate dinoflagellates, the naked forms are easily damaged by net sampling, cells lysis or the morphology is distorted due to the manipulation and common fixatives. Modern methods such as the cultures provide abundant material fresh material that allow reducing the gap of knowledge between naked and thecate species. Although the bias remains for the heterotrophic species that predominate in the open oligotrophic ocean (Gómez 2014). In the earlier studies, most of the free-living unarmored dinoflagellates were placed in the genus *Gymnodinium*, and later new genera were

proposed based on characters such as the degree of cingular displacement (*Gyrodinium*), number of turns of the cingulum (*Cochlodinium*), or the relative height of the cingulum (*Amphidinium*, *Torodinium*) (Kofoid and Swezy 1921). The molecular data revealed that genera such as *Gymnodinium*, *Amphidinium* or *Cochlodinium* were not monophyletic, and these classical diagnostic criteria were not supported (Daugbjerg et al. 2000, Jørgensen et al. 2004a, Gómez et al. 2017a). The modern generic diagnoses are based on a combination of features with an especial relevance of the shape of the apical groove (Takayama 1985, Daugbjerg et al. 2000).

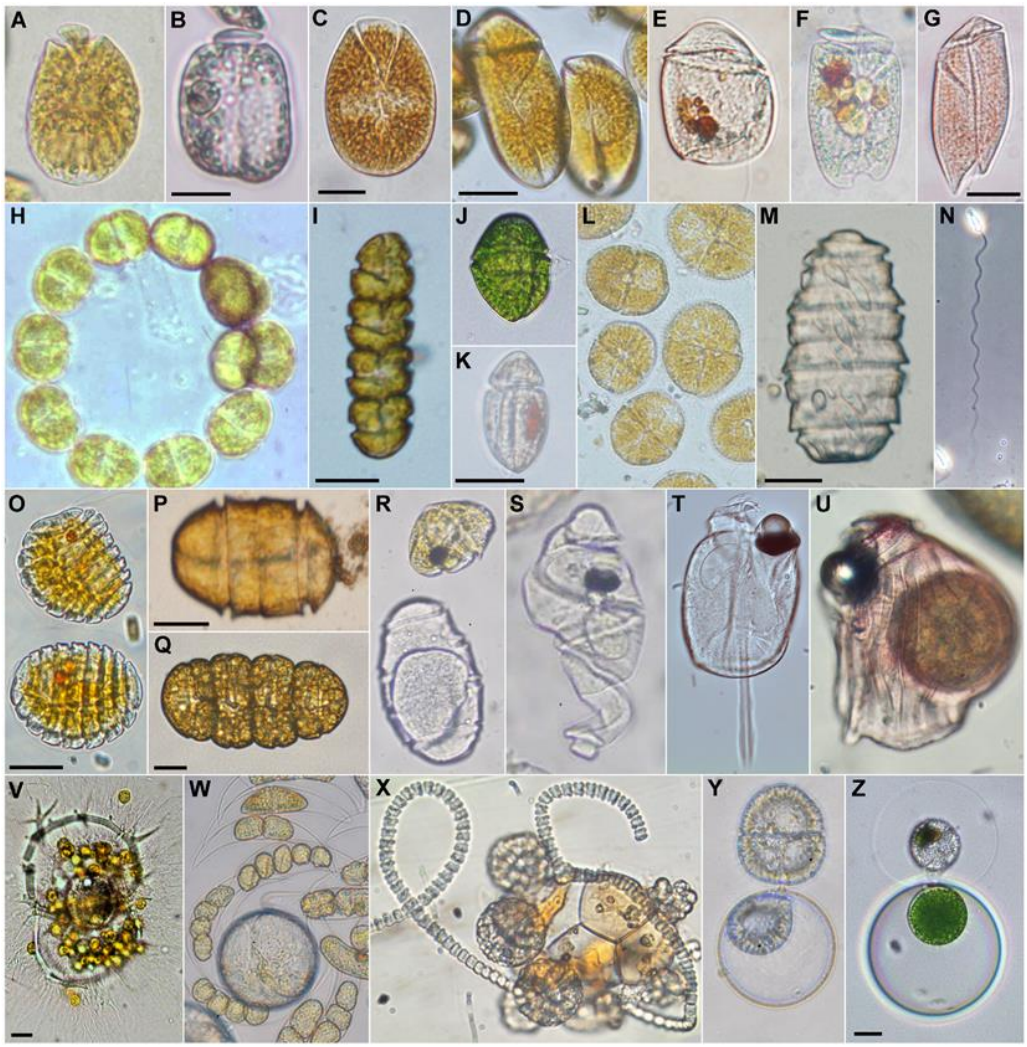

Figure 4. Light micrographs of unarmored dinoflagellates: Amphidinioids and Gymnodiniales *sensu stricto*. A. *Amphidinium* (photosynthetic). B. *Amphidinium* (heterotrophic). C. *Testudodinium*. D. *Togula*. E. *Ankistrodinium*. F. Apicoporus. G. '*Amphidinium'* scissum. H. *Gymnodinium catenatum*. I. *G. impudicum*. J. *Lepidodinium viride*. K. *Gymnodinium venator*. L. *Spiniferodinium*. M. *Polykrikos kofoidii*. N. nematocyst. O. *Polykrikos lebouriae*. P. *Polykrikos hartmannii*. Q. *Pheopolykrikos beauchampii*. R. *Warnowia* sp. with chloroplasts and *Polykrikos*. S. *Nematopsides*. T–U. *Erythropsidinium*. V. *Gymnoxantella*. W. *Dissodinium*. X. *Chytriodinium*. Y. *Syltodinium*. Z. *Myxodinium*. Scale bar = 20 μm.

The unarmored dinoflagellates with a very anterior cingulum have been placed under the genus *Amphidinium* (Kofoid and Swezy 1921). The earlier life stages of *Kofoidinium* (Figure 2N) and *Spatulodinium* (Figure 2O) resemble species formerly placed in the genus *Amphidinium* (Figure 4D–F), and we could speculate that the amphidinioids represent the most ancestral morphology of the dinokaryotes. In the earlier evolutionary schemes, *Amphidinium* was placed as a basal dinoflagellate after the prorocentroids (Kofoid and Swezy 1921, p. 86). Based on the ribosomal DNA such as the SSU rRNA gene, the sequences of the clade of the type species *Amphidinium operculatum* are characterized by long branches, and often clusters closer to the basal dinoflagellates that have also long branches, raising the question of whether *Amphidinium* is really a basal group among the dinokaryotes or its position is artificial due to the long-branch attraction (Figure 3). Based on multigene (Zhang et al. 2007) and transcriptome phylogenies (Janouškovec et al. 2017), *Amphidinium* is considered as the most basal lineage of the dinokaryotes clustering between the Noctilucales and the other dinokaryotes. As occurred with other earlier genus of naked dinoflagellates, under the genus *Amphidinium* were placed unrelated genera of unarmored dinoflagellates that share a very anterior cingulum and consequently a small episome (Jørgensen et al. 2004a) (Figure 3). The clade *Amphidinium sensu stricto* (Figure 4A–B) is a basal group, and species such as *Gymnodinium venator* (Figure 4K) belongs to the Gymnodiniales or other former members of *Amphidinium* are currently placed in new genera such as *Testudodinium* (Figure 4C), Togula (Figure 4D), *Ankistrodinium* (Figure 4E), Apicoporus (Figure 4F), and new amphidinioid genera have been proposed (i.e., *Bispinodinium*). Other species of *Amphidinium* still needs a new generic placement (Figure 4G) (Jørgensen et al. 2004b, Sparmann et al. 2008, Hoppenrath et al. 2012, Horiguchi et al. 2012, Yamada et al. 2013). According to Janouškovec et al. (2017) *Amphidinium sensu stricto* is the most basal lineage of the dinokaryotes, and Togula is a sister group of clade of Gymnodiniales *sensu stricto*. There are no transcriptome sequences of the other amphidinioid genera that in the SSU rRNA gene phylogenies are dispersed in the dinokaryote core without a clear relationship with other dinoflagellates to establish a suprageneric classification (Figure 3).

The order Gymnodiniales is restricted to the clade that contains the type species of *Gymnodinium*, *G. fuscum* (Figure 3, Daugbjerg et al. 2000). This is the most specious, and morphologically and ecologically diverse clade of unarmored dinoflagellates. Blooms of the colonial species *Gymnodinium catenatum* have received attention as responsible of Paralytic Shellfish Poisoning (Figure 4H), and sometimes confused with other colonial non-toxic species such as *G. impudicum* (Figure 4I). In addition to the typical peridinin-containing chloroplast, other species possess a chloroplast derived from a chlorophyte with chlorophyll *b* (*Lepidodinium*, Figure 4J), and the members of the family of *Nusuttodinium* and *Spiniferodinium* (Figure 4L) have a chloroplast derived from a cryptophyte. The polykrikoid dinoflagellates are constituted of multinucleate ‘pseudocolonies’ of zooids (Figure 4M–P). *Polykrikos* possesses two nuclei and the species are heterotrophic (Figure 4M, R) or with chloroplasts (Figure 4O–P). All the species have ejectile organelles or extrusomes with analogies to the nematocyst found in metazoans such as the cnidarians (Figure 4N; Gavelis et al. 2017). Other genus constitutes by zooids is *Pheopolykrikos* that possesses an equal number of nuclei and zooids (Figure 4Q). The warnowiids possess an elaborate photoreception organelle with analogies the ocelloid of pluricellular organisms (Figure 4R–U; Gómez et al. 2009b, Gavelis et al. 2015). The described species are heterotrophic, but at least one species may contain chloroplasts (Figure 4R). The highest degree of ultrastructural sophistication is

found in *Erythropsidinium* that also has a piston (Figure 4T–U, Gómez 2017). Among the symbiotic forms, *Gymnoxantella* lives in symbiosis with pelagic radiolarians and it was overlooked with the thecate dinoflagellate *Zooxanthella* (Figure 4V, Yuasa et al. 2016). The members of the family Chytriodiniaceae are parasites of copepod eggs (*Dissodinium*, Figure 4W; *Chytriodinium*, Figure 4X; *Syltodinium*, Figure 4Y; Gómez et al. 2009c, 2019a), and *Myxodinium* (Figure 4Z) that feeds on the microalgae *Halosphaera*, probably as an alternative host in absence of eggs. The rRNA gene sequences of parasitic forms have often longer branches than the free-living relatives. This results in unstable topologies in the phylogenetic trees as often occurs with the sequences of *Chytriodinium* (Figure 3). Mixotrophic species such as *Gymnodinium aureolum* are closely related to the Chytriodiniaceae (Figure 3).

Based on currently available transcriptome phylogeny, the brachidiniaceans (Brachidiniales) are placed between the Amphidiniales and the Gymnodiniales *sensu stricto* (Janouškovec et al. 2017). The members of the Brachidiniales are photosynthetic species containing fucoxanthin, a typical accessory pigment of haptophyte. Recently, a new genus, *Gertia*, with a chloroplast containing peridinin and lacking fucoxanthin has been described (Takahashi et al. 2019). The brachidiniaceans cluster into two groups in the SSU rRNA gene phylogenies (Figure 3). One group for the species which apical groove is linear straight such as *Asterodinium* (Figure 5A), *Brachidinium* and *Karenia* (Figure 5B; Daugbjerg et al. 2000, Gómez et al. 2005, Benico et al. 2019), and other group for species with a sigmoidal apical groove such as *Karlodinium* and *Takayama* (de Salas et al. 2003). The order Ptychodiscales was a catch-all of species with a supposed strongly developed cell covering or pellicle, pooling thecate forms such as *Kolkwitziella*, unarmored forms such as *Balechina* and *Ceratoperidinium*, the Brachidiniales, and species with a endoskeleton such as *Monaster* (=*Achradina*) (Fensome et al. 1993). The order Ptychodiscales remains only for *Ptychodiscus* (Figure 5C–D), with straight apical groove and yellow pigmentation, tentatively with fucoxanthin-containing plastids, that suggests an affinity with the Brachidiniales that is not well supported in the molecular phylogenies (Figure 3; Gómez et al. 2016a).

The family Ceratoperidiniaceae contains up to date species with a circular apical groove, which is also present in other lineages of unarmored dinoflagellates (*Cochlodinium*, *Cucumeridinium*). All the described species of the Ceratoperidiniaceae are photosynthetic with the typical peridinin-containing plastids, a smooth cell surface, and the cells are often enclosed in a hyaline membrane. There are species with the typical gymnodinioid outline, *Kirithra*, and cells with body extensions and ventro-dorsally compressed (*Ceratoperidinium*, Figure 5E), laterally compressed (*Gynogonadinium*, Figure 5F) or slightly or non-compressed such as *Pseliodinium* (Figure 5G–I) (Reñé et al. 2013, Boutrup et al. 2017). The torsion or the cingular displacement, features traditionally used for the diagnoses, have low diagnostic value for the generic split as evident for two closely related species such as *Pseliodinium fusus* (Figure 5G–H) and *P. pirum* (Figure 5I) (Gómez 2018). The latter species was placed in the new genus *Torquentidium* using as diagnostic character that the cingulum encircled the cell 1.5 times (Shin et al. 2019). The use of this arbitrary numeric morphometric character, also found in *Polykrikos geminatum* or the warnowiids, is not supported by the molecular data. This new genus proposal only contributes to the excessive proliferation of new genera of dinoflagellates.

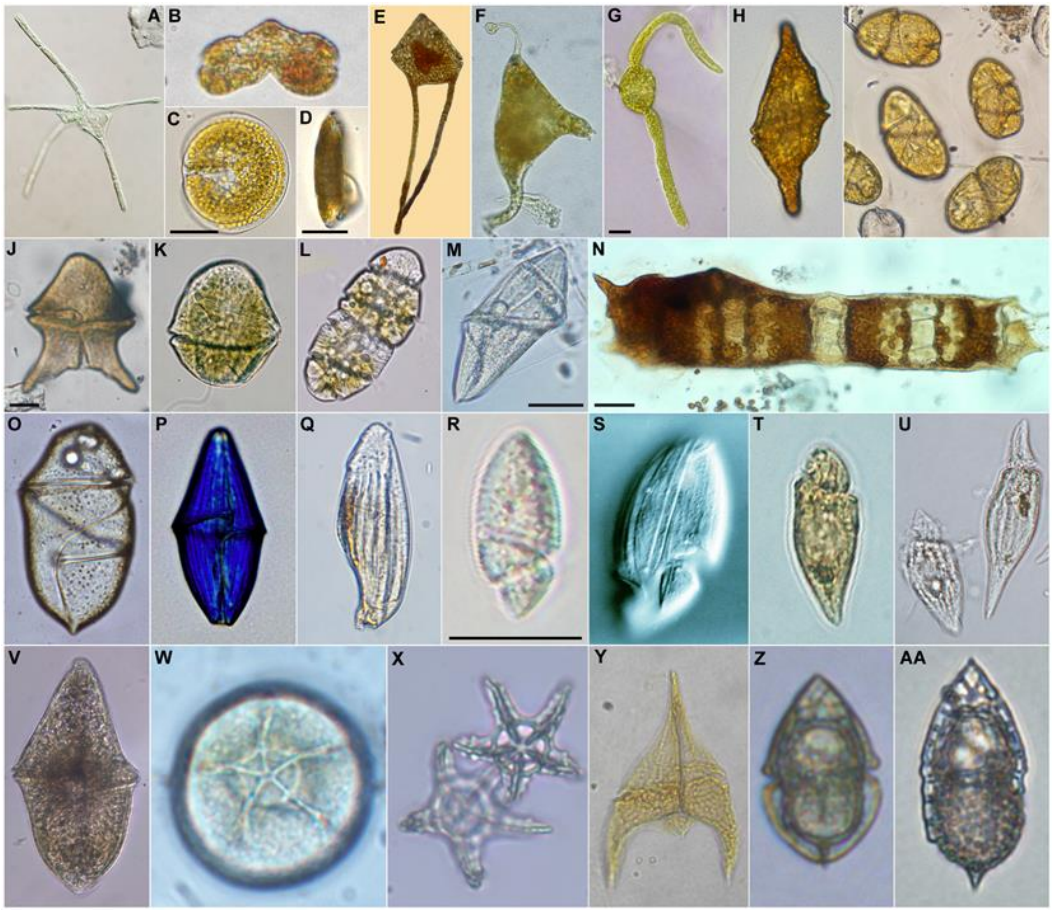

Figure 5. Light micrographs of unarmored dinoflagellates. A. *Asterodinium*. B. *Karenia*. C–D. *Ptychodiscus*. E. *Ceratoperidinium*. F. *Gynogonadinium*. G–H. *Pseliodinium fusus*. I. *Pseliodinium pirum*. J. *Akashiwo*. K. *Levanderina*. L. *Margalefidinium*. M–N. *Gyrodinium*. O. *Cochlodinium*. P. *Cucumeridinium*. Q. *Torodinium*. R–S. *Lebouridinium*. T. *Kapelodinium*. U. *Kapelodinium* and '*Amphidinium*' sphenoides. V. *Balechina*. W–X. Actiniscus. Y. Dicroerisma. Z–AA. Monaster. Scale bar = 20 μm.

Other species that were first described as members of the genus *Gymnodinium* or *Cochlodinium* constitute independent lineages of photosynthetic species with a smooth cell surface such as *Akashiwo* (Figure 5J), *Levanderina* (Figure 5K) or *Margalefidinium* (Figure 5L, Gómez et al. 2017a). Based on the available transcriptome phylogenies, *Akashiwo* is the closer relative to the thecate dinoflagellates (Janouškovec et al. 2017). Longitudinal striae in the cell surface and encapsulated nucleus are common features in several heterotrophic genera. This could be an adaptation to engulf large preys. We have to be cautious to consider the surface striation as a diagnostic character for a clade. For example, nearly all the members of the Gymnodiniales *sensu stricto* have a smooth surface, while *Erythropsidinium*, able to ingest large preys, has reinforcements of the cell surface that can be interpreted as longitudinal striae (Figure 4U, Gómez 2017). Longitudinal striae are found in *Gyrodinium* (Figure 5M–N), *Cochlodinium* (Figure 5O), *Cucumeridinium* (Figure 5P), and taxa with a reduced episome such as *Torodinium*, that also have chloroplasts (Figure 5Q), and *Lebouridinium* for the species previously known as *Katodinium*/*Gyrodinium glaucum* (Figure

5R–S) (Hansen and Daugbjerg 2004, Takano and Horiguchi 2004, Gómez et al. 2015a,d). Boutrup et al. (2016) proposed *Kapelodinium vestifici* for the cold water species *Gymnodinium vestifici* (Figure 5T–U), and they considered that *Katodinium*/*Gyrodinium glaucum* and *Amphidinium extensum* as synonyms. The synonymy of *Gymnodinium vestifici* and *Katodinium/Gyrodinium glaucum* (now *Lebouridinium*) is unsupported. *Gymnodinium vestifici* is an amphidinioid cell, with a small episome and missing the cap-like structure, fine longitudinal striation and closely related to species such as '*Amphidinium*' *extensum* or '*Amphidinium*' *sphenoides* (Figure 5U). The cells of *Katodinium*/*Gyrodinium glaucum* have a small hyposome, an apical cap-like structure, and coarse longitudinal striation (Figure 5R–S). The true *Gymnodinium vestifici* has not been investigated by modern methods and the molecular data labelled as *Kapelodinium vestifici* correspond to *Lebouridinium glaucum* (Figure 3). The longitudinal striation is not the exclusive of the phagotrophic species. The genus *Balechina* (=*Gymnodinium gracile*) possesses a thick and smooth cell covering that is able to sudden changes of shape (Figure 5V, Gómez et al. 2015d).

Other unarmored dinoflagellates have an internal skeleton. *Actiniscus* possesses a pair of endoskeletal elements of silicate (Figure 5W–X), *Dicroerisma* has an inverted Y-shaped endoskeleton of unknown nature (Figure 5Y), and *Monaster* (=*Achradina*) has as endoskeleton of celestite, strontium sulfate. This type of skeleton is only known in Acantharia and some radiolarians, opening the hypothesis of a gene transfer as numerous Rhizaria live in symbiosis with dinoflagellates (Figure 5Z–AA; Gómez et al. 2017b). The cell covering of *Monaster* may contain thin thecal plates, and it may constitute other group of the so-called thin-walled dinoflagellates among the thecate dinoflagellates.

THECATE DINOFLAGELLATES (PERIDINIPHYCIDAE)

Prorocentroids and Dinophysoids

One century ago, the prorocentroids were considered as the most primitive forms of dinoflagellates, even more basal dinoflagellates than *Noctiluca* and *Amphidinium* (Kofoid and Swezy 1921, p. 86). The most typical armored and unarmored dinoflagellates are dinokonts, in which the two flagella are inserted ventrally, and the wavy transversal flagellum is usually placed in the cingulum, and the smooth longitudinal flagellum is placed in the sulcus. In contrast, the prorocentroid dinoflagellates are desmokonts, with both smooth flagella and inserted anteriorly. The grooves such as the sulcus or cingulum are not evident. This configuration is closer to some flagellate groups (e.g., Cryptophyta) suggesting that *Prorocentrum* could constitute the most basal group representing the ancestral morphology of the dinoflagellates (Kofoid and Swezy 1921). However, in the ribosomal RNA gene phylogenies, *Prorocentrum* is well-placed among the dinokaryotic core (Figure 6), and in the transcriptome phylogenies is, together with the dinophysoids, a basal group of the thecate dinoflagellates (Janouškovec et al. 2017).

The theca of prorocentroids is composed of two large plates joined by a sagittal suture, and numerous tiny plates near the flagellar pore (periflagellar platelets). In molecular phylogenies based on a single ribosomal RNA gene marker, the prorocentroids clustered into three clades within the dinokaryotic core (Figure 6), while the monophyly appears using

several genes (Zhang et al. 2007, Murray et al. 2009). We have to take into account that these multigene phylogenies are built with a less taxonomically representative dataset of different species of other dinoflagellates. The clade of Prorocentrales *sensu stricto* with the type species, *P. micans*, contains planktonic or tychoplanktonic species taxa, often with an apical spine or tooth (Figure 7A–C). Benthic or epiphytic species (Figure 7D–E), often with toxic species (i.e., *Prorocentrum lima*) constitute a second clade. The third clade also contains several benthic species, which first described member was *P. panamense* (Grzebyk et al. 1998).

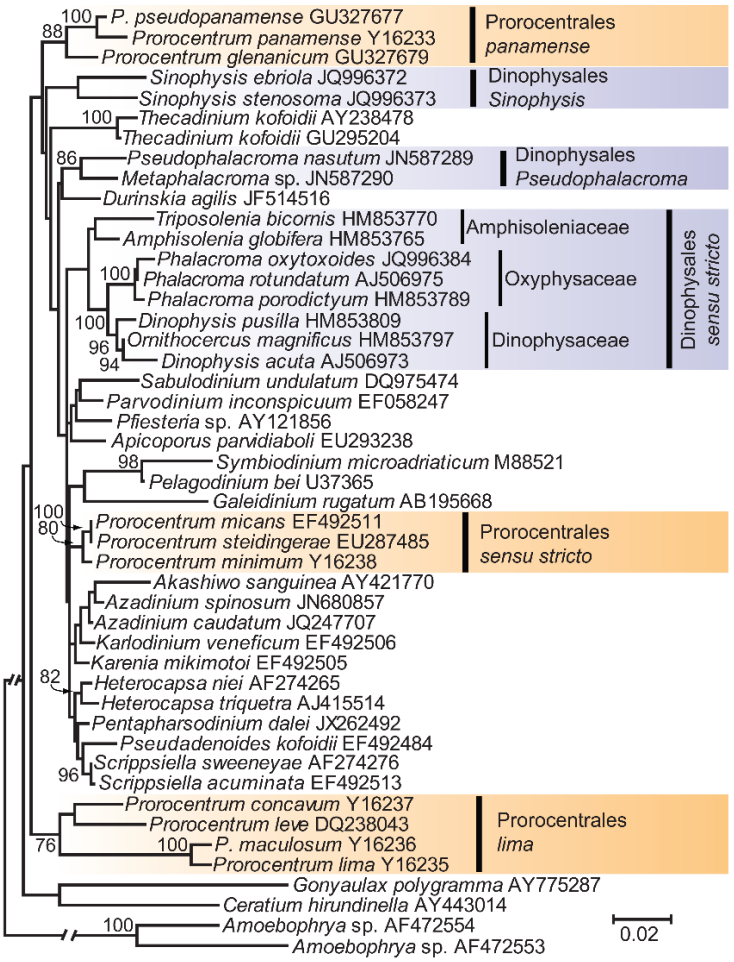

Figure 6. The phylogenetic position of dinoflagellates based on Maximum Likelihood analysis inferred from SSU rRNA gene sequences, with especial focus on prorocentroids and dinophysoids. Bootstrap values ≥70 are noted to the left of internodes. The basal dinoflagellate *Amoebophrya* was used as outgroup. Scale bar = 0.02 substitutions site^{-1}.

The dinophysoid dinoflagellates possess prorocentroid organization because the theca is fundamentally divisible into two halves by a sagittal suture, but they have a cingulum separating the epitheca and hypotheca, with the typical wavy flagellum that corresponds to the dinokont organization. Consequently, the dinophysoids could be regarded as morphologically intermediate between the desmokonts and dinokonts (Figure 7). In the rRNA

gene phylogenies, the dinophysoids cluster into three clades. The Dinophysales *sensu stricto* comprised the most specious and ecologically diverse group. The genus *Dinophysis* (Figure 7F) comprises numerous toxic species that are responsible of Diarrhetic Shellfish Poisoning. These species contains chloroplasts with a controversy on whether they are permanent or kleptoplastids that need a periodical replacement. Other species of *Dinophysis* in a more basal position are heterotrophic and have an antapical spine (Figure 7G). Several oceanic taxa such as *Citharistes* (Figure 7H), *Histioneis* (Figure 7I–J) and *Ornithocercus* (Figure 7K) survive to the prevailing oligotrophic conditions in circumtropical seas with ectosymbiotic bacteria in the 'phaeosome' chamber or attached to the cingular or sulcal lists. The family Oxyphysiaceae is highly diverse with most of the species placed in the genus *Phalacroma*, mainly composed of heterotrophic and some species with plastids of different origins (Figure 7L–O). The most basal group contains the highly elongated cells of *Triposolenia* (Figure 7P) and *Amphisolenia* (Figure 7Q) with a much reduced epitheca, and endosymbionts in numerous taxa. A second clade of dinophysoids contains the planktonic heterotrophic genera *Metaphalacroma* (Figure 7R) and *Pseudophalacroma* (Figure 7S), and the third clade contains the benthic heterotrophic species of *Sinophysis* (Figure 7T–U) that in some species may also harbor photosynthetic endosymbionts (Gómez et al. 2011b, 2012b).

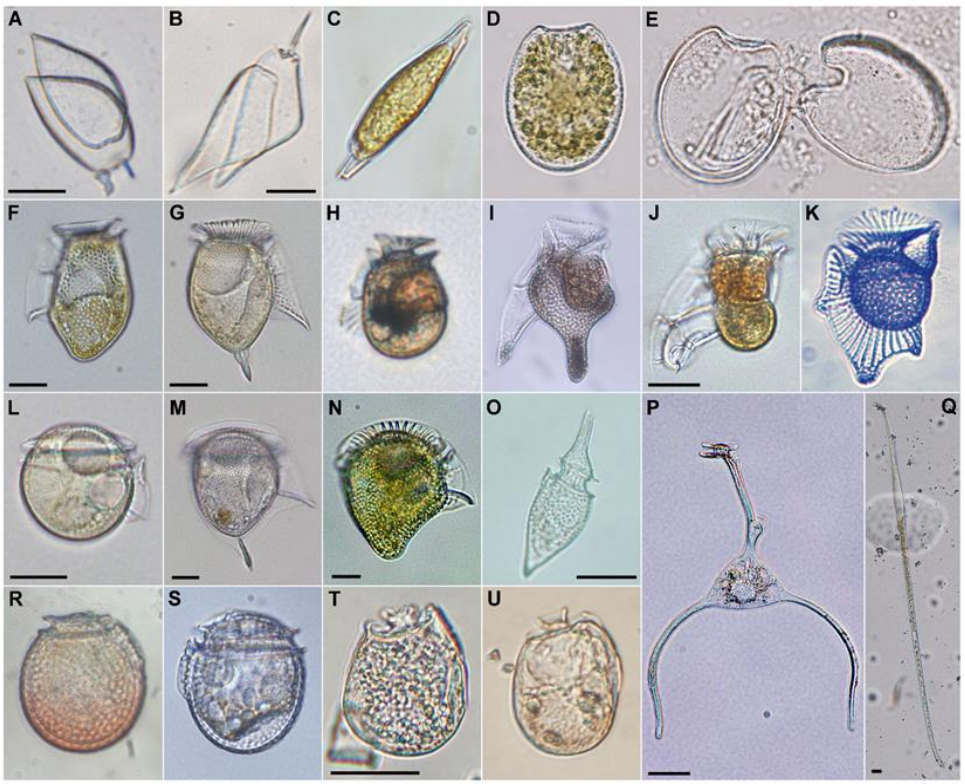

Figure 7. Light micrographs of thecate dinoflagellates: prorocentroids and dinophysoids.
A. *Prorocentrum micans* (dissociated valves). B. *P. gibbosum* (dissociated valves). C. *Prorocentrum rostratum*. D–E. *Prorocentrum* sp. (dissociated valves). Note the excavation of the right valve.
F. *Dinophysis acuta.* G. *D. alata*. H. *Citharistes*. I. *Histioneis highleyi*. J. *H. gubernans*.
K. *Ornithocercus*. L–O. *Phalacroma*. P. *Triposolenia*. Q. *Amphisolenia*. R. *Metaphalacroma*.
S. *Pseudophalacroma*. T–U. *Sinophysis*. Scale bar 20 μm.

Gonyaulacales

The most evolved dinoflagellates lack the two large valves, and the theca shows latitudinal series of plates (apical, anterior intercalary, precingular, cingular, postcingular, posterior intercalary, antapical) that reach a higher number of series >10 in the so-called thin-walled dinoflagellates. The number of plates is variable, and we can observe a high plate multiplication or fragmentation of plates in *Pyrophacus* (Figure 9L). The Gonyaulacales is the more recently proposed classical order, previously considered as a peridinioid family (Taylor 1980, Fensome et al. 1993). It should be noted that *Pyrocystis* is the type of the order Pyrocystales Apstein 1909 that was proposed before the Gonyaulacales Taylor 1980. The article 11.3 of the I. C. N. states that the correct name is the earliest legitimate, but from family to genus. The type genus of the Gonyaulacales is *Gonyaulax* that shows a marked asymmetry due to the left-handed torsion of the epitheca as well as an asymmetrical first apical plate (Figure 9M), while the Peridiniales tend to show a bilateral symmetry, with a more-or-less symmetrical first apical plate (Figure 11A). Among other characteristics, the Peridiniales have additional plates such as the canal plate near the apex that is missing in the gonyaulacoids (Fensome et al. 1993).

Up to date, the Gonyaulacales is the only classical major order that is monophyletic, although species such as *Peridiniella catenata* with a gonyaulacoid appearance clusters within the short-branching dinokaryotic core together with peridinioid taxa (Daugbjerg et al. 2000). In the molecular phylogenies, the rRNA gene sequences of the Gonyaulacales are characterized by long branches, especially for the genera *Pyrophacus*, *Ostreopsis* and *Gambierdiscus*. This problem is evident in the case of *Pyrophacus* and *Ostreopsis* that artificially cluster together due to the long-branch attraction artefact (Figure 8). If *Ostreopsis* is removed, *Pyrophacus* clusters with *Fragilidium* and *Pyrocystis*. The most diverse clade of gonyaulacoids includes *Centrodinium* (Figure 9A) that is a tropical open ocean form of the best known genera *Alexandrium*, *Gessnerium* and *Protogonyaulax* (Figure 9B). Contrary to all previous taxonomical schemes, Gómez (2012b) placed *Centrodinium* in the same subfamily of the toxicologically important genus *Alexandrium*. The morphological and molecular data of *Centrodinium punctactum* (Li et al. 2019), and typical species of *Centrodinium* such as *C. eminens* (Figure 8) evidended that *Alexandrium* is not monophyletic, and the need of the re-instatement of the genera *Protogonyaulax* and *Gessnerium*, and a new genus for the clade of *Alexandrium affine* (Gómez and Artigas 2019). Within the family Ostreopsidaceae, these planktonic genera are closely related to benthic genera also associated with harmful events such as *Coolia* (Figure 9C) and *Ostreopsis* (Figure 9D). Other clade contains the planktonic and benthic genera such as *Pyrrhotriadinium* (nom. illeg.) [formerly *Goniodoma* (nom. rejic.)] (Figure 9E), *Psammodinium* (Figure 9F), *Fukuyoa* (Figure 9G), *Gambierdiscus* (Figure 9H), and the more distantly related bioluminescent *Pyrodinium* (Figure 9I) that may be also placed in the Ostreopsidaceae or an independent family (Gómez et al. 2015b, Reñé and Hopperanth 2019). Family names such as Goniodomataceae or Triadiniaceae are not considered valid because they are based on a peridinioid type (Kretschmann et al. 2015). *Pyrocystis* (Figure 9J) is other bioluminescent genus closely related to genera with an exceptional multiplication/fragmentation of thecal plates such as *Fragilidium* (Figure 9K), and very especially *Pyrophacus* (Figure 9L).

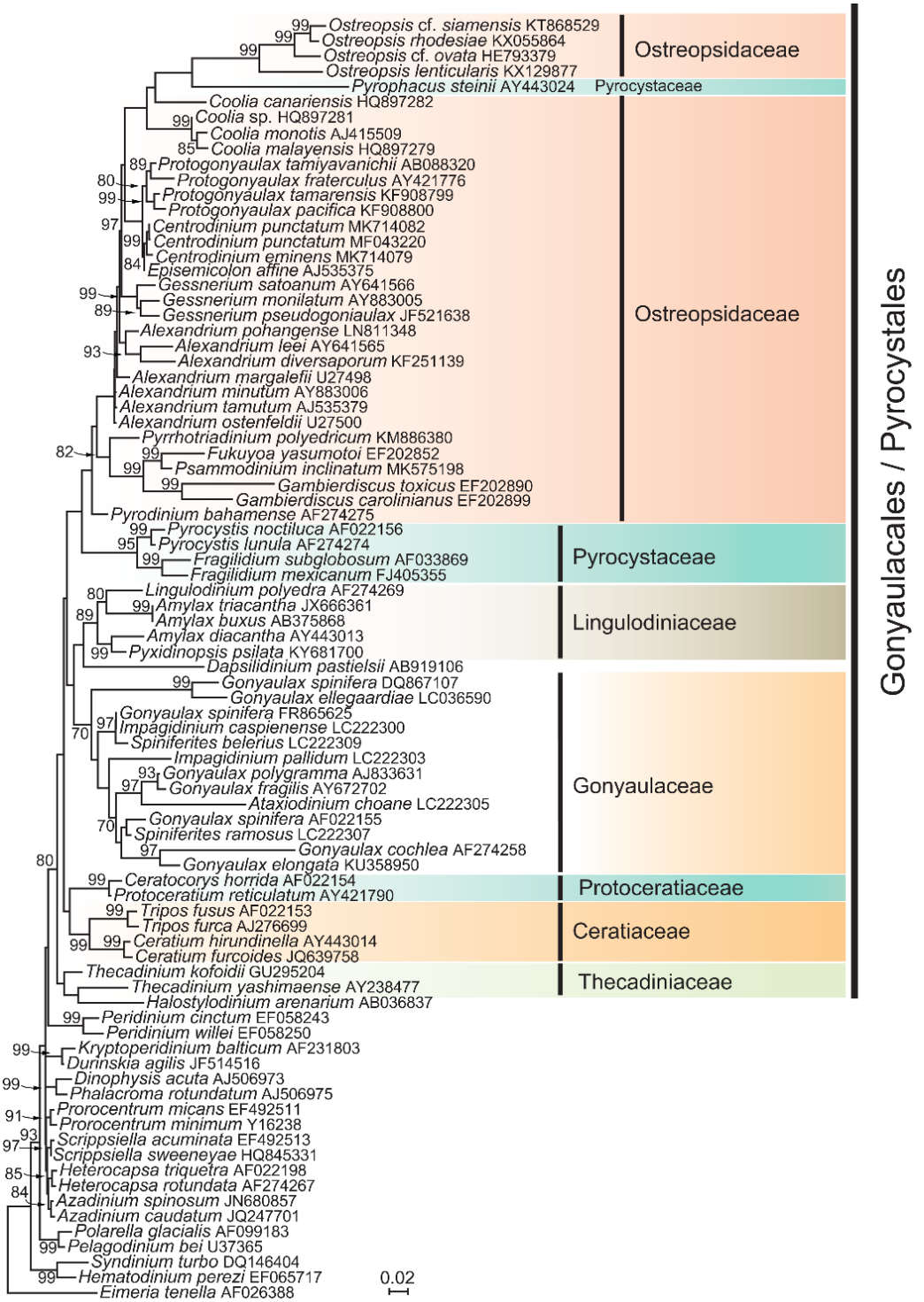

Figure 8. The phylogenetic position of dinoflagellates based on Maximum Likelihood analysis inferred from SSU rRNA gene sequences, with especial focus on gonyaulacoid dinoflagellates. Bootstrap values ≥70 are noted to the left of internodes. The apicomplexan *Eimeria* was used as outgroup. Scale bar = 0.02 substitutions site^{-1}.

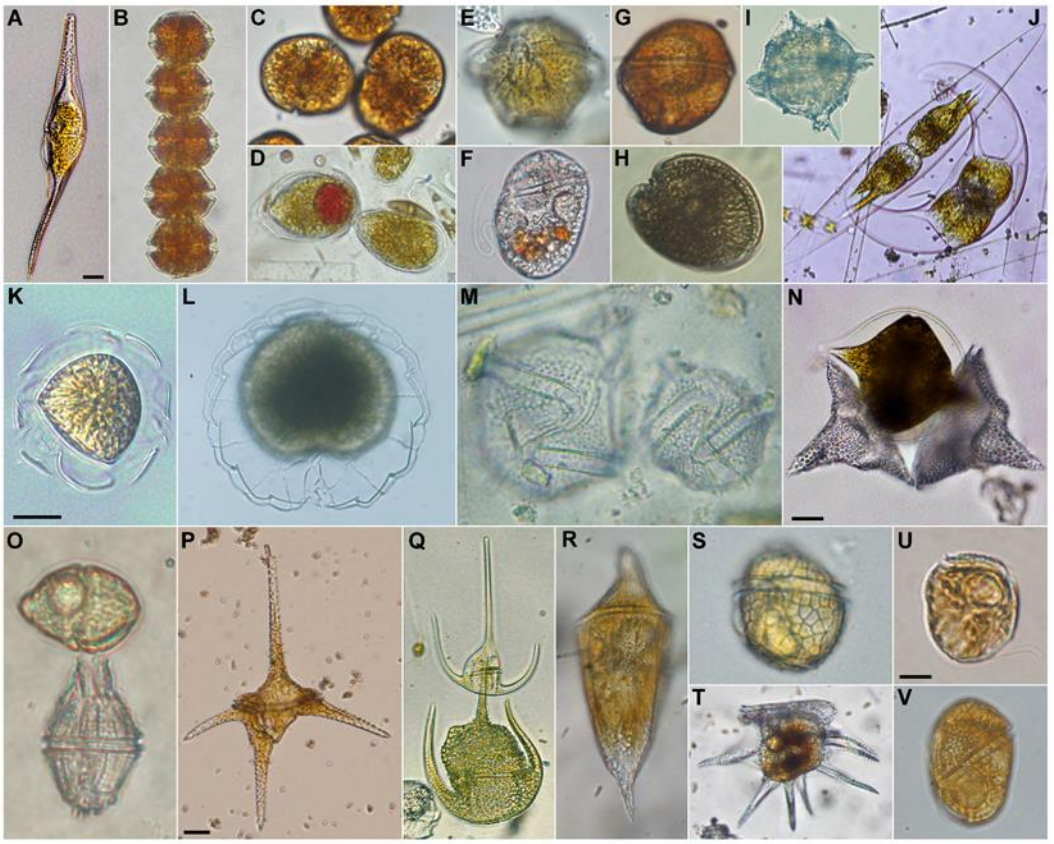

Figure 9. Light micrographs of gonyaulacoid dinoflagellates. A. *Centrodinium.* B. *Protogonyaulax*. C. *Coolia*. D. *Ostreopsis*. E. *Pyrrhotriadinium*. F. *Psammodinium* G. *Fukuyoa*. H. *Gambierdiscus*. I. *Pyrodinium*. J. *Pyrocystis*. K. *Fragilidium*. L. *Pyrophacus*. M. *Gonyaulax*. N. *Spiraulax* (*ecdysis).* O. *Lingulodinium* (ecdysis). P. *Ceratium*. Q. Two species of *Tripos*. R. *Schuettiella*. S. *Protoceratium*. T. *Ceratocorys*. U–V. Two species of *Thecadinium*. Scale bar 20 μm.

The second main group of gonyaulacoids contains the type *Gonyaulax* (Figure 9M), and *Spiraulax* (Figure 9N) and generic names based on fossils such as *Ataxiodinium*, *Bitectatodinium*, *Impagidinium* or *Spiniferites*. It should be noted that the fossil names have not priority if a name of an extant taxa is available (I. C. N. Art. 11.8). Other closely related clade is composed of *Lingulodinium* (Figure 9O) and *Amylax* that could be placed in the family Lingulodiniaceae or alternatively also placed in the Gonyaulacaceae (Figure 8). Based on the molecular data, other lineages are *Dapsilidinium*, the Ceraticeae for *Ceratium* (Figure 9P) and *Tripos* (Figure 9Q), and in a more divergent position the Protoceratidaceae with *Schuettiella* (Figure 9R), *Protoceratium* (Figure 9S), *Pentaplacodinium* and *Ceratocorys* (Figure 9T). The placement of the benthic genus *Thecadinium* (Figure 9U–V) among the Gonyaulacales is unstable (Figure 8). In some phylogenies, the genera *Amphidiniella* or *Halostylodinium* may cluster among the basal gonyaulacoids, but this is uncertain whether this reflects a real phylogenetic relationship or more likely an artefact of long-branch attraction as also occurred between *Protoperidinium* and the long-branch group of the Gonyaulacales.

Peridinioids

A more restricted tabulation scheme for the Peridiniales is an epitheca with seven precingular plates (7″), several anterior intercalary plates (1–3a), four apical plates (4′), and often additional plates such as the canal plate or ×-plate near the apical pore. This is the most extended scheme for planktonic peridinioids that shows a more or less globular cell shape with an equatorial cingulum. When the cell is compressed and the cingulum is more anterior, as usual in benthic forms, this pattern is altered and the interpretation of the tabulation is more difficult. The Peridiniales are polyphyletic, and we must restrict the Peridiniales *sensu stricto* to the clade that contains *Peridinium* (Figure 11A), a clade of freshwater photosynthetic taxa with few species (*P. cinctum*, *P. bipes*, *P. willei*), and tentatively we can add other freshwater genera such as Glochidinium, Palatinus, Parvodinium or Thompsodinium (Figure 10). The marine species of *Peridinium* were placed in the genus *Protoperidinium*, being the most specious genus that was inflated to over 200 described species (Figure 11B–D). Most of the species of *Protoperidinium* cluster together, with the exception of several species such as *P. depressum* (section Oceanica) that cluster with the members of the *Diplopsalis*-group (Figure 11E), the latter also divided into two groups (Figure 10). The members of the *Diplopsalis*-group show variations in the number of hypothecal and epithecal plates that have resulted on the description of numerous genera (*Diplopsalis*, *Gotoius*, *Huia*, *Niea*, *Preperidinium*, *Qia*, etc.). The nomenclature of the diplopsalid genera is also complicated because sometimes a genus has been proposed with different names under the Botanical and Zoological Codes of Nomenclature. Other species of *Protoperidinium* such as *P. americanum*, *P. monovellatum* and *Archaeperidinium* (*P. minutum*) cluster with benthic genera such as *Amphidiniopsis* (Figure 11F–G) and *Herdmania* (Figure 11H) that can be placed in the Amphidioniopsidaceae (Figure 10; Gómez et al. 2011a, Yamaguchi et al. 2011). All the members of the Protoperidiniaceae are heterotrophic forms known as pallium-feeders (Figure 11B), and at least one species, *Protoperidinium* cf. *diabolum* possesses photosynthetic endosymbionts with similar morphology to those in *Podolampas* (Figure 11I–J).

The shape of *Podolampas* (Figure 11I–J) reminds some species of *Protoperidinium*, but with an important difference because *Podolampas* and its relatives **(**Gaarderiella, Lissodinium, Mysticella, *Heterobractum*, *Blepharocysta*; Figure 11K) lack of a series of depressed or sunken plates that harbor the transversal flagellum that could be interpreted as the cingulum, although the transversal flagellum encircles the cell at the position where is expected to find the cingulum. As occurred with *Herdmania* or *Amphidiniopsis*, the molecular phylogenies have unexpectedly revealed a relationship of the pelagic podolampadaceans and the benthic genera *Roscoffia* (Saldarriaga et al. 2003b; Gómez et al. 2010b). The available sequences of the sand-dwelling dinoflagellate *Cabra* (Figure 11L) are highly divergent and it is not easily to confirm the affinity with the Podolampadaceae proposed by Yamaguchi et al. (2018). The sequences of other sand-dwelling genera such as *Planodinium* (Figure 11M), *Plagiodinium* and *Chrysodinium* have similarities with the Podolampadaceae, but this feature is poor supported in the molecular phylogenies (Figure 10, Gómez et al. 2019b). *Lessardia*, with a fusiform antero-posteriorly elongated cell (Figure 11N), was related to the Podolampadaceae in the earlier phylogenies (Saldarriaga et al. 2003b), but more complete phylogenies support the placement in its own family as suggested by Carbonell-Moore (2004). It is not common to find highly antero-posteriorly elongated cells in the planktonic

peridinioids such as in some species of *Oxytoxum* (Figure 11O), that together with *Corythodinium* (Figure 11P) conforms the family Oxytoxaceae (Gómez et al. 2016b).

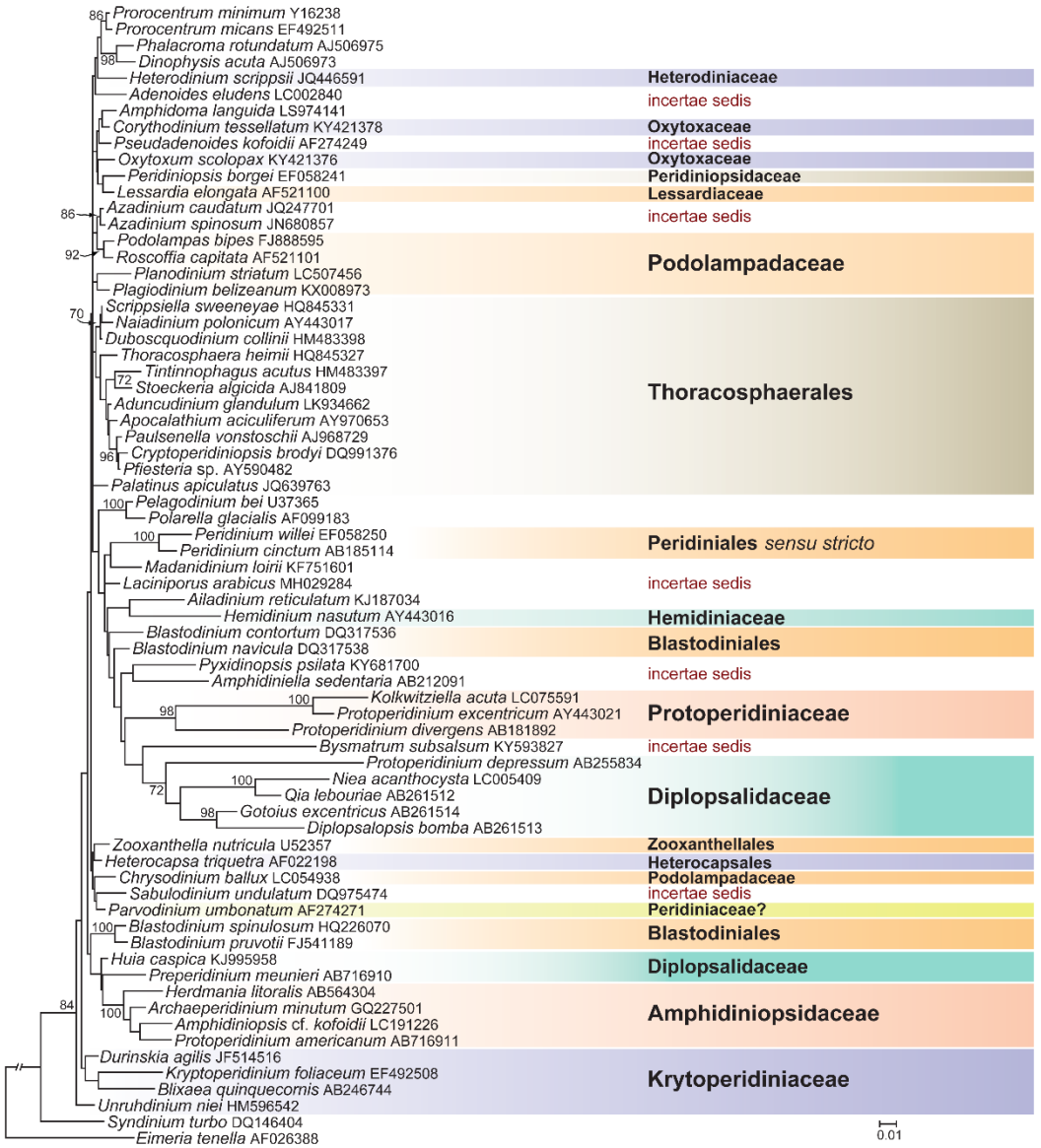

Figure 10. The phylogenetic position of dinoflagellates based on Maximum Likelihood analysis inferred from SSU rRNA gene sequences, with especial focus on peridinioid dinoflagellates. Bootstrap values ≥70 are noted to the left of internodes. The apicomplexan *Eimeria* was used as outgroup. Scale bar = 0.02 substitutions site^{-1}.

Other peridinioids with endosymbionts are the members of the Kryptoperidiniaceae, the so-called 'dinotoms' because they contains a diatom as endosymbiont (Yamada et al. 2017). Up to date this family remains unrepresented in the open ocean, all the known members live in coastal, brackish or freshwater environments such as the genera *Blixaea* (Figure 11Q), *Dinothrix*, *Durinskia* (Figure 11R), *Galeidinium*, *Kryptoperidinium* and *Unruhdinium*. One of

the most specious and ecologically diverse clade of peridinioid dinoflagellates are the members of the family of *Scrippsiella* (Figure 11S) that is able to produce calcareous cysts that have been described as different genera names (*Calcigonellum*, *Pernambugia*, *Leonella*, *Posoniella*). The whole family and order is placed in the Thoracosphaerales, based on *Thoracosphaera heimii* that was first described as a coccolithophorid. In addition to the free-living planktonic photosynthetic species (*Scrippsiella*), there are other planktonic forms **(**Apocalathium, Caladoa, *Calcicarpinum*, Chimonodinium, *Fusiperidinium*, *Laciniporus*, Naiadinium, Sperоidinium, *Stoeckeria*, Theleodinium, Tyrannodinium), and also heterotrophic benthic forms (*Aduncodinium*, Figure 11T). Many of new planktonic genera derived from freshwater taxa first described as species of *Peridinium*, *Peridiniopsis*, *Glenodinium* or *Glenodiniopsis*. An important group of species is the pfiesterids (*Pfiesteria*, *Pseudofiesteria*, *Luciella*, *Cryptoperidiniopsis*) (Figure 11U) that cause fish mortalities in estuaries (Steidinger et al. 1996). The Thoracosphaerales also includes parasitic forms, such as parasites of diatoms (*Paulsenella*, Figure 11V), parasites of ciliates (*Duboscquodinium*, *Tintinnophagus*, Figure 11W; Coats et al. 2010) and fishes (*Amyloodinium*, Figure 11X; Levy et al. 2007, Gómez and Gast 2018). In a more basal position of the Thoracosphaerales are marine species *Pentapharsodinium* and *Ensiculifera*, but the phylogenetic position is unstable.

In the classical taxonomic schemes, most of parasitic dinoflagellates were placed in the orders Syndiniales and Blastodiniales, placing the dinokaryotic parasites in the latter order. Currently, the Blastodiniales must be restricted to the clade that contains the type species of *Blastodinium*, *B. pruvotii*, and even the monophyly of the genus is not always evident (Figure 10, Skovgaard et al. 2007). Nearly all the species of *Blastodinium* contain chloroplasts and carry out the photosynthesis inside the copepod hosts (Figure 11Y). The members of the Heterodiniaceae are free-living heterotrophic species that live preferentially in deep waters of warm oceans, and the most distinctive species are characterized by highly flattened cell body as typical in other members of the 'shade flora' (Figure 11Z, Gómez et al. 2012a). The order Heterocapsales was proposed for the genus *Heterocapsa* (Figure 11AA; Fensome et al. 1993). Based on the combination of several genes, *Heterocapsa* was placed as a basal dinoflagellate, a sister group of *Amphidinium* (Zhang et al. 2007), but recent analyses based on dinoflagellate transcriptomes placed *Heterocapsa* within the Peridiniales *sensu lato* (Janouškovec et al. 2017).

The nomenclatural issues also affect *Symbiodinium*, a dinoflagellate genus with a high ecological interest for the functioning of the coral reefs that have recently split in several genera (LaJeunesse et al. 2018). The symbionts of the coral reefs invertebrates are commonly referred as zooxanthellae, and this is source of confusion with the genus name *Zooxanthella*. The type of the genus *Zooxanthella*, *Z. nutricula*, is a symbiont of pelagic radiolarians such as *Collozoum* (Figure 12A; Gast and Caron 1996). *Zooxanthella nutricula* is a thecate dinoflagellate, and it was unnecessarily transferred to *Scrippsiella* and *Brandtodinium* (nom. illeg.) (Probert et al. 2014). Planktonic radiolarians may also contain an unarmored dinoflagellate symbiont such as *Gymnoxanthella* (Figure 3V). The genus *Zooxanthella* has been placed among the Thoracosphaerales, but this is not well supported in the molecular phylogenies (Figure 10). In that case, the order Zooxanthellales proposed in 1970 and Thorascophaerales in 1982 may have the priority, although the article 11.3 of the I. C. N. states that the correct name is the earliest legitimate, but from family to genus. The type species of *Symbiodinium* was already transferred into *Zooxanthella* (Loeblich and Sherley 1979), and more recently all the species of *Symbiodinium* have been placed into *Zooxanthella*

(Guiry and Andersen 2018). *Zooxanthella* is a thecate genus that lives in pelagic radiolarians (Figure 12A), and *Symbiodinium* is a thin-walled dinoflagellate that live in benthic invertebrates (Figure 12M). The species of these unrelated genera are placed together (Guiry and Andersen 2018) although there is no a morphological or phylogenetic relationship (Figure 13). The parasites of fishes *Amyloodinium* (Figure 11V) and *Piscinoodinium* have been placed in the family Oodiniaceae (Cachon and Cachon 1987, Fensome et al. 1993), but they are members of the order Thoracosphaerales and Symbiodiniales, respectively (Levy et al. 2007). The Oodiniaceae is restricted to the type *Oodinium*, an ectoparasite of appendicularians (Figure 12B, Gómez and Skovgaard 2015). The rRNA gene sequences of *Oodinium*, as occurred with other parasites, have long branches in the phylogenetic trees, which resulted in unstable topologies, often clustering in basal positions.

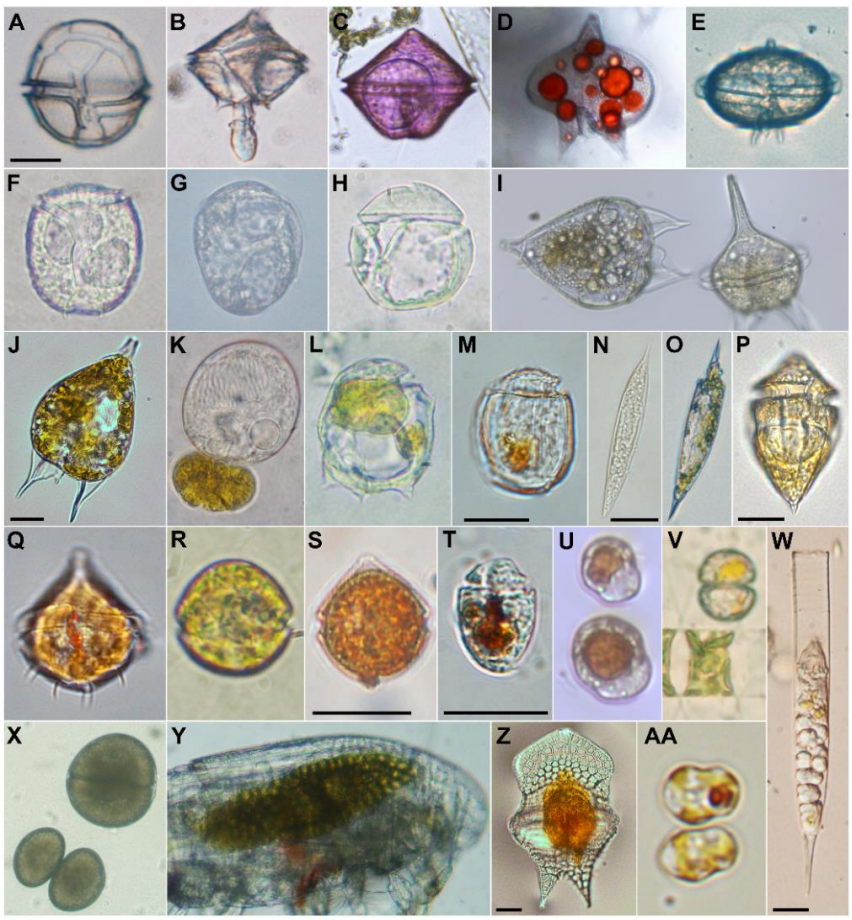

Figure 11. Light micrographs of peridinioid dinoflagellates. A. *Peridinium*. B–D. *Protoperidinium.* B. See the pallium, a feeding organelle. C. Purple pigmentation. D. Carotenoid globules. E. A member of the *Diplopsalis*-group. F–G. *Amphidiniopsis* spp. H. *Herdmania* with hyposomal spines. I. *Podolampas bipes* and *Protoperidinium* cf. *diabolum* with the same unidentified photosynthetic endosymbiont. J. *Podolampas bipes*. K. *Blepharocysta* feeding on a microalga. L. *Cabra*. M. *Planodinium*. N. *Lessardia*. O. *Oxytoxum*. P. *Corythodinium.* Q. *Blixaea*. R. *Durinskia*. S. *Scrippsiella*. T. *Aduncodinium*. U. Unidentified pfiesterid species. V. *Paulsenella*. W. *Tintinnophagus*. X. *Amyloodinium.* Y. *Blastodinium.* Z. *Heterodinium.* AA. *Heterocapsa*. Scale bar = 20 μm.

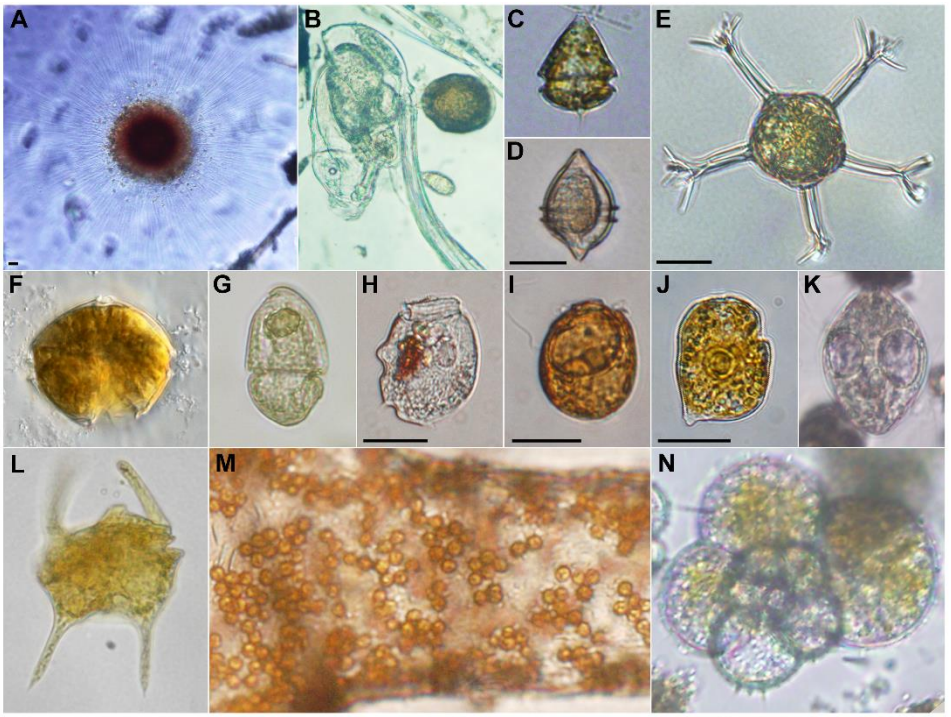

Figure 12. Light micrographs of peridinioid and thin-walled dinoflagellates. A. *Zooxanthella nutricula*. B. *Oodinium*. C. *Azadinium caudatum*. D. *Amphidoma nucula*. E. *Cladopyxis*. F. *Bysmatrum*. G. *Rhinodinium*. H. *Sabulodinium*. I. *Pseudadenoides*. J. *Adenoides*. K. *Thaumatodinium*. L. Undescribed thecate dinoflagellate. M–N. *Symbiodinium s.l.* Scale bar = 20 μm.

Since the proposal of the genus *Azadinium* (Tillmann et al. 2009), more than 10 new species have been added. The species *Amphidoma caudata* (Figure 12C) was transferred into *Azadinium*, and more recently new of species of *Amphidoma* have been proposed, placing *Amphidoma* and *Azadinium* in the Amphidomataceae. A genus and family is defined by the type species, and *Amphidoma nucula* (Figure 12D) has not been investigated by modern methods. Although *A. nucula* and *A. caudata* showed a superficial resemblance (Figure 12C–D), they are unrelated species, and *Azadinium* and the recently described species of *Amphidoma* do not belong to the Amphidomataceae. The members of the Cladopyxiaceae have been classified among the Gonyaulacales (Fensome et al. 1993). Molecular data are missing of genus such as *Clapopyxis* (Figure 12E) with abundant information in the fossil records. Numerous genera of thecate dinoflagellates cluster as independent lineages within the dinokaryotic core, especially benthic dinoflagellates. Clear affinities with pelagic genera have been found for *Amphidiniopsis*, *Herdmania* or *Roscoffia*, but numerous benthic genera cluster as independent lineages of uncertain suprageneric classification (Figure 10). Some species have more typical peridinioid morphology with an median cingulum such as *Bysmatrum* (Figure 12F) and *Rhinodinium* (Figure 12G), while other have an anterior cingulum that remind the dinophysoids such as *Sabulodinium* (Figure 12H), and a very reduced episome (*Pseudadenoides*, Figure 12I), and without an apparent cingulum such as *Adenoides* (Figure 12J) that reminds the prorocentroids. Other thecate dinoflagellates, even with distinctive morphology, remain unreported since the original description such as *Thaumatodinium* (Figure 12K) or undescribed (Figure 12L).

Thin-Walled Dinoflagellates

A thecate life stage has been reported in the life cycle of the unarmored dinoflagellate *Margalefidinium polykrikoides* (Kim et al. 2007), but this supposed thecate life stage is a misidentification for an *Alexandrium* species. It is common to observe how armoured dinoflagellates leave behind the theca and continue as unarmored forms (i.e., Figure 9N–O), but from a phylogenetic point of view armoured and unarmoured dinoflagellates are distinct groups. At a first sight, the so-called 'thin-walled' dinoflagellates could be considered as intermediate between unarmored and thecate dinoflagellates, and consequently showing the characteristics of the hypothetical ancestor of the thecate dinoflagellates. The recent transcriptome phylogenies suggest that the thin-walled dinoflagellates such as *Symbiodinium* have evolved from thecate dinoflagellates (Janouškovec et al. 2017). This could be interpreted as that the thin-walled dinoflagellates are thecate dinoflagellates that have reduced the thickness of the thecal plates, and the plate multiplication or fragmentation resulted in numerous latitudinal plate series. The proliferation of plates also occurs in other thecate dinoflagellates such as *Pyrophacus* (Figure 9L). If the thecal plates are considered as defensive structures, thick thecal plates are not necessary when living as an endosymbiont, but not all the genera of the thin-walled dinoflagellates are endosymbionts. The thin-walled dinoflagellates are not a monophyletic group (Figure 13), suggesting that the loss of the thick thecal plates that are replaced by more numerous delicate plates have occurred independently several times in the evolution.

The symbiotic *Symbiodinium* and its recently derived new genera are essential for the life of the coral reefs (LaJenneuse et al. 2018). It has been intensively investigated by ecologists, more interested on aspects as the host specificity than in the morphological differences among the species. *Symbiodinium* has been recently split into several genera (*Breviolum*, *Durusdinium*, *Effrenium*, *Fugacium*, *Gerakladium*; Figures 12M–N) based on molecular data (Figure 13) and ecological aspects such as the type of host or geographical information, and missing information on the morphology such as the plate arrangement in the generic diagnoses (LaJeunesse et al. 2018). The ecologists do not always know or respect the rules of the Nomenclature, and the species of *Symbiodinium* have experienced a convulse history of typifications and validations, and finally the generic name *Symbiodinium* have been attributed to three different authorities. The recent proposal by Guiry and Andersen (2018) placing all the species of *Symbiodinium* into the distantly related thecate genus *Zooxanthella* is other source of confusion. The family Symbiodiniceae also contains *Pelagodinium*, a symbiont of pelagic Foraminifera, parasites of fishes such as Haidadinium and Piscinoodinium, free-living species such as *Polarella*, *Protodinium* and numerous new genera have been recently added (*Ansanella*, *Asulcocephallum*, *Biecheleria*, *Biecheleriopsis*, *Dactylodinium*, *Leiocephalium*, *Yihiella*). This clade is often reported as the Suessiaceae, based on a fossil genus *Suessia*. The article 11.8 of the International Code of Nomenclature (I. C. N) reports that fossil type has no priority over names of extant species, and at the family level the use of Symbiodiniaceae seems to be more coherent because we cannot verify with molecular data the relationship of these taxa with the extinct *Suessia*. Other thin-walled dinoflagellate clades are mainly freshwater taxa placed in recently erected families as the Borghiellaceae (*Baldinia*, *Borghiella*) and the Tovelliaceae/Tovelliales (*Bernadinium*, *Esoptrodinium*, *Jadwigia*, *Tovellia*) (Moestrup and Calado 2018). Nearly all these families and orders are based on species described from accessible localities in Europe, but new names that can be superfluos

are proposed without a re-investigation of the type of other existing suprageneric names (Cystodiniaceae, Desmomastigales, Dinamoebidiales, Dinamoebales, Dinococcales, Dinotrichales, Dinosphaeraceae, Hemidiniaceae, Glenodiniopsidaceae, Lophodiniales, Phytodiniales, Woloszynskiaceae). The thin-walled dinoflagellates are often named woloszynskioid dinoflagellates, being based on species that were previously described as *Woloszynskia*, *Gymnodinium* or *Glenodinium*. New genera, families and even orders are proposed without a re-investigation of the genus *Woloszynskia*, type of the family *Woloszynskiaceae*.

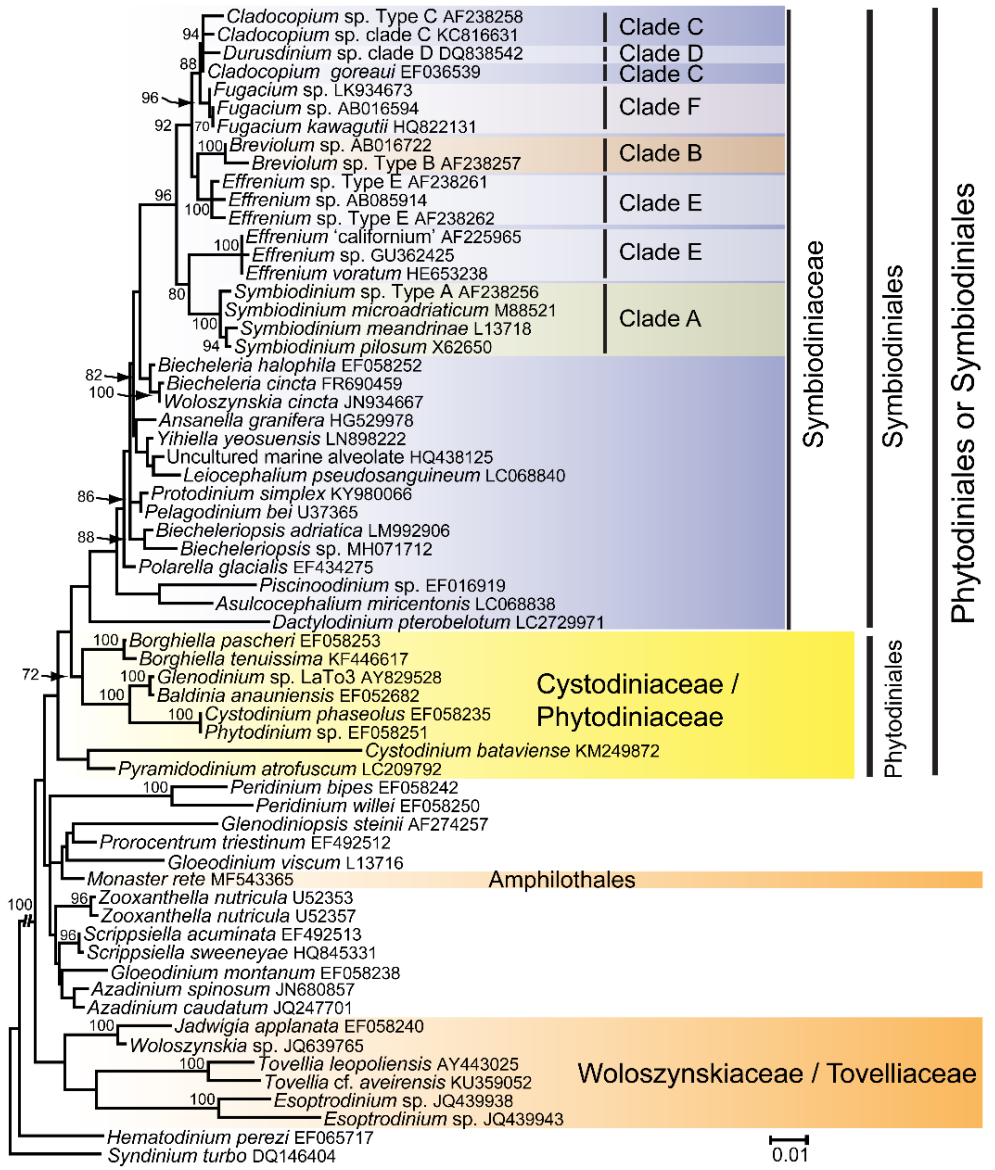

Figure 13. The phylogenetic position of dinoflagellates based on Maximum Likelihood analysis inferred from SSU rRNA gene sequences, with especial focus on thin-walled dinoflagellates. Bootstrap values ≥70 are noted to the left of internodes. The syndinean *Syndinium* was used as outgroup. Scale bar = 0.01 substitutions site^{-1}.

CURRENT ISSUES

The most important advances in our knowledge of the dinoflagellate classification are due to the molecular phylogeny that has allowed to test the hypotheses in the evolution and the morphological characters of diagnostic value. Since the surprising relationship between dinoflagellates and human parasites such as the apicomplexans (Gunderson et al. 1987), the molecular phylogenies have modified the diagnostic criteria used for the classification (Saldarriaga et al. 2004). The examples are evident in the unarmored dinoflagellates with split of the genera *Gymnodinium*, *Amphidinium* and *Cochlodinium* (Daugbjerg et al. 2000, Jørgensen et al. 2004a, Gómez et al. 2017a). Among the thecate dinoflagellates, the tabulation is considered a stable diagnostic character, but again we fail in the interpretation of the tabulation when the species have lost the typical globular morphology with an equatorial cingulum of the pelagic species. The cell compression and the small epitheca, as usually occurred in the benthic species, is associated with a re-arrangement of the thecal plates. Only with the aid of the molecular phylogeny, we are able to relate *Roscoffia* and *Podolampas* (Saldarriaga et al. 2003b, Gómez et al. 2010b) or *Herdmania* and *Amphidiniopsis* with species of *Protoperidinium* (Gómez et al. 2011a, Yamaguchi et al. 2011). The use of molecular markers (SSU-, LSU- and ITS rRNA genes) is unable to resolve the evolution and suprageneric classification of dinoflagellates, although the resolution is slightly better in a few groups (Gymnodiniales *sensu stricto*, Thoracosphaerales, Gonyaulacales). Sequences of transcriptomes are a promising tool to resolve the deep branching relationships, but at the present the number of available sequences of different species is low (Janouškovec et al. 2017). As usual, the application of the new tools shows a strong bias towards the cultivable species or easily accessible coastal species (Gómez 2014). The open ocean is mainly studied with techniques such as the metabarcoding (Le Bescot et al. 2016). These short environmental sequences allow a view of the relative abundance of groups of dinoflagellates, but the studies are not accompanied with microscopic observations. The microscope is an instrument to banish together with the taxonomists. Research and academic positions are offered for molecular biology and bioinformatics. Beyond the research groups focused on the monitoring of the harmful algae blooms, the general taxonomy of dinoflagellates persists in a few groups in Denmark, Germany and Japan, and as it occurs in other research fields such as the ciliates with an important increase of the taxonomical studies in China.

There are no significant advances on several topics. The molecular phylogeny reveals a huge diversity of syndinean dinoflagellates (Guillou et al. 2008), but in the last decade there is no advance because the sequences are not identified at the species level, and the syndineans remains restricted to five genera. Other tentative syndinean genera such as *Actinodinium*, *Atlanticellodinium*, *Caryotoma*, *Coccidinium*, *Dogelodinium*, *Keppenodinium*, *Merodinium* or *Sphaeripara* do not receive the amount of attention that they deserve. Coats et al. (2012) proposed the transfer of the species associated with the sequences previously identified as *Duboscquella* in the new genus *Eduboscquella*, but they did not solve the identity of the dinokaryotic genus *Duboscquella*. Coats et al. (2012) proposed the family *Eduboscquellaceae*, but this clade of *Eduboscquella* and *Ichthyodinium* needs to be ranked at least at the order level. We can use the order Coccidinales following Cachon and Cachon (1987), although cautiously because *Coccidinium* has not been investigated by modern methods. Our knowledge on the phylogeny of Noctilucales remains limited to the few partial

sequences in Gómez et al. (2010a). It is uncertain if the leptodiscaceans are really basal dinoflagellates or they should be placed among the dinokaryotic dinoflagellates (Figure 1). The nature of chloroplasts of *Spatulodinium* or the endosymbionts of *Pomatodinium* remain unknown.

In the last two decades, there is an increase of the descriptions of new taxa, mainly of unarmored and thin-walled dinoflagellates, while numerous species described by Wołoszyńska, Skvortsov, van Goor, van Meel, Conrad & Kufferath, or Schiller remains in most of the cases restricted to the original descriptions (Thessen et al. 2012). The proposals of new genera, often based on a single species, have largely increased, revealing the inability to relate the morphology of the new taxa with other existing species. In other cases, superflous new genus names are proposed without molecular support such as the case of Torquentidium (Shin et al. 2019). Several attempts have tried to propose new genus names for species such as *Gymnodinium catenatum* and other species of *Gymnodinium* just only based on the distance in the molecular phylogenies to the type species *G. fuscum*. The question could be the reverse, why to place all the species of the Gymnodiniaceae into *Gymnodinium*? Similarly, we could place the species of the genera Alexandrium, Episemicolon, Gessnerium and Protogonyaulax into *Centrodinium*. On the other hand, numerous benthic species of *Prorocentrum* have been described in the last two decades, but many of them should be placed into another genus because they are distantly related to the type species, the spine-bearing planktonic *Prorocentrum micans*. Sometimes, we are splitters in the Gymnodiniaceae, *Alexandrium*/*Centrodinium* or the Pfiesteriaceae (*Pfiesteria*, *Pseudofiesteria*, *Luciella*, *Cryptoperidiniopsis*), while lumpers in *Prorocentrum*. This is the dilemma of being a splitter or a lumper taxonomist.

New species are described from coastal isolates or germination of cysts that are able to grow well with the standard culture media. In contrast, most of the oceanic species are uncultivable with the current methods, and the studies must be based on the few cells available. This paucity of material renders the detailed studies that are not comparable to the morphological, ultrastructural and molecular studies when billions of cells are available in a culture. Unfortunately, this limitation is not well understood, and the requirements for publication discourage researchers to study the heterotrophic species that predominates in the open ocean (Gómez 2014). It is embarrassing that we have not solved classical issues such as whether *Pronoctiluca* is or is not a dinoflagellate and the molecular affiliation of *Cladopyxis*.

In addition to the excessive proliferation of new genus names (i.e., *Torquentidium*), there is an increase of the proposal of suprageneric names, families and orders, based on the characteristics of a single genus. If we are unable to find relatives based on the ribosomal RNA gene sequences, it does not mean that each taxon constitutes a new family or even order. Moestrup and Calado (2018) proposed new families (Gyrodiniaceae, Amphidiniaceae, Sphaerodiniaceae) or even an order (Amphidiniales) based on a single genus. If this practice extends, new suprageneric names will appear everywhere. Several new families or even orders of freshwater taxa have been proposed such as Borghiellaceae, Sphaerodiniaceae or Tovelliales (Moestrup and Calado 2018). Tens of new taxa of the so-called ‘woloszynskiods’ are proposed without a study of the identity of *Woloszynskia*. The detail of the earlier species descriptions is not comparable to the current description, but we have the option of the typification.

The consideration of the earlier descriptions is important, but sometimes they have scarce detail that may result in subjective interpretations. Gottschling and collaborators have recently re-interpreted the identity of species illustrated by Ehrenberg in the 1830’s that are the

basionyms of the Stein's genera *Goniodoma*, *Heterocapsa* or *Blepharocysta* (Stein 1883). Proposals appears such as the consideration that the description of *Peridinium splendormaris* in the Mediterranean Sea, basionym of the type of the genus *Blepharocysta*, corresponds to an earlier description of *Alexandrium balechii*, a species that lives in the mangroves of the Caribbean Sea. Ehrenberg's *Peridinium splendormaris* looks like *Lingulodinium polyedra*, a common bioluminescent dinoflagellate in the Mediterranean Sea. Gottschling et al. (2019) considered that the basionym of the type species of *Heterocapsa*, *Peridinium triquetrum*, is a species of the genus *Kryptoperidinium*. There is no a phylogenetic relation between *Kryptoperidinium* and the species that we know as *Heterocapsa* (Figure 10). Then, the genus, family and order of Heterocapsales is dismantled when the type is placed in another genus. Kretschmann et al. (2015) re-interpreted that the basionym of the type of *Goniodoma* is a peridinioid cell of the genus *Scrippsiella* instead of a gonyaulacoid dinoflagellate. The genus named *Goniodoma* is rejected, as well as related families such as Goniodomatadaceae or Triadiniaceae (Prud'homme van Reine 2017), without reporting an alternative because *Pyrrhotriadinium* is an illegitimate name. The changes may not stop here. The most common species of *Scrippsiella*, *S. trochoidea*, is now *S. acuminata* (Kretschmann et al. 2015). In the molecular phylogenies, *Duboscquodinium* is very closely related to the type species of *Scrippsiella*, *S. sweeneyae* (Figure 10). In case of congenerity, *Duboscquodinium* Grassé 1952 has the priority over *Scrippsiella* Balech 1959. There is no molecular data of *Duboscquella*, type genus of the Duboscquellaceae, which could be closely related to *Duboscquodinium*. Then, the family name Duboscquellaceae has the priority over the Thoracosphaeraceae. As consequence of nomenclatural changes, the free-living genus *Goniodoma* traditionaly used for gonyaulacoid dinoflagellates is a posterior synonym of a peridinioid parasite of ciliates. Other studies are not good examples of the observance of the recommendations in the Nomenclature. Gottschling et al. (2017) proposed *Blixaea* and *Unruhdinium* for species of the family Kryptoperidiniaceae, when other tentative related taxa such as *Dinothrix* have not been investigated. According the Recommendation 20A of the I. C. N.: "Not dedicate genera to persons quite unconnected with botany, mycology, phycology, or natural science in general". *Blixaea* and *Unruhdinium* are in honor of musicians. At least, I will prefer the use of the orthography '*Unruhidinium*'. Based on an inexistent proposal to reject the name *Zooxanthella*, Probert et al. (2014) proposed the new name *Brandtodinium* to replace *Zooxanthella*, the thecate endosymbiont of pelagic radiolarians. The conflicts on nomenclature must be solved by the Committee for Algae of International Association for Plant Taxonomy. Most of the members of the Committee that vote the recommendation or rejection of proposals are not experts on taxonomy of dinoflagellates and decision remains in the hands of a few people. The decision to recommend or to reject a proposal may delay for several years being associated with uncertainty during that period. John et al. (2014) proposed to reject the *Gonyaulax catenella*, basionym of an important toxicologically species of *Protogonyaulax*. In the decision, Prud'homme van Reine (2017) reported "*Alexandrium fundyense* and *A. catenella* are certainly conspecific, and then 'catenella' has nomenclatural priority". Is it really the same species responsible of the blooms in the Canadian Atlantic and California? Members of the Committee placed all the species of *Symbiodinium* into *Zooxanthella* (Guiry and Andersen 2018), when the morphology and molecular phylogeny evidence that *Zooxanthella* and *Symbiodinium* are independent genera (Figure 13).

CLASSIFICATION

The names that we use for the classification of dinoflagellates are in continuous evolution due to the new taxonomical innovations, re-classifications based on the molecular data, and the nomenclatural changes. There is nothing more risky for an author than to propose a suprageneric classification when the evolutionary relationships of numerous dinoflagellates remain unresolved yet. With the exception of some groups (Gymnodiniales *sensu stricto*, Thoracosphaerales, Gonyaulacales), the words *-incertae sedis-* are everywhere, and we cannot propose a new family or order for each independent lineage of dinoflagellates. A principle that I follow in the classifications is to avoid adding taxonomical innovations, or suprageneric new names and using the existing published names (Fensome et al. 1993). New terminology can be found in other classifications (Hoppenrath 2017). The next classification is an updated version of that in Gómez (2012b), but in this case from genus to class because the citation of all species names needs more than 100 pages. A tentative scheme that summarises the dinoflagellate evolution is proposed in the Figure 14.

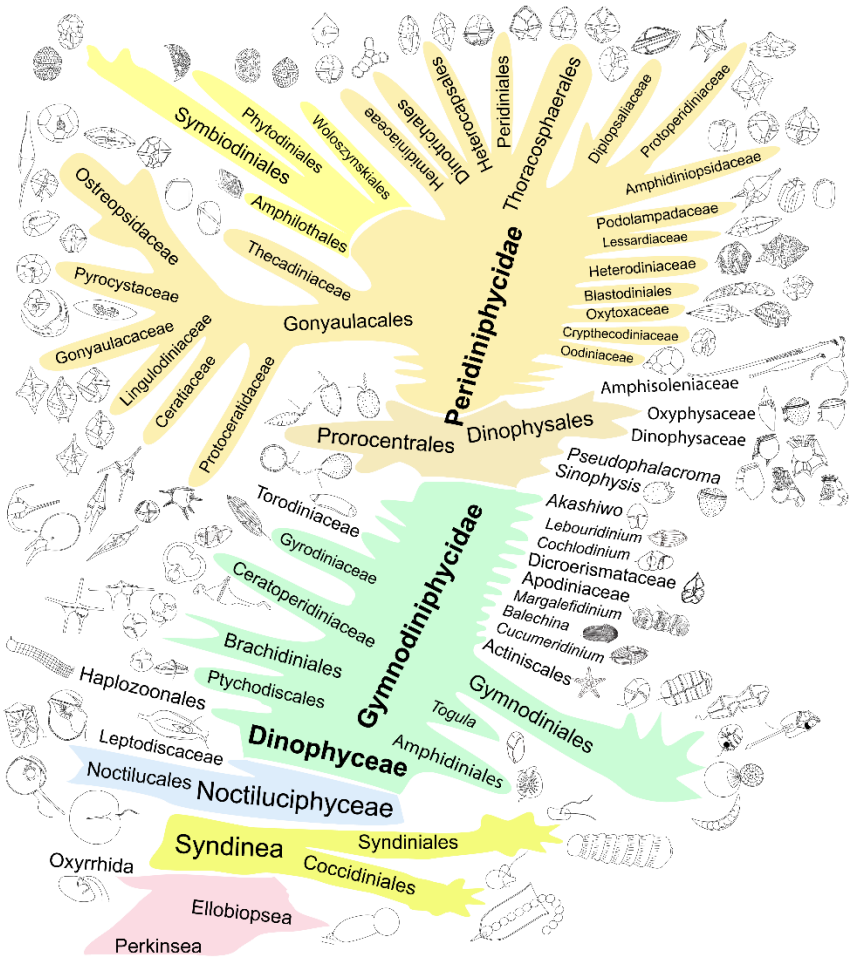

Figure 14. Tentative evolutionary relationships of dinoflagellates.

Infraregnum Alveolata, Phyllum Dinoflagellata

Class Ellobiopsea / Ellobiophyceae

Order Thalassomycetales: Ellobiopsidae / Thalassomycetaceae: Ellobiocystis, Ellobiopsis, Parallobiopsis, Rhizellobiopsis, Thalassomyces

Class Oxyrrhea

Order Oxyrrhida / Oxyrrhinales

Oxyrrhinaceae: Oxyrrhis

Class of Pronoctiluca, incertae sedis. The affinity with the dinoflagellates needs molecular data.

Protodiniferaceae: Pronoctiluca

Class of Coccinidiales (Marine Alveolate Group I)

Order Coccinidiales: Coccidinium, Dogelodinium, Eduboscquella, Ichthyodinium, Keppenodinium

Class Syndinea/Syndiniophyceae (Marine Alveolate Group II)

Order Syndiniales

Syndiniaceae: Actinodinium, Caryotoma, Merodinium, Syndinium, Hematodinium

Sphaeriparaceae: Atlanticellodinium, Sphaeripara

Amoebophryidae / Amoebophryaceae: Amoebophrya

Class Noctilucea / Noctiluciphyceae (Dinokaryota)

Order Noctilucales

Noctilucaceae: Noctiluca, Pomatodinium, Spatulodinium

Kofoidiniaceae: Kofoidinium

Order of Leptodiscaceae

Leptodiscaceae: Abedinium, Cachonodinium, Craspedotella, Leptodiscus, Petalodinium, Scaphodinium

Class Dinoflagellata / Dinophyceae (Dinokaryota)

Subclass Gymnodiniphycidae

Order Amphidiniales

Amphidiniaceae: Amphidinium s.s., Schillingia, Trochodinium

Order Brachidiniales

Brachidiniaceae: Asterodinium, Brachidinium, Gertia, Karenia, Karlodinium, Microceratium, Takayama

Order Ptychodiscales

Ptychodiscaceae: Ptychodiscus

Order Haplozooidea / Haplozoonales

Haplozoonaceae: Haplozoon

Order Gymnodiniales

Gymnodiniaceae: Barrufeta, Gymnodinium, Gymnoxantella, Lepidodinium, Paragymnodinium, Polykrikos, Spiniferodinium, Nussuttodinium, Pellucidodinium, Wangodinium

Chytriodiniaceae: Chytriodinium, Dissodinium, Myxodinium, Schizochytriodinium, Syltodinium, Gymnodinium s.l.

Warnowiaceae: Erythropsidinium, Nematodinium, Nematopsides, Proterythropsis, Warnowia

Gymnodiniales sensu stricto, incertae familiae: *Gyrodiniellum*, Pheopolykrikos

Torodiniales
Torodiniaceae: *Torodinium*
Family of *Lebouridinium*: *Lebouridinium*
Order Amphilothales
Amphilothaceae: Monaster
Order Actiniscales
Actiniscaceae: Actiniscus
Subclass Gymnodiniphycidae, incerti ordinis.
Dicroerismataceae: Dicroerisma
Apodiniaceae: Apodinium, Parapodinium
Ceratoperidiniaceae: Ceratoperidinium, Gynogonadinium, Kirithra, Pseliodinium
Kapelodiniaceae: Kapelodinium
Gyrodiniaceae: Ceratodinium, Gyrodinium, Plectodinium, Sclerodinium
Gymnodiniphycidae, incerti ordinis, incertae familiae: Akashiwo, Ankistrodinium, Apicoporus, Balechina, Bispinodinium, Cachonella, Cochlodinium, *Cucumeridinium*, *Filodinium*, *Grammatodinium*, Levanderina, Katodinium, *Margalefidinium*, Moestrupia, Testudodinium, Togula
Subclass Peridiniphycidae
Order Prorocentrales
Haplodiniaceae: Haplodinium
Prorocentraceae: Prorocentrum s.s., Mesoporos
Family of Prorocentrum lima: Exuviaella, Prorocentrum s.l.
Family of Prorocentrum panamense: Genus of Prorocentrum panamense
Order Dinophysales sensu stricto
Amphisoleniaceae: Amphisolenia, Triposolenia
Oxyphysaceae: Dinofurcula, Latifascia, Phalacroma, Proheteroschisma
Dinophysaceae: Citharistes, Dinophysis, Histioneis, Histiophysis, Metadinophysis, Ornithocercus, Parahistioneis
Order Dinophysales sensu lato
Family of Pseudophalacroma: Metaphalacroma, Pseudophalacroma
Family of Sinophysis: Sinophysis
Order Gonyaulacales / Pyrocystales
Ostreopsidaceae: Alexandrium, Centrodinium, Coolia, Episemicolon, Gambierdiscus, Gessnerium, Fukuyoa, *Goniodinium*, Ostreopsis, Pachydinium, Protogonyaulax, *Psammodinium*, Pyrodinium, Pyrrhotriadinium
Pyrocystaceae: Fragilidium, Pyrocystis, Pyrophacus
Gonyaulacaceae: Gonyaulax, Spiraulax
Lingulodiniaceae: Amylax, Lingulodinium
Ceratiaceae: Ceratium, Tripos
Protoceratidaceae: Ceratocorys, Pentaplacodinium, Protoceratium, Schuettiella
Thecadiniaceae: *Thecadinium*
Order Gonyaulacales, incertae familiae: *Dapsilidinium*, *Halostylodinium*, *Pseudothecadinium*, Thecadiniopsis

Order Peridiniales *sensu stricto*

Peridiniaceae: Peridinium, Bagredinium, Glochidinium, Palatinus, Parvodinium, Staszicella, Thompsodinium

Order Peridiniales sensu lato

Perinidiniopsidae: Peridiniopsis

Glenodiniopsidaceae: Glenodiniopsis

Protoperidiniaceae: Kolkwitziella, Matvienkodinium, Protoperidinium s.s.

Diplopsaliaceae: Boreadinium, Diplopelta, Diplopsalis, Diplopsalopsis, Dissodium, Gotoius, Huia, Lebouraia, *Niea*, *Qia*, Preperidinium, Protoperidinium depressum-group

Amphidiniopsidaceae: Amphidiniopsis, Archaeperidinium, Herdmania, Protoperidinium s.l.

Podolampadaceae: Blepharocysta, Gaarderiella, *Heterobractum*, Lissodinium, Mysticella, Podolampas, Roscoffia. The genera Cabra, Chrysodinium, Plagiodinium and Planodinium may be related, but the placement in this family needs further research.

Lessardiaceae: Lessardia

Heterodiniaceae: Dolichodinium, Heterodinium

Oodiniaceae: Oodinium

Heterocapsaceae: Heterocapsa

Oxytoxaceae: Oxytoxum, Corythodinium

Amphidomataceae: Amphidoma s.s.

Cladopyxidaceae: Cladopyxis, Palaeophalacroma

Endodiniaceae: Endodinium

Family of *Azadinium*: *Azadinium*, *Amphidoma s.l.*

Crypthecodiniaceae: Crypthecodinium

Order Blastodiniales

Blastodiniaceae: Blastodinium

Order Thoracosphaerales or Duboscquellales?

Apocalathium, Amyloodinium, Caladoa, Cachonella, Calcigonellum, Chalubinskia, Chimonodinium, Crepidoodinium, Cryptoperidiniopsis, Cystodinedria, Duboscquodinium, Duboscquella, Ensiculifera, *Fusiperidinium*, Lebessphaera, Leonella, Luciella, Naiadinium, *Oodinioides*, Paulsenella, Pentapharsodinium, Pernambugia, Pfiesteria, Protoodinium, Pseudopfiesteria, Scrippsiella, Theleodinium, Tyrannodinium, Speroidinium, Staszicella, Stylodinium, Stoeckeria, Tintinnophagus, Thoracosphaera

Order Dinotrichales

Dinotrichaceae/Kryptoperidiaceae: *Blixaea*, Dinothrix, Durinskia, Galeidinium, Kryptoperidinium, *Unruhdinium*

Order Symbiodiniales/Dinococcales

Symbiodiniaceae: Ansanella, Asulcocephallum, Aureodinium, Biecheleria, Biecheleriopsis, Breviolum, Dactylodinium, Durusdinium, Effrenium, Fugacium, Gerakladium, Haidadinium, Leiocephalium, Pelagodinium, Piscinoodinium, Polarella, Protodinium, Symbiodinium, Yihiella

Order Phytodiniales

Phytodiniaceae: Baldinia, Borghiella, Cystodinium, Dinamoebidium, Dinastridium, Dinoclonium, Dinococcus, Hypnodinium, Manchudinium, Phytodinium, Prosoaulax, Sphaerodinium, Tetradinium

Order Tovelliales

Woloszynskiaceae/Tovelliaceae: Bernardinium, Esoptrodinium, Jadwigia, Opisthoaulax, Tovellia, Woloszynskia

Peridiniphycidae, incerti ordinis

Dinosphaeraceae: Dinosphaera

Hemidiniaceae: Hemidinium, *Nottbeckia*

Glenodiniaceae: Glenodiniopsis, Nephrodinium

Peridiniphycidae, incerti ordinis, incertae familiae: Adenoides, Amphidiniella, Archaeosphaerodiniopsis, Berghiella, Bysmatrum, *Grammatodinium*, Halophilodinium, Laciniporus, *Madanidinium*, Micracanthodinium, Peridiniella, *Pileidinium*, Pseudoactiniscus, Pseudadenoides, Pyramidodinium, Rhinodinium, Sabulodinium, Stylodinium, Vulcanodinium, Thaumatodinium

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Chapter 137

PHOTOSYNTHETIC PIGMENTS IN DINOFLAGELLATES

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ABSTRACT

Dinoflagellates exhibit the richest pigment composition among microalgae. Their diverse trophic modes and evolutionary histories, with multiple losses and acquisition of plastids, turn them into a sample book of many other protist, and even prokaryote, pigment suites. Unfortunately, pigment analyses have not always been documented, either due to the lack of cultures or the opportunity in obtaining them as a complementary information in addition to morphological and molecular characters. Here we review the major pigment groups previously reported in dinoflagellates, and also discuss the plastid types found within them (with or without pigment data), described as obligate endosymbionts fully integrated in the host, temporary kleptochloroplasts or in endo/ectocytobionts of eukaryotic and prokaryotic nature.

Keywords: carotenoids, chlorophylls, chloroplast, dinoflagellates

INTRODUCTION

Dinoflagellates are an abundant and diverse group of protists in freshwater and marine ecosystems, with a cosmopolitan distribution from polar to tropical latitudes. There are approximately 2,400 named extant species (Gómez, 2012). They display three major trophic modes: (primarily) photoautotrophic, mixotrophic and heterotrophic (Jeong et al. 2010).

Parasite forms include both photosynthetic (order Blastodiniales) and heterotrophic members (Stoecker 1999).

A comprehensive review of their lifestyle, habitat and trophic modes (Gómez, 2012) states that most species can be found in marine waters (82%). Overall, photosynthetic dinoflagellates account for ~50% of the described species so far, with a vast predominance of the photosynthetic component in the freshwater environment.

From the available literature, it becomes evident that relative proportion of dinoflagellates having permanent/constitutive plastids (either autotrophic or mixotrophic species) is considerably larger than mixotrophic ones with non-permanent temporal organelles that need to be replenished during feeding (Stoecker 1999; Stoecker et al. 2017).

With that in mind, five plastid types were described by various authors based on their pigment types (Dodge 1989; Jeffrey et al. 2011; Egeland 2016). These authors considered fucoxanthin-type, peridinin-type, 19'-hexanoyloxyfucoxanthin-type, phycobilin-type and chlorophyll (Chl) *b*-type pigment groups. Zapata et al. (2012) refined this classification introducing an additional sixth group (Table 1) splitting those having 19'-acyloxifucoxanthins using different Chl *c* compounds and the presence/absence of gyroxanthin-diesters. The six groups as a whole represent the highest diversity among algal classes.

The latter study stands out as the most comprehensive review on photosynthetic pigments in dinoflagellates up to date. Since then limited information on pigment patterns have been accumulated from the already known or newly described species. Our aim here is to provide an updated overview of pigment composition in dinoflagellates, expanding as much as possible the data provided by Zapata et al. (2012).

CHLOROPHYLLS AND CAROTENOIDS DESCRIBED IN DINOFLAGELLATES

Plastids found in dinoflagellates are overwhelmingly dominated by those belonging to the red lineage. In consequence, pigment composition is characterized by the presence of chlorophylls (Chls) *c* and associated carotenoids distributed among diverse microalgal groups. Given the promiscuous nature of several dinoflagellate genera that have acquired their plastids from secondary algae, a wide array of pigment suites can be found in them.

However, most plastid-containing dinoflagellates display the canonical pigment type including peridinin (Per) as main accesory carotenoid, considered to be ancestral to all photosynthetic members of the group (Delwiche, 2007). It is not the purpose of this chapter to discuss the evolutionary relationships within dinoflagellates and the putative ancestral or derived state of Per-containing organisms, but it must be kept in mind that actual pigment composition in dinoflagellates reflects both the origin of their plastids and the transformations and new pigments then synthesized by the host that integrated these plastids as permanent organelles during evolution.

Chlorophylls in Dinoflagellates

Table 1 summarizes the actual chlorophylls found in dinoflagellates. Besides the constant presence of Chl *a* and the widespread occurrence of its biosynthetic precursor Magnesium-2,4-Divinylpheoporphyrin a_5 monomethyl ester (MgDVP), the Chl *c* family compounds are frequent and very diverse. For instance, among the polar Chls *c* with known structure, only monovinyl-Chl c_3 has not been recorded in dinoflagellates. This compound is restricted to some haptophytes from the genera *Emiliania*, *Imantonia* and *Prymnesium* (Zapata et al. 2004) but it has never been observed in dinoflagellates with tertiary plastids from haptophyte origin.

Several additional polar Chls *c* with unknown nature, whose UV-VIS absorption properties match those of Chl c_1, c_2 and c_3, have been also found in dinoflagellates, although these are not exclusive from this group (Zapata et al. 2012). However, we will not cover here these compounds that are minor and still pending of deeper studies to reveal their structure and putative functions in the photosynthetic machinery.

Non-polar Chls *c* (i.e., those with a galactolipid moiety containing two fatty acid chains, Garrido et al. 2000, Zapata et al. 2006, Porra et al. 2011), which are characteristic from haptophytes, display a wide distribution in dinoflagellates as discussed hereafter. Two non-polar Chls *c* have been characterized until date in haptophytes (Garrido et al. 2000; Zapata et al. 2001), that can be found also in dinoflagellates (Table 1). Additionally, a third non-polar unknown Chl *c* has been also detected in *Takayama* and *Karlodinium* (Zapata et al. 2012).

Chl *b*-containing dinoflagellates are represented by two unique -and very distinct- examples to our knowledge. First, *Lepidodinium chlorophorum*, originally described as *Lepidodinium viride* (Watanabe et al. 1990), later emended as *L. chlorophorum* by Hansen et al. (2007) on the basis of its identical ultrastructure and phylogenetic characterization to *Gymnodinium chlorophorum* (Elbrächter & Schnepf, 1996). Second, the green form of *Noctiluca scintillans* containing endocytobionts that mainly occurs in tropical waters in Southeast Asia (see below).

Regarding Chls *a* and *b* the unique variations of these compounds are their divinylic counterparts (DV Chl *a* and *b*) found in the cyanobacteria *Prochlorococcus marinus*. Both pigments were considered since their discovery as exclusive markers for prochlorophytes (Goericke and Repeta, 1992). Nevertheless, it was recently detected the presence of DV Chl *a* in a Per-containing species, *Alexandrium ostenfeldii* from the Baltic Sea (Rodríguez et al. 2016). These authors suggested that a mutation and/or epigenetic change in the expression of divinyl reductase gene/s could explain this altered pigment composition. DV mutants of *A. ostenfeldii* exhibited reduced fitness in comparison to normally pigmented cultures, due to a slower growth and stronger sensitivity to high irradiance (evidenced from pulse amplitude modulated fluoresence (PAM) measurements), and lower ^{14}C assimilation rates.

This fact, together with the gradual loss of DV-chl *a* content, made necessary to continuously isolate and raise new clonal cultures from the mutant lineage to avoid losing high DV Chl *a*/ Chl *a* ratios. In any case, not a single mutant was found to lack Chl *a* and the loss of DV Chl *a* proceeded irreversibly in every case.

Last, Yamada et al (2014) reported the presence in six species of benthic dinoflagellates of 13^2,17^3-cyclopheophorbide *a* enol (cPPB-aE), a chlorophyll catabolite derived from Chl *a* previously found in heterotrophic protists and in marine and lacustrine sediments. This non fluorescent compound was later found in shrunken/abnormal zooxanthellae (symbiotic algae harbored by reef-building corals) where its formation was considered as a mechanism for

avoiding oxidative stress (Suzuki et al. 2015). Further chlorophyll catabolites have been reported in cultures of *Prorocentrum micans* and *Amphidinium tamarense* with the detection of cPPB-aE in *A. tamarense* in a culture exposed to copepod grazers supporting a possible stress response mechanism (Talbot et al. 2000).

Carotenoids in Dinoflagellates

The main carotenoids found in dinoflagellates are summarized in Table 1. In addition to most of those already known in diatoms, haptophytes and cryptophytes, dinoflagellates display exclusive pigments both in Per-type organisms and those with tertiary plastids.

Among the first, dinoflagellate exclusive carotenoid compounds are peridinol, peridinin, cis-peridinin, dinoxanthin and pyrroxanthin (Zapata et al. 2012; Egeland 2016). Also, the unique carotenoid lactoside, P457, was characterized from Per-containing dinoflagellates, namely a *Symbiodinium* sp. strain isolated from a sea anemone, two strains of *Amphidinium carterae* and *Prorocentrum triestinum*, as well as in many other *Symbiodinium* strains, reef-building corals and other cnidarians harbouring dinoflagellate symbionts in their tissues (Wakahama et al. 2012).

In addition, a new unknown carotenoid has been detected in a recently described Suessial species, *Biecheleria tirezensis* (Raho et al. 2018), which was not mentioned in that study, as shown in the corresponding HPLC pigment analyses in Figure 1A. The UV-VIS properties and chromatographic behaviour of this compound do not coincide with those of any known carotenoid and suggest that its molecule could harbor some very polar group and have a reduced conjugated system (around eight double bonds) restricted to the aliphatic part of the molecule.

Among dinoflagellates with “non-canonical” peridinin plastids, a special mention must be given to the presence of the acetylenic allenic gyroxanthin-diesters. Gyroxanthin-diesters are present in those organisms with tertiary plastids and haptophyte (also possibly pelagophyte) pigment signature. Gyroxanthin-diester was first characterized in *Gymnodinium galatheanum* (at present *Karlodinium veneficum*) by Bjørnland et al. (2000), then detected, together with two new different forms, in other members of the genera *Karenia* and *Karlodinium*, but not in *Takayama* (Bjørnland et al. 2003; Zapata et al. 2012). This structural diversity can be explained by the different chain lengths of the fatty acids (e.g., lauric, myristic, Bjørnland et al, 2000) that are part of the molecules.

Regarding Chl *b*-containing dinoflagellates of the genus *Lepidodinium*, their plastid was initially attributed to a prasinophyte endosymbiont, as prasinoxanthin was tentatively identified (Elbrächter and Schnepf 1996). Further studies demonstrated that *Lepidodinium chlorophorum* contains permanent plastids acquired by serial endosymbiosis from green algae (core chlorophytes; Matsumoto et al. 2011). Later, these were traced to the pedinophyte *Pedinomonas minor* (Kamikawa et al. 2015), the occurrence of prasinoxanthin was dismissed and the expected carotenoid profile of a chlorophyte (excepting lutein) was confirmed (Matsumoto et al. 2012; Gavalás-Olea et al. 2016).

L. chlorophorum has been recently demonstrated to contain a new carotenoid, lepidoxanthin (Gavalás-Olea et al. 2016), with a unique 19,19′-diacyloxy structure ((3S,5R,6S,3′R,6′R)-5,6-epoxy-19-(2-decenoyloxy)-19′-acetoxy-4′,5′-didehydro-5,6,5′,6′-

tetrahydro-β,ε-carotene-3,3′-diol), absent from parallel pigment analyses in *Pedinomonas minor*.

Table 1. Distribution of chlorophylls (chl) and carotenoids among pigment-based chloroplast types in Dinophyta. Black circle: consistent occurrence; white circle: occasional occurrence

Pigment	Pigment-based chloroplast type					
	1	**2**	**3**	**4**	**5**	**6**
Chlorophylls	----------Chl *c*----------					Chl *b*
Chl c_2-like *P. gyrans*-type				●		
Chl c_1-like *K.foliaceum*-type				●		
Chl c_3		●	●	○		
MgDVP	●	●	●	●	●	●
Chl c_2	●	●	●	●	●	
Chl c_1	○			●		
Chl *b*						●
Chl *a*	●	●	●	●	●	●
MGDG-chl c_2*		●	○			
Carotenoids	Per-type	Fuco-type			Allo-type	Vio/Neo-type
Peridininol	●					
P457	●					
Peridinin	●					
cis-Peridinin	●					
19’-but-keto-fuco-like		●				
19’-but-4-keto-fuco		●				
19’-but-fuco		●	●			
Fucoxanthin		●	●	●		
19’-hex-keto-fuco-like		●				
19’-hex-4-keto-fuco		●	○			
19’-hex-fuco		●	●			
Pyrrhoxanthin	●					
Violaxanthin				○		●
Diadinochrome	●					
Diadinoxanthin	●	●	●	●	●	
Dinoxanthin	●					
Alloxanthin					●	
Diatoxanthin	○	○	○	○	○	
Zeaxanthin	○	○	○	○		○
Lepidoxanthin						●
Lycopene-like	○	○	○	○		○
Gyroxanthin diester 1		●	●			
Gyroxanthin diester 2		●	●			
Gyroxanthin diester 3		●	●			
Crocoxanthin					●	
β,Ψ-carotene				●		●
β,ε-carotene		●	●	●	●	●
β,β-carotene	●	●	●	●	○	●

*Up to three different compounds detected in dinoflagellates from types 2 and 3. Modified from Zapata et al. (2012).

In the case of the green form of *Noctiluca scintillans*, this heterotrophic species harbours endosymbiotic populations constituted by free-swimming phototrophic endocytobionts of *Protoeuglena noctilucae* (Chlorophyta, Pedinophyceae; Wang et al. 2017). Those green *Noctiluca* are responsible of massive outbreaks (green tides) in the open ocean (Gomes et al. 2014). Pigment composition of *Protoeuglena noctilucae* (as *Pedinomonas noctilucae* in

Furuya and Lirdwitayaprasit, 2000) showed a typical pigment pattern in Chl *b*-containing algae, matching previous analyses from *Pedinomonas* as mentioned herein.

Finally, in a recent study relating pigment composition and habitats in benthic dinoflagellates, Yamada et al. (2015) reported the occurrences of various sets of unknown carotenoids either in some peridinin-containing and certain fucoxanthin-bearing dinoflagellates. Carotenoids are highly prone to isomerisation and oxidation indicating the possibility of carotenoid catabolite production within benthic dinoflagalles. Further detailed analyses of carotenoids in benthic dinoflagellates is required to determine phylogenetic distribution and the functional role of such undetermined carotenoids.

CHLOROPLAST PIGMENT TYPES AND DINOFLAGELLATE TAXA

Zapata et al. (2012) defined six types of chloroplasts in dinoflagellates, depending on their pigmentary composition (Tables 1 and 2). Since then, some additional pigments have been detected in a few cases but in general, they do not imply in our opinion essential modifications as to vary the number of types proposed. These pigmentary types are constructed according to a presence/absence criterion, since the relative contents of the different compounds vary depending on environmental and physiological conditions (Kana et al. 1997, Brunet et al. 2011, Islabão, 2016). Dinoflagellates may benefit from photosynthesis by three groups of associations (Schweikert and Elbrächter 2004): obligate endosymbionts (i.e., permanent plastids), kleptochloroplasts (temporal organelles acquired by feeding) and endo/ectocytobionts (where symbionts are identical to their free-living counterparts and are separated from the host cytoplasm). The following pigmentary types belong to the first and second groups.

Type-1: Peridinin-Containing

In the last years numerous studies have dealt with the characterization of dinoflagellate species harbouring typical type 1 chloroplasts. These plastids are characterized by the constant presence of peridinin and dinoxanthin, and occasionally of peridininol, phyrrhoxanthin and P457, all of them being dinoflagellate-exclusive carotenoids. Additional compounds are the xanthophylls diadinoxanthin and diatoxanthin (implied in photoprotection mechanisms), diadinochrome and β, β-carotene.

Accessory chlorophylls in this pigmentary type are chlorophyll c_2 (major) and MgDVP at trace level. Chlorophyll c_1 appears occasionally in this pigmentary type (Zapata et al. 2012, Yamada et al 2015). An interesting example is the recently described *Pentaplacodinium saltonense* (Mertens et al. 2018; =*Ceratocorys mariaovidiorum*, Salgado et al. 2018), with appreciable amounts of chlorophyll c_1 (this work). It has been suggested that chlorophyll c_1 can be produced from chlorophyll c_2 whenever the latter is present, although the amount of chlorophyll c_1 is variable and frequently it cannot be detected (Yamada et al. 2015).

Pigment type 1 is the most frequently found among photosynthetic dinoflagellates, comprising the orders Amphidiniales, Gonyaulacales, Gymnodiniales, Peridiniales, Prorocentrales, Suessiales and Thoracosphaerales (Zapata et al. 2012). In that study HPLC

analyses of any member of the order Suessiales were not detailed, but these authors proposed the extension of chloroplasts with peridinin within them by generalizing the composition described for *Polarella glacialis* by Montresor et al. (2003). In an attempt to add information about this order, in this work we have analyzed *Symbiodinium natans* (Class Symbiodiniaceae), *Biecheleriopsis adriatica* and *Biecheleria tirezensis* (Class Biecheleriaceae), and the chromatographic traces of these additional species are similar (Figure 1A) and support the presence of type 1 chloroplasts in Suessiales. Furthermore, the genera *Amphidoma* and *Azadinium*, which have not been adscribed to any extant order, also appear to display type 1 plastids (Tillmann et al. 2010, 2012, 2014, this study).

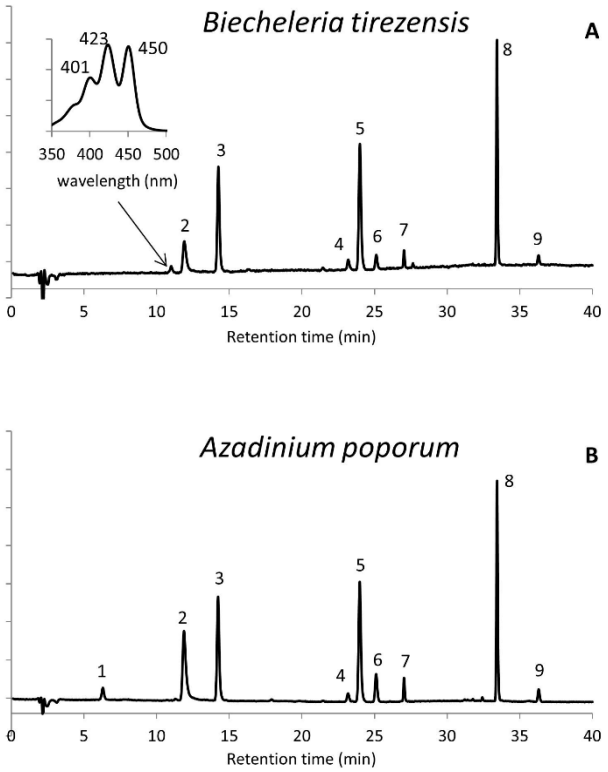

Figure 1. A) HPLC chromatogram of a pigment extract from *Biecheleria tirezensis* (BEA 1549B). The insert shows the VIS spectrum of an unknown carotenoid. B) HPLC chromatogram of *Azadinium poporum* (UTH D4). Peak identification: 1) peridininol, 2) chlorophyll c_2, 3) peridinin, 4) diadinochrome, 5) diadinoxanthin, 6) dinoxanthin, 7) diatoxanthin, 8) chlorophyll *a*, 9) β,β-carotene.

The descriptions of *Azadinium spinosum* (Tillman et al. 2009) and *A. obesum* (Tillmann et al. 2010) reported a peridinin-containing profile which surprisingly included prasinoxanthin in minor amounts. *A. caudatum* was also shown to bear type 1 chloroplasts with no prasinoxanthin but minor amounts of violaxanthin (Tillmann et al. 2014). However, the HPLC profiling of *A. poporum* performed for this work yielded the typical type 1 pigment suite without any additional carotenoid (Figure 1B).

Interestingly, a new and minute Kareniaceae species displaying a Per-containing plastid, belonging to a new genus (*Gertia stigmatica*) has been recently discovered (Takahashi et al. 2019). To our knowledge, this is the only exception within such family characterized by

permanent plastids of tertiary origin acquired from haptophytes (Type-2, see below). These authors suggested that this undescribed kareniacean replaced the former acyloxifucoxanthin-containing chloroplast by a Per-type one.

Type-2: 4-Keto-Acyloxifucoxanthin-Containing

The maximum diversity of carotenoids related to fucoxanthin has been detected in certain dinoflagellates, constituting type 2. In this type, restricted to the genus *Karenia*, six of these xanthophylls characteristically appear: fucoxanthin itself, 19'-hexanoyloxyfucoxanthin, 19'-butanoyloxyfucoxanthin, 19'-hexanoiloxy-4-keto-fucoxanthin, and three unidentified carotenoids (as far as we know, restricted to members of the genus *Karenia*) with identical absorption spectrum to the latter (Zapata el al. 2012). The different proportions of these fucoxanthin-related carotenoids seems to be very influenced by the culture irradiance as it has been observed in the coccolitophoride *Emiliania huxleyi* (Garrido et al. 2016). Work on this topic in several *Karenia* species is currently under progress (Barreiro et al. *in preparation*).

Diadinoxanthin (and diatoxanthin at high irradiances) appear as main photoprotective carotenoids. In addition, various forms of gyroxanthin have been detected in different species of the genus *Karenia* (Zapata et al. 2012). Accompanying these xanthophylls, several chlorophyll *c* compounds (chlorophyll c_3, chlorophyll c_2, MgDVP and MGDG-chlorophyll c_2-*Chrysochromulina polylepis*-type) complete the pigment suite of type 2 (Zapata et al. 2012).

Type-3: Acyloxifucoxanthin-Containing

Type 3 chloroplasts, contained in species of the *Asterodinium*, *Karlodinium* and *Takayama* genera, lack the acyloxy derivatives of 4-keto-fucoxanthins. The major carotenoids in this pigment type are fucoxanthin, 19'-acyloxifucoxanthin derivatives, a fucoxanthin-related compound of unknown structure and the diadinoxanthin and diatoxanthin pair. The occurrence of 19'-butanoyloxyfucoxanthin cannot be generalized in this Type after the recent finding that *Asterodinium gracile* lacks this pigment (Benico et al. 2019). Although absent in *Takayama* cf. *helix*, gyroxanthin derivatives are abundant in species of the genus *Karlodinium* analyzed in Zapata et al. (2012) and in *Asterodinium gracile* (Benico et al. 2019). The composition in chlorophylls is very similar to type 2, with chlorophylls c_2, c_3 and MgDVP always present, while MGDG-chlorophyll c_2-like pigments have not been always detected (Zapata et al. 2012). Recent analyses in new *Karlodinium* species raised a very first contradiction in the pigment composition of members of that genus. *K. gentienii* (Nézan et al. 2014) could be classified as a type 3 member as it contains acyloxy fucoxanthins, despite it lacks gyroxanthin derivatives and MGDG-chlorophylls. However, *K. zhouanum* (Luo et al. 2018b) contains fucoxanthin but lacks its acyloxy derivatives, thus placing this species into chloroplast type 4 (see below).

Type-4: Fucoxanthin-Containing

The dinoflagellates with chloroplast type 4, coming from a tertiary endosymbiont derived from a diatom (the so-called "dinotoms" due to the early stage of integration of their endosymbiont, Imanian et al. 2010), display fucoxanthin without any acyloxyderivatives. The monophyletic "dinotoms" harbor a chloroplast of diatom origin, but their chloroplasts are polyphyletic belonging to one of four genera: *Chaetoceros*, *Cyclotella*, *Discostella*, or *Nitzschia* (Yamada et al. 2017). It is worth highlighting the characteristical presence in this group of β, ψ-carotene (Zapata et al. 2012; Yamada et al. 2015) that, together with diadinoxanthin and diatoxanthin, complete the set of carotenoids. On the composition of chlorophylls there is a certain discrepancy: although the presence of chlorophylls c_2 and c_1 in all the species examined has been detected in several studies, Zapata et al. (2012) found chlorophyll *c* type pigments characteristic of the haptophytes *Pavlova gyrans* and *Exanthemachrysis gayraliae* in *Durinskia dybowskii* (=*D. baltica*) and *Kryptoperidinium foliaceum*. Moreover, Yamada et al. (2015) reported the occurrence of chlorophyll c_3 in the sand-dwellers *Galeidinium rugatum* and *Durinskia* cf. *baltica*. Yamada et al. (2017) also described *D. kwazulunatalensis*, that had been analyzed as *Durinskia* sp. in their previous work, showing a type 4 pattern without chlorophyll c_3 (Yamada et al. 2015).

Type-5: Alloxanthin-Containing

Type 5 is composed of mixotrophic dinoflagellates that harbor heterotrophically acquired temporal cryptophyte chloroplasts (kleptoplastids). Among them, *Dinophysis* is the most studied and representative example, whose chloroplasts were traced to a cryptophyte by pigment and ultrastructural analyses (e.g., Schnepf and Elbrächter 1988, Meyer-Harms and Pollehne 1998) long before confirming their identity by molecular means (Hackett et al. 2003) and the establishment of cultures (Park et al. 2006). Their temporary or permanent nature held a long debate, now settled in favor of the first hypothesis due to the evidences accumulated from culture and field studies (see Reguera et al. 2012). In the field, photosynthetic species of *Dinophysis* display kleptoplastids from cryptophyte origin (typically *Teleaulax* spp.), most probably obtained from the ciliate *Mesodinium rubrum*. This specific food chain is the unique identified so far to grow *Dinophysis* spp., but plastids from multiple origins have been also retrieved in the field suggesting occasional feeding on other preys (Kim et al. 2012a).

In the genus *Dinophysis*, HPLC pigment data from cultures (fed with the *Teleaulax/ Mesodinium* food chain) have become available for *D. acuminata, D. acuta, D. caudata* and *D. tripos* (Zapata et al. 2012, Rial et al. 2013, García-Portela et al. 2018) with alloxanthin and crocoxanthin as the characteristic carotenoids, accompanied by chlorophyll c_2, MgDVP and biliproteins (Jeffrey et al 2011, Zapata et al., 2012, Egeland 2016). Cryptophytes also contain the pigment-protein phycobilin complex, phycoerythrin. All these pigments are contained in the engulfed plastids originating from cryptophytes, that undergo important structural modifications in *Dinophysis* (Kim et al. 2012b).

But neither all the members of type 5 are Dinophysiales nor all of them show kleptochloroplasts of cryptophyte origins (Qiu et al. 2011, Nishitani et al. 2012), as it will be discussed afterwards.

There are other taxonomic groups of dinoflagellates having kleptoplastids from cryptophyte preys which are temporary and photosynthetically active. Members of the genera *Amphidinium* (*A. poecilochroum, A. latum*), *Amylax* (*A. buxus, A. triacantha*), *Gymnodinium* (*G. aeruginosum, G. acidotum, G. eucyaneum, G. gracilentum*), *Cryptoperidiniopsis* and *Pfiesteria* (*P. piscicida*) have been reported (see refs. in Nishitani et al. 2012, Onuma et al. 2013).

In the case of the genus *Amylax*, *A. triacantha* cultures were established feeding the ciliate *Mesodinium rubrum* (Park et al. 2013) and its pigment composition is expected to be identical to *Dinophysis*. Although no pigment data are available in the literature, *A. busus and A. triacantha* are known to contain cryptophyte kleptochloroplasts of *Teleaulax amphioxeia* (Koike and Takishita 2008).

Similarly, within the order Gymnodiniales, the recently described genus *Nusuttodinium* that comprises both freshwater and marine species retains plastids from cryptophyte origin (Takano et al. 2014).

Table 2. Distribution of pigment-based chloroplast types across Dinophyta taxa

		Pigment type					
Order	Representative species	1	2	3	4	5	6
Amphidiniales	*Amphidinium carterae*[a]	●					
Dinophysiales	*Dinophysis acuminata*					●	
Gonyaulacales	*Alexandrium minutum*	●					
	Amylax triacantha[b]					●	
	Pentaplacodinium saltonense[c]	●					
	Tripos fusus[c]	●					
Gymnodiniales	*Bispinodinium angelaceum*[d]	●					
	Gymnodinium catenatum	●					
	Nusuttodinium myriopirenoides[e]					●	
	Paragymnodinium shiwhaense[f]	●					
	Spiniferodinium galeiforme[g]	●					
	Wangodinium sinense[h]	●					
	Karenia mikimotoi		●				
	Karlodinium veneficum			●			
	Takayama helix			●			
	Karlodinium zhouanum[i]				●		
	Lepidodinium chlorophorum						●
Peridiniales	*Heterocapsa triquetra*	●					
	Durinskia baltica				●		
	Galeidinium rugatum[j]				●		
	Kryptoperidinium foliaceum				●		
Prorocentrales	*Prorocentrum lima*	●					
Suessiales	*Biecheleria tirezensis*[c]	●					
	Symbiodinium natans[c]	●					
Thoracosphaerales	*Ensiculifera loeblichii*[c]	●					
	Thoracosphaera heimii	●					
Tovelliales (freshwater)	*Tovellia diexiensis*[k]	●					
	Woloszynskia coronata[l]	●					
Dinophyceae ordo incertae sedis	*Azadinium poporum*[c]	●					
	Amphidoma languida[m]	●					
	Levanderina fissa[c]	●					

Data from Zapata et al. (2012) and other authors (for superscripts see references): [a]Haxo et al. (1976), [b]Based on kleptoplast origin by Koike and Takishita (2008), [c]This study, [d]Yamada et al. (2013), [e]Based on kleptoplast origin by Yamaguchi et al. (2011), [f]Kang et al. (2010), [g]Takano et al. (2014), [h]Luo et al. (2018a), [i]Luo et al. (2018b), [j]Tamura et al. (2005), [k]Zhang et al. (2016), also reported high amounts of astaxantin and astaxanthin diester in a natural bloom, [l] Lindberg et al. (2005), [m]Tillmann et al. (2012).

Type-6: chl *b*-Containing

Type 6 chloroplast, restricted to the genus *Lepidodinium*, shows pigments characteristic of green algae, including chlorophyll *b*, and the carotenoids 9'-*cis*-neoxanthin, violaxanthin, antheraxanthin and β, β-carotene, together with a new major carotenoid, lepidoxanthin, unique in this type of chloroplast (Gavalás-Olea et al. 2016). Lepidoxanthin coeluted with lutein found in *Pedinomonas minor* in the Zapata et al. (2000) method, although both carotenoids displayed different UV-VIS properties. The chromatographic method proposed by Sanz et al. (2015) achieves good separations of lutein from lepidoxanthin.

Kleptoplasts and Endo/Ectocytobionts from Multiple Origins

The organisms considered below could provide, in some instances, additional pigment types in dinoflagellates. These would originate from their kleptoplasts acquired by feeding or through their association with endo/ectocytobionts from different phytoplankton groups.

It must be kept in mind that the nature and origin of these associations remains fairly unknown and *pigment data have not been reported*, to our knowledge, due to the absence of cultures or their scarce abundance in field samples (excepting, as described above, the green forms of *Noctiluca* that develop extensive blooms).

Some examples of Dinophysiales that retain plastids not only from cryptophyte origin are *Dinophysis mitra* (haptophytes, Nishitani et al. 2012), *D. miles* (cryptophyte, haptophyte and cyanobacteria, Qiu et al. 2011), and species of the genera *Sinophysis* (cyanobacterial endosymbionts, Escalera et al. 2011, García-Portela et al. 2017), *Amphisolenia* and *Triposolenia* (cyanobacterial and eukaryotic endosymbionts, Tarangkoon et al. 2010), *Citharistes*, *Histioneis*, *Parahistioneis* and *Ornithocercus* (cyanobacterial ectosymbionts like *Synechococcus* and *Synechocystis*, Ossipov et al. 1997, Tarangkoon et al. 2010).

In the order Peridiniales plastids of Dictyochophycean origin have been detected in *Podolampas bipes* (Schweikert and Elbrächter 2004).

Finally, this group of dinoflagellates that retain the photosynthetic machinery from other organisms is completed with the already mentioned green forms of *Noctiluca* which enclose intact cells of *Protoeuglena noctilucae* (Wang et al. 2017).

The systematic pigment analysis of all these species is still needed to contribute to tracing the exact origin of the foreign plastids and cyanobionts.

LINKING PIGMENT AND PLASTID DIVERSITY

Despite the evolutionary and ecological importance of dinoflagellates, their morphological diversity and complicated genetics has made phylogenic resolution highly challenging. This is compounded by the ability of dinoflagellates to lose, replace or gain new plastids. However rapid advances have been made in recent years. This has been made possible with the availability of genome and transcriptome sequencing data resulting in a clearer understanding of dinoflagellate phylogeny, plastid diversity, endosymbiosis and evolutionary trends (Bachvaroff et al. 2015, Janouškovec et al. 2017, Waller and Koreny

2017; Gornik et al. 2019). However, as highlighted by Hoppenrath (2017) natural classification reflecting evolutionary processes and phylogenetic relationships for dinoflagellates whilst on a promising path is still at an extremely early stage.

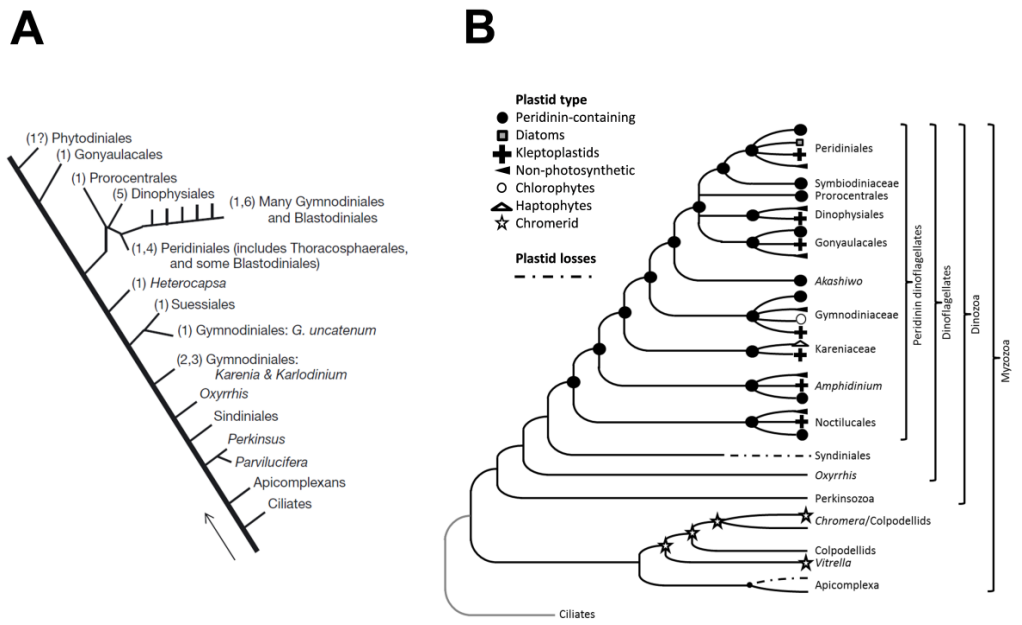

Figure 2. Mapping and comparing pigment types with plastid diversity and phylogeny: A. Taken from Zapata et al. (2012). Numbers indicate chloroplast types (1-6). B. Adapted from Waller and Koreny (2017).

Historical chemotaxomic pigment analysis spearheaded by the seminal work of Jeffrey and Zapata appears remarkably consistent with this more recently emerging understanding on dinoflagellate phylogenomics and plastid diversity. In Zapata et al. (2012), pigment based chloroplast types were mapped to a proposed evolutionary history based on molecular data scheme by Saldarriaga et al. (2004) (Figure 2A). This can be compared to a more recent schematic (Figure 2B) where current knowledge on plastid diversity has been mapped onto a phylogenomic tree (Waller and Koreny, 2017) derived using protein transcript information (Janouškovec et al. 2017).

As dinoflagellate phylogeny is still fluid detailed comparisons are challenging. Four pigment plastid types plus kleptoplasty are presented by Waller and Koreny (2017). Detailed pigment analysis can help discrimate further plastid diversity patterns. One example being for the alloxanthin containing, classified as Type 5, derived from cryptophyte plastids (also containing phycoerythrin) present within the Dinophysiales and Gonyaulacales clades. Another example being the gyroxanthin diesters, possibly derived from coccolithophytes and or pelagophytes, and the acyloxyfucoxanthin (excluding keto-derivatives typical of haptophytes) grouped as Type 3 as discussed in this chapter within the Kareniaceae clade. In addition a peridinin-containing plastid has also been reported for the first time within Kareniaceae, as mentioned before (Takahashi et al. 2019).

Further detailed analysis of pigments in now required in conjunction with recent molecular data to gain further insight into phylogenic diversity and evolutionary and ecological adjustment of dinoflagellates. This could be further developed to incorportate understanding on dinoflagellate metabolome with wider metabolomic analysis including at the single cell level. The ability to discriminate physiological status using metabolomic analysis at the single cell level in diatoms and chlorophytes has recently been achieved (Baumeister et al. 2019).

PIGMENT COMPOSITION AND HABITAT TYPES

Studying pigment profiles of 40 dinoflagellate species (45 strains) isolated from three habitat types (sand-dwelling benthic forms, tidal pool inhabitants and planktonic species), Yamada et al. (2015) proposed that far greater diversity of pigments are produced by the dinoflagellates living in sand regardless of chloroplast types relative to those of planktonic and tidal pool forms.

In sand-dwellers with type-1 chloroplasts a set of minor unidentified carotenoids, together with cPPB-aE, were found in addition of the common pigment profile. Also, sand-dwellers with type-4 chloroplasts showed a characteristic set of unknown carotenoids and chl c_3. These results were claimed by Yamada et al. (2015) as a link between pigment profile and habitat types, but given that these pigments have not been reported elsewhere, further work would be needed to confirm the native occurrence of these compounds.

A BRIEF METHODOLOGY OF PIGMENT ANALYSES

Analytical methods for pigment analysis date back from colorimetric techniques like the Forel Ule scale, employed since the last decade of s. XIX to build up maps of water color, still useful at present, such as those of the Atlantic Ocean during the Plankton Expedition (1889). Further methodological developments enabled to quantify the absorption and fluorescence signals of pigment mixtures (both in natural samples and organic extracts), by means of spectrophotometric and fluorometric methods (e.g., Lorenzen, 1967, Jeffrey and Humphrey, 1975; Neveux and Lantoine, 1993). These approaches provided complementary results and, with proper data processing, good estimates of main carotenoids and chlorophyll forms (total chls *a*, *b*, *c* and phaeopigment derivatives) in aquatic ecosystems.

However, the precise calculation of individual compounds in phytoplankton field samples and cultures, such as those detailed in the previous sections, were only acquired after the implementation of modern chromatographic techniques. These evolved from thin-layer chromatography (e.g., Jeffrey 1968) towards more resolutive and quantitative methods like HPLC and UPLC incorporating diode-array and fluorescence detectors, which streamlined with mass-spectrometry analysis allow to gain further insights on the nature of unknown pigments (Airs and Garrido 2011).

Since the pioneer work of Mantoura and Llewellyn (1983), many HPLC methods were developed and applied for the quantitation and estimation of pigments both in laboratory cultures and phytoplankton populations in the field. It is not our task here to provide a

comprehensive overview of these methods: these have already been compiled by other authors (Garrido et al. 2011, Sanz et al. 2015). Nevertheless, it must be stressed that the chemotaxonomic application of pigments –and its utility to characterize the composition of phytoplankton groups– required (as cited in the case of spectrophotometric and fluorometric methods), the application of mathematical tools to extract additional information from HPLC data to reconstruct phytoplankton groups by means of marker pigments.

These tools include five methods (Higgins et al. 2011), among which multiple linear regression and CHEMTAX software, based on iterative adjustments of pigment ratios (Mackey et al. 1996), have been widely applied in the last twenty years. To give an idea, only HPLC combined with CHEMTAX data processing accounts for around 300 studies, increasing steadily in number since 1997.

In parallel, the fast development of molecular tools and high-throughput sequencing provided more "meticulous" ways (both in qualitative and quantitative terms) to scrutinize the taxonomic composition of aquatic microorganisms, including dinoflagellates (e.g., Stern et al. 2010, Le Bescot et al. 2016), superseding the dominant place of pigments in previous decades.

The chemotaxonomic characterization by pigments (whichever the mathematical approach used) is mostly restricted in dinoflagellates to illustrate a single pigmentary group (type-1), based on the exclusive carotenoid marker, peridinin (characteristic of pigmentary type-1, found in most photosynthetic dinoflagellates). Notwithstanding, additional pigmentary groups, built up on the presence of fucoxanthin-related compounds and gyroxanthin-diester have been also considered in some studies (Örnólfsdóttir et al. 2003, Rodríguez et al. 2003), although these do not fit always to the pigment types shown herein and can integrate mixed contributions from other groups (mainly diatoms and haptophytes). The reverse situation frequently occurs when pigments shared by a certain dinoflagellate type and other algal groups are attributed to the latter (this being the case of type-4 dinoflagellates and diatoms or type-5 and cryptophytes). This stresses the relevance of other methods, such as microscopic cell counts, to discern the dominant taxa in natural samples minimizing misidentification of pigment patterns originating from tertiary dinoflagellates (Irigoien et al. 2004).

CHEMTAX calculations require the input of pigment ratios (accessory marker pigments to chl *a*) in order to estimate the relative contribution of defined pigment groups to total chl *a*. In this sense, the availability of pigment ratios for each pigmentary type is needed and those from organisms isolated in the study area was originally suggested by Mackey et al. (1996) as an adequate strategy to gain a more accurate approach. In addition, splitting data prior to CHEMTAX processing has been also employed to reconstruct pigment groups and adjust pigment ratios following geographical areas or depth (light) ranges (e.g., Llewellyn et al. 2005, van De Poll et al. 2016, Araujo et al. 2017).

In this sense, despite the relative paucity of data about the influence of distinct environmental factors on dinoflagellate pigments, there exists at least a review about the effects of variable light conditions on the ratios of accessory pigments to chlorophyll *a* (Higgins et al. 2011). Namely, these authors compiled field and culture data about pigment:chl *a* ratios at low, medium and high light for type-1 and type-2 dinoflagellates, which provide good estimates of initial pigment ratios to reconstruct these groups by means of CHEMTAX processing of HPLC data.

STRATEGIC UTILITY OF PIGMENTS

The quantitative estimation of the contribution of a certain dinoflagellate (group) to the planktonic biomass can be a crucial information when studying the onset, development and decline of a bloom, specially if it involves species with negative effects towards the marine fauna, public health and/or human activities. If a pigment can be employed as a proxy for the causative organism, this compound has a strategic utility, facilitating the detection and monitoring of the bloom. This approach usually relies on the previous knowledge of the recurring proliferations in a region that can be linked to the presence of a marker pigment reasonably attributable to a given species (Johnsen et al. 2011).

The most remarkable example is the use of gyroxanthin diester for quantifying the abundance of *Karenia brevis* in natural waters, either HPLC measured (Örnólfsdóttir et al. 2003, Richardson and Pinckney, 2004) or *in situ* detected through optical sensors exploiting the unique absorption characteristics that this pigment confers (Johnsen et al. 2011). In this sense, satellite techniques have been successfully applied to monitor not only *K. brevis* (Stumpf et al. 2009) but also other species such as *K. mikimotoi* (Kurekin et al. 2014) and even, but with a lower specificity, the proliferations of the chl *b*-containing species *Lepidodinium chlorophorum* (Sourisseau et al. 2016).

The main drawback of this approach could be the presence of other algal groups that contain the same reference pigments. For example, gyroxanthin diester has been detected in other dinoflagellates (genus *Karlodinium*, type-3) and certain haptophytes and pelagophytes (Bjørnland et al. 2003, Zapata et al. 2005). The quest for other pigments with such strategic utility in dinoflagellates should consider compounds with appropriate marker quality, in amount enough to be easily detected even at low cell abundances. Among the different pigments found in dinoflagellates, only a few can heretofore be unequivocally assigned to a certain type: peridinin and dinoxanthin for type-1, keto-acyloxy-fucoxanthins for type-2 and lepidoxanthin for type-6.

The separation of these pigments in field samples and their use as chemotaxonomic markers of dinoflagellates requires highly resolution HPLC methods. The discrimination of peridinin and dinoxanthin is achieved by most methods but the separation of fucoxanthin from all its related acyloxy- and keto- derivatives in the genus *Karenia* (as described in Zapata et al. 2012) is difficult for methods focused on the analysis of gyroxanthin diester (Örnólfsdóttir et al. 2003). On the other hand, lepidoxanthin is hard to differentiate from lutein except with certain specialized methods (Sanz et al. 2015)

CONCLUSION

As highlighted the pigment diversity in dinoflagellates is the widest across any group of microalgae reflecting the complexity of plastid endosymbiosis. It is clear that whilst there have been detailed studies of carotenoids and chlorophylls across the dinoflagellates, the systematic data collection across more species would be advantageous. This should be combined with on-going advances in transcriptomic and genetic research and with improved resolution in dinoflagellate phylogeny. Single cell analysis techniques provide exciting opportunities and will be particularly important in determining physiological response of

dinoflagellates to environmental variables such as light and nutrients. Combining knowledge on pigment distribution and function with a systems biology approach will help build deeper understanding on this important and amazingly diverse and beautiful group of microorganisms.

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Chapter 138

SPECIES ASSEMBLIES AND SEASONAL SUCCESSION OF DINOFLAGELLATES

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ABSTRACT

Seasonality of dinoflagellate blooms and dinoflagellate assemblies are not random. Yet, bloom events and the specific appearance of species is difficult to predict and sometimes appears stochastic. Nevertheless, it has been established that both abiotic and biotic factors determine species assemblies, as well as the available species pool. The physical environment, especially nutrients and mixing/light depth largely determines when and where dinoflagellates are present. As a response to the environment, adaptive strategies (C,S,R) have evolved, allowing different species to be favored in different conditions. Based on functional traits, with cell size being a so called master trait, species can be categorized into different life-forms, which in turn can be predicted for different conditions. Among biotic factors, mortality due to natural enemies, including both grazers and parasites are involved in species succession. In addition both competition among dinoflagellates and with other phytoplankton groups can determine which species occur. Finally, life-cycle transitions, especially for cystproducing (meroplanktonic) species can be used to explain fine-scale species replacement. The latter together with species-specific parasite infections provide promise to untangle the processes behind apparently stochastic events.

Keywords: dinoflagellate blooms, seasonal succession, community assembly, adaptive strategies, life-forms

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INTRODUCTION

Years of monitoring dinoflagellates in limnic and marine environments have revealed that there is a pattern to the seasonal occurrence of certain taxa and of bloom events. However, to the frustration of ecologists and managers, the timing of blooms, as well as the specific species that will dominate are not entirely predictable. By identifying controlling factors, relevant traits, and the processes involved, it is possible to develop concepts and build models to better understand and predict dinoflagellate occurrence.

The dinoflagellate species present at any given point of time in a specific aquatic habitat, form a species assembly or community. This assembly of species is the result of various factors and processes including the physical environment, interactions among the biota, inherent traits of the present species, as well as stochastic processes. The change of species assemblies with time throughout a year, and the yearly reoccurrence of specific taxa, is what here is loosely referred to as seasonal succession. Thus, both shifts due to physical alterations as well as true ecological succession due to species replacement (Odum 1969), as determined by community-driven processes, will be included in this chapter.

In the following paragraphs I will discuss the concepts and theory regarding how dinoflagellate species assemblies are formed. This includes the abiotic and biotic factors that determine the community compositions, and the mechanisms involved in species replacement with time. In relation to these, I will describe the different adaptive strategies and the role of functional traits. Finally, I will also include examples of the patterns observed in different habitats.

SPECIES ASSEMBLY

Species Do Not Assemble Randomly

Species assemblages or communities have been widely studied in terrestrial plants, and much of the conceptual basis can be found in this literature (e.g., Kraft and Ackerly 2014). The two overarching concepts are that of a "species pool," which contains the suite of potential species that can colonize a habitat, and a "filter," the abiotic and biotic barriers to establishment (Figure 1). Overall, species communities are considered to be formed by deterministic processes, such as environmental and biotic filtering, and stochastic processes, such as dispersal, which regulates the species pool (Cornell and Harrison 2014). In contrast to perennial terrestrial plants, phytoplankton have a generation time that is very short and they must remain in suspension. In addition, the pelagic zones of aquatic habitats are highly dynamic, and vary rapidly both spatially and seasonally. As a consequence plankton communities change frequently throughout a year. Since the biotic and abiotic filters change with time, some species will disappear (die or encyst), while other species will be recruited from the species pool (Figure 1). The replacement of species will result in new assemblies, thereby generating a succession pattern.

Thus, to understand dinoflagellate succession, I will first focus on species assembly, regardless of whether differences are due to spatial or temporal factors. The major ecological filters to consider that define when and where dinoflagellates will be present in the water

column are the physical environment, resources and resource competition (both abiotic and biotic factors), and natural enemies as well as positive interspecies interactions (biotic interactions).

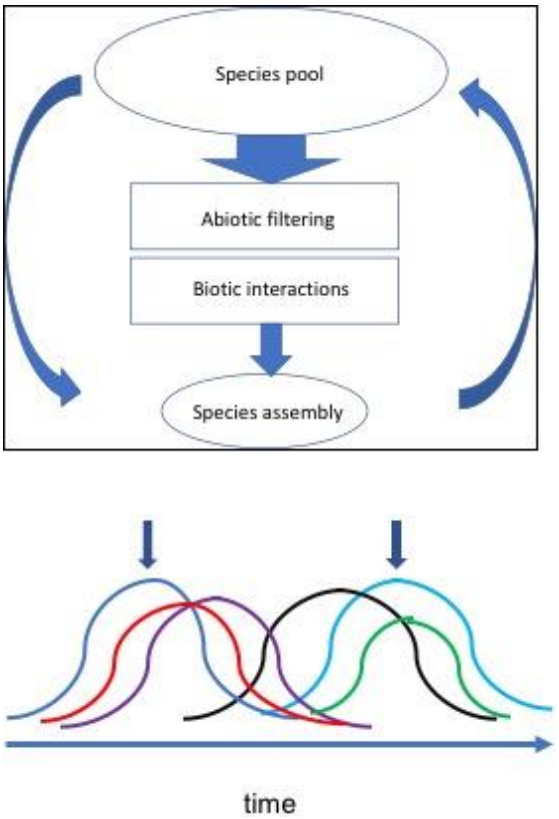

Figure 1. Schematic view of species assembly and seasonal succession of phytoplankton species. Abiotic and biotic conditions will change with time, and some species will be replaced, resulting in a seasonal succession of different taxa.

The Species Pool

The species compositions of a specific habitat will be determined by the pool of species available, on which the environmental filtering acts. Dinoflagellate species may originate from three main sources; (1) from holoplanktonic species, i.e., those present in the water column all the time, (2) from meroplanktonic species, which alternate growing in the water column and resting on the sediments, and provide an inoculum when cysts germinate or (3) species derived from stochastic introduction by advection (in marine habitats) (Anderson and Rengefors 2006) or other means of dispersal (marine and inland waters). As for dispersal to freshwater environments, dinoflagellates can potentially disperse by water (e.g., streams and rivers), air, or by animal vectors, likely as resting cysts (Kristiansen 1996, Tesson et al. 2016, 2018). In addition, cysts can be dispersed as resting cysts by ballast water and other human introduction (Hallegraeff and Bolch 1992, Lilly et al. 2002, Bolch and de Salas 2007, Briski et al. 2013).

Functional Traits and Seasonal Succession

While abiotic and biotic factors can be viewed to shape when and where which type of dinoflagellate communities form, species have responded by evolving a variety of functional traits. Functional traits are features that can be measured and influence processes such as growth and reproduction (Weithoff 2003). The fundamental traits that affect fitness can be categorized as life history-related, behavioral, physiological, and morphological (e.g. Litchman et al. 2006). These in turn have a function related to reproduction, resource acquisition, or predator avoidance (Litchman and Klausmeier 2008). In dinoflagellates, these traits include traits such as motility, resting cysts, mixotrophy, and toxin production, where cell-size is considered a master trait that affects several other traits (Litchman and Klausmeier 2008). For instance, small dinoflagellates tend to have higher growth rates but be more susceptible to grazing. Smayda and Reynolds (2001, 2003), also recognized cell size as the master trait, that could be related to dinoflagellate species assembly. By categorizing dinoflagellates (and other phytoplankton) by functional traits (as in Smayda and Reynolds 2001, 2003), models for assembly, dynamics, and succession can be tested.

THE ROLE OF ABIOTIC FACTORS IN SPECIES ASSEMBLY

Filtering by the Physical Environment

For planktonic assemblages, Margalef was among the first to argue that it is the physical environment, i.e., advection and turbulence, which controls plankton communities. Water movement, he reasoned, in turn affects light climate, suspension, and nutrient availability. Margalef developed a a model that became known as *Margalef's Mandala* that utilizes habitat mixing and nutrient concentration to explain selection of taxa and their seasonal succession (Figure 2) (Margalef et al. 1978). Based on this model, Margalef predicted round-shaped dinoflagellates in high nutrient – high stability habitats, and flattened dinoflagellate species in oligotrophic high stability habitats.

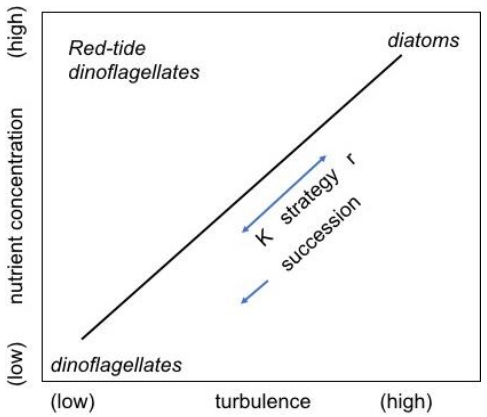

Figure 2. Margalef's Mandala, modified from Margalef et al. (1978). This conceptual model utilizes habitat mixing and nutrient concentrations to explain the presence of taxa and their seasonal succession. *K* refers to *K*-strategists and *r* to *r*-strategists, the former indicating species that are slow growers but good competitors, and the latter, species with fast growth rates.

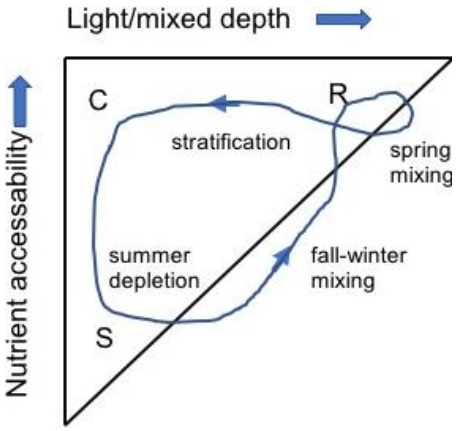

Figure 3. *Reynolds´ Intaglio*, redrawn from figure 6 in Smayda & Reynolds (2001), shows species selection based on nutrient accessibility and mixing/light depth. The year-long trace of the selective trajectory imposed by temperate seasonal habitats is added on top of the intaglio. C, S, and R refer to the primary adaptive life-cycle strategies. C-strategist are competitors or opportunistic colonists, S-strategists are nutrient stress-tolerant, while R-strategists are disturbance-tolerant.

However, while Margalef's Mandala predicted the presence of "red" rounded bloom-forming dinoflagellates in high-nutrient, low turbulence environments, this was contradicted by many coastal and freshwater harmful algal bloom (HAB) events, appearing in oligotrophic oceans or relative turbulent conditions (Reynolds 1988a, Smayda 2000, Steidinger et al. 1998). Reynolds resolved this by showing that nutrients and turbulence act independently and further developed Margalef's concept into *Reynolds' Intaglio*, with nutrient accessibility on one axes, and light/mixed depth on the other axis (Reynold 1998a) (Figure 3). Later, Smayda and Reynolds (2001) reasoned that the importance of turbulence is the degree of vertical structure it offers, rather than direct effect on nutrients, which allows the flagellated dinoflagellates to utilize their ability to swim to exploit light and nutrients. The current understanding is thus that the abiotic factors that shape dinoflagellate communities are centered around mixing-irradiance-nutrients.

Dinoflagellate Adaptive Strategies in Response to the Physical Environment

In order to predict and explain which dinoflagellates will occur in different physical environments, a first step is to consider which type of adaptive strategy will be favored in the different environments. Reynold's reasoned that phytoplankton are adapted to survive prolonged periods of suboptimal conditions. He hypothesized that (freshwater) phytoplankton have evolved three types of adaptive strategies based on Grime's C, S, R-strategists (Grime 1979, Reynolds 1988a) (Figure 3). Dinoflagellates exploit all three of these basic adaptive strategies: C-strategist are competitors or opportunistic colonists, S-strategists are nutrient stress-tolerant, while R-strategists are disturbance-tolerant. Within each, both r- (high growth rates) and K-selected (good competitors in crowded niches) species occur (Reynolds 1988a).

Life-Forms Based on Functional Traits and the Physical Environment

Based on Margalef's and Reynold's models, Smayda and Reynolds (2001) could group assemblages of marine dinoflagellates on a continuum from the shore to the open ocean. This continuum involves a gradient of decreasing nutrient levels, reduced mixing, and a deepening euphotic zone, and is based on morphotype and habitat preference (Figure 4). The general trend is for an increase in cell size along this gradient, with classical eridinoid/gymnoid/prorocentroid forms in high nutrient high mixing environment, towards more attenuated and chain-forming, and with the most elongated and elaborated in low nutrient highly stratified water masses (Smayda and Reynolds 2001). Within each sub-group of life-forms (nine types) species thus have a similar size/shape. Also, there is a general decrease of surface area to cell volume in each life-form type with reduced nutrients, mixing, and a deepened euphotic zone. Consequently, depending on the conditions, which can vary both spatially and temporarily, one of the nine different type communities will form.

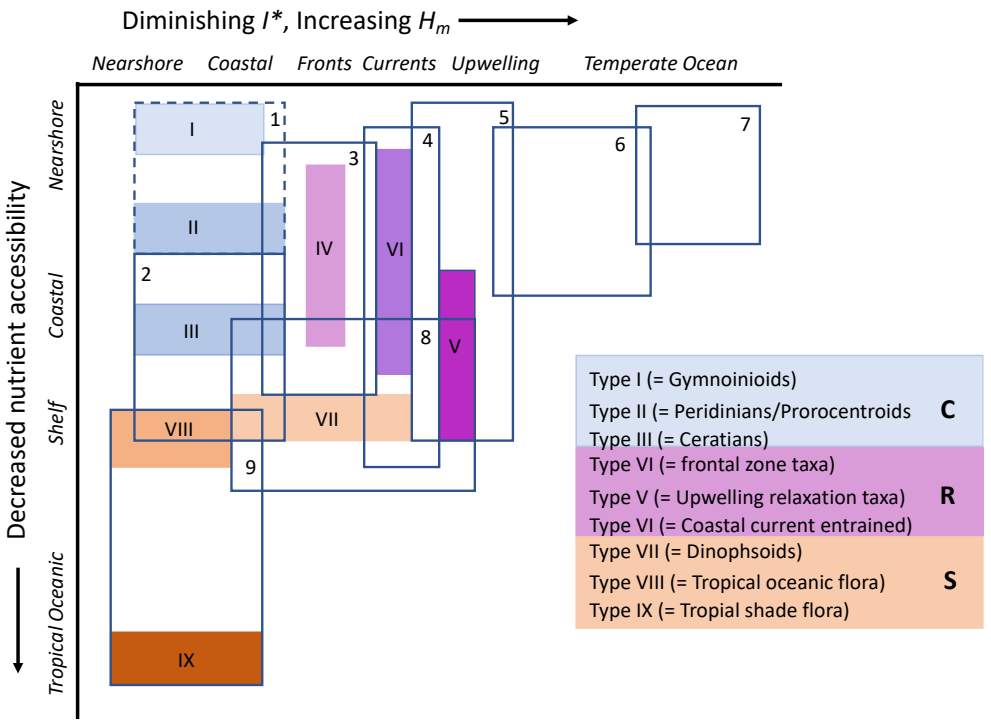

Figure 4. Smayda & Reynold's life-form types displayed on a schematic matrix of pelagic marine habitats. The marine habitats (numbers 1-10, see below), are defined by nutrient accessibility (y-axis) and turbulence/light climate (x-axis). *I** refers to the irradiance level that reaches cells, and *Hm* is the depth of the mixed layer. Smayda & Reynold's life-form types (roman numerals I-IX) are represented in colored boxes. Types I-II represent Reynolds' C types, i.e., invasive, small fast growing, requiring high nutrients. Types IV-VI are R-strategists, which are adapted to moderate shear stress and have efficient light harvesting ability. Types VIII-IX are typically large, ornamented species, often with endosymbionts, highly mixotrophic, and with high motility. Type III encompasses mainly C, but also S and R-strategists. The marine pelagic habitats are; 1 = increasingly eutrophic coastal waters, 2 = shallow shelf waters, 3 = frontal zones, 4 = off-shore currents, 5 = major upwelling, 6 = temperate ocean (spring), 7 = temperate ocean (winter), 8 = post upwelling relaxation, 9 = highly stratified tropical ocean. Redrawn and combined from Smayda & Reynolds (2001) Figures 4 & 5.

Smayda and Reynolds (2001, 2003) categorized their different dinoflagellate life-form types into the C, S, R adaptive strategies. In habitats that are relatively shallow and are high nutrient-enriched, C-strategists dominate. These include types I and II, and are invasive, small, fast growing species that thrive in high nutrient conditions. The species assemblages are composed of small to intermediate-sized gymnoid (Type I) and peridinoid (Type II) species (Figure 4). Habitats such as frontal zones, upwelling relaxation, and coastal currents are instead dominated by R-strategists. The latter are dinoflagellates adapted to elevated shear stress (but vulnerable to turbulence) and effective light harvesting. Many are strong vertical migrators and resting cyst-producers. They encompass types IV, V, VI. Type IV include typical frontal zone species that require high nutrients but are adapted to entrainment in coastal currents. Type V are adapted to upwelling habitats and bloom during upwelling relaxation, and have high tolerance to elevated shear. Type VI, in turn, are coastally entrained, and tolerate the shear/stress of coastal currents. Some examples are *Karenia mikimotoi* (IV), *Gymnodinium catenatum* (V), and *Karenia brevis* and *Alexandrium fundyense* (VI). Finally, towards the off-shore, with reduced mixing, low nutrients, and deep euphotic zones, types VII-IX are found, which are classified as S-strategists. Most are large and ornamented, highly motile, have endosymbionts, and are mixotrophic. These includes *Dinophysis* spp. (VII) and tropical flora, e.g., *Ceratium* spp. (VIII) and *Pyrocystis* spp. (IX).

DINOFLAGELLATE ASSEMBLY RULES

The life-form classification model allowed Smayda and Reynolds (2003) to propose five rules of assembly that govern marine dinoflagellate communities. These can be summarized as follows:

1. Specific habitat conditions select for specific life-forms, i.e., assemblages are not random, and all species are not capable of sustaining growth everywhere/all the time.
2. Life-forms are selected primarily on a physical-chemical habitat template of turbulence-irradiance-nutrients.
3. Dinoflagellates employ the C-, S- and R- strategies to exploit abiotic habitat conditions, with r-(fast-growing) and K-selected species (slow-growing, resource tolerant) within each strategy
4. Selection follows the pathway: phylogenetic to genera to species. In other words, it may be uncertain whether diatoms or flagellates will bloom, or which flagellates. But if it is dinoflagellates, the candidate genera are known.
5. Selection of species within a life-form is stochastic.

Currently, there are few studies that thoroughly test the Rules of Assembly. However, in a study of dinoflagellates in an estuary, Anderson and Rengefors (2006) concluded that the dominant species were those predicted by the Rules of Assembly, but also that the seasonal succession of cyst-forming species was not stochastic, thereby challenging Rule 5.

DINOFLAGELLATE SPECIES ASSEMBLY IN FRESHWATER LAKES

For freshwater dinoflagellates, there is no equivalence to the Smayda and Reynolds model and Assembly rules. However, Reynolds's classification system with C, S, R strategies and more fine-scaled subdivisions can, and has to a limited extent been applied to freshwater dinoflagellate species (Reynolds et al. 2002). Moreover, for temperate lakes, there is a general model for phytoplankton seasonal succession. The PEG (Plankton Ecology Group)-model (Sommer et al. 1986, 2012), predicts a high contribution of dinoflagellates in late summer when the water column is stratified and both silica and orthophosphate has decreased down below levels of detection. The large dinoflagellates that dominate have a low growth rate, but are considered to have a selective advantage due to high grazer resistance and their ability to migrate vertically to retrieve nutrients in the meta/hypolimnion. Built on the PEG model, the Lake Erken model (Blomqvist et al. 1994) describes a similar situation in late winter/early spring, when the column stability is high due to ice-cover in high latitude/altitude lakes, and dinoflagellates can form blooms under ice. Nevertheless, finer-scale models are lacking.

BIOLOGICAL FACTORS SHAPING DINOFLAGELLATE COMMUNITIES

While Reynolds and Smayda proposed that selection of species within a dinoflagellate life-form type is stochastic (see above), there is evidence that deterministic biotic factors play an important role. Although the physical environment explains much of general phytoplankton assembly and succession, biotic interactions perhaps are more important in determining the specific dinoflagellate species replacement. The biotic filtering factors include natural enemies (grazers and parasites), but also resource competition (with other phytoplankton, including other dinoflagellates). These processes, similar to the abiotic filters, have resulted in the evolution of different adaptive traits.

COMPETITION

Resource Competition

Resource competition occurs when reproduction is limited by consumption of a commonly needed resource (Tilman 1981). For dinoflagellates (and other phytoplankton) these include light, inorganic carbon, phosphorus, nitrogen, silica (not all), trace elements, and vitamins. Dinoflagellates are generally considered poor competitors for phosphorus and have low maximum uptake (V_{max}) rates and high half-saturation (K_s) constants. For example, the freshwater *Peridinium* sp. had a V_{max} of 0.11 compared to green algae that range from 5-25 in V_{max}, and a K_s of 6.3 compared to cyanobacteria that have K_s of 0.2-0.3 µmol phosphorus L^{-1} at half V_{max} (Reynolds 1988a). Likewise, marine dinoflagellates also generally have high K_s, ranging from 0.01 to 2.7 (Cembella et al. 1984). Interestingly, in a natural freshwater community bioassay, the dinoflagellates were not nutrient-resource limited in 54% of the cases, or else only slightly limited. When limited, *Ceratium hirudninella* was usually nitrogen limited, while *Peridinium bipes*, *P. cinctum*, *P. umbonatum*, and

P. inconcpicuum were more often P-limited (Sommer 1988). These findings illustrate that dinoflagellates have other nutrient acquisition strategies.

Because they are poor competitors for nutrient assimilation, dinoflagellates have evolved a number of adaptations to compete. These include luxury storage (polyphosphates) (Cembella et al. 1984), use of alkaline phosphatase (AP) to utilize organically bound phosphorus (Cembella et al. 1984, Dyhrman and Palenik 1999, González-Gil et al. 1998), vertical migration to access nutrients in the meta- and hypolimnion (Heaney and Eppley 1981, James et al. 1992), and mixotrophy (Stoecker 1999). Together, these adaptations allow dinoflagellates to flourish when nutrient conditions are limiting phytoplankton growth.

Diatom-Dinoflagellate Competition

Because of their generally low competiveness for nutrients, dinoflagellates usually do not dominate during seasons characterized by relatively high nutrient conditions, often coupled to high turbulence (see figure 4 for marine species). In temperate lakes, they tend to dominate during periods of high water column stability (under-ice later winter or summer stratification) when nutrients are low in the epilimnion but available at depth (e.g. Sommer et al. 1986, Rengefors 1998, Heaney et al. 1988, Kremp et al. 2005). On the other hand, diatoms dominate during spring and fall turnover, when turbulence is and nutrients are high (Sommer et al. 1986, Blomqvist et al. 1994). In tropical lakes, dinoflagellates are often abundant during the dry season, when turbulence is low (Meyer et al. 1997).

In the field it is, however, difficult to tease apart column stability from nutrients. In the Baltic Sea for instance, recent evidence indicates a trend of an increased proportion of dinoflagellates relative to diatoms (Klais et al. 2011), which was originally suggested to be coupled to a reduction of silica. Nevertheless, in a mesocosm experiments, changed nutrient ratios of N, P, and Si did not lead to dinoflagellate dominance (Kremp et al. 2008). Instead, the dinoflagellate dominance depended mainly on the size of the inoculum (from cysts), suggesting that bloom initiation strategies and initial conditions were more important (Tesson et al. 2018, Klais et al. 2011, Kremp et al. 2008).

In another study, observations together with numerical modelling indicated that phosphate, but not light, temperature, silicate or nitrate, is critical in seasonal succession from diatoms to dinoflagellates in the China Sea (Zhou et al. 2017). While not important for species replacement, nitrate was implicated as important in determining the intensity and duration of the dinoflagellate bloom.

Mutshinda et al. (2016) analyzed a set of dinoflagellate and diatom species during seven consecutive years and found repeated seasonal patterns, where biomass was mainly explained by temperature and irradiance. While the diatoms had a higher growth rate, the dinoflagellates responded more to increased temperature, and dominated during warmer stratified conditions (Mutshinda et al. 2016). However, the sequence of particular species was not predictable, suggesting again that stochastic variation governs species dynamics.

Interference Competition – Alleopathy

While dinoflagellates can compete by exploitative competition, as described above, they can also compete by interference competition. The latter refers to when the competitor growth is inhibited, for instance by chemical interference, aka allelopathy (Lampert and Sommer 1997). It is likely that allelopathy may also contribute to seasonal succession, by reducing competitor density, and increasing nutrient availability. Keating (1977,1978) was the first to provide some evidence that algal succession may be connected to allelopathic interactions. Many dinoflagellates have been shown to produce allelochemicals that suppress other competitor species, including members of diatoms, dinoflagellates, and cyanobacteria (see reviews by Gross 2003, Legrand et al. 2003). For example, *Karenia brevis*, produces allelopathic compounds that inhib many co-occurring species (Prince et al. 2008), and which could help explain its dominance. However, microcosm studies show that the allelopathic effect on co-occurring natural assemblies is species-specific and dependent on conditions of cells (Poulson et al. 2010). While recent modelling efforts demonstrate that certain phytoplankton communities are susceptible to allelochemicals, and that alleopathy can help explain species replacement (Muhl et al. 2018), studies from the natural communities are still largely lacking.

Dinoflagellate – Cyanobacteria Competition in Lakes

In temperate lake ecosystems, dinoflagellates and cyanobacteria often occupy the same niche during late summer stratification (Sommer et al. 1986). For instance, in both Rostherne Mere, England, and Lake Erken, Sweden, the late summer bloom altered between dinoflagellate (*Ceratium* sp.) and cyanobacterial dominance (Blomqvist et al. 1994, Reynolds and Bellinger 1992). Also in the oligotrophic Lake Njupfatet, Sweden, dominance switched between the cyanobacterium *Merismopedia tenuissima* and the dinoflagellate *Peridinium inconspicuum* (Blomqvist et al. 1994). Evidence from mesocosm experiments show that this competition is driven by nutrients, with cyanobacteria being favored by ammonium-nitrogen and dinoflagellates by nitrate-nitrogen (Blomqvist et al. 1994). Similarly, in subtropical Lake Kinneret (Sea of Galilee), Israel, there is a strong negative correlation between the bloom-forming *Peridinium gatunense* and the cyanobacterium *Microcystis* sp. (Vardi et al. 2002). However, here experimental studies show that this interaction is likely due to density-dependent allelochemical cross-talk, where each species' allelochemicals can inhibit or lyze the other (Susenik et al. 2002, Vardi et al. 2002). To summarize, the evidence to date suggests that both resource availability and intereference competition plays a role in the dynamics of dinoflagellate-cyanobacteria competition.

NATURAL ENEMIES

Grazing by Metazoan Zooplankton

Another biotic interaction that has a very strong effect on phytoplankton is grazing by zooplankton, both as a selective force leading to adaptations to minimize losses due to

grazing, and by affecting seasonal succession (Sommer et al. 1986). In freshwater, the PEG-model (Sommer et al. 1986, 2012) predicts and explains the dominance of large dinoflagellates (and other canopy species) as a response to high water-column stability, low nutrient conditions, and high predation due to metazoans (mostly calanoid copepods). Modelling by Scheffer et al. (1997) was able to explain seasonal patterns in phytoplankton biomass using predator-prey models. Klausmeier and Litchman (2012) were subsequently able to model species replacement of an edible species leading to a grazer-induced clear-water phase, followed by the dominance of an inedible species. They also found that nutrients and the length of the season could influence the trajectories, and that a bimodal seasonality of inedible species before and after the grazer peak, could be modelled. These models suggest that grazing can have a significant effect on dinoflagellate succession, potentially including species-specific dynamics.

Predation occurs to some extent all year round and in all habitats, but the composition of predators varies, as do the numbers. For instance, zooplankton abundance is very low in lakes under the ice during winter, a time period when round peridinid dinoflagellates flourish (Rengefors 1998). Rengefors et al. (1998) showed that *Peridinium* (now *Apocalathium*) *aciculiferum* cyst germination was reduced by zooplankton exudates, and suggested that growth during winter was a temporal escape from predation. In a marine estuary, the decline of *Alexandrium tamarense*, *Gonyaulax verior*, and *Scrippsiella* spp due to cyst formation, occurred when nutrients and temperature were conducive of growth, but grazers increased (Anderson and Rengefors 2006) . Other correlative evidence shows high dinoflagellate biomass when calanoid population numbers are low, and vice-versa (e.g. Calic et al. 2013). In Vancouver Lake, the cyploid copepod *Diacyclops thomasi* grazed selectively on dinoflagellates, reducing the standing stock by 30% per day (Rollwagen-Bollens et al. 2013), and likely contribute to take-over by other phytoplankton.

Predation by Dinoflagellates and Mixotrophy

A large percentage of dinoflagellates have been shown to be mixotrophic, and to a lesser or larger extent photoautrophic (see Chapter 7, Hansen & Tillmann). Given that constitutive and non-constitutive mixotrophic dinoflagellates (see chapter 7) can have the role as grazers on autotrophic species, there is also a complex correlation and interrelationship between dinoflagellates taxa, which affects seasonal succession. Hansen (1991) found that large heterotrophic dinoflagellates coincided with, and fed on large dinoflagellate species in late summer (August/September). The small heterotrophs, in contrast, dominated in the summer (May to July) when nanoplankton were most abundant. Nevertheless, it is difficult to unravel whether the system is top-down or bottom-up governed.

In a modelling study, Berge et al. (2017) proposed that there is a seasonal aspect to the degree of mixotrophy in dinoflagellates. In the spring, mixotrophic populations are more photoautorophic, i.e., invest in photosynthesis and resource uptake, while in the summer populations have a higher investment in phagotrophy (Berge et al. 2017). Based on modelling and experimental studies with *Karlodinium armiger* and *K. veneficum*, Berge et al. (2017) constructed a seasonal cycle with high nutrients in the spring and recycled nutrients in the summer. *K. armiger* which has invested mostly in phagotrophy was favored in the summer,

when optimal investment in photosynthesis and nutrient uptake was low, while *K. veneficum* (high investment in inorganic resource uptake) was favored in the spring and end of summer.

Parasites and Dinoflagellate Dynamics

Microparasites, including viruses, bacteria, fungi, and protists, have long been known to infect dinoflagellates. However, their importance in dinoflagellate population dynamics and succession is still not fully understood. Because of the species specific nature of many microparasites, this may explain some of the variation which has been attributed to stochasticity.

Zoosportic parasitoids, are aquatic parasites which produce numerous asexual spores, kill their hosts and have a significant top down effect (Jephcott et al. 2016). These include several protist groups, and but especially fungi and alveolates are parasitic on dinoflagellates. Fungal parasites (chytrids) were described in freshwater dinoflagellates early on (Canter 1968), and were shown to be involved in the dynamics of *Ceratium* spp. (Canter and Heaney 1984, Heaney et al. 1988, Sommer et al. 1984) . A long-term data set shows that periods of high infections were associated with marked decreases in *Ceratium* populations, and in addition affected next year's inoculum as they infected resting cysts (Heaney et al. 1988, Sommer et al. 1984). Thus, if losses to parasites are large, this will help end the boom of the infected species, while stimulating the replacement of another. In marine dinoflagellates, a chytrid parasite (*Dinomyces*) was isolated and described in *Alexandrium minutum*, and was shown to preferentially infect *Alexandrium* species, but also strains of *Heterocapsa*, *Ostreopsis*, and *Scrippsiella* (Lepelletier et al. 2014). While mortality was high in some strains, their role in the population dynamics of these species remain to be explored.

In marine dinoflagellates, the taxa *Parvilucifera* (Perkinsea, Alveolata) and *Amoebophrya* (Syndiniales, Alveolata) are the most dominant and well studies parasitoids (Jephcott et al. 2016). Because each infection generates hundreds of spores, these parasitoids have the potential to control the sizes of host populations, and thereby affect seasonal succession. In a study of the dinoflagellate succession in Chesapeake Bay, USA, Coats et al. (1996) showed that the species *Gymnodinium sanguineum*, *Gyrodinium uncatenum*, and *Scrippsiella* sp. showed temporal differences in peaks during the summer season. Infection of the parasite *Amoebophrya ceratii* in these species, showed similar fluctuations, and high infection levels were often followed by a population decline in the host. Consequently, the authors postulated that the parasites were the main drivers of species succession. Similarly, Chambouvet et al. (2008) investigated the seasonal succession of dinoflagellates in an estuary in Brittany, France. Here *Heterocapsa rotundata* typically blooms at the end of May, and is followed by *Scippsiella trochoidea*, *Alexandrium minutum*, and *H. triquetra*. While population dynamics were not correlated to physical-chemical parameters, each species was infected by a species-specific parasitoid (Syndiniales), and the decline of each population preceded by high infection rates and subsequently high number of parasite spores. Montagnes et al. (2008) next constructed a mathematical model that could predict the decline of dinoflagellate populations due to microparasites. Likewise, *Parvilucifera* parasitoids appear to be involved in bloom termination of *Alexandrium minutum*, as they regularly peaked in abundance after the dinoflagellate bloom peak, and were shown to contribute with up to 18% of the mortality (Alacid et al. 2017).

Many bacteria have been shown to be algicidal to a number of dinoflagellates but their role in natural populations are still unclear (reviewed in Meyer et al. 2017). For example, it has been shown that associated alpha, beta and Gammaproteobacteria can inhibit growth the dinoflagellate *A. tamarense* (Hu et al. 2015). However, the importance and role of bacteria in seasonal succession has not been established.

Lytic viruses have been found to control phytoplankton either by causing population decline or by preventing populations from reaching high densities (Brussard 2004). It is also known that algal viruses are very host specific (Short 2012). As such, viral-mediated mortality can affect both interspecific and intraspecific phytoplankton succession. One example of viruses that are thought to be able to control the biomass of dinoflagellate populations, is the RNA-virus HcRNAV in *Heterocapsa ciruclarisquama* (Nakayama and Hamaguchi 2016, Nagasaki et al. 2004, Tarutani et al. 2001).

THE ROLE OF LIFE-CYCLE TRANSITIONS IN SEASONAL SUCCESSION

While most functional traits have been treated above in relation to environmental factors determining species assembly, a remaining trait, unrelated to cell size, but closely coupled to seasonal succession, is life-history traits.

The bloom dynamics of many dinoflagellates are regulated by the transition between resting cysts and motile planktonic cells (Anderson et al. 1983, Rengefors 1998, Bravo et al. 2010, Krempand Heiskanen 1999, Kremp and Anderson 2000). In other words, life-cycle events determine the seasonal occurrence of cyst-forming (meroplanktonic) dinoflagellates. The two main events framing the seasonal occurrence of meroplanktonic species are cyst germination (excystment), providing the population inoculum, and cyst formation (encystment) determine the end of the planktonic phase (Anderson et al. 1983). Early studies addressing the role of life cycle transitions in bloom initiation and termination were on *Ceratium hirundiella* in freshwater (Chapman et al. 1982, Heaney et al. 1983) and *Alexandrium tamarense* (Anderson et al. 1983). The timing of these events have been shown to depend on a combination of inherent traits of individual species, and external environmental cues (Anderson 1980, Binder and Anderson 1987, Rengefors and Anderson 1998). Reliance on these cues is necessary to avoid periods that are generally unfavorable to growth.

Studies on the role of life-history stages on succession are mostly focused on single species, although there are a few examples of multiple species, addressing species replacement. For example, the summer bloom sequence of six taxa of cyst-producing dinoflagellates in Perch Pond, USA, could be explained by the length of the cyst dormancy period and the temperature window in which they could germinate (Anderson and Rengefors 2006) (Figure 5). Similarly, the timing of excystment was described for the brackish water species *Scrippsiella hangoei* (now *Biecheleria baltica* and *Apocalathium malmogiense*), which germinate and grow during the winter. In freshwater, the appearance of *Ceratium hirundinella* in early summer and the growth of *Apocalathium aciculiferum* (previously *Peridinium aciculiferum*) during winter was explained by cyst dormancy, together with an endogenous clock timing excystment together with a germination temperature window (Rengefors and Anderson 1998).

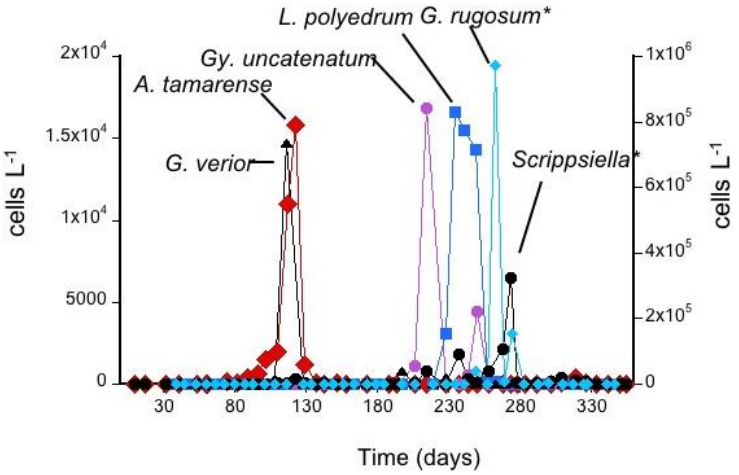

Figure 5. Seasonal succession of six cyst-forming dinoflagellate species in Perch Pond, USA. Note the sequential biomass peaks (n.b. species with * are plotted against the Y2-axis). The seasonal appearance of each species could be explained by dormancy period, temperature window for germination, and threshold for germination. Data replotted from Anderson & Rengefors (2006).

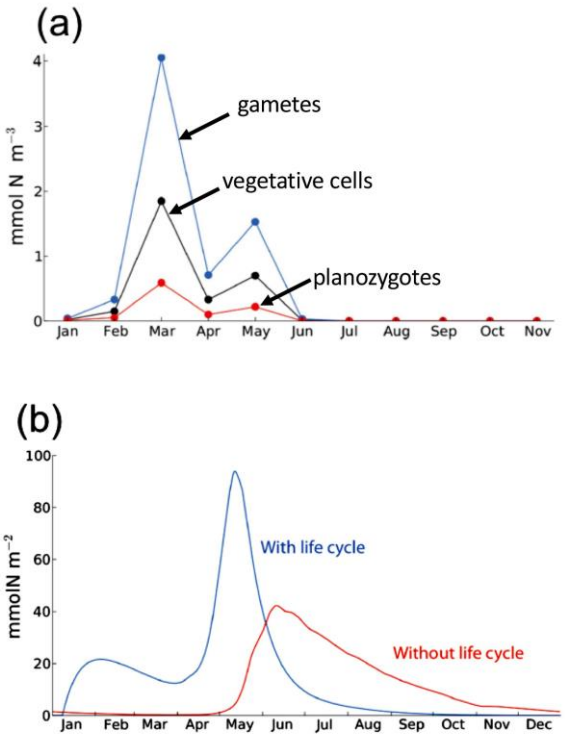

Figure 6. Illustration of data from a dinoflagellate bloom and a model of the same species, with and without the inclusion of life-cycle events. Panel (a) shows cell biomass concentrations in moles nitrogen (converted from cell numbers) from field observations of the brackish-water dinoflagellate *Bicheleria baltica*. The different colored lines represent vegetative cells (black), gametes (blue), and planozygotes (red). Panel (b) displays model results of the same system showing dinoflagellate cells in the water with and without including life-history events. These results illustrate that the timing, magnitude, and duration of a bloom is affected by life-cycle events, and the that by including the life-cycle the model is much improved. Re-printed from Warns et al. 2013, Figure 8a and 7a, with permission from the authors.

The end of the bloom, on the other hand, is usually controlled by cyst formation in these species. Encystment has been shown to be important in bloom termination in a number of marine dinoflagellate species (e.g. Anderson et al. 1984, Kremp and Heiskanen 1999, Garces et al. 2004, Wang et al. 2007). In laboratory experiments, encystment can usually be induced by nutrient stress (von Stosch 1973, Anderson and Lindquist 1985, Figueroa et al. 2005), while lower nutrient conditions are typically not associated with cyst formation in the field, neither in marine or freshwater habitats (Anderson et al. 1983, Kremp and Heiskanen 1999, Rengefors 1998). Instead, Kremp et al. (2009) provided data that indicated that dinoflagellates utilize reliable token cues (in this case temperature), even if the ultimate causes may be nutrient depletion.

Some efforts have been made at modelling population dynamics based on life-cycle transitions. For example, Yamamoto et al. (2002) used both encystment and excystment to model the population dynamics of *Alexandrium tamarense* in Japan. Similarly, based on cyst distribution and germination, McGillicuddy and colleagues have developed a model explaining the temporal (and spatial) distribution pattern of *Alexandrium fundyense* (Stock et al. 2005, McGillicuddy et al. 2005). Warns et al. (2013) modeled the seasonality of the cold-water brackish species *Biecheleria baltica* based on the whole life cycle (germination, encystment, growth rates) and temperature and nitrogen concentrations, resulting in seasonal patterns similar to those observed in the field. When the removed life cycle transitions from the model, they could demonstrate that the life cycle affects the timing, magnitude and duration of the bloom (Figure 6). On the other hand, Estrada et al. (2010) found in a simulation study that excystment rates were only important in the beginning of the bloom, but failed to affect final bloom magnitude. Also, fieldwork on the lake-dwelling *Ceratium hirundinella*, indicated that the size of the germinating inoculum did not correlate with the final maximum bloom densities (Rengefors 1998). Nevertheless, overall it can be concluded that species with resting cysts are more likely to re-occur at specific sites, less prone to stochastic processes compared to non-cyst producers.

CONCLUSION

General models for dinoflagellate succession are largely lacking, but the building blocks are there, based on functional traits, knowledge of seasonal changes in the physical environment, species pools, and the factors determining assembly. From these building blocks, and models produced so far, it should be possible to make better predictions in other systems. Although conclusions to date indicate that the succession patterns of specific species are difficult to predict, and that stochastic events rule these, there is some promise of improving the resolution. In my opinion, this lies in improved incorporation of mainly the biological factors in conceptual and mathematical models. These include species-specific life-cycle transitions, better understanding of species-specific mortality due to natural enemies, and allelopathic interactions. Moreover, by utilization of molecular tools it should be easier to better establish the potential species pool in an area.

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Chapter 139

CULTURE AND GROWTH OF DINOFLAGELLATES

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ABSTRACT

In this chapter, following a brief historical review of dinoflagellate cultures, methods of culturing athecate, thecate, toxic dinoflagellates and composition of 22 media are presented. About a total of 100 species of photosynthetic, heterotrophic, mixotrophic and symbiotic dinoflagellates are amenable for culturing. Division rates of natural assemblages of dinoflagellates, and as well as those cultured reported from various publications are summarized. In general, dinoflagellates have lower division rates compared to several other microalgae. The impact of salinity, temperature, and light, nutrients such as phosphate, nitrate, ammonia and iron on division rates is discussed. Mixotrophy appears to be common amongst dinoflagellates and resulted in higher growth rates than cells grown as autotrophs. The lower division rates in dinoflagellates are possibly due to channelling of much energy into the production of cellulose skeleton and toxins; this needs to be substantiated with detailed observations on the Carbon: Nitrogen dynamics in a variety of dinoflagellate cultures.

Keywords: growth, media, nutrients, divison rates

INTRODUCTION

Culturing marine microalgae dates to 1893 [1]. Since culturing the photosynthetic diatom *Thalassiosira gravid* in artificial seawater as a feed for animal life [2], interest has grown in culturing microalgae in nutrient media as feed for invertebrates and commercially important

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organisms.The pioneering contributions of Pringsheim [3] in establishing pure cultures of algae and usage of soil extract for enrichment, Venkataraman [4] on culturing and cultivation of tropical algae, Provosoli [5] in developing artificial culture media and treatment with antibiotics to obtain bacteria free cultures, Tamiya [6] in developing synchronous cultures for investigating individual life cycles and Burlew [7] in developing pilot plant cultures gave rise to several interesting culture studies. Studies of Stein [8], Fogg [9] and Richmond and Hu [10] provide an excellent introduction to algal culturing, metabolism and growth. Results obtained on algal cultures established from various geographical regions have been utilized for various purposes including scientific enquiry, to advance our knowledge on their structure and physiological functioning in relation to their environmental conditions, wastewater treatment, enhancing primary production in oligotrophic ocean expanses by ocean fertilization with nitrogen or iron, providing microalgal feed for animal husbandry, specifically in aquaculture, and in discovering natural bioactive compounds and nutraceuticals that will advance microalgal biotechnology and biomedicine [11-13]. This increase in interest in culturing microalgae has reflected in worldwide institutionalized culture centers (Table 1). These centers are engaged in the collection of microalgae, isolation and maintenance, characterization of algal strains from all over the world, from polar to tropical waters, marine, freshwater, brackish, and hypersaline environments and supply of strains for scientific research, teaching, and biotechnology development.

Table 1. Microalgal culture collection centers

Center	Country	Address	Strains
CCMP	USA	National Center for Marine Algae and Microbiotancma@bigelow.org	2100
UTEX	USA	The UTEX Culture Collection of Algae at the University of Texas at Austin includes over 3,000 different strains of living algae. www.bio.utexas.edu/research/utex	3000
UTCC	Canada	Canadian Phycological Culture Centrewww.botany.utoronto.ca/utcc	258
CCAP	Scotland	The Culture Collection of Algae and Protozoa of The Scottish Association for Marine Science www.ife.ac.uk/ccap	3000
RCC	France	Roscoff Culture Collectonvaulot@sb-roscoff.fr	5000
SAG	Germany	The Culture Collection of Algae at The Gottingen University www.epsag.uni-goettingen.de/html/sag.html	2300
NIVA CCA	Norway	Norwegian Culture Collection of Algae	2000
CSIRO NACC	Australia	The Australian National Algae Supply Servicewww.csiro.au	3000
CICCM	New Zealand	The Cawthron Institute Culture Collection of Micro-algae only toxic algae	450
NIES	Japan	The National Institute for Environmental Studies Collection www.nies.go.jp/biology/mcc/home.htm	935
CCVIEO	Spain	https://vgohab.es/coleccion-de-cultivos	
CODIMAR	Mexico	https://www.cibnor.gob.mx/investigacion/colecciones-biologicas/codimar (dinoflagellates only)	149
IOUSP	Brazil	Marine Microalgae Culture Collection secrdob@netuno.io.usp.br	
CCTCC	China	Chinese Centre for Type Cultures Collections	
IPPAS	Russia	Culture Collection of Microalgaekuptsova@ippras.ru	

Table 2. Enrichments in culture media for dinoflagellates in Excel format

MEDIUM →	AQUIL	ASP M	ESAW	ESNW	f/2	K	L1	ANTIA	SOILWATER	GPM	OPTIMAL	OPTIMAL 2	DAIGO MK	MLH	NH15	NR12	NS	M9	CS1	B+	SM
Enrichment ▼in µ Mols																					
Agar																		18.00 g lit^{-1}#	18.00 g lit^{-1}#	15.00 g lit^{-1}	
Adenine Sulfate															2.50						
DL-Alanine																				1.12 x 10^3	
DL-Aspartic acid																				7.51 x 10^2	
DL-Asparagine																				7.57 x 10^2	
$BaCl_2$															3.60 x 10^{-1}						
Betaine HCl														9.70 x 10^3							
Biotin (Vitamin H)	2.25 x 10^{-3}	1.00 x 10^{-3}	4.09 x 10^{-3}	4.09 x 10^{-3}	2.05 x 10^{-3}	2.05 x 10^{-3}	2.05 x 10^{-3}	4.09 x 10^{-3}	4.09 x 10^{-3}	4.09 x 10^{-3}	1.78 x 10^{-2}	2.97 x 10^{-3}	6.00 x 10^3	8.20 x 10^{-3}	2.10 x 10^{-3}			4.30 x 10^{-3}	4.30 x 10^{-3}	1.02 x 10^{-3}	
Calcium Pantethenate		1.00 x10^{-1}																4.20 x10^{-2}	4.20 x10^{-2}	2.10 x10^{-1}	
$Ca(NO_3)_2.4H_2O$																					
$CaCl_2$														7.5 x 10^3				7.49 x 10^3	9.98 x 10^3	3.75 x 10^3	7.48 x 10^3
$CaCl_2$. $2H_2O$	1.05 x 10^4	1.00 x 10^4	9.14 x 10^3												4.80 x 10^3	1.00 x 10^2	5.00 x 10^3				
Choline H_2 citrate															1.70 x 10^{-1}			1.69 x 10^{-1}	1.69 x 10^{-1}	8.47 x 10^{-1}	
Citric acid .H2O											32.50	65.00									
$CoCl_2$																		5.09 x 10^{-1}	1.70 x 10^{-1}	5.09 x 10^{-2}	5.09 x 10^{-1}

Table 2. (Continued)

MEDIUM →	AQUIL	ASP M	ESAW	ESNW	f/2	K	L1	ANTIA	SOILWATER	GPM	OPTIMAL	OPTIMAL 2	DAIGO MK	MLH	NH15	NR12	NS	M9	CS1	B+	SM
$CoCl_2$. H_2O											4.20×10^{-2}										
$CoCl_2$. $6H_2O$	5.03×10^{-2}	3.00×10^{-1}			4.20×10^{-2}		5.00×10^{-2}		5.04×10^{-1}	5.04×10^{-1}		14.03×10^{-3}				4.00×10^{-2}	5.00×10^{-1}				
$CoSO_4$. $7H_2O$			5.69×10^{-2}	5.69×10^{-2}		5.00×10^{-2}		5.00×10^{-2}					49.80×10^{3}								
$CuCl_2$																				1.89×10^{-2}	
$CuCl_2$. $2H_2O$																					
$CuSO_4$. $5H_2O$	1.96×10^{-2}	3.00×10^{-1}			3.93×10^{-2}	1.00×10^{-2}	1.00×10^{-2}	1.00×10^{-1}					1.00×10^{4}								
Cyanoco-balamine (B12)	3.70×10^{-4}	1.00×10^{3}	1.48×10^{3}	1.48×10^{-3}	3.69×10^{-4}	3.69×10^{-4}	3.69×10^{-4}	1.48×10^{-3}	1.48×10^{-3}	$1,48 \times 10^{-3}$	1.33×10^{-3}	25.00×10^{-5}	1.00×10^{3}	7.49×10^{-4}	7.40×10^{-4}		7.40×10^{-4}	7.42×10^{-4}	7.42×10^{-4}	9.22×10^{-5}	
Cytidylic acid																			92.80		
EDTA (anhydrous)	10.00	48.00														1.50×10^{-1}					
$Fe(C_4H_4O_6)_3$															4.50						
$Fe(NH_4)_2$ $(SO_4)_2$. $6H_2O$																					
$FeCl_3$																		23.60	20.00	23.30	5.46
$FeCl_3$. $6H_2O$	1.00	2.00	6.55	6.55	11.70	11.70	11.70	10.00	5.37	5.37	9.36	9.36				4.00×10^{-1}	5.40				
$FeNH_4$ Citrate																					
Folic Acid		2.00×10^{-3}													5.70×10^{-4}			5.67×10^{-4}	5.67×10^{-4}	2.83×10^{-3}	
Folinic aid																		3.91×10^{-5}	3.91×10^{-5}	1.96×10^{-4}	
$FeSO_4.7H_2O$																					
D-Glucose														22.00 x 10^{3}							

MEDIUM →	AQUIL	ASP M	ESAW	ESNW	f/2	K	L1	ANTIA	SOILWATER	GPM	OPTIMAL	OPTIMAL 2	DAIGO MK	MLH	NH15	NR12	NS	M9	CS1	B+	SM
Glycerol								5.00×10^{5}													
Glycylglycine		5.00×10^{3}																			
H_2SeO3						1.00×10^{-2}	1.00×10^{-2}				7.50×10^{-3}	5.00×10^{-3}	1.32×10^{4}		6.40×10^{-1}						
H_3BO_3	48.50	4.00×10^{2}	3.72×10^{2}						5.53×10^{2}	5.53×10^{2}		400.00			23.00		5.53×10^{2}	5.55×10^{2}	1.85×10^{2}		5.55×10^{2}
HOEDTA																					
Histidine HCl														8.00 x 10^{2}							
L-Histidine																				64.40	
Inositol															5.60×10^{-1}			5.55×10^{-1}	5.55×10^{-1}	2.78	
Iodine		2.00×10^{-1}																			
K_2CrO_4							1.00×10^{-2}				1.00×10^{-2}	1.00×10^{-2}			1.90×10^{-1}						
K_2HPO_4									2.01×10^{2}	2.01×10^{2}			28.70×10^{6}		57.00	10.00			57.40	5.74	
$K_2HPO_4.3H_2O$																					
KBr	8.40 x 10^{2}	5.00 x 10^{2}	7.25 x 10^{2}																		
KCl	9.39 x 10^{3}	1.00 x 10^{4}	8.03 x 10^{3}											9.00 x 10^{3}	8.00 x 10^{3}		1.00 x 10^{4}	8.05 x 10^{3}	8.05 x 10^{3}	8.05 x 10^{3}	9.39 x 10^{3}
KH_2PO_4															7.40×10^{-1} *						
KNO_3								2.47×10^{3}	1.98×10^{3}	1.98×10^{3}					1.00 x 10^{2}						
Lactic acid																			3.33×10^{2}		
Li		10.00																			
MES														8.00 x 10^{3}							
DL-Methionine																				67.00	

Table 2. (Continued)

MEDIUM →	AQUIL	ASP M	ESAW	ESNW	f/2	K	L1	ANTIA	SOILWATER	GPM	OPTIMAL	OPTIMAL 2	DAIGO MK	MLH	NH15	NR12	NS	M9	CS1	B+	SM
$MgCl_2$. $6H_2O$	5.46 x 10^4	2.00 x 10^4	4.71 x 10^4												2.20 x 10^4				1.97 x 10^4		
$MgSO_4$														28.00 x 10^3							
$MgSO_4$. $7H_2O$		2.00 x 10^4													2.40 x 10^4	50.00	2.50 x 10^4	3.65 x 10^4	2.43 x 10^4	2.43 x 10^4	3.65 x 10^4
Mn EDTA													8.71 x 10^5								
$MnCl_2$. H_2O							9.09 x 10^{-1}					20.00									
$MnCl_2$. $4H_2O$	1.21 x 10^{-1}	10.00			9.10 x 10^{-1}	9.00 x 10^{-1}			21.80	21.80			9.10 x 10^5		9.00 x 10^{-1}	5.00 x 10^{-2}	21.80	21.80	7.28	21.80	21.80
$MnSO_4$. $7H_2O$																					
$MnSO_4.4H_2O$			2.42	2.42				4.56													
NTA																		1.05 x 10^3		5.23 x 10^2	
Na NO_3	100.00	1.00 x 10^3	5.49 x 10^2	5.49 x 10^2	8.82 x 10^2	8.82 x 10^2	8.82 x 10^2		2.35 x 10^3		6.30 x 10^3	8.50 x 10^3	2.35 x 10^3			1.50 x 10^2	3.50 x 10^3	5.88 x 10^2	1.18 x 10^2	1.18 x 10^2	5.88 x 10^2
$Na_2B_4O_7$. $10H_2O$																					
Na_2CO_3 H_2O																		2.42	2.42	4.03 x 10^2	
Na_2CO_3											100.00	40.00									
Na_2EDTA															27.00			80.60	26.90	80.60	80.60
Na_2EDTA. $2H_2O$			6.56-8.30	6.56-8.30	11.70	1.00 x 10^2	11.70	21.80	1.02x 10^2	1.02x 10^2	109.38	145.84	1.00 x 10^4				1.03 x 10^2				
Na Fe EDTA $3H_2O$		2.00				11.70					7.02	9.36	12.30 x 10^6								
NaH glutamate																			1.77 x 10^2	5.91 x 10^2	
Na_2HPO_4													9.86 x 10^6								

MEDIUM →	AQUIL	ASP M	ESAW	ESNW	f/2	K	L1	ANTIA	SOILWATER	GPM	OPTIMAL	OPTIMAL 2	DAIGO MK	MLH	NH15	NR12	NS	M9	CS1	B+	SM
Na_2HPO_4. H_2O							36.20														
Na_2HPO_4. $2H_2O$									55.80		115.20										
Na_2MoO_4. $2H_2O$	1.00 x 10^{-1}	5.00	6.12 x 10^{-3}	6.12 x 10^{-3}	2.60 x 10^{-2}	3.00 x 10^{-1}	8.22 x 10^{-2}	1.00			8.04 x 10^{-1}	1341.65 x 10^{-3}	3.10 x 10^4			2.00 x 10^{-1}					
Na_2SeO_3.	1.00 x 10^{-2}		1.00 x 10^{-3}	1.00 x 10^{-3}												7.00 x 10^{-4}					
$Na_2SiO_3.9H_2O$	100.00	1.00 x 10^2	1.06 x 10^2	1.06 x 10^2	1.06 x 10^2	5.04 x 10^2	1.06 x 10^2	2.96 x10^2			169.60	110.00			8.90	4.00		3.52 x 10^2	70.40		
Na_2SiO_4. $9H_2O$																					
Na_2SO_4	2.88 x 10^4		2.50 x 10^4																		
Na_3VO_4							1.00 x 10^{-2}					2.00 x 10^{-2}									
NaCl	4.20 x 10^5	4.00 x 10^5	3.63 X 10^5											34.20 x 10^4	4.10 x 10^5		3.30 x 10^5	4.45 x 10^5	4.79 x 10^5	4.79 x 10^5	4.28 x 10^5
NaF	71.50		66.70																		
$NaH_2PO_4.H_2O$	10.00	1 x 10^2	22.40	22.4	36.20																
$NaH_2PO_4.2H_2O$								2.50 x 10^2				20.00									
$NaHCO_3$	2.38 x 10^3	2.0 x 10^3	2.07 x 10^3												2.40 *	1.60 x 10^3	1.00 x 10^3				
$NaVO_3$											2.00 x 10^{-2}										
Na_2S															1.90						
NH_4Cl						50.00							50.10 x 10^6		3.70 *						
$(NH_4)_2SO_4$														1.50 x 10^3				7.57			
NH_4VO_3															4.90 x 10^{-1}						
$NH_4Mo_2O_{24}$. $4H_2O$												14.56 x 10^{-3}									

Table 2. (Continued)

MEDIUM →	AQUIL	ASP M	ESAW	ESNW	f/2	K	L1	ANTIA	SOILWATER	GPM	OPTIMAL	OPTIMAL 2	DAIGO MK	MLH	NH15	NR12	NS	M9	CS1	B+	SM
$NiCl_2$. $6H_2O$			6.27 x 10^{-3}	6.27 x 10^{-3}																	
Niacin (nicotinic acid)		1.00 x 10^{-1}													8.10 x 10^{-2}			8.12 x 10^{-2}	8.12 x 10^{-2}	4.06 x 10^{-1}	
$NiSO_4$. $6H_2O$							1.00 x 10^{-2}				2.00 x 10^{3}	8.00 x 10^{-3}									
Orotic acid															1.70 x 10^{-1}			1.28 x 10^{-2}	1.28 x 10^{-2}	6.41 x 10^{-2}	
p-Aminobenzoic Acid		1,0 x 10^{-2}																			
PABA															7.30 x 10^{-3}			7.29 x 10^{-3}	7.29 x 10^{-3}	3.65 x 10^{-2}	
Putrescine															9.10 x 10^{-3}			4.54 x 10^{-2}	4.54 x 10^{-2}	2.27 x 10^{-1}	
Pyridoxine															2.00 x 10^{-2}			1.95 x 10^{-2}	1.95 x 10^{-2}	9.73 x 10^{-2}	
Pyridoxamine															8.30 x 10^{-3}			8.30 x 10^{-3}	8.30 x 10^{-3}	4.15 x 10^{-2}	
Riboflavin (Vitamin B2)															1.30 x 10^{-3}			1.33 x 10^{-2}	1.33 x 10^{-2}	6.64 x 10^{-2}	
Ru		2.00																			
Sodium acetate														15.00 x 10^{3}							
Sod.glycerol phosphate $C_3H_7Na_2O_6P{\cdot}5H_2O$												110.00									
Sodium glycero-phosphate						10.00								7.90 x 10^{2}			3.30 x 10^{2}	63.5	9.52		2.54 x 10^{2}
$SrCl_2$		1.00 x 10^{2}																			
$SrCl_2$. $6H_2O$	63.80		81.80																		
Thiamine HCl (B1)	2.97 x 10^{-1}	5.00 x 10^{-1}	2.96 x 10^{-1}	2.96 x 10^{-1}	2.96 x 10^{-1}	2.96 x 10^{-1}	2.96 x 10^{-1}	1.48	2.96 x 10^{-1}	2.96 x 10^{-1}	9.88 x 10^{-1}	4941.65 x 10^{-3}	5.93 x 10^{5}	2.96 x 10^{-2}	30.00		2.90	1.54	3.55 x 10^{-1}	1.48 x 10^{3}	

MEDIUM →	AQUIL	ASP M	ESAW	ESNW	f/2	K	L1	ANTIA	SOILWATER	GPM	OPTIMAL	OPTIMAL 2	DAIGO MK	MLH	NH15	NR12	NS	M9	CS1	B+	SM
TiO_2															5.20						
Tris															6.30 x 10^2		5.00 x 10^3	3.17 x 10^3	6.33 x 10^3	3.17 x 10^3	6.33 x 10^3
Tri HCl								8.31													
Tris base		5.00 x 10^3				1.00 x 10^3															
Thymine		3.00													6.30 x 10^{-1}			6.34 x 10^{-1}	6.34 x 10^{-1}	3.17	
Urea											99.20	10.00						16.70	83.30		
Yeast autolyzate																				0.10 mg lit^{-1}	
$ZnCl_2$																5.00 x 10^{-1}	2.30	2.29	7.65 x 10^{-1}	2.29	2.29
$ZnCl_2$. H_2O									2.29	2.29											
$ZnSO_4$. $7H_2O$	7.97 x 10^{-2}	35.00	2.54 x 10^{-1}	2.54 x 10^{-1}	7.65 x 10^{-2}	8.00 x 10^{-2}	8.00 x 10^{-2}	2.00			1.528	63.667 x 10^{-2}	8.00 x 10^4								
$ZrOCl.8H_2O$															1.10						
Soil extract ml									15.00-50.00	15.00											
pH														6.60	8.00 ± 0.1	8.10	7.90	7.80-8.00	7.80-8.00	8.00-8.30	8.20
F Metals														10 ml							
Reference→	Morel et al. 1979	McLachlan 1964; Goldman & McCarthy 1978	Harrison et al. 1980; Berges et al. 2001	Harrison et al. 1980; Berges et al. 2001	Guillard and Ryther 1962; Guillard 1975	Keller et al. 1987	Guillard and Hargraves 1993	Antia et al. 1969	Tompkins et al. 1995	Loeblich, 1975	L.Lopez-Rosales et al./Algal Research 10 (2015)	Garcia-Camacho F et al., 2011	Yamaguchi et al. 2012	Tuttle and Loeclich 1965	Provasoli, 1964	Lindstrom and Rodhe, 1978	Loeblich, 1975	McLaughlin and Zahl, 1959 (for Zooxanthella sp.)	McLaughlin and Zahl, 1959 (for Zooxanthella sp.)	McLaughlin and Zahl, 1957 (for Zooxanthella sp.)	Loeblich and Sherley, 1979 (for Zooxanthella
														* Part of the sulphide mix, probably of no nutritional significance in the midium because of the low conc.				# Optional	# Optional		

This paper summarizes information on the sources of athecate, thecate, toxic dinoflagellate cultures, culturing methods and division rates of natural assemblages of dinoflagellates, as well as those of cultured photosynthetic, heterotrophic, mixotrophic and symbiotic dinoflagellates. Further, a brief discussion on the factors regulating their growth is provided. Dinoflagellates have lower division rates compared to other microalgae such as diatoms and flagellates and studies on the carbon: nitrogen dynamics in a variety of dinoflagellate cultures would be instructive.

The Centre of Excellence for Dinophyte Taxonomy (CEDIT), part of the herbarium Senckenbergianum of the Senckenberg Research Institute and Natural History Museum, located in Wilhelmshaven (curator Dr. Mona Hoppenrath - mhoppenrath@senckenberg.de) is a source of information on dinoflagellates but does not handle live cultures. It is an excellent source for taxonomic knowledge of 2500 living dinoflagellate species and for holotypes, epitypes and isotypes. It maintains checklists, descriptions of taxa, type collection reference material, literature, Max Taylor library, identification keys, photocollections and video clips.

BRIEF HISTORY OF DINOFLAGELLATE CULTURES

There are 2377 dinoflagellate species belonging to 259 genera [14] with diverse nutritional requirements. A large number of species are autotrophic (photosynthetic) while a few are heterotrophic (holozoic) and symbiotic species. Küster [15] made the first successful attempts to grow the colorless heterotrophic dinoflagellate *Gyrodinium fucorum* isolated from rotting *Fucus* in simple artificial seawater but could not obtain pure cultures. He concluded *G. fucorum* was osmoheterotrophic and assimilated carbon through its cell membrane. *G. fucorum,* now revised as *Crypthecodinium cohnii* (Seligo) [16], is widely cultured for commercial production of polyunsaturated fatty acids. *Gloeodinium montanum* an autotrophic freshwater dinoflagellate was the first to be cultured [17]. For culturing marine autotrophic dinoflagellates i.e., *Prorocentrum micans, P. gracile, Exuviella* sp. *Gonyaulax* species, *Ceratium* sp., *Dinophysis,* Barker [18] utilized enriched aged seawater and, to culture the holozoic *Oxyrrhis marina,* used the diatom *Nitzschia* sp. as feed and studied their physiology. A detailed historical review of the importance of microalgae and techniques in algal culturing utilized in the 19th and 20th centuries is given by Anderson [12]. Today there are 97 species of dinoflagellates cultured in about 22 media (Table 2) and most of the efforts are on toxigenic harmful dinoflagellates.

PROTOCOL FOR CULTURES

For culturing dinoflagellates, in general, the protocol detailed for microalgal culture can be used. Excellent step-by-step guides describing in detail the sterilization techniques, cleaning the culture ware, preparation of the nutrient media, inoculations and precautions for maintenance of culture facilities are provided [12, 19-24]. Also, Lavens and Sorgeloos [25] discussed culture production schemes for batch cultures, carboy cultures, mass production of micro-algae in 20,000 liter tanks and 150 liter cylinders and 40 liter continuous cultures

techniques. These are not repeated here but based on our experience a few helpful pointers for culturing dinoflagellates are given below.

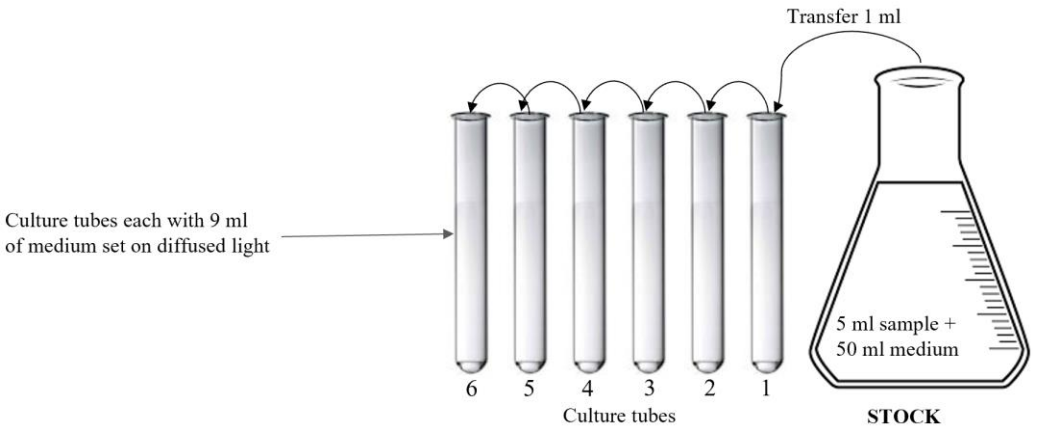


Figure 1. Serial dilution method to isolate single cells for cultures.

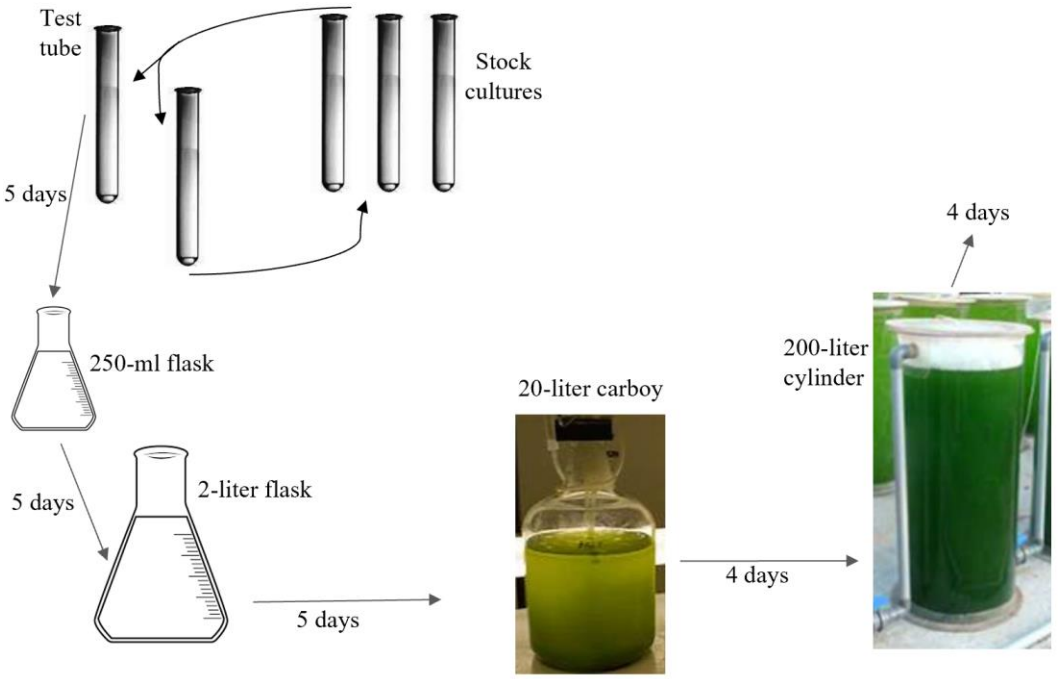


Figure 2. Scaling up stock cultures to 200 liter cultures.

Marine dinoflagellate cultures are grown in suitably enriched seawater. To avoid batch to batch variations in the composition of sea water, it is best to obtain offshore seawater in bulk and age it in plastic barrels. Aged seawater filtered through Glass fibre filter (GF/F) and steam sterilized at 125ºC for 20 minutes is enriched with nutrients. Nutrients are refrigerated individually in Teflon bottles and added aseptically.

To establish cultures from cysts of *Gonyaulax tamarensis*, Anderson et al. [26] used extensive precautions to avoid precipitation, adsorption, desorption or chemical contamination. All plastic and Pyrex glassware was washed with Micro detergent, rinsed several times, soaked in 2N HCl, rinsed again with deionized distilled, UV –irradiated water. Glassware was coated with Surfasil (Pierce Chemical Co) and recoated after 3 or 4 washings.

Local seawater was filtered through Gf/f filters, and was autoclaved in Teflon bottles. After depressurization, the bottles were recapped and cooled immediately and cooled for more than 24 hours. Nutrients, except the iron/chelator kept in separate autoclaved Teflon, were used aseptically for enrichment. Pyrex tubes (25x 150 mm), with the 25ml f/2-Si medium, were used. Following greater than 30 days of growth, vegetative cells were destroyed by sonication of the culture (Branson S-75) for 1min at a 1.4 setting and the cysts were counted. In another experiment [27] such cysts were incubated in medium without silicate, phosphate, nitrate and vitamins with chelator ranging from 0 to $10^{-4.3}$ M EDTA.

Most investigators used f/2 medium without silicate enrichment. Anderson et al. [26] using f/2 –Si medium reported the lowest cyst yield in *Gonyaulax tamarensis*; the yield dropped to 41% of the control when the inocula and media were prepared in glass containers. Because of suspected leaching of silica from borosilicate culture ware, and because of no apparent requirement of silica by dinoflagellates, the investigators used inert tissue culture ware (Table 3) i.e., polyethylene [28], polycarbonate bottles [29-31], microplate wells [32] and polystyrene tissue culture ware [33]. Guillard and Keller [19] reported more reliable survival of *Ceratium tripos* cultures over a number of years in f/2 media in polycarbonate rather than in glass. For culturing dinoflagellates, the LATZ Laboratory (Scripps Institution of Oceanography) uses sterilized glassware if autoclave is available, otherwise uses disposable tissue culture flasks and Guillard's (f/2) marine medium with macro- and micronutrients, but without silicate.

Table 3. Range of nutrient enrichment in media utilized for culturing dinoflagellates

Enrichment	Range µM
Phosphorus	5.74-28.70 $x10^6$
Nitrogen	1 $x10^2$ – 2.47 x 10^3
Silica	3.52 $x10^2$ – 5.04 x 10^2
Iron	4.0 x 10^{-1} -11.70
Copper	1.00 x 10^{-2} – 1.00 x 10^4
Cobalt	9.24 x 10^{-7} – 5.00 x 10^{-1}
Zinc	7.97 x 10^{-2} -8 x 10^4
Manganese	5 x 10^{-2} – 9.10 x 10^5
Vitamin B_{12}	9.22 x 10^{-5} – 3.69 x 10^{-4}
Thiamine hydrochloride	49.42 x 10^{-1} -30.0
Thiamine	6.30 x 10^{-1} – 3.00
Biotin	1.0 x 10^{-3} – 1.78 x 10^{-2}

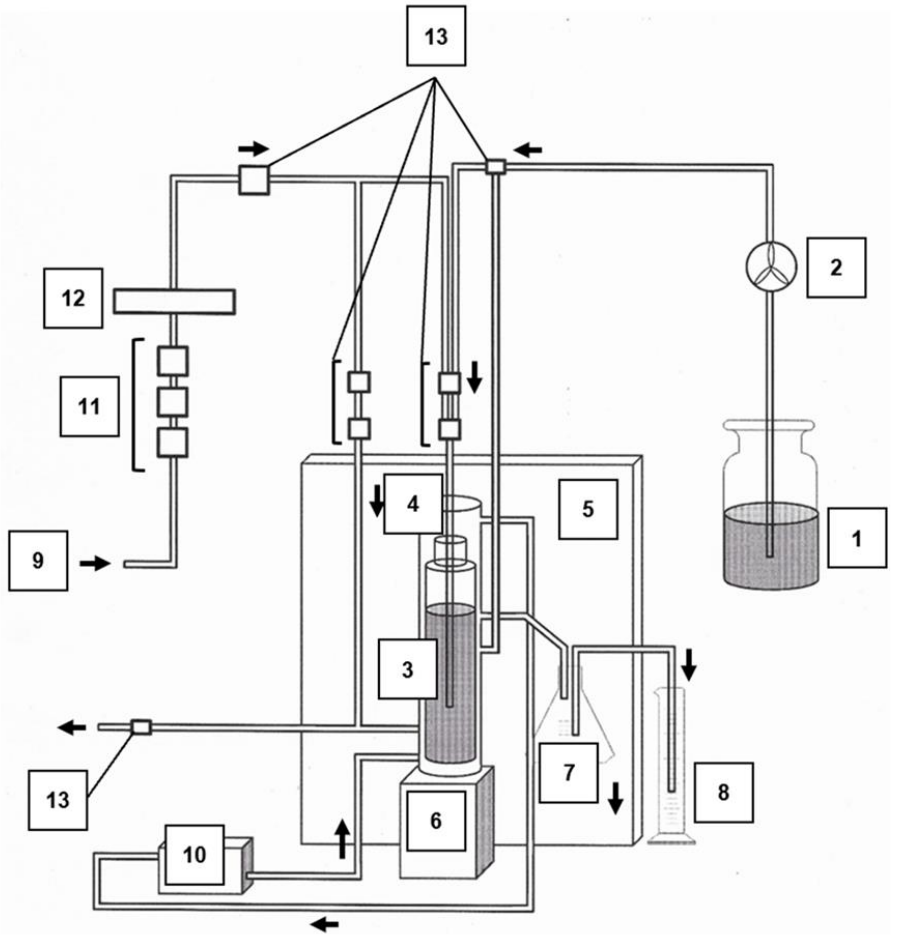
Composition of various media is given in Table 2. Selected major nutrient enrichments in 22 media (Table 3) show a wide range for phosphate and nitrogen. Trace elements Fe, Co, Cu and Zn varied over several orders of magnitude thus suggesting certain dinoflagellates grow in a wide range of nutrients.

Although several investigators used media without silicate [24], a few others used media enriched with silica: for example f [35], f/2 [36], Aquil [37], ESAW [38], ASP [39] and NH

[19, 40] and optimal medium [41] (Table 2). Loeblich [42] used GPM medium enriched with 15 ml l^{-1} soil extract which has an unknown composition but fortified with 4.0 g $NaNO_3$ and 0.6 g Na_2HPO_4 $12H_2O$ per liter.

Categories of Cultures

Cultures are grown either indoor under controlled conditions of light and temperature and outdoors under ambient light and temperatures. Based on their sterility they are axenic (sterile/xenic) and non-axenic cultures. It is best that cells in the exponential growth phase are used as inocula and the inoculum is 2-10% of the final volume. Sub-culturing is done once a week and parent cultures are retained for 30 days as a fallback. Cotton gauze plugs are used as stoppers.



1. Sterile medium reservoir 2. Medium pump 3, Culture vessel 4. Temperature regulated bath
5. Light source 6. Magnetic stirrer 7. Collecting receptacle 8. Measuring cylinder
9. Inflow of fresh sterile air 10. Water bath 11. Air flow meter 12. Cotton filter 13. Regulatory valve

Figure 3. Continuous culture set up.

There are 3 types of cultures 1. Batch cultures, 2. Continuous cultures 3. Semi-continuous cultures.

1) In batch culture, cells are inoculated and as the culture grows the nutrients in the medium change. Considered as the most reliable method, cells go through lag, accelerated, exponential, retardation, stationary and decline phases. Cells use up the nutrients and produce their own waste products. Batch cultures can be scaled from test tubes to 250 ml- 500ml-1000ml flasks to 5 and 20 liter carboys, 160 liter indoor cylinders to 5000 liter to 25,000 liter outdoor tanks.
2) In continuous culturing, the cells are kept growing indefinitely by supplying fresh nutrient medium and automatically an equal volume of the culture is removed. The steady supply of nutrients maintains the cells at their maximum growth rate. Two categories of continuous cultures can be recognized a) Turbidostat and b) Chemostat. .In the former, fresh medium is added at a predetermined turbidity point of the culture determined by the extinction of light passing through the culture. In the chemostat, fresh medium is added to the culture at a steady predetermined rate. The level of biomass is adjusted by an inflow of fresh sterile medium; the growth rate is equal to the dilution rate. When the algal cell density of the culture is held constant the culture is designated as a steady state culture.
3) In semi-continuous cultures, a predetermined volume of fresh nutrient flows into the culture vessel and an equal volume of culture flows out.

Photobioreactors

When massive algal biomass is required for biotechnological production of high-value products, photobioreactors are utilized. There are excellent reviews on the current status, recent developments and detail designs of photobioreactors for different microalgae, both lab-scale and upscale [43, 44]. However, not many dinoflagellates were cultured in photobioreactors. For culturing benthic dinoflagellates, *Amphidinium carterae* (JHWAC), *Prorocentrum rhathymum* (JHWPMX1) and *Symbiodinium* sp. (JHLSD1) Shah et al. [45] used a photobioreactor. It had two rows of 6 columns each, placed adjacently. The towers were 35cm diameter, 160 cm high (#1 to 6) and 145 cm high (#7-12) and were connected at the bottom for circulation of the medium through aeration. Cool-white fluorescent lights (38W) provided 40-50 μmol photons m^{-2} sec^{-1} with a 12:12 h light/dark (L/D) cycle. The water used for culturing was 30PSU, UV treated, heat sterilized at 110^0C, cooled and filtered through 0.2 μm. For 20 L carboy cultures, water was chemically sterilized with sodium hypochlorite solution containing 9%active chlorine for 30min followed by neutralization with sodium thiosulphate (0.12g l^{-1} seawater). Exponentially growing stock cultures were incrementally scaled up from 30ml -300ml-1L-3Land 20L. Each tower contained 60L of the medium. Two media, f/2 and IMK, were used as needed. Twenty liters of exponentially growing culture was introduced in tower 1 and filtered air (0.2 μm) was used for aeration. Cultures were grown at 20±1°C.

Protoceratium reticulatum cultures raised in a 15L semicontinuous perfusion photobioreactor were more productive with 5228 cell mL^{-1} day^{-1} similar to those obtained in a 2L stirred tank [41]. For *Karlodinium veneficum* mass culture two types of photobioreactors

with a working volume of 80 liters were used [46]: a bubble column and a rectangular flat-panel device. The bubble column consisted of clear plastic (polymethyl methacrylate) cylindrical tube; cell production was more in a semi continuous operation in a bubble column photobioreactor [47].

Turbulence: Photobioreactors

Cultures raised in Photobioreactors experience turbulence which inhibits growth. Sullivan and Swift [48] investigated the impact of high turbulence ($\varepsilon \sim 10^{-4}$ $m^2.S^{-3}$), low turbulence ($\varepsilon \sim 10^{-8}$ $m^2.S^{-3}$), and unstirred cultures on cell growth. Of the 10 species tested, in *Lingulodinium polyedrum, Gymnodinium catenatum* and *Alexandrium fundyense* division rates increased in high turbulence while *Alexandrium tamarense, Pyrocystis fusiformis, Alexandrium catenella* and *Gymnodinium* species were unaffected. Shaking of cultures affected growth rate in *Amphidinium klebsii*, lowered the division rate in *Scripsiella trochoidea* from 0.53 to 0.35 div d^{-1} and even killed *Gyrodinium aureolum* cells [49]. Growth of dinoflagellates is inhibited by shear forces and turbulence shear rate of about 0.3 s^{-1} for 24-h resulted in an elevation in the levels of chlorophyll *a*, peridinin and dinoxanthin in *Protoceratium reticulatum* [50].

Though the response of dinoflagellates in photo bioreactors is relatively unknown, a few advancements have been made. Gallardo-Rodríguez et al. [51] evaluated growth kinetics, micronutrient requirements and toxin production in two strains of *Protoceratium reticulatum;* in low-shear stirred tank photobioreactors quite different quantities (ranging from 0 to 34 pg $cell^{-1}$) of yessotoxins (YTX) are produced. Results of Garcia-Camacho et al. [23, 24] with a semi-continuous perfusion culture of *Protoceratium reticulatum* in a 15 L photo bioreactor yielded 5228 cell mL^{-1} day^{-1}, or about 3.7 times more than the best attainable cell density in static flask cultures; however, the productivity of yessotoxin was 9.16 μg L^{-1} day^{-1} comparable to that obtained in a 2L stirred tank. López-Rosales et al. [52] designed and utilized a bubble column photobioreactor to culture the shear-sensitive marine dinoflagellate *Karlodinium veneficum*. To prevent the damage of the cells, López-Rosales et al. [46] designed a photobioreactor (80L) with a transparent methyl methacrylate tube (internal diameter 0.242m, with a gas-free liquid height of 1.85 m). Red, green, blue and warm white light emission-diodes provided light spectrally similar to sunlight in the photosynthetically active (PAR) range. Nearly 12x 10^5 cells ml^{-1}, similar to densities in laboratory cultures, were sustained. Further, López-Rosales et al. [46] modelled the impact of turbulence caused by the nozzle sparger diameter, air flow. Because of its potential to yield several high-value metabolites, *Amphidinium carterae* was cultured in a 5cm light path, a 320 L photobioreactor [53] and obtained a growth rate (0.29 d^{-1}), and dry biomass productivity (78 mg L^{-1} d^{-1}). An indoor LED-lighted raceway photobioreactor was used for long-term culture of the marine dinoflagellate microalga *Amphidinium carterae for* production of carotenoids and fatty acids [54]. Biomass productivity was $2.5\ g\ m^{-2}\ day^{-1}$ and carotenoid peridinin was $19.4 \pm 1.35\ mg\ m^{-2}\ L^{-1}$, nearly 1% of the biomass dry weight. Additionally, these cells had several polyunsaturated fatty acids. Continuous cultures of the toxigenic *Alexandrium ostenfeldii* grown in a100 liter photobioreactor in L1 medium fortified with soil extract had a division rate of 0.16 day^{-1} [55].

Isolation of Cells

Cells are isolated with a modified one ml plastic disposable syringe; the needle is removed and the needle hub is suitably modified (with a neoprene O ring) to accommodate a finely drawn haematocrit capillary (0.11 to 0.12cm diameter) pipette. Single cells or cysts are isolated under a bright field microscope from natural assemblages of phytoplankton or directly from seawater and transferred to polycarbonate tubes with sterile media. To avoid osmotic shocks to cells and to avoid a high nutrient concentration shock, media are diluted (50, 25, 10 and 2%) and used. The seeded cultures are incubated in a temperature controlled facility under light 3-10% of full daylight or ~ <170 μ mol m^{-2} s^{-1} supplied by daylight fluorescent lights. Light and dark cycle of 12: 12 or 14: 10 and 16:8 h are maintained. The time-honoured serial dilution (1:10) technique was used successfully to isolate algal cells.

As selection of cells manually is labor intensive, multiparametric flow cytometry is used successfully to sort out cells [56] and to establish axenic cultures of *Karenia brevis* from a bloom sample [57].

Cellamare et al. [58] discussed the utility of flow cytometry to sort freshwater phytoplankton.Trauma to electronically sorted cells was not a limiting factor, as fragile dinoflagellates, such as *Karenia brevis* (Dinophyceae), survived electronic cell sorting to yield viable cells. However, one of the disadvantages of electronic cell sorting is the need for manual post-sort culture maintenance and assessment of a large number of isolated cells. The rapidity of cell sorting would potentially accelerate the discovery of new algal strains [57].

Antibiotics

Complex bacterial flora is associated with harmful algae and is known to form an intrinsic component of phytoplankton physiology and ecology. Growth of *Gymnodinium catenatum* was modified by a complex of one, two, or three-bacterial associates. The microbial community induced up to four-fold changes in the dinoflagellate growth rate and a three-fold change in stationary phase cell concentration and death rate, probably mediated by cell lysis [59]. Hence, dinoflagellate cells are washed several times usually in penicillin and streptomycin [19]. Tropical and temperate species are exposed for 18-36 hours and 36-72 hours for cold water species. In general penicillin G (K or Na salt), streptomycin (SO_4) and chloramphenicol are used. To eliminate gram-positive bacteria broad spectrum antibiotics i.e., erythromycin, novobiocin are used and for gram-negative bacteria streptomycin and polymixin B are effective. Other antibiotics (ampicillin, kanamycin and neomycin) were also used to reduce the bacterial activity in *C. polykrikoides* cultures [60]. Su et al. [61] developed a method to obtain axenic cultures of *Alexandrium tamarense* in sterile f/2 medium without antibiotics; cells were repeatedly washed in lysozyme/SDS and antibiotic treatment with a mixture of gentamycin, streptomycin, cephalothin and rifampicin. Sterility tests were carried out on *Cochlodinium polykrikoides* cultures using the f/2pm medium [62] and fluorochrome 4′, 6-diamidino-2-phenylindole staining [63]. As treatment with antibiotics may alter the cell physiology, maintaining the cultures in active exponential phase is recommended.

Bacterial involvement in toxicity has been hypothesized in the dinoflagellate *Alexandrium tamarense* [64]. A great diversity of bacterioplankton, either attached or intercellular, is usually associated with dinoflagellates. Based on pure cultures obtained by

repeated streaking on nutrient seawater agar, from the benthic toxic dinoflagellates, *Ostreopsis lenticularis* and *Gambierdiscus toxicus*, six bacterial genera- *Nocardia, Pseudomonas, Vibrio, Aeromonas, Flavobacterium,*and *Moraxella*- were isolated in addition to coryneform bacteria but extracts of dinoflagellate-associated bacteria grown in pure culture were not toxic [65].

From *A. tamarense* culture, bacteria were cultured in Zobell 2216E medium based on aged seawater enriched with GSe trace elements and vitamins [66]. Using DNA extraction and PCR amplification, 17 bacteria were identified i.e., *Paracoccus* sp. zao 3-3,*P*. sp 3-6, *P*.sp. 3-10, *P.homiensis* DD-R11, *Roseibacteriuim* sp. Zao 3-2, *R.elongatum* DSM, *Nitratireductor* sp. Zao 3-1, *N.basaltis* J3(T)'Amorphus sp. Zao 3-6'A.sueedae'Marinobacter sp. Zao3-5'M. algicola DG893'M.sp Zao3-7, *M.adhaerens* HP15(T), *M*.sp. zao 3-4, *M*.sp.zao 3-B' and *Arthobacter* sp. Zao 3-A members of *Roseobacter* and *Alteromonas* clades, associated with *A. tamerense* [66].

Earlier methods employed to test the bacterial toxicity hypothesis were time consuming or imprecise and involved extraction of DNA, followed by sequencing and dot blot hybridization [67]. Molecular studies on cultures of toxic and non-toxic dinoflagellates, *Alexandrium* spp. and *Scrippsiella trochoidea* [68] showed that the phylogenetic diversity of the bacteria was limited to the Proteobacteria and the Cytophaga-Flavobacter-Bacteroides (CFB); while some bacteria were common, some were unique to a particular dinoflagellate.

Several advanced techniques such as double hybridization (TSA-FISH) using monolabeled probes and confocal microscopy were developed to identify the bacteria [69]. In the non-thecate dinoflagellate *Gyrodinium instriatum,* endocytoplasmic and endonuclear bacteria were successfully identified but in either toxic or nontoxic strains of the thecate *Alexandrium* spp intracellular bacteria were not observed. There are 3 main constraints to establish the bacterial involvement of toxicity: 1. Establish whether the intercellular and those attached bacteria are the same 2. Establishing culturability of these bacteria and 3. Screening these bacterial cultures for toxins.

MEDIA FOR AUTOTROPHS

Most media (Table 2) used for dinoflagellate cultures are based on enrichment of sea water (ESAW) and f/2 [70]. Derivatives of f/2 have been used for a long time in several laboratories but the National Center for Marine Algae and Microbiota (CCMP) is using L1 and L1 derivatives. There have been several versions for preparation of media using artificial seawater [39, 71]. An artificial seawater medium tested on 83 strains of phytoplankton represented by 11 algal classes [39] resulted in reduced final cell yields of Prymnesiophyceae and Dinophyceae compared to those grown in enriched natural seawater. The Aquil medium [37] and ESAW medium [72], unlike other media, are based on enrichment of artificial seawater water and are therefore highly useful in critical evaluation of specific nutrient requirements on the growth of dinoflagellates. More attention is paid to culturing toxin producing dinoflagellates due to their importance as constituents of algal blooms and producers of harmful phycotoxins with consequential impact on commercial fisheries and the consumers.

Dinoflagellate cultures go through an initial lag phase; a log phase with exponential growth, a stationary phase after attaining a maximum, followed by a senescent phase as in *Karlodinium micrum* [73], *Dinophysis caudata* [74], *Alexandrium tamarense* [75], and *A. fundyense* [33].

To suit individual experimental needs such as nutrient uptake studies, perturbation (spiking) techniques are used [76]. Several media including medium *f* are enriched to evaluate the impact of nutrients on growth and include carbon dioxide [77], phosphate [78], nitrogen compounds [49, 51], ammonia [79], $NaNO_3$, urea, glycerophosphate [80], lipoperoxides, ascorbic acid [78] and iron [81].The medium utilized seems to affect the toxin profile as in *Alexandrium ostenfeldii* [82], and in *A. Tamarense* [75] and the production of bioactive compounds in several strains of *Protoceratium reticulatum* [51].

Several media contain a wide range of micronutrients, trace elements and a vitamin mix consisting of cyanocobalamin (vitamin B_{12}), thiamine hydrochloride (B_1) and biotin (H) as in medium f [35], f/2 [36], Aquil [37], ESAW [38], ASP [39], Optimal Culture medium [80], IMK [83-85], GPM [42], NH15 [40], MLH medium [86] (Table 2). The vitamin concentrations in these media vary widely, *ca*. 3.7 pM - 7.4 nM, 0.3 nM - 3 mM and 3.27 nM to 0.2 μM for B_1, B_{12} and H, respectively (Table 2). Growth of axenic dinoflagellate *Lingulodinium polyedrum* cultures in L1medium was studied at different vitamin concentrations ranging from 3.33×10^{-2} to 3.33×10^{1} pM of B_1 thiamine and 5.25×10^{-2} to 5.25×10^{1} pM of B_{12} cobalamin (0.033 pM [83]. In their lowest range, 0.033 pM of B_1 and 0.053 pM of B_{12}, cell growth was limited. When co-cultured with the bacterium *Dinoroseobacter shibae*, a known B_1 and B_{12} producer, *L. polyedrum* grew at the same rate as in culture media supplemented with B_1 and B_{12}.

CULTURING MIXOTROPHS

The history of culturing the toxic mixotrophic dinoflagellate *Dinophysis* was elusive and fascinating [88]. It was done in two steps. First, the marine ciliate *Myrionecta rubra* isolated from the bay is cultured. The salinity of the local water from Inokushi Bay, Japan was adjusted to 30PSU to grow *M. rubra* in f/2 medium [36], modified with 1/3 nitrate, phosphate, and metals and 1/10 vitamins [89]. Polycarbonate Erlenmeyer flasks were incubated under 100–150 μmol m^{-2} s^{-1} light provided by cool-white fluorescent lamps with a 12: 12 h L: D cycle. Next, *M. rubra*, in turn, was fed with the cryptophyte *Teleaulax amphioxeia* (3,000 cells) maintained similarly as *M. rubra*. *D. caudata* were isolated from the bay and maintained as a clonal culture and 150μl (2,500-3,000 cells) were added to 750 μl of *M. rubra* but incubated at 25^0C. The growth rate of *D. caudata* was 1.03 kd^{-1}. A mix of both *M.rubra* and *Teleaulax amphioxeia* was essential to sustain *D. caudata* culture.

For culturing mixotrophic dinoflagellates, media f/2 without silica, ES, L1 and SWM are utilized supplemented by a variety of microalgal flagellates, diatoms, dinoflagellates, the ciliate *Mesodinium rubrum* and the blue green *Synechococcus* as feed. Several autotrophic dinoflagellates were cultured in a mixotrophic mode using the ciliate *Mesodinium rubrum* and the blue green *Synechococcus* as feed.

A review by Jeong et al. [90] listed 29 dinoflagellate species feeding on a variety of prey including heterotrophic bacteria, autotrophic bacteria, pico-eukaryotes, Cryptophytes,

Haptophyta (Prymnesiophyta), Ciliates, Chlorophytes (Dunaliella), Prasinophytes, Euglenophytes, Raphidophytes, Bacillariophytes, Dinoflagellates, Heterotrophic nanoflagellates, Ciliates, Naupliar stages of metazoans, Bloods of metazoans and flesh of metazoans. The feed included *Pseudomonas, Alteromonas, Acinetobacter, Teleaulax amphioxieia, Rhodomonas salina, Chroomonas*, *Pyramimonas* sp, *Myrionecta rubra (Mesodinium rubrum)* and *Thalassiosira.* Of interest are the other feed organisms i.e., dinoflagellates *Ostreopsis* cf. *ovata*. *Alexandium lucitanicum, A.tamarensis, Heterocapsa rotundata. H. triquerea, Heterocapsa* sp, *Scripsiella trochoidea, Margalefidinium (Cochlodinium) polykrikoides, Dinophysis accuminata, D. caudata* and *Ostreopsis* cf. *ovata, Heterosigma akashiwo, Amphidinium carterae* and the ciliate *Mesodinium rubrum.* Blood cells of the perch *Lateolabrax japonicus* served as feed for *Stoeckeria changwonensis* [91].

Unlike most heterotrophic dinoflagellates which play a significant role in the transfer of organic matter, the predatory dinoflagellate *Pfiesteria* has unique nutritional preferences. Skelton et al. [92] cultured 3 strains of *Pfiesteria shumwayae* monoxenically on fish cells [93] and then axenically in a biphasic culture medium containing chicken egg yolk as a major component [94]. The development of a semi-defined culture medium (PSD) is a significant step as it facilitates understanding the biochemical requirements of this heterotrophic dinoflagellate [94]. PSD is a mix of Instant Ocean (75%, v/v; Aquarium Systems, Atlanta, GA), Medium 199 (25%, v/v; Sigma-Aldrich), amicase (0.5 g/L; Sigma-Aldrich), soy lecithin (0.5 g/L; MP Biomedicals, Solon, OH, #102147), L1 trace metals and 2 × L1 vitamins [95].

DIVISION RATES

Monophasic

Division rates are calculated when the cells are in their exponential growth phase. The exponential increase of cell numbers in a population (N_0) to a proportionally (N_t) is expressed as:

$$Nt= N_0e^{kt}$$

where *k* is the specific growth rate and t is the time period expressed in days. The growth coefficient (*k*) is calculated from changes in cell numbers over a period of days as:

$$k\ (day^{-1}) = [\ln(N_t/N_0)]$$ [96] (Furnas, 2002)

Growth of culture is usually monitored by determining changes in biomass measured in terms of cell numbers and pigments [97]. Cells are examined under a microscope. Cell enumeration is done using a Sedgwick Rafter cell, or Utermohl sedimentation chamber or using an electronic particle counter .The initial numbers(N_0) increase exponentially (N_1) over a generative period (t); the cell growth rate(μ) is calculated [96], i.e.,

$$G= 24/(((\ln (Nt/N0))/\delta t))/0.693$$

derived from k (day^{-1}) = [ln(Nt/N0)]/δt
μ (divisions day^{-1}) = *k* (day^{-1})/ln 2
= *k* (day^{-1})/0.693
G= 24/μ

where G is generation time, n_1 and n_2 represent cell numbers corresponding to day1 (t_1) and day 2 (t_2) and k is the doubling time day $^{-1}$. Division rates (μ divisions day^{-1}) reported by the authors are used in this study.

Doblin et al. [98] used fluorescence yield of cells to calculate division rates as:

$$k\ (day^{1}) = \ln (F_1 / F_0)/(t_1 - t_0)$$

where F_1 and F_0 are fluorescence reading at each time period t_1 and t_0 were corrected for fluorescence of control tubes at the same time. It should be noted that fluorescence depends on the past growth history (temperature and light) of the algal cells.

Bayesian Generalized Logistic Model

The effects of temperature and light on the comparative growth kinetics of five strains of cultured dinoflagellate *Symbiodinium*, *S. microadriaticum* (cp-type A194; strain 04–503), *S. microadriaticum* (cp-type A194; strain CassKB8), *S. minutum* (cp-type B184; strain Mf 1.05b.01.SCI.01), *S. psygmophilum* (cp-type B224; strain Mf 11.05b.01) and *S. trenchii* (cp-type D206; strain Mf 2.2b were studied [99]. Cultures were grown in medium f/2, 38 PSU at 26°C under 14:10 light: dark70–90 μmol photons m^{-2} s^{-1} light. Results of growth modelled using Bayesian generalized logistic model showed faster growth reaching a higher asymptote at 26°C than at 18°C.

Klueter et al. [99] formulated the biphasic model to simultaneously estimate growth parameters and quantify the uncertainty of monophasic or biphasic growth with a binary switching parameter, *w*, similar to the model averaging method described by Carlin and Chib [100].

The likelihood of observing a given cell density, *D* (x 10^4 cells ml^{-1}), *n* days after the experiment began was defined as:

$$D \sim Poisson(\mu \cdot \rho)\ \mu = 1 + \frac{K_1 - 1}{1 + e^{-B_1(n - M_1)}} + w\left(\frac{k}{1 + e^{-B_2(n - M_1 - m)}}\right)\ \rho \sim Gamma(\alpha, \alpha)$$

where (prior distributions in parentheses):

1) μ = Expected density,
2) K_1 = Asymptotic density of the first curve (*Uniform*(1, 800)),
3) *k* = Increase in the density of the asymptote of the second curve above K_1 (*Uniform*(1, 500)),
4) B_1, B_2 = Logistic growth rates of the first and second curves respectively (*Uniform*(10^{-1}, 1)),

5) M_1 = Number of days to the maximum growth rate of the first curve (*Uniform*(2, 40)),
6) m = Number of days to the maximum growth rate of the second curve above M_1 (*Uniform* (2, 40)),
7) w = A switching parameter determining if the model describes a monophasic logistic (= 0), or biphasic logistic (= 1) curve (*Bernoulli* (0.5),
8) ρ = Gamma distributed error term,
9) α = Gamma shape and scale parameters (*Lognormal* ($\mu = 0$, $\tau = 10^{-4}$)).

Biphasic growth was especially evident for *S. minutum* and *S. psygmophilum* across all treatments. When the cell density was ~ 200 million cells ml^{-1}, monophasic growth was more common but final asymptotic densities were relatively low. All species tended to grow faster and / or reached a higher asymptote at 26°C than at 18°C. *S. minutum*, exhibited the fastest growth with an approximately four-fold increase in estimated cell density after 60 days. We do not know whether the biphasic growth was due to diauxic growth i.e., when two nutrients are present in the medium, the easily metabolized nutrient is consumed faster than the other. This cellular growth may take place in two phases: the first is the fast growth phase and the second a slower growth phase similar to that described by Monad with the bacterium *Escherichia coli*. Alternately, the biphasic growth may be due to bi-phasic life cycles in algae discussed by Thornber [101] where two organisms produce haploid gametes, fuse to form a diploid zygote which undergoes meiosis resulting in haploid cells. Haploid cells have reduced nutrient requirement unlike the diploid cells [102].

DIVISION RATES (GK) OF CULTURED ATHECATE AND THECATE DINOFLAGELLATES

In Situ Division Rates

Division rates of dinoflagellates given in publications are assembled in Table 4. There are few reports on the *in situ* division rates based on natural bloom populations; for *Ceratium tripos* from a 45 km patch in the sub-surface chlorophyll maximum in August 1978 on the Southern California shelf, it averaged 0.25 d^{-1} [103]. Potential division rates based on 'post mitotic index' of samples during a cruise in the Galician rias yielded division rates 0.09 – 0.28 for *Dinophysis acuminata*, 0.17-0.20 for *D. acuta* , 0.24 for *D. caudata* and 0.50 for *D. tripos* [104]. For *Gymnodinium stellaltum*, *G. nelsoni*, *Gonyaulax tamarensis, G. polyedra, Prorocentrum marie-lebouriae, Pyrocystis noctiluca, P. fusiformis* division rates calculated on species–specific carbon assimilation (C^{14} method) of natural samples from Chesapeake Bay corresponded to 0.3, 0.5, 0.5, 0.3, 0.5, 0.1 and 0.2 d^{-1} [105]. Division rates for *Pyrocystis noctiluca, P. fusiformis* in the Sargasso Sea were 0.33 [106]. Along the Karachi coast, growth rates (μ_{max} d^{-1}) of the dominant species were: *Prorocentrum gracile*, *Prorocentrum minimum, Prorocentrum arcuatum* (1.0-1.10), *Protoperidinium steinii* (0.92), *Gonyaulax spinifera* (0.69), *Dinophysis acuminata* (2.3), *Dinophysis caudata* (0.92), *Ceratium lineatum, Prorocentrum micans* (1.95), *Gyrodinium* sp. (1.88*), Ceratium (revised as Tripos) furca* (1.70), and *Alexandrium ostenfeldii* (1.34) [107] and suggested that the prevailing higher

temperature induced higher division rates but compared to the division rated (Tables 3) these values are quite high, even higher than those for several microalgae [108]. Wilkerson et al. [109] based on mitotic index reported *in situ* growth rates of intracellular zooxanthellae higher than those in oligotrophic waters and attributed it to the assimilation of higher ambient nutrient concentrations. It is of interest to note the maximum growth rates of many phytoplankton taxa measured *in situ* are like those observed in culture [96, 110].

Table 4. Dinoflagellate culture conditions and division rates

Taxa	TO, AT, T	Nutrition (Ps, Holo, Mixo)	Medium	˚C	µmol m^{-2} s^{-1}	Gk div d^{-1}	Reference
Akashiwo sanguinea	TO/AT		L1	20	90-490	0.26-0.76	Islabao and Odebrecht [115]
Akashiwo sanguinea			L1	20	90-490	0.26-0.33	Islabao et al. [122]
Akashiwo sanguinea	TO/AT	Ps	f/2 no silicate	20	78.14	Nutrients	Chen et al. [120]
Alexandrium catenella	TO/T		f/2	20	200	0.61-0.69	Sullivan and Swift [48]
A. fundyense	TO/T		f/2	20	200	0.37-0.38	Sullivan and Swift [48]
A.fundyense	TO/T	Ps	K	16	160	0.35	John and Flynn [126]
A.fundyense	TO/T		f/4 -Si	15	15-130	0.11	Juhl et al. [112]
A. tamarense	TO/T		f/2	20	200	0.46-0.61	Sullivan and Swift [48]
A. tamarense	TO/T		k modified	23.5	80-220	0.5	Wang and Hsieh [125]
A. ostenfeldii	TO/T		L1, f/2	16-22	155-199	0.12-0.14 0.08-0.10 0.13 0.16	Medhioub et al. [55]
Amphdinium klebsii	AT		Aquil	20	80	0.87 div at 80	Dixon and Syrett [121]
A.carterae	AT	Ps	Aquil	20	80	0.87	Dixon and Syrett [121]
A.carterae	AT	Ps	L1	23.3	150	0.29	Fuentes-Grünewald et al. [53]
A. carterae	AT		INK- f/2	20	180	0.317	Shah et al. [85]
A.operculatum	AT		INK- f/2	20	180	0.317	Shah et al. [85]
Ceratium tripos	T					0.25	Eppley et al. [103]
C. tripos	T		f/2	20	200	0.13-0.33	Sullivan and Swift [48]
C. fusus	T		f/2	20	200	0.21-0.39	Sullivan and Swift [48]
Dinophysis caudata	TO/T		f/2 Mixo			0.66+ temp18-32.5C	Basti et al. [74]
Gymnodinium catenatum	AT		f/2	20	200	0.15-0.23	Sullivan and Swift [48]
G.catenatum	TO		f/2		11-22-28	0.53 +11-28C	Bravo and Anderson [119]

Taxa	TO, AT, T	Nutrition (Ps, Holo, Mixo)	Medium	˚C	µmol m^{-2} s^{-1}	Gk div d^{-1}	Reference
G. catenatum	TO		f/2	20	150	0.18-0.21 0.28-0.31 at 28-348 PSU	Band-Schmidt et al. [113]
Gyrodinium aureolum	AT		f/2+ tris	20	80	0.15-0.29	Dixon and Syrett [49]
Gymnodinium galatheanum			f/2	20	30-170	0.03-0.26	Johnsen and Sakshaug [133]
Gyrodinium aureolum	TO		K	20	35-380	0.07-0.44	Garcia and Purdie [134]
Gyrodinium aureolum	TO		f/2	20	30-170	0.09-0.33	Johnsen and Sakshaug [133]
Gyrodinium sp.	AT		f/2	20	200	0.40-0.49	Sullivan and Swift [48]
Gyrodinium moestrupii	AT		f/2	20	20	0.862	Yoo et al. [31]
Heterocapsa psammophila	TO/T		INK- f/2	20	180	0.344	Shah et al. [85]
Heterocapsa sp.	Non tox T		f/2	22	75	0-.149	Montero and Lubian [127]
Heterocapsa verruculosa *H. circularisquama*	TO/T	Ps	SWM-3	15 30	150	1.74 1.3	Yamaguchi et al. [135]
Karlodinium brevis	TO/AT		f/2	22	50-300	0.024-0.249	Li et al. [128]
Karlodinium veneficum	TO/AT	Ps	L1 Best #6 Other media ESAW	18	200	0.27-0.53 0.33 0.11-0.35 0.17-0.36	López-Rosales et al. [80]
K. veneficum	TO/AT	Ps	L1	18	200		López-Rosales et al. [80]
K. veneficum	TO/AT	Ps	L1	21	300		López-Rosales et al. [136]
K. (Karenia) veneficum	TO/AT	Ps	f/2	22	300 50	0.237-0.321 0.150-0.187	Li et al. [128]
K. micrum	TO/AT		f/2-Si	20	30-400	0.5 -1.5 20C1.03	Adolf et al. [73]
K. veneficum	TO/AT		f/2	22	50-300	0.150-0.321	Li et al. [128]
K. verneficum	TO/AT	Ps	L1	21	220	0.17-0.53	López-Rosales et al. [80]
Lingulodinium polyedra	TO		L1	18		0.2120.240	Rodriguez et al. [78]
L. polyedra	TO		f/2	20	200	0.33	Sullivan and Swift [48]
Ostreopsis sp. *5*	T		IMK	25	90-100	0.394	Yamaguchi et al. [83]
Ostreopsis sp. *5*	T		IMK	25	90-100	0.353	Yamaguchi et al. [83]
Ostreopsis sp. *1*	T		IMK	25	90-100	0.629	Yamaguchi et al. [83]
Ostreopsis sp. *1*	T		IMK	25	90-100	0.608	Yamaguchi et al. [83]
Ostreopsis sp. *6*	T		IMK	25	90-100	1.09	Yamaguchi et al. [83]
Ostreopsis sp. *6*	T		IMK	25	90-100	0.918	Yamaguchi et al. [83]

Table 4. (Continued)

Taxa	TO, AT, T	Nutrition (Ps, Holo, Mixo)	Medium	˚C	µmol m^{-2} s^{-1}	Gk div d^{-1}	Reference
Ostreopsis ovata	TO		Daigo IMK	25	90-100	0.802	Yamaguchi et al. [83]
Ostreopsis ovata	TO		f/2	25	90-100	0.866	Yamaguchi et al. [83]
Ostreopsis ovata	TO	Holo	f/2	20	20		Yoo et al. [31]
Ostreopsis ovata	TO		INK- f/2	20	180	0.15 – 0.25	Shah et al. [85]
Pfiesteria piscicida		Holo	f/2-Si	Room temp and ambient lt			Marshall et al. [137]
Polykrikos kofoidii			f/2	20	20	0.725	Yoo et al. [31]
Prorocentrum micans	TO/T		L1	20	90-490	0.11-0.41	Islabao and Odebrecht [115]
P.micans			L1	20	90-490	0.25-0.31	Islabao et al. [123]
P. lima	TO/T			18.1	90	0.10 -0.15	Pan et al. [138]
P. rhathymum			INK- f/2	20	180	0.204	Shah et al. [85]
Prorocentrum sp			f/2	20	30-170	0.35-0.39	Johnsen and Sakshaug [133]
Prorocentrum donghaiense			f/2	22	50-300	0.116-0.380	Li et al. [128]
Prorocentrum minimum			f/2	22	50-300	0.232-0.485	Li et al. [128]
P. minimum			f/2	22	50-300	0.038-0.276	Li et al. [128]
P. minimum str 2			f/2 modified	20	80-110	0.23-0.42	Micheli et al. [122]
P.minimum		Mixo	f/2	22	300 50	0.377-0.485 0.235 - 0.277low lt	Li et al. [128]
P. reticulatum			L1	18	100	0.37	Rodríguez et al. [139]
Protoceratium reticulatum	Th	Ps	L1	18	50	Shear stress	Garcia Camacho et al. [142]
P. reticulatum	TO/T		L1 -Si	17	100-150		Paz et al. [141]
	TO/T		L1	17	50	0.3	Rodriguez et al. [78]
P. reticulatum	TO/T		L1	18	100	0.1909	Rodriguez et al. [139]
Pyrocystis fusiformis			f/2	20	200	0.10-0.14	Sullivan and Swift [48]
Pyrocystis noctiluca			f/2	20	200	0.07-0.10	Sullivan and Swift [48]
Scripsiella trochoidea	TO		Aquil	20	80	0.27-0.29	Dixon and Syrett [49]
S. trochoidea	TO		f/2 modified	20	80-110	0.02-0.27	Micheli et al. [122]
Symbodinium sp.			IMK- f/2	20	180	0.242-0.30	Shah et al. [85]
Symbiodinium sp.			IMK- f/2(½ ¼)	20	40-50	0.304	Shah et al. [140]
Amphidinium carterae			IMK- f/2(½ ¼)		40-50	0.161-0.473	Shah et al. [140]
Prorocentrum rhathymum			IMK- f/2(½ ¼)		40-50	0.176 -0.312	Sinigalliano et al. [57]

Taxa	TO, AT, T	Nutrition (Ps, Holo, Mixo)	Medium	˚C	µmol m^{-2} s^{-1}	Gk div d^{-1}	Reference
Ostreopsis cf. *ovata*			f/2, K/2, L1	26 30	200	0.59 0.74-83	Ben-Gharbia et al. [143]
Prorocentrum lima						0.33	Ben-Gharbia et al. [143]
Coolia monotis						0.35	Ben-Gharbia et al. [143]
Ostreopsis ovata	TO				up to 460	0.695	Yamaguchi et al. [84]
Cochlodinium polykrikoides	TO/AT		f/2 -Si	10-30	up to 230	0.3 -0.41	Kim et al. [34]

TO=Toxic,T-Thecate, AT-Athecate; Ps – Photosynthetic , Holo- Holozoic, Mixo- Mixotrophic.

Division Rates: Cultures

Results on the division rates of cultured a) athecate, b) thecate dinoflagellates, c) non-toxic and d) toxic dinoflagellates are presented (Table 4). As calculation of division rates are based on exponential growth coefficient of cells, maximal division rates of the species are compared. Thus, the division rates ranged between 0.16 for *Alexandrium ostenfeldii* [55] and 1.50 for *Karlodinium veneficum* [111] and both are toxin producers as well. Division rates for athecate dinoflagellates ranged from 0.10 div $d^{-1}$for *Pyrocystis noctiluca* [48] to 1.5 for *Karlodinium micrum* [73]; for the thecate taxa 0.11 for *Alexandrium fundyense* [112] and 1.09 for *Ostreopsis* sp.6 [83]. The range of division rates between the non-toxic and toxic species seems to be comparable.

Repetitive measurements of division rates available for a few species(Table 4) permit comparison within the species and between the species. For example, these rates ranged as in *Alexandrium fundyense* 0.11-0.38, *Amphidinium carterae* 0.29-0.87, *Gymnodinium catenatum* 0.15-0.53, *Gymnodinium aureolum* 0.29-0.44, *Karlodinium veneficum* 0.32-1.5, *Lingulodinium polyedra* 0.24-0.33, *Ostreopsis* sp. 0.25-1.09, *Prorocentrum micans* 0.31-0.41, *Prorocentrum minimum* 0.276-0.485, and *Protoceratium reticulatum* 0.19-0.37. Band-Schmidt et al. [113] reported 0.18 – 0.31 division rates of *G. catenatum* under a variety of growth conditions ; 0.14–0.21 day^{-1} between 15 and 29°C, the highest rate (0.18–0.21 day^{-1}) between 21 and 29°C. Between 15-36 PSU, the highest growth rates (0.24 day^{-1}) were at salinities from 26 to 30 PSU. Growth also varied with the source of the sea water and was 0.28-0.31 in seawater from Bahía Concepcion; growth was more in medium f/2 enriched with selenium at 10^{-8} M than at 10^{-7}. In the Tasmanian waters influx of dissolved organic matter (humic substances) via rainfall triggered the growth of blooms of the toxic dinoflagellate *Gymnodinium catenatum* [98]. Culture studies showed the trace element selenium (as 10 M selenite) limited growth rate and biomass. Also, review of Band-Schmidt et al. [113] concluded the possible existence of different ecotypes strains from Galacia-Spain, Vigo-Spain, Derwent estuary-Tasmania, Bahía Concepción and Mexico seem to have evolved physiological and nutritional requirements.

Division Rates: Benthic Dinoflagellates

Benthic dinoflagellates *Amphidinium carterae* (JHWAC), *Prorocentrum rhathymum* (JHWPMX1) and *Symbiodinium* sp. were grown in f/2 and IMK media (1/2X and 1/4X) in vertical column photobioreactors (35cm diameter x 160 cm high) 20°C 40-50 μ mol photons m^{-2} sec^{-1} [85, 114]. Medium f/2 and IMK/2 supported higher division rates than did f/4 and IMK/4. From Bizerte Bay (Tunisia, Mediterranean) monoclonal cultures of thermophilic epiphytic, benthic toxigenic dinoflagellates (*Ostreopsis* cf. *ovata*, *Prorocentrum lima* and *Coolia monotis*) had maximum growth rates of 0.59 ± 0.08 d^{-1} for *O.* cf. *ovata*, 0.35 ± 0.01 d^{-1} for *C. monotis* and 0.33 ± 0.04 d^{-1} for *P. lima*. The toxic benthic dinoflagellate *Ostreopsis* sp. grew rapidly at 90–199 μmol photons m−2 s−1 but growth was inhibited at ≥263 μmol photons m^{-2} s^{-1} [84]. In IMK and/or f/2 media, maximum growth rates (div d^{-1}) were 0.8334 for *O.* cf. *ovate*, 0.619in *Ostreopsis* sp.1and 1.04 for *Ostreopsis* sp.6 [83] much higher than the epiphytic *Gambierdiscus toxicus*, *Prorocentrum lima*, and *Coolia monotis* cultures. Klueter et al. [99] reported faster growth rate in *Symbodinium microadriaticum* (cp-type A194; strain 04–503), *S. microadriaticum* (cp-type A194; strain CassKB8), *S. minutum* (cp-type B184; strain Mf 1.05b.01.SCI.01), *S. psygmophilum* (cp-type B224; strain Mf 11.05b.01) and *S. trenchii* (cptype D206; strain Mf 2.2b) reaching a higher asymptote at 26^{0}C than at 18^{0}C and attributed due to modification of growth-related nutritional physiology.

CULTURE CONDITIONS: DIVISION RATES

Salinity and Temperature

In their growth response to changes in salinity (PSU), temperature, light, and nutrients, dinoflagellates are similar to other microalgae. Kim et al. [34] studied growth of *Cochlodinium polykrikoides*- a harmful dinoflagellate, at 60 different combinations of temperature (10–30°C) and salinity (10–40PSU) and irradiance. The maximum growth rate of 0.41 was at 25^{0}C and 34 Pusan their optimum temperature corresponded to 21 to 26°C and at salinities from 30 to 36 units PSU. *Akashiwo sanguinea* had a broad tolerance to salinity PSU (10 to 35), more than *Prorocentrum micans* (25-35) [115]. The optimum irradiance for growth for *Akaqshiwo sanguinea* was >90 μmol m^{-2} $s^{-1}$and photoinhibition did not occur at 230 μmol m^{-2} s^{-1}.

Growth rates of *Alexandrium fundyense* [116] at five temperatures (5, 10, 15, 20, 25ºC), six irradiance levels (6, 25, 50, 100, 175, 42 μmol m^{-2} s^{-1}), and five salinities (15, 20, 25, 30, 35 PSU) showed that temperature exerted the greatest impact; the highest growth rate was 0.68 div d^{-1} at 15ºC; at 25ºC cells failed to grow. Growth rates generally increased with increasing irradiance up to 450μmol $m^{-2}s^{-1}$. Salinity did not impact growth significantly. In the harmful red tide dinoflagellate, *Cochlodinium polykrikoides* growth was monitored at 60 combinations of 10-30˚C and salinity 10-40 PSU under 130 μmol m^{-2} s^{-1} cool-white fluorescent illumination on a 12 h:12 h light: dark cycle . At a combination of 25ºC and 34 PSU, specific growth rate was 0.41 and optimum growth rates occurred between 21 to 26°C and at salinities from 30 to 36. *C. polycrikoides* did not grow below 10˚C and salinities >30 if the temperature was >15ºC. The optimum irradiance for growth was >90 μmol m^{-2} s^{-1}.

In *Karenia brevis* (Florida clone) growth occurred under various combinations of irradiance (19, 31, 52, 67, and123 μmol m^{-2} s^{-1}), salinity (25, 30, 35, 40 and 45 PSU), and temperature (15, 20, 25, and 30˚C) [117]. Maximum growth rates varied from 0.17 to 0.36 div day^{-1} with exponential growth rates increased with increasing irradiance. Growth was limited at 19 μmol m^{-2} s^{-1}. Maximum growth rates at 15˚C were much lower than at other temperatures. Of interest is the similarity between Florida clone and Texas clone in their temperature tolerance (15-30˚C) and light saturation 65 μmol m^{-2} $s^{-1.}$

From 42 different combinations of temperature (10–31˚C) and salinity (10–40 PSU) under saturated irradiance, *Prorocentrum donghaiense* had a maximum specific growth rate of 0.77 d^{-1} at a combination of 27˚C and salinity of 30 PSU [118]. The range for optimum growth rate (0.60 d^{-1}) was from 20 to 27˚C and salinities from 25 to 35 PSU. Cells did not grow at salinities <10 and temperatures 10-20˚C.

Temperature also affected the division rates in *Peridinium* sps, *Prorocentrum micans* and *P. gracile* [18] with an optimum at about 20˚-27˚C. In *Dinophysis caudate,* the optimum was in the range 18-30˚C and division rates increased from 0.21 to 0.67 between 15˚C and 30˚C and decreased at >30˚C [74]. This was also the case in *Gymnodinium catenatum;* cells grew well in the temperature range >11˚C to 28˚C and attained a 0.53 division rate; cells did not grow below 11˚C or at > 30˚C [119], *Akashiwo sanguinea* [120], *Amphidinium carterae* [49], *Alexandrium ostenfeldii* [55]. In *Prorocentrum minimum* strain 2 at μmol m^{-2} s^{-1} and20˚C growth was 0.42 and at 110 μmol m^{-2} s^{-1}27˚C division decreased to 0-.23 -0.38 [122]. Of interest are the growth rates of *Heterocapsa circularisquama* which did not grow at 10˚C but had 1.3 Gk d^{-1}at 30˚C and 30 PSU. *Chattonella verruculosa* did not grow at 25˚C and 10PSU but divided 1.74 times at a combination of 15˚C and 25 PSU [135]. Thus this interaction of growth to temperature and salinity may result in harmful algal blooms contributed by different taxa.

Light

Dinoflagellates seem to prefer low light <170 μmol m^{-2} s^{-1}. Yamaguchi et al. [84] using white light-emitting diodes, devised a new photoirradiation-culture system to study the growth of the benthic dinoflagellate *Ostreopsisovate* cultured in irradiances up to 460 μmol m^{-2} s^{-1}. The threshold irradiance for growth was 29.8 μmol m^{-2} $s^{-1.}$ At the optimum light 196 μmol m^{-2} s^{-1} division rate was 0.659 day^{-1} and decreased to 95% at 130– 330 μmol m^{-2} s^{-1}, further decreased to 80% at 90 μmol m^{-2} s^{-1}. Photoinhibition was at 460 μmol m^{-2} s^{-1} and the division rate was 80% of the maximum [84]. Results on *Akashiwo sanguinea*, *Prorocentrum micans* and *Scrippsiella trochoidea* are instructive [123]. Division of cells grown at low (LL, 87–90 μmol photons m^{-2} s^{-1}) and high (HL, 450–490 μmol photons m^{-2} s^{-1}) were similar for *A. sanguine* (μ=0,26–0,33 d^{-1}) and *P. micans* (μ=0,25–0,31 d^{-1}) while they were significantly higher in *Scripsiella trochoidea* under HL (0,29±0.02; p b 0,001) than LL (μ=0,23±0.01 d^{-1}). Cells grown at higher irradiance had higher lipid that served as a protective mechanism. Using a monochromator, cell divisions of *Heterocapsa pygmaea* were similar and averaged 0.7 d^{-1} [124].

Nutrient Phosphate and Nitrate

In *Alexandrium fundyense* cultures grown in f/2 medium the phosphate, nitrate and ammonia levels decreased as growth progressed and were abundant by the 21st or 29th day, the time at which the cells attained their peak growth; compared to the initial levels remaining phosphate levels were 38–43%, nitrates were 82–98% and 14–97% ammonium chloride [33]. Even at the stationary phase, these nutrients were not exhausted and by the 50th day were reduced to 38–51% phosphate, and 10–97% nitrate. In *Alexandrium tamarense* grown in K medium, when nitrate exceeded 264 μM, cell division decreased to 0.5 div d^{-1} compared to 0.6 div d^{-1} with K medium [125]. High salinity, low light and high nitrate and low phosphate favored toxin production in this species. *A. fundyense* can remove dissolved free amino acids (DFAAs) during early exponential growth, at a rate 0.8 pmol-N cell h^{-1}), equivalent to ~20% of the total N requirement but this does not enhance toxin content [126]. Production of some of these mycosporine-like amino acids (MAAs) is more when exposed to PAR+/UVA without UVB as in *Heterocapsa* sp. and may be photoprotective [127].

Observations on cultures of nearshore dinoflagellates *Prorocentrum minimum*, *Prorocentrum donghaiense*, *Karlodinium veneficum*, and an off-shore *Karenia brevis* grown in f/2 medium with NO_3 as nitrogen source are interesting [128]. Growth rates of *Karlodinium veneficum* were 0.32 div d^{-1} did not differ significantly between low and high N: P ratios (12 and 145) suggesting excessive or luxury consumption of nutrients unrelated to growth. When urea was used as a nitrogen source there was a long lag phase followed by a growth phase of one week with a decrease in growth rate to 0.24 div d^{-1}. In phosphorus minimum with nitrate as a source, growth rate was 0.17-0.27 d^{-1} in low N: P and 0.23 to 0.33 in high N: P ratio. Growth rates of *K.brevis* and *Prorocentrum donghaiense* were reduced in a mixed culture with *Synechococcus* [128]. Lopez-Rosales et al. [80] in a study to optimize medium formulation for culturing *Karlodinium veneficum* and used 25 components including the macronutrients, trace elements and vitamins and compared 30:1 N:P ratio to the control L1 medium with 25:1 N:P; the former enhanced cell density by 120%, 14% greater division rate (0.0- 0.33 d^{-1}) and the ichthyotoxic (haemolytic) activity of the culture filtrate by 190% compared to (0.29 d^{-1}) L1 medium.

Iron

More than phosphate and nitrate, iron appears to be important for the growth of dinoflagellates. In phytoplankton growth is limited by lack of iron [129, 130]. *Alexandrium fundyense* cells exposed to 366 mm iron (Harrison medium – Ha) reached a peak in 18 days while in the absence of iron (f/2 – Fe – control) they did not grow [33]. In Harrison's medium in a gradient of Fe (36.6, 73.2, 146.4, and366 mMFe) cell division corresponded to 0.25, 0.208, 0.172 and 0.21 d^{-1}. At 732 mM Fe, however, cells divided slowly (0.159 d^{-1}). In *Scrippsiella trochoidea* that produces harmful algal blooms off Japan, Korea and China [131], results from a gradient experiment (low iron: 0.063 mg L^{-1}, medium iron: 0.63 mg L^{-1} and high iron: 6.3 mg L^{-1}) showed under high Fe and high light cells divided 0.22 d^{-1} compared to 0.06 d^{-1} in low Fe and low light [81].

Protoceratium reticulatum was grown in nutrient-enriched seawater (1/10 GP medium) without iron or with 0.076 and 0.0076 μM iron added; the growth rate in treatments without

added iron was also significantly reduced to (μ=0.16 day^{-1}) compared to μ=0.23 day^{-1} with iron [132].

MIXOTROPHIC CULTURES

Mixotrophy, a combination of phototrophy and phagotrophy, appears to be common amongst dinoflagellates [144, 145] and it enhanced division rates. Stoecker lists 46 mixotrophic dinoflagellate species belonging to Prorocentrales (4), Dinophysiales (2), Gymnodiniales (13), Noctilucales (1) Gonyaulacales (15), Peridiniales (2), Blastodiniales (5), Phytodiniales (3) and Dinamoebales (1). Several are to be added to the mixotrophs listed in Table 5. Plausible advantages of being mixotrophic include a) it extends the range of nutritional substrates to meet growth requirements as in *K. brevis*, particularly in N-poor waters [146] b) it acts as a survival strategy during the time when photosynthesis cannot support growth as in *D. norvegica* [32, 88] or c) it serves as a primary source of carbon as in *D. acuminata* [147] and d) it meets inorganic nutrient limitation during summer stratification of the water column as in *Prorocentrum minimum feeding* on cryptophytes material [145]. *Pfiesteria shumwayae*, the predatory dinoflagellate axenically grown in dark on a semi-defined biphasic culture medium rich in certain dissolved and particulate organic compounds, including amino acids and lipid particles, attained cell densities ranging from 0.1×10^5 to 4.0×10^5 cells/ml and 0.5 to 1.7 div/day [93]. Feeding on insoluble lipid particles suggests phagocytosis may be a requirement in this dinoflagellate.

Dinoflagellates grown heterotrophically had higher growth rates (with prey versus autotrophic, Table 5). In *Karlodinium micrum* fed on *Storeatula major* (Cryptophyceae) (0.52 to 0.75 vs 0.55) [148], *Yihiella yeosuensis* fed on *Pyramimonas* sp. *Teleaulax* sp. (1.32 *vs* 0.026) [149], *Protoperidinium hirobis*, a thecate heterotrophic dinoflagellate fed on the diatom *Leptocylindrus danicus* reached a maximum 1.7 vs 1.0 [150], *Polykrikos hartmannii* fed on *C. polykrikoides* (0.03 vs 0.023) [151], *Alexandrium andersonii* fed on *Pyramimonas* sp., the cryptophyte *Teleaulax* sp., and the dinoflagellate *Heterocapsa rotundata* (0.432 vs 0.243) [29], *Alexandrium pohangense* fed on *Cochlodinium polykrikoides*(0.487 vs 0.091) [30], *Karenia brevis* fed on *Synechococcus* (0.53 vs 0.25) [146], *Dinophysis acuminata* fed on *Mesodinium rubrum* (0.17-0.51 vs 0.2) [147], *Fragilidium duplocampanaeforme* fed on *D. acuminata, D. caudata, Mesodinium* (0.07-0.6 vs 0.21-0.26) [152], and *Dinophysis caudata* fed on *Myrionecta rubra, Teleaulax amphioxeia* (1.03 vs 0. 28) [30]. Phagotrophy probably serves as an alternative option to acquire nutrients.

In a mixed culture (Table 5) *Prorocentrum minimum* with 0.377-0.485 Gk out grew *Karlodinium veneficum* and *Prorocentrum donghaiense* outgrew *Karenia brevis* [128]. When fed with the microalga *Rhodomonas*, growth of *P. minimum* was inhibited [128]. In the presence of *Synechococcus* growth rates of both *K. brevis* and *P. donghaiense* were reduced.These reciprocal inhibitory effects may be due to allelochemical potency observed between the toxic *Alexandrium ostenfeldii* and other dinoflagellates *Kryptoperidinium foliaceum, Levanderina fissa* and *Heterocapsa triquetra* [153] or allelopathic effects between the species [120].

Table 5. Cultures and growth of mixotrophic dinoflagellates

Taxa	Medium	Temperature (ºC)	µmol m^{-2} s^{-1}	Gk div d^{-1}	Reference
Karlodinium micrum	f/2-Si	20		0.55 auto 0.52-0.75 mixo	Adolf et al. [148]
Dinophysis caudata	f/2			0.66	
Karenia brevis	L1	20	43-300	0.25-0.53	Glibert et al. [146]
Yihiella yeosuensis	f/2	20	20	0.026 1.32	Jang et al. [149]
Takayama helix	f/2 -si	20	20	0.272-0.268 0.152	Jeong et al. [155]
Gymndinium smaydae	f/2	20	20	1.053- 2.226	Lee et al. [154]
Polykrikos hartmannii		20	100	0.030 0.023	Lee et al. [151]
Alexandrium andersonii,	*f/s-Si*	*20*	*20*	*0.243 (Ps)* *0.432 (hetero)*	Lee et al. [29]
Prorocentrum minimum	*f/2*	*22*	*300* *50*	*0.377-0.485 high light* *0.235 -0.277 low light*	Li et al. [128]
Stoeckeria changwonensis	f/2	20	20	0.354 0.376 -0.12 -0.11 -0.09	Lim et al. [91]
Alexandrium pohangense	f/2	20	100	0.091 0.487	Lim et al. [30]
Dinophysis caudata	f/2	18	100-150	0.28 auto 1.03 hetero	
	SWM-3	25			Nishitani et al. [32]
D.caudata	SWM-3			0.68 auto	Nishitani et al. [88]
Fragilidium duplocampanaeforme	f/2-Si	20	100	0.07 -0.6 0.21-0.26	Park et al. [152]
D. acuminate	f/2	20	100	0.17-0.51	Riisgaard and Hansen [147]
Ostreopsis ovata	f/2	20	20		Yoo et al. [31]
Gyrodinium moestrupii				0.862	
Polykrikos kofoidii	f/2			0.725	
Pfiesteria shumwayae	PSD-10	21	Dark	0.5-1.7	Parrow et al. [93]

Gymnodinium smaydae preyed on *Heterocapsa rotundata. H.triquerea, Heterocapsa* sp., *Scripsiella trochoidea* but did not on *Heterosigma akashiwo, Teleaulax, Rhodomonas salina and Amphidinium carterae* [154]. Their mixotrophic growth rates (d^{-1}) were higher (1.053-2.226), compared to the autotrophic without prey (0.005 -0.0051). *Gyrodinium dominans*, *G. moestrupii*, *Oxyrrhis marina*, *Pfiesteria piscicida*, and *P. kofoidii* were able to feed on the toxic epiphytic benthic dinoflagellate *Ostreopsis cf. ovata*; but not on *Gyrodinium spirale, Protoperidinium bipes*, *Stoeckeria algicida*, and *Strobilidium* sp. [31]. Growth rates *G. moestrupii* and *P. kofoidii* on *O*. cf. *ovate* corresponded to 0.86 and 0.73 coinciding with high ingestion rates.

Takayama helix another dinoflagellate that causes fishkills feeds on diverse algal species [155]. It ingests large dinoflagellates ≥15 µm, on *Alexandrium minutum, A. lusitanicum, A. tamarense, A. pacificum, A.insuetum, Cochlodinium polykrikoides, Coolia canariensis, Coolia malayensis, Gambierdiscus caribaeus, Gymnodinium aureolum, Gymnodinium catenatum, Gymnodinium instriatum, Heterocapsa triquetra, Lingulodinium polyedrum*, and *Scrippsiella trochoidea* except for the dinoflagellates *Karenia mikimotoi*, *Akashiwo sanguinea*, and *Prorocentrum micans*. However, it does not feed on flagellates and dinoflagellates <13 µm in size (i.e., the prymnesiophyte *Isochrysis galbana*; the cryptophytes

Teleaulax sp., *Storeatula major*, and *Rhodomonas salina*; the raphidophyte *Heterosigma akashiwo*; the dinoflagellates *Heterocapsa rotundata*, *Amphidinium carterae*, *Prorocentrum minimum*; or the small diatom *Skeletonema costatum*). However, *T. helix* did not feed on small flagellates and dinoflagellates <13 μm in size (i.e., the prymnesiophyte *Isochrysis galbana*; the cryptophytes *Teleaulax* sp., *Storeatula major*, and *Rhodomonas salina*; the raphidophyte *Heterosigma akashiwo*; the dinoflagellates *Heterocapsa rotundata*, *Amphidinium carterae*, *Prorocentrum minimum*; or the small diatom *Skeletonema costatum*). Growth rates in the mixotrophic mode were 0.268- 0.272 d^{-1} compared to 0.094 – 0.152 d^{-1} in the autotrophic growth.

CONCLUSION

About 100 autotrophic, heterotrophic, toxigenic and symbiotic dinoflagellate species are amenable for culturing. Their growth rates are studied in enriched sea water media or enriched artificial seawater media. Dinoflagellate division rates are low compared to 14 pennate diatoms with maximum division rates of 3.2 div d^{-1} [156], and 2.47 for *Phaeodactylum tricornutum* [157] or *Leptocylindrus danicus* with 2.7 div day^{-1} [158], or the green flagellate *Dunaliella tertiolecta* with high growth rates 0.8- 2.64 [35]. Physiological difference between the dinoflagellates and other microalgae exist. Photosynthetic and thecate dinoflagellates which have a cellulose cell wall are significantly more carbon-dense than diatoms [159] evident from their Carbon: Nitrogen that ranged from 3.44 to 6.456. Log phase dinoflagellates although have lower photosynthetic rates per unit chlotophyll *a* than diatoms which have lower carbon: chlorophyll *a* ratio [160,161]. A review of 31 dinoflagellates species showed lower growth rates which are related to lower chlorophyll *a* to carbon ratio; these two variables explain 68% of variation in growth [161]. Because of these physiological differences, it is possible that dinoflagellates expend much energy into the production of cellulose skeleton and toxins which result in their lower division rates. In mixotrophic and heterotrophic dinoflagellates which prey on high energy biota, their division rates are higher than when grown autotrophically (controls) or compared to other autotrophic dinoflagellates. If this conclusion is correct, then it needs to be substantiated with detailed observations on the Carbon: Nitrogen dynamics in a variety of dinoflagellate cultures.

Dinoflagellates are influenced by a variety of abiotic (e.g., temperature, salinity, stratification, nutrient enrichment) and biotic (e.g., grazing) factors and no single factor accounts for their blooming, a conclusion drawn by Vargo [162]. Vargo based his conclusion on a review of 24 thoughts and hypotheses to account for the initiation, growth and maintenance, and termination of Karenia *brevis* blooms on the West Florida shelf. It is possible sheltered bays or coastal waters get seeded by large numbers of cysts by advection [163]. Based on a study of 27 estuaries between Prince Edward Island, Canada and Maine to Delaware, Price et al. [164] distinguished four main estuary types i.e., riverine, lagoon, coastal embayment, and fjord, in which tidal range, sea surface temperature (SST), and sea surface salinity (SSS) are statistically significant parameters influencing cyst assemblages. Since cysts are resistant to adverse environmental conditions, encystment enables the survival of cells. With their slow asexual reproduction (binary fission), the rapid ephemeral massive occurrences of monospecific dinoflagellate blooms in a short time are intriguing.

Studies from 23 northeast USA estuaries and 9 estuaries from Prince Edward Island, Canada [165], and in the Tunisian Gulf of Gabes, Mediterranean Sea [166] suggest the association of dinoflagellate blooms with environmental variables. Redundancy analysis of 10-year data using a generalized linear mixed-effect model (GLMM) suggested that in the Gulf of Gabes, blooms of *Karenia selliformis* are related to the different spatial gradients caused by anthropogenic activity- positively with nitrate and negatively with total phosphorus [165]. Results from the NW Atlantic estuaries [164] showed that the overall abundance of cysts of heterotrophic dinoflagellates correlates with modelled nitrogen loading. In view of this, studies on the Carbon: Nitrogen dynamics utilizing community dinoflagellate cultures vs individual species (axenic), particularly those rich in lipids, carotenoids and bioactive compounds with potential biotechnological utility, using unprocessed or partially processed sewage water and in post-bloom water would be instructive.

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Chapter 140

GROWTH AND FEEDING BEHAVIOUR OF MIXOTROPHIC *DINOPHYSIS* SPECIES IN LABORATORY CULTURES

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ABSTRACT

Since Park et al. (2006) succeeded in cultivating the toxic dinoflagellate *Dinophysis acuminata* by feeding them the ciliate *Mesodinium rubrum* grown with a cryptophyte *Teleaulax* sp., we succeeded in establishing six *Dinophysis* species, i.e., *D. acuminata*, *D. caudata*, *D. fortii*, *D. infundibulum*, *D.* cf. *ovum* and *D. tripos* under laboratory conditions and maintaining clonal strains kept at relatively high abundance (>2,000 cells mL^{-1}) for a long period (>6 months) when fed on *M. rubrum* with the addition of *Teleaulax amphioxeia*. The six *Dinophysis* species grew at growth rates of 0.50-1.03 day^{-1}, reaching maximum concentrations of ca. 2,200-11,000 cells mL^{-1} at temperature ranges of 18-25ºC, suggesting that these *Dinophysis* species are able to grow as rapidly as other red-tide forming species. In the transmission electron microscopic observation of *D. fortii* cells, which had fully fed on the ciliate prey, well-developed chloroplasts (5–12 μm in length) were seen and three thylakoids were usually arranged in most of the chloroplasts observed, but chloroplasts having two thylakoids were sometimes confirmed. In cells starved for 4 weeks, a decrease in chloroplast numbers and disappearance of large chloroplasts were observed, and only a few small chloroplasts (0.5–2 μm in length) remained in the marginal regions. In the observation of the sequestration process of the

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chloroplasts ingested from *M. rubrum* by *Dinophysis*, within 15 min after *Dinophyis* captured *M. rubrum*, incorporation of almost all of the chloroplasts was observed, while most of the other cell contents still remained in the *M. rubrum* cell. After that, dispersion of the ingested chloroplasts toward the marginal regions was confirmed, suggesting that chloroplasts of *M. rubrum* are ingested and dispersed in *Dinophysis* cells in advance of the ingestion of the other cell contents to prevent them from being digested in food vacuoles. The ingested chloroplasts can function as kleptoplastids. Molecular analyses of *Mesodinium rubrum* and several *Dinophysis* species plastid genes from natural samples showed a simple food web among *Teleaulax*/*Mesodinium*/*Dinophysis*. However, *Dinophysis* may still utilize other unknown prey species as the success rate in establishing *Dinophysis* cultures is still variable, and thus, a further study is needed.

Keywords: cryptophyte, diarrhetic shellfish poisoning (DSP), *Dinophysis acuminata*, *Dinophysis caudata*, *Dinophysis fortii*, *Dinophysis infundibulum*, *Dinophysis* cf. *ovum*, *Dinophysis tripos*, feeding behavior, growth rate, kleptoplastids, *Mesodinium rubrum*, Phylogeny, *Teleaulax amphioxeia*

1. INTRODUCTION

Diarrhetic shellfish poisoning (DSP) is a severe gastrointestinal disease caused by the consumption of shellfish contaminated with DSP toxins (Yasumoto et al. 1978, 1980). DSP toxins are produced by dinoflagellates belonging to the genera *Dinophysis* Ehrenberg and benthic *Prorocentrum* Ehrenberg (Yasumoto et al. 1980; Lee et al. 1989) and result in serious economic losses to shellfish culture industries worldwide (Della Loggia et al. 1993). At present, 10 *Dinophysis* and 2 *Phalacroma* species are known to be toxic (Lee et al. 1989; Maestrini 1998; Moestrup 2004; Reguera and Pizarro 2008; Moestrup, et al. 2019, Figure 1). In phylogenetic analyses, the *Dinophysis* species cluster into two or three main groups depending on the gene and/or region targeted (Edvardsen et al. 2003; Guillou et al. 2002; Handy et al. 2009; Jensen & Daugbjerg 2009; Nagai et al. 2008; Nishitani et al. 2008a; Papaefthimiou et al, 2010; Qiu et al, 2011; Raho et al, 2008, 2013; Stern et al, 2014, Figures 2, 3). Both the ribosomal RNA and mitochondrial genes, however, lack the sufficient resolution to differentiate between all *Dinophysis* species or strains of different geographic origin (Edvardsen et al., 2003; Hart et al., 2007; Raho et al., 2008, 2013; Rodríguez et al., 2012; Stern et al., 2014). Thus, morphology-based observations facilitate more detailed species identification compared to molecular methods.

Field investigations on the population dynamics of *Dinophysis* species have been carried out in various areas (Koike et al. 2001; Nishitani et al. 2002, 2005; Lindahl et al. 2007). Despite extensive studies on the distribution and population dynamics of *Dinophysis* species in the last two decades (e.g., Maestrini, et al. 1998; Hällfors, et al. 2011; Sjöqvist & Lindholm, 2011; Reguera, et al. 2012; Díaz, et al. 2013, 2016, 2019; Singh, et al. 2014; Hattenrath-Lehmann, et al. 2015; MacKenzie, et al. 2019), many aspects of ecophysiology and bloom mechanisms of *Dinophysis* species are still unknown, primarily due to the inability to culture them (Sampayo 1993; Jacobson & Andersen 1994; Maestrini 1998; Nishitani et al. 2003). Non-photosynthetic species of *Phalacroma* feed by myzocytosis, a process whereby a peduncle (or feeding tube) sucks up the cytoplasm from the prey, leaving behind the plasmalemma (Hansen 1991). Photosynthetic species of *Dinophysis* share this structure and,

although they had not been observed feeding (but see Park et al. 2006), food vacuoles were often found in their cytoplasm, clearly indicating mixotrophy (Jacobson and Andersen 1994; Koike et al. 2000). Many phototrophic species have an orange autofluorescence under blue or green light suggesting the presence of phycobilin (Geider and Gunter 1989; Hallegraeff and Lucas 1988; Lessard and Swift 1986; Schnepf and Elbrächter 1988; Imai and Nishitani 2000) and phycoerythrin (Geider and Gunter 1989; Hewes et al. 1998; Vesk et al. 1996). Based on ultrastructural analyses, the plastids have paired stacked thylakoids with electron-dense contents in the relatively wide lumen, similar to cryptophyte thylakoids (Schnepf and Elbrächter 1988; Lucas and Vesk 1990). These observations suggested that the *Dinophysis* plastids originated from cryptophytes (Schnepf and Elbrächter 1988; Meyer-Harms & Pollehne 1998). Molecular analyses of several *Dinophysis* species using plastid sequences such as *psbA*, 16S-rDNA, and *rbcL* (encoding the large subunit of RuBisCO) genes also proved the *Dinophysis* plastid to be of cryptomonad origin (Takishita et al. 2002; Hackett et al. 2003; Janson and Granéli 2003; Janson 2004; Takahashi et al. 2005). This led to the suggestion that the plastid of *Dinophysis* might be a kleptoplastid, a temporary but functioning plastid captured from a prey (Melkonian 1996; Janson 2004; Minnhagen & Janson 2006). However, whether the plastids of *Dinophysis* result of a permanent endosymbiosis or predation of a cryptophyte was not clarified until 2006.

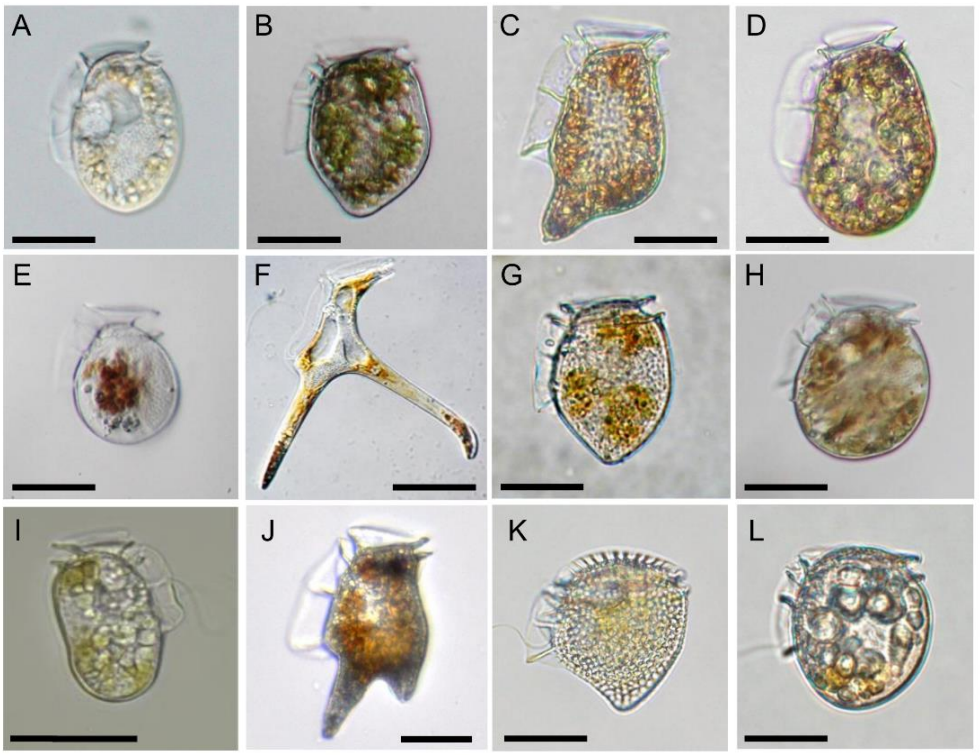

Figure 1. Light microphotographs of ten toxic *Dinophysis* and two toxic *Phalacroma* species taken by normal light microscopy. A, *D. acuminata*; B, *D. acuta*; C, *D. caudata*; D, *D. fortii*; E, *D. infundibulum*; F, *D. miles*; G, *D. norvegica*; H, *D. ovum*; I, *D. sacculus*; J, *D. tripos*; K, *P. mitra*; L, *P. rotundatum.* All scale bars = 30 µm. B, E, H were provided by H. Shimada. F, G, and I were provided by M. Iwataki, M. Selina and F. Rodríguez, respectively.

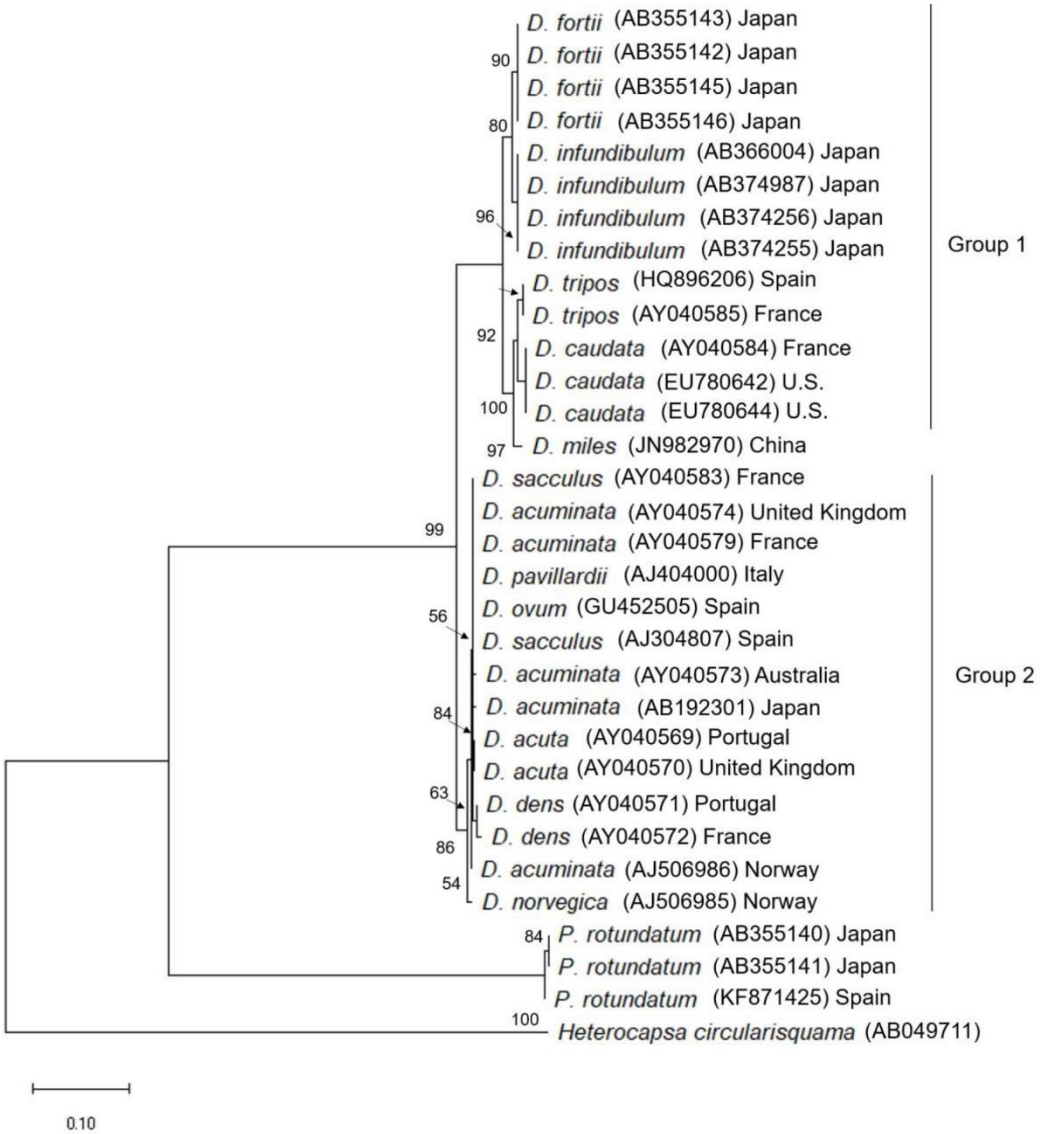

Figure 2. A maximum-likelihood tree based on ITS alignment (579 bp, incl. insertions/deletions) including 31 sequences and *Heterocapsa circularisquama* serving as an outgroup. Bootstrap values > 50% are shown.

Since Park et al. (2006) succeeded in cultivating the toxic dinoflagellate *Dinophysis acuminata* by feeding them the ciliate *Mesodinium rubrum* grown with a cryptophyte *Teleaulax* sp., other seven species were also successfully cultured, namely *D. acuta* (Jaén et al. 2009), *D. caudata* (Nishitani et al. 2008b) *D. fortii* (Nagai et al. 2008), *D. infundibulum* (Nishitani et al. 2008a), *D.* cf. *ovum* (Mafra et al. 2016), *D. sacculus* (Riobó et al. 2013) and *D. tripos* (Nagai et al. 2013). Although further supportive data are clearly required, this evidence strongly suggests that *Dinophysis* species depend on predator-prey interactions with *M. rubrum* and *Teleaulax* or *Teleaulax*-related species (Nagai et al. 2008; Nishitani et al. 2008a) for their photosynthetic capability. Cultured strains of *Dinophysis* species can be

established and maintained by feeding with the ciliate prey grown with *Teleaulax* or *Teleaulax*-related species (Nagai et al. 2008; Nishitani et al. 2008a; Kamiyama & Suzuki 2009). Here we report on the feeding behavior and the growth characteristics of several *Dinophysis* species under laboratory conditions and the sequestration process of the chloroplasts ingested from *M. rubrum* by *Dinophysis* on the basis of transmission electron microscopy (TEM) and epifluorescence microscopy (EMF).

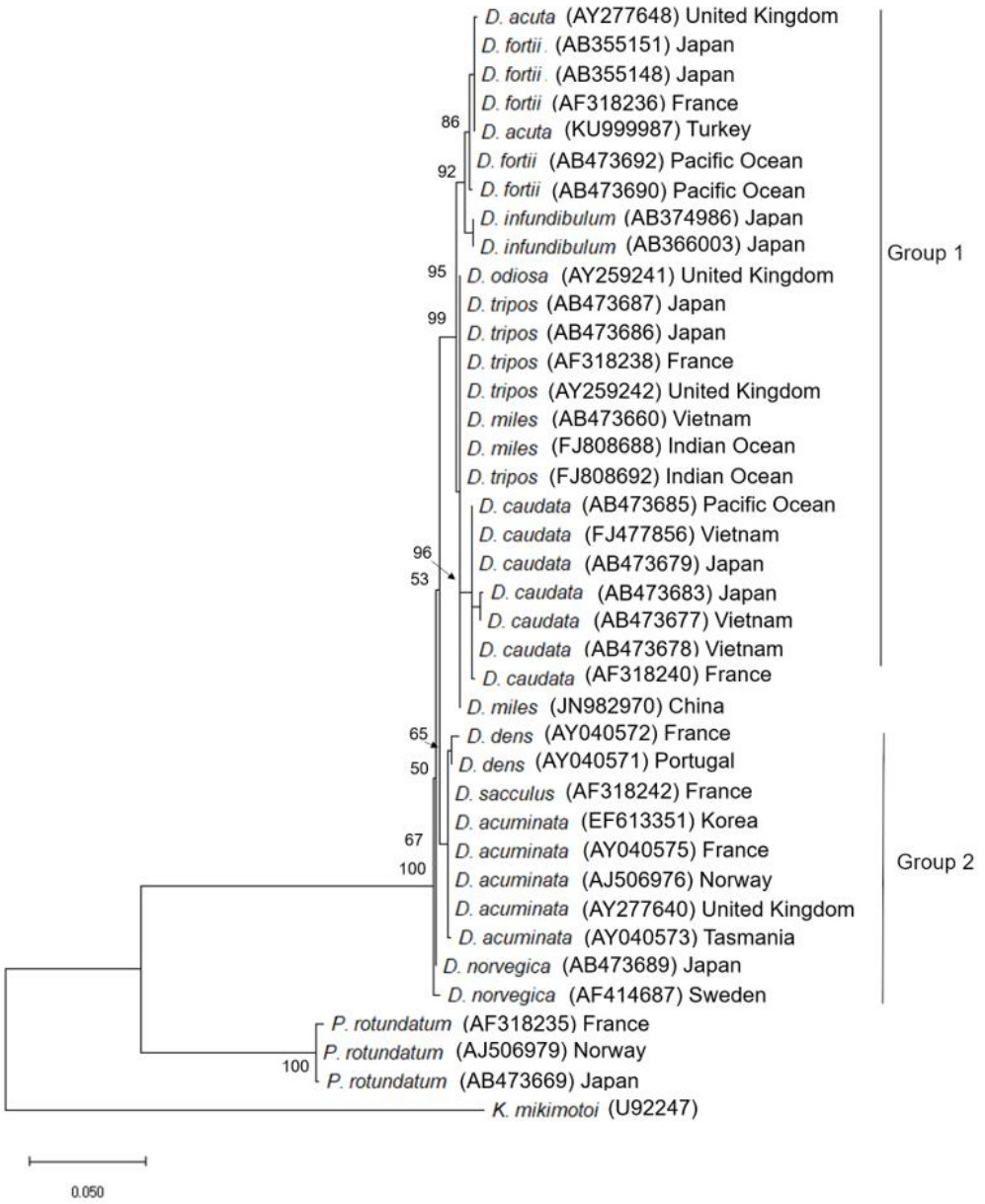

Figure 3. A maximum-likelihood tree based on LSU D1-D2 alignment (593 bp, incl. insertions/deletions) including 38 sequences and *Karenia mikimotoi* serving as an outgroup. Bootstrap values > 50% are shown.

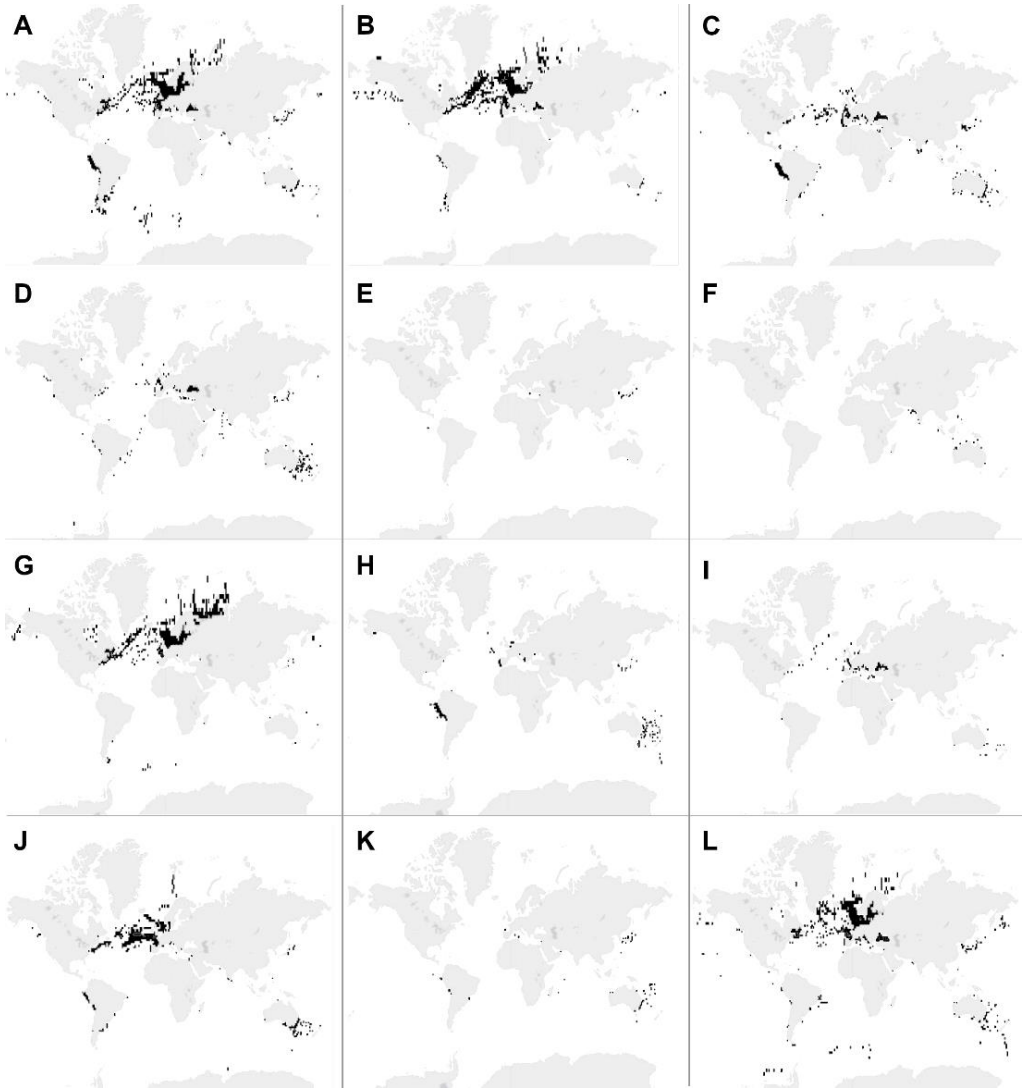

Figure 4. Distribution of toxic *Dinophysis* and *Phalacroma* species (OBIS, 2019): A, *D. acuminata*; B, *D. acuta*; C, *D. caudata*; D, *D. fortii*; E, *D. infundibulum*; F, *D. miles*; G, *D. norvegica*; H, *D. ovum*; I, *D. sacculus*; J, *D. tripos*; K, *P. mitra*; L, *P. rotundatum*.

2. BIOGEOGRAPHY OF *DINOPHYSIS* AND *PHALACROMA* SPECIES ASSOCIATED WITH DSP EVENTS

The *Dinophysis* and *Phalacroma* species listed as toxic in the IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae (Moestrup, et al. 2019) are widely distributed, however, six species display more global distribution (*D. acuminata*, *D. acuta*, *D. caudata*, *D. norvegica*, *D. tripos*, *P. rotundatum*: Figure 4, OBIS, 2019). Majority of those *Dinophysis* and *Phalacroma* species have been detected from a wide salinity range of 0 to 35 with the highest number of reports from the salinity of 30-35 (Table 1, OBIS, 2019). At the same time,

three species: *D. infundibulm*, *D. miles,* and *P. mitra*, occur in a narrower salinity range (25-35 or 30-35), which can be explained by their appearance in the fully oceanic conditions (Figure 4, OBIS, 2019). The temperature range for the majority of the species is similarly broad, from -5 to 30°C and with the highest number of reports from 10 to 20°C (Table, 1, OBIS, 2019). Effect of temperature on the growth of *Dinophysis* species in laboratory conditions has been currently only investigated for *D. caudata*, *D. acuminata* and *D. tripos* (Kamiyama, et al. 2010; Rodríguez, et al. 2012; Basti et al. 2015; 2018; Gao, et al. 2017). *Dinophysis tripos* showed growth under the three tested temperatures (15, 19 and 25°C) with growth rates of 0.32, 0.37 and 0.35 day^{-1} (d^{-1}, Rodríguez, et al. 2012). Growth in *D. caudata* and *D. acuminata* was confirmed in the range of 15-32.5°C and 8-32°C and no growth was observed below 10 and 5°C, respectively (Basti, et al. 2015; 2018). Growth rates and maximum cell yields in *D. caudata* were 0.21 (15°C) - 0.67 (30°C) d^{-1} and 840 (15°C) - 7,000 (32.5°C) cells mL^{-1}, respectively (Basti, et al. 2015). The growth rate at 15°C was significantly lower than at other temperatures, suggesting that *D. caudata* prefers a relatively high temperature between 27-32.5°C. Similarly, the growth rates and the maximum cell yields in *D. acuminata* were 0.27 (8°C) - 0.93 (26°C) d^{-1} and 400 (8°C) - 6,900 (20°C) cells mL^{-1}, respectively (Basti, et al. 2018). The results are consistent with their distributions as *D. caudata* is distributed in tropical and temperate waters (Okaichi 1967, Figure 4C) and *D. acuminata* is a cosmopolitan species present in the Pacific and Atlantic Oceans, and in the Mediterranean Sea (Figure 4A, OBIS, 2019).

Table 1. Water temperature and salinity ranges for toxic *Dinophysis* and *Phalacroma* species based on global phytoplankton monitoring data (OBIS, 2019)

Species		Water temperature (°C) range	Temperature (°C) - highest number of reports	Salinity range	Salinity - highest number of reports
Dinophysis acuminata	Claparède & Lachmann, 1859	-5 to 30	5 to 15	0 to 35	5 to 10, 30 to 35
Dinophysis acuta	Ehrenberg, 1839	-5 to 25	10 to 15	0 to 35	30 to 35
Dinophysis caudata	Saville-Kent, 1881	0 to 30	15 to 20	10 to 35	30 to 35
Dinophysis fortii	Pavillard, 1924	-5 to 30	15 to 20	10 to 35	15 to 20, 30 to 35
Dinophysis infundibulum	J. Schiller, 1928	10 to 25	20 to 25	25 to 35	30 to 35
Dinophysis miles	Cleve, 1900	20 to 30	25 to 30	30 to 35	30 to 35
Dinophysis norvegica	Claparède & Lachmann, 1859	-5 to 30	10 to 15	0 to 35	5 to 10, 25 to 30
Dinophysis ovum	(F. Schütt) T. H. Abé	5 to 30	15 to 20	15 to 20, 25 to 35	30 to 35
Dinophysis sacculus	F. Stein, 1883	5 to 30	10 to 20	10 to 35	30 to 35
Dinophysis tripos	Gourret, 1883	-5 to 30	10 to 20	10 to 35	30 to 35
Phalacroma mitra	F. Schütt, 1895	10 to 30	15 to 20	30 to 35	30 to 35
Phalacroma rotundatum	(Claparéde & Lachmann)	-5 to 30	10 to 15	0 to 35	30 to 35

3. ISOLATION OF CLONAL STRAINS, GROWTH AND FEEDING PROCESS

3.1. Importance of the Establishment of Clonal Culture Strains

Regarding toxin production by *Dinophysis* species, pectenotoxin-2 (PTX-2), dinophysistoxin-1 (DTX-1), and okadaic acid (OA) have been detected from several species obtained from natural samples from Japanese coastal waters (Suzuki et al. 1997, 1998, Uchida et al. 2018). OA and DTX-2 are the dominant toxins in some European *Dinophysis* species (Lee et al. 1989; Draisci et al. 1999; Pavela-Vrančič et al. 2001; Fernández-Puente et al. 2004a,b; Vale 2004; Fernández et al. 2006)., *Dinophysis acuminata*, *D. acuta*, *D. caudata*, *D. norvegica* and *D. sacculus* have caused DSP outbreaks around the world (Table 1 in Uchida et al. 2018). Further, PTX-11, which is a dominant toxin in *D. acuta* in New Zealand (MacKenzie et al. 2002, 2005; Suzuki et al. 2004, 2006), has not been detected in any *Dinophysis* species in Hokkaido, Japan (Suzuki et al. 2009). Although it is well known that various shellfish species exhibit different patterns of biotoxin uptake, biotransformation, and depuration (MacKenzie et al. 1997), another potential cause of this variability is the presence of qualitative and quantitative differences in the toxin profiles of different species of *Dinophysis* at different locations and times (MacKenzie et al. 2005). To explain the wide variation in toxin content of the same species of *Dinophysis* collected at the same site, it has been suggested that *Dinophysis*-toxin precursors actually originate from other microorganisms (Imai & Nishitani 2000; Nishitani et al. 2002). However, the production of DTX-1 and PTX-2 by a clonal culture of *D. acuminata* isolated from Japan and fed with the ciliate *M. rubrum* was conclusively proven by liquid chromatography (LC)-mass spectrometry (MS) analysis (Kamiyama & Suzuki 2009). The studies on the toxin profiles of several *Dinophysis* species obtained from samples from Hokkaido, Japan, have indicated significantly higher cellular content of DTX-1 in *D. fortii* than in other *Dinophysis* species, suggesting that *D. fortii* is the species responsible for DSP toxin contamination in shellfish in Hokkaido (Miyazono et al. 2008; Suzuki et al. 2009; Nagai et al. 2011). Thus, establishing clonal strains is very important to understand not only feeding behaviors or growth characteristics but also toxin production in *Dinophysis.*

3.2. Establishment of *Dinophysis* Cultures

In *D. fortii*, forty-eight cultures from 60 single cell isolates were grown with the addition of the marine ciliate *M. rubrum* and the cryptophyte *T. amphioxeia* as prey species. They were successfully established as clonal strains and the success rate for isolation was 80.0% (48/60). The cultures reached maximum cell densities of 417-2,550 cells mL^{-1} (1,048 ± 99) (mean ± SD, n = 48) at the end of one-month incubation (Figure 5A). In contrast, reduced growth of *D. fortii* (max. of 3-4 divisions) and formation of small cells was observed in the absence of the ciliate or when provided only with *T. amphioxeia*, showing that *D. fortii* can not utilize *T. amphioxeia* as prey (Nagai et al. 2008). *D. acuminata* cultures were also successfully established as clonal strains and the success rate for isolation was 78.0% (47/60). The cultures reached maximum cell densities of 700-7,500 cells mL^{-1} (2,229 ± 1,549) at the

end of the one-month incubation (Figure 5B) (Nagai & Kamiyama 2016). Park et al. (2006), Nishitani et al. (2008a, b), Kamiyama et al. (2010), Nagai et al. (2008, 2011, 2013) and Mafra et al. (2016) showed that *D. acuminata*, *D. caudata*, *D. fortii*, *D. infundibulum*, *D.* cf. *ovum* and *D. tripos* required *M. rubrum* grown with *Teleaulax* as prey for their propagation. Although further examples are required, the evidence suggests *Dinophysis* species photosynthetic capacity dependence on the prey-predator interactions occurring among *Dinophysis*, *M. rubrum* and *Teleaulax/Geminigera*. In short, culture strains of *Dinophysis* species could potentially be established and maintained by feeding the dinoflagellates with ciliate prey grown with *Teleaulax/Plagioselmis/Geminigera*. Interestingly, in *D. fortii* sampled from the Okhotsk Sea (144° 20' E, 44° 03' N), the percentage of isolation success was 26/192 (13.5%) (Nagai et al. 2008) which was much lower than for the Hiroshima Bay population (48/60, 80.0%). Although most of the single cells isolated from the Okhotsk Sea grew once, the maintenance of the culture was unsuccessful due to poor growth after the *Dinophysis* cells were re-inoculated into *M. rubrum* culture. In addition, trials for the establishment of clonal cultures in *D. tripos* sampled from the Okhotsk Sea were unsuccessful, although a phylogenetic analysis inferred from plastid genome supported chloroplast origin from the cryptophytes *Teleaulax/Plagioselmis*/*Geminigera* clade (Koike et al. 2005; Takahashi et al. 2005). Thus, with regard to *D. fortii* or *D. tripos* in the Okhotsk Sea, the predator and prey cultures may be incompatible or the prey species may be slightly different from those found in the northern part of Japan.

3.3. Growth under Laboratory Conditions

To allow a preliminary investigation of the effect of nitrogen and phosphate levels on the growth of *D. fortii*, growth experiments were conducted under different nutrient conditions (Nagai et al. 2008). Cell numbers of *D. fortii* increased exponentially until Day 16 with a growth rate of 0.45 d^{-1} over the first 6 days (Figure 6A, Table 2) under nutrient conditions of 1/3 nitrate, phosphate and metals and 1/10 vitamins of f/2 medium. The initial abundance of *M. rubrum* was around 1,000 cells mL^{-1} and increased until reaching the peak of ca. 7,500 cells mL^{-1} on Day 10 (0.48 d^{-1} during Day 6-10), although *M. rubrum* did not grow smoothly during Day 2-6. After the peak, *M. rubrum* declined rapidly in cell number and disappeared completely on Day 16 with the increase in the abundance of *D. fortii* by feeding or by the death due to unknown reasons, as seen in Figure 6A. After the full consumption of *M. rubrum*, *D. fortii* slowly continued to increase in number until Day 20 and reached a maximum cell density of ca. 2,470 cells mL^{-1}. The cell number of *T. amphioxeia* increased exponentially until Day 6 with a growth rate of 1.78 d^{-1} over the 4 days after Day 2. After the peak, the abundance of *T. amphioxeia* decreased rapidly and the decrease continued until the end of incubation. As controls, *D. fortii* was incubated with or without the addition of *T. amphioxeia* at the initial concentrations of ca. 180 (*D. fortii*) and 1,000 or 0 (*T. amphioxeia*) cells mL^{-1} (Nagai et al. 2008). *M. rubrum* alone was also incubated at the initial concentration of ca. 1,000 cells mL^{-1}. Cell numbers of *D. fortii* declined slightly in both the presence and absence of *T. amphioxeia*, showing that *D. fortii* can not utilize *T. amphioxeia* as prey (Figure 6B). Most *D. fortii* cells survived during 41 days of starvation including 21 days of the pre-incubation period. On the other hand, the rapid growth of *T. amphioxeia* was observed until Day 6 (2.0 d^{-1}) and the number of cells declined gradually thereafter. In the control of *M.*

rubrum without the predator, they grew exponentially for the first 10 days, with a growth rate of 0.45 d^{-1} during Day 6-10, and a gradual reduction in the number of cells thereafter due to unknown reasons. Yet, ca. 670 cells mL^{-1} of *M. rubrum* still survived at the end of incubation (Figure 6C).

Similarly, growth experiments in *D. caudata* and *D. infundibulum* were carried out (Nishitani et al. 2008a, b). The number of cells of *D. caudata* increased exponentially until Day 10 with a growth rate of 1.03 d^{-1} during Days 2-5 (Figure 6D, Table 2). The initial abundance of *M. rubrum* was ca. 1,500 cells $well^{-1}$ (810 μL) and grew until reaching a peak of ca. 8,900 cells mL^{-1} on Day 4 (0.74 d^{-1}). After the peak, the number of cells of *M. rubrum* declined rapidly and disappeared by Day 8 due to active feeding by *D. caudata* and natural death. Even after the disappearance of *M. rubrum*, *D. caudata* continued to increase in number until Day 10 and reached a maximum cell density of ca. 5,200 ± 550 cells mL^{-1} (mean ± SD) (Nishitani et al. 2008b). In *D. infundibulum*, a clonal strain grew actively when *M. rubrum* cells were added as prey (Figure 6E). The number of *D. infundibulum* cells increased exponentially with a growth rate of 0.87 (Day 1-4, Table 2) d^{-1}. Even after the disappearance of *M. rubrum*, *D. infundibulum* continued to increase in number until Day 19-22, and the cell densities reached its maximum yields, 2.3×10^3 cells mL^{-1} (Figure 6E).

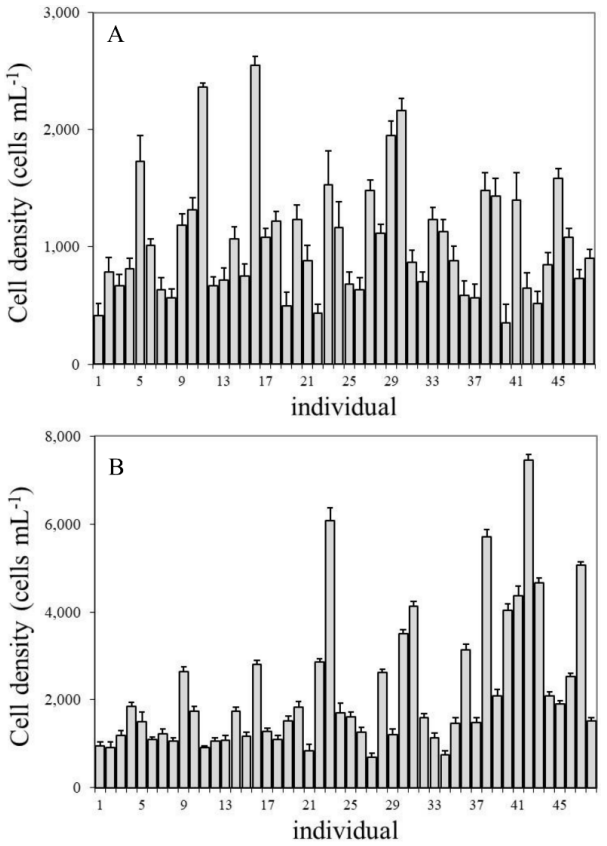

Figure 5. Maximum cell yields of *Dinophysis fortii* (A) and *Dinophysis acuminata* (B) isolated from single cells in Hiroshima Bay, Japan and established as clonal strains by feeding on the ciliate prey *Mesodinium rubrum* grown with the cryptophyte *Teleaulax amphioxeia*. A, Nagai et al. (2008); B, Nagai, and Kamiyama (2016).

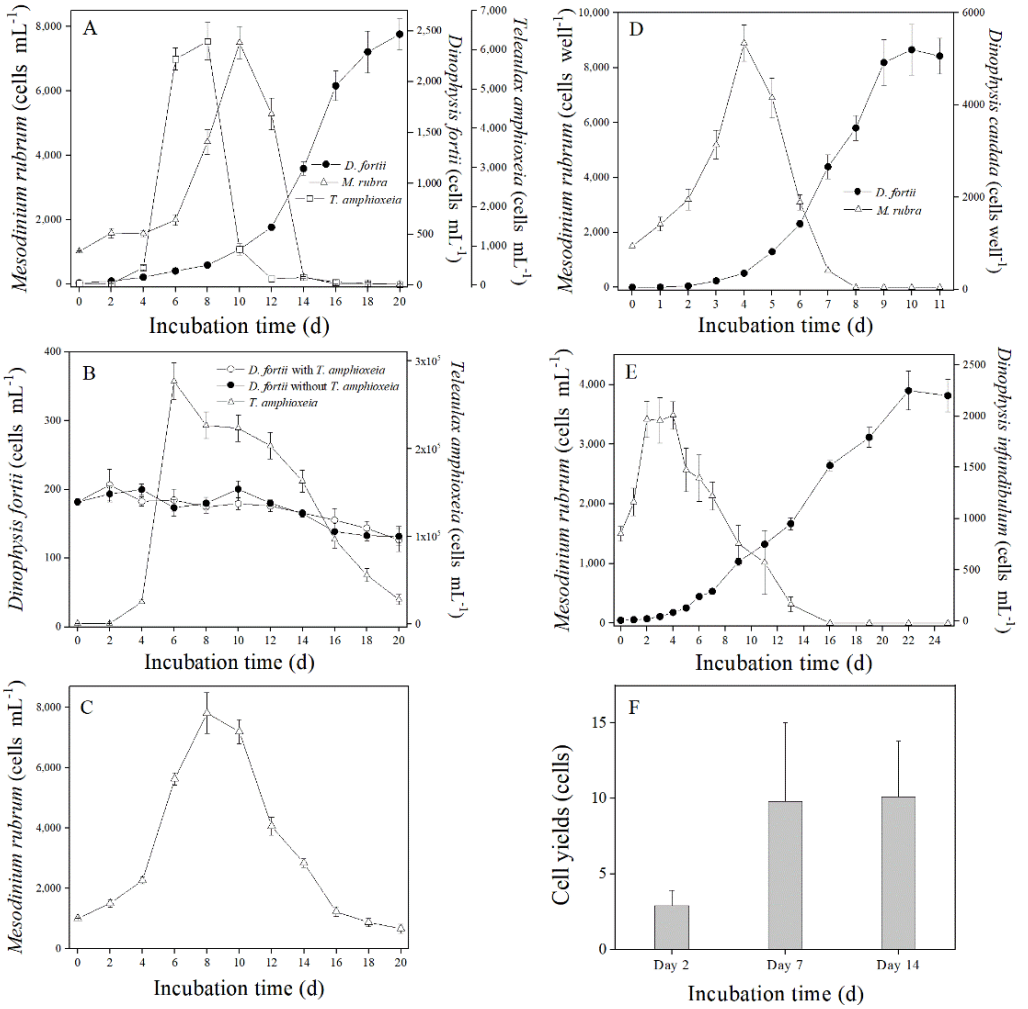

Figure 6. Growth experiments of *Dinophysis fortii*, *D. caudata*, and *D. infundibulum* fed on the ciliate prey *Mesodinium rubrum* grown with the cryptophyte *Teleaulax amphioxeia* (modified from Nagai et al. 2008 and Nishitani et al. 2008a, b). *D. fortii* was grown in 48 wells microplates under two different nutrient conditions; A) 1/3 nitrate, phosphate and metals and 1/10 vitamins in f/2 medium. Initial concentrations of *D. fortii*, *M. rubrum* and *T. amphioxeia* were 10-20, 1.0 x 10^3 and 50 cells mL^{-1} respectively; B) As controls, *D. fortii* was grown with/without *T. amphioxeia* in 48 wells microplates. Initial concentrations of *D. fortii* and *T. amphioxeia* were 180 and 1,000 or 0 cells mL^{-1} respectively; C) As a control, *M. rubrum* was grown alone without the predator *D. fortii* and the prey *T. amphioxeia*. An initial concentration of *M. rubrum* was 1,000 cells mL^{-1}. Averages of triplicate counts and the standard deviation were plotted; D) Growth of *D. caudata* given 5,000 cells $well^{-1}$ (48 well microplate) of *M. rubrum* as the initial concentration, E) Growth of *D. infundibulum* given 1,500 cells mL^{-1} of *M. rubrum* as the initial concentration.

Growth rates of several *Dinophysis* species, estimated under *in situ* and laboratory conditions, are summarized in Table 2; rates varied from 0.13 to 1.37 d^{-1}, due to the different methods and conditions used for growth rate estimation. For example, growth rates of *D. caudata* grown with *M. rubrum* (Nishitani et al. 2008b; Basti et al. 2015) were very high in comparison with previous reports for *D. caudata*, which were estimated from field

observations (Reguera et al. 2003) and a cultivation trial under laboratory conditions (Nishitani et al. 2003). When *D. fortii* is grown in the absence of ciliate prey, after feeding heavily on *M. rubrum*, the growth only occurs during the first 7 days at a rate of 0.73 d^{-1} (Table 2, Figure 6F). High photosynthetic activities of several *Dinophysis* species were reported by Berland et al. (1994) and Granéli et al. (1995, 1997). Based on ^{14}C uptake during light and dark periods, the growth rates of *D. acuminata*, *D. norvegica* and *D. acuta* were 0.52-0.73, 0.26-0.91 and 0.50-0.59 d^{-1}, respectively. These experimental data suggest that they were able to grow for the first few days utilizing either the accumulated nutrients or the stolen chloroplasts from the ciliate prey ingested during previous incubations or in the field. The growth rates of *D. fortii* obtained in this study varied markedly among conditions (Nagai et al. 2008), including differences in the nutrient levels or predator-prey interactions (Nagai et al. 2008; Nishitani et al. 2008b). The maximum yields also depended on the clonal strain, presumably due to their differences in feeding activity (Figure 5).

Table 2. Comparison of the growth rate in several *Dinophysis* species estimated from natural and incubation conditions (modified from Nagai et al. 2008)

Species	Growth rate (as day^{-1})		Maximum yield cells mL^{-1}	Conditions for estimation	References
	m*[1]	m*[2]			
D. acuminata		1.37	2,500	culture[*]	Park et al. (2006)
D. acuminata		1.36	5,000	culture[*]	Kim et al. (2008)
D. acuminata		0.58-1.01	167	culture[*]	Kamiyama and Suzuki (2009)
D. acuminata		0.20-0.40	4,200	culture[*]	Kamiyama et al. (2010)
D. acuminata		0.70-0.94	11,000	culture[*]	Nagai et al. (2011)
D. acuminata		0.65	ca. 1,000	culture[*]	Riisgaard and Hansen (2009)
D. acuminata		0.61		culture	Sampayo (1993)
D. acuminata		0.52-0.73		culture[***]	Granéli et al. (1995)
D. acuminata	0.78-0.97			*in situ*	Chang and Carpenter (1991)
D. acuminata	0.13-0.40			*in situ*	Reguera et al. (2003)
D. acuta		0.50-0.59		culture[***]	Granéli et al. (1997)
D. acuta	0.48-0.94			*in situ*	Reguera et al. (2003)
D. caudata		0.93-1.03	5,200	culture[*]	Nishitani et al. (2008b)
D. caudata		0.68	7	culture[**]	Nishitani et al. (2008b)
D. caudata		0.22		culture	Nishitani et al. (2003)
D. caudata	0.35			*in situ*	Reguera et al. (2003)
D. fortii		0.41-0.85	2,500	culture[*]	Nagai et al. (2008)
D. fortii		0.73	10	culture[**]	Nagai et al. (2008)
D. fortii		0.58-0.70	2,760	culture[*]	Nagai et al. (2011)
D. infundibulum		0.29-0.59	2,300	culture[*]	Nishitani et al. (2008a)
D. cf. ovum		0.50	2,200	culture[*]	Mafra et al. (2016)
D. norvegica		0.26-0.91		culture[***]	Granéli et al. (1997)
D. tripos	0.72			*in situ*	Reguera et al. (2003)
D. tripos		0.54	2,190	culture[*]	Nagai et al. (2013)
D. sacculus	0.29-0.61			*in situ*	Garcés et al. (1997)

[*1], The values were estimated according to the model of Carpenter and Chang (1988). [*2], The values were estimated according to the model of Guillard (1973). [*], The ciliate *Mesodinium rubrum*, grown with the cryptophyte *Teleaulax* sp./*T. amphioxeia*, was added as prey. [**], *Dinophysis* grown in the absence of prey, after being fed heavily with *M. rubrum*; [***], The growth rate was estimated by a ^{14}C method.

3.4. The Feeding Behavior, Cell Division and Other Biological Observations in *Dinophysis*

Similar to the case of *D. acuminata* (Park et al. 2006), *D. fortii* (Figure 7 A-D), *D. caudata* (Figure 8 A, C), *D. infundibulum* (Figure 9 A, B), *D. tripos* (data not shown) and *D.* cf. *ovum* (data not shown) were able to feed on *M. rubrum* (Nagai et al. 2008, 2013; Nishitani et al. 2008a, b; Mafra et al. 2016). *Dinophysis* cells swam around the prey (Figures 5B, 2D) and inserted a peduncle, which extends from around the flagellar pore region, into the cell of *M. rubrum* (Figures 7A, 7C, 8A, 9B), as has been previously reported for *P. rotundatum* (Hansen 1991) and *D. acuminata* (Park et al. 2006). The peduncle of these three *Dinophysis* species was narrower and shorter than those described in *P. rotundatum* (Hansen 1991) and *D. acuminata* (Park et al. 2006) and its length and width was 15-20 μm and 2-3 μm, respectively (Figure 7A, 7C, 8A, 9B). No peduncles were visible externally to the cell of these *Dinophysis* species, indicating that the peduncle extended from the cell just before capturing the prey. Therefore, it was difficult to show the morphology of the peduncle and the transfer of cell content from the prey through the peduncle into the *Dinophysis* cell. Soon after *Dinophysis* inserted the peduncle into the cell of *M. rubrum* (Figure 7A, C), the ciliate became immobile and their cilia were shed from the cell within 1-5 minutes (Figure 7A-D, 9B), suggesting that *Dinophysis* injects some kind of toxin into the cell of the ciliate using the peduncle (Nagai et al. 2008). The captured prey was closely tethered around the flagellar pore region of *Dinophysis* cells (Figures 7A-D, 8A, 9B) and the cytoplasm of the prey was actively ingested through the peduncle. Transfer of small portions of cytoplasm into the food vacuole of *Dinophysis* cells was observed through the transparent peduncle (Figure 7A). *D. fortii* fed heavily on *M. rubrum* (Figure 7E) i.e., even when the cell was fully expanded by the active ingestion of prey, the active feeding behavior still continued (Figure 7A). *D. fortii* cells connecting with *M. rubrum* by the peduncle, lying on the bottom of the plates, were often observed during the ingestion of prey (Figure 7A). It took 45-90 min (n = 15) until the whole cell content of *M. rubrum* was consumed. Propagation of these *Dinophysis* species was observed by frequent vegetative cell divisions (Figures. 7F, 8B, 9C), and sequential binary fission was sometimes observed without the cell separation from the previous cell division (Figures 7G, 8C, 9D). These four cells were united by their cingular lists and were still able to swim actively. After the separation, each cell was able to feed on prey and grow normally. The tetrad state is also regarded as the rejuvenation process from a resting cyst reproduced through karyogamy (Escalera & Reguera 2008).

When exposed to high *Dinophysis* cell densities (> 500 cells mL^{-1}), *M. rubrum* cells tended to form clumps, became tangled with each other by their flagellae, swam helicoidally or rotated at the same position on the bottom of the microplate (Figures 7B, 8D, 8E), suggesting the release of an allelopathic chemical from *Dinophysis* cells. *Dinophysis* cells aggregated around these abnormally behaving *M. rubrum* and actively fed on them (Figures 7B, 8D). *M. rubrum* is an organism that can move rapidly; however, *Dinophysis* was able to capture the prey without any apparent difficulty. Therefore, it is reasonable to assume that *Dinophysis* species must have evolved a very effective capture mechanism. We observed that *M. rubrum* cilia were sometimes intertwined with the *Dinophysis* cell surface due to mucilaginous secretions on the dinoflagellate cell surface, suggesting that *Dinophysis* has various strategies to increase the chances of capturing the ciliate prey. Several mixotrophic

microalgae produce substances that deter grazing and/or inhibit the growth of competitors (allelochemicals) (Stoecker et al. 2006; Hansen, 2011), such that the additional role of these compounds in prey capture has been increasingly considered (e.g., Blossom et al. 2012).

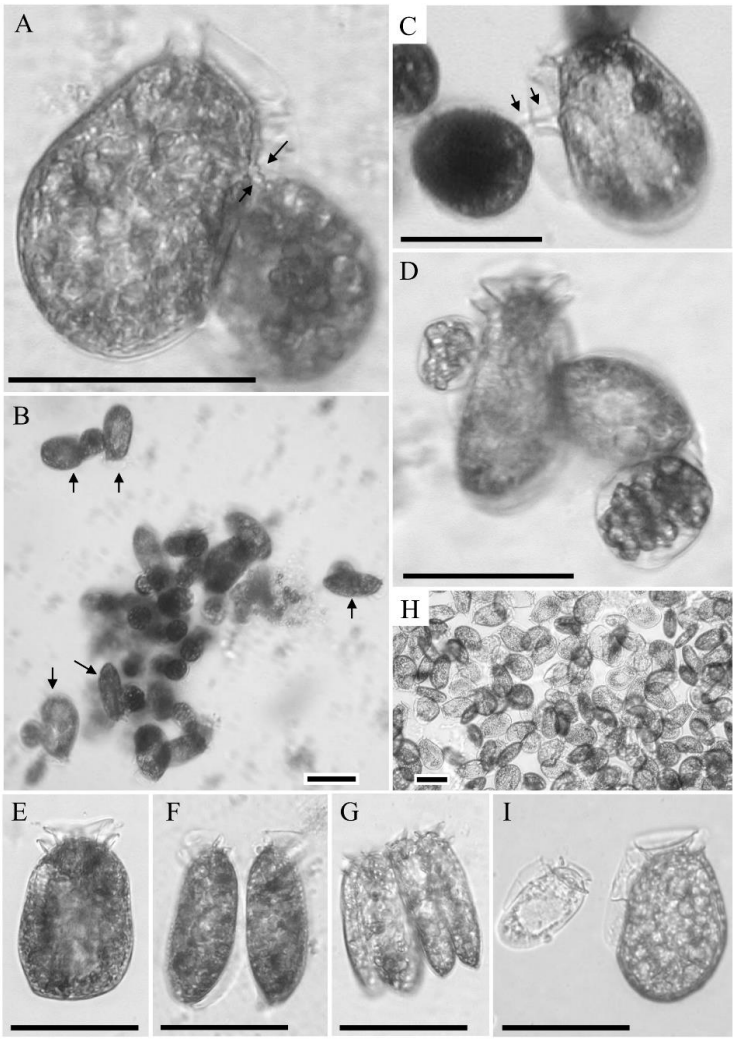

Figure 7. Observations of the feeding behavior and propagation in *Dinophysis fortii*, which fed on the ciliate prey *Mesodinium rubrum* using the peduncle (Nagai et al. 2008). (A) *D. fortii* cell actively ingesting the prey, which shows a round shape and loss of cilia. Transport of cell contents of *M. rubrum*, captured by the *Dinophysis*, is visible through the transparent peduncle (arrows). (B) Clumping of *M. rubrum* cells, which may be caused by the release of an allelopathic chemical from *D. fortii* cells. *D. fortii* aggregated, and actively fed on, these abnormally behaving *M. rubrum* (arrows). (C) *D. fortii* cell, which has just inserted the peduncle into an *M. rubrum* cell. Arrows indicate the peduncle. (D) *D. fortii* cells actively ingesting the prey, which shows a round shape and loss of cilia. (E) An expanded cell that had fed heavily on the ciliate prey (ventral view). (F) Vegetative cell division by binary fission. (G) Sequential binary fission is seen without the cell separation of the previous cell division. (H) Harvested cells after growth experiments, showing the successful cultivation. (I) A dwarf cell produced as the result of depauperating division (cell shape was similar to that of *D. acuminata*, left) and a normal size cell (right). Scale bar = 50 μm (all at the same magnification).

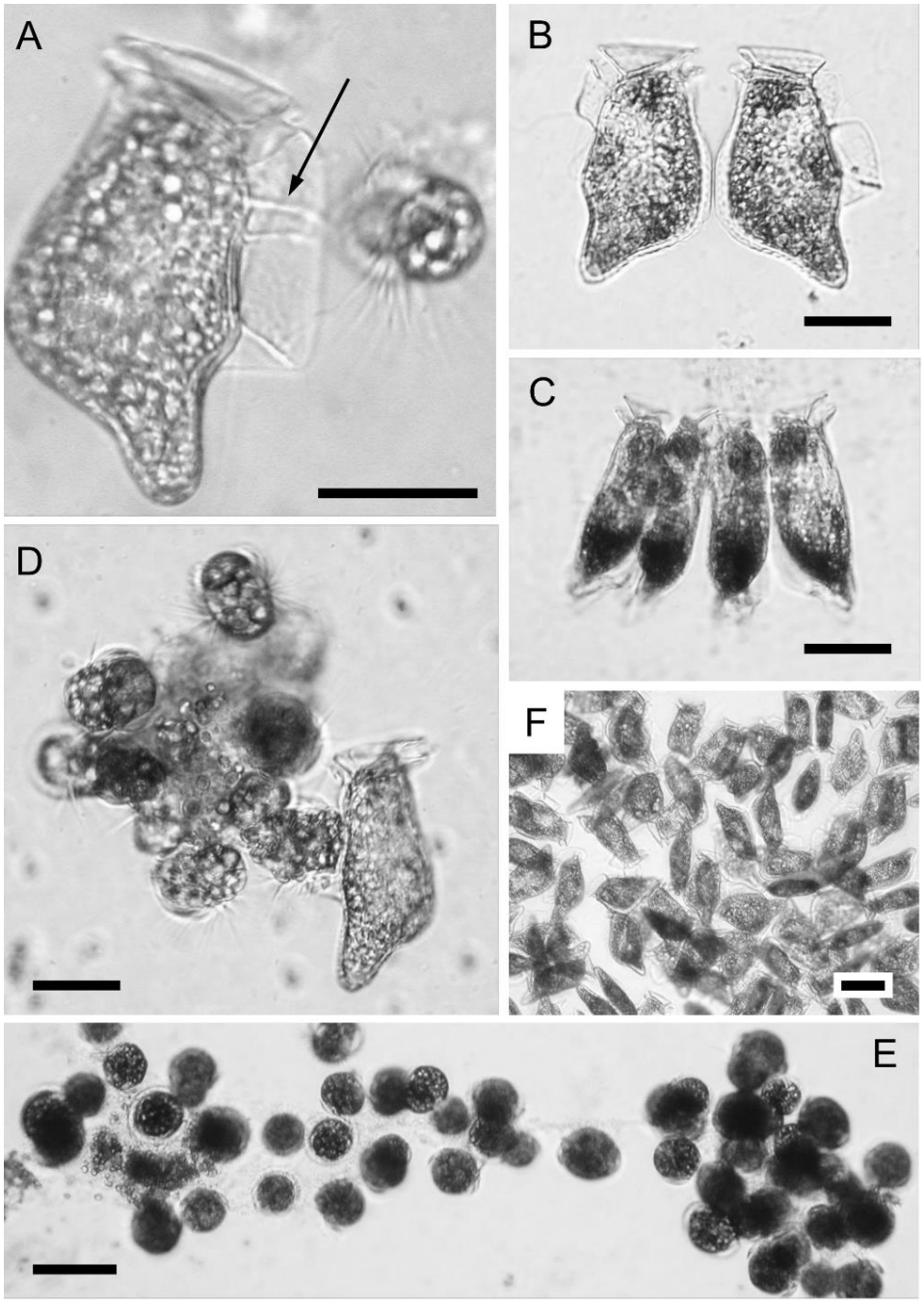

Figure 8. Observations of feeding behavior and propagation in *Dinophysis caudata* seen during the growth experiment (Nishitani et al. 2008b). A) A *D. caudata* cell actively ingesting prey, showing the round shape and loss of cilia in the prey; B) Vegetative cell division of *D. caudata* by binary fission; C) A sequential binary fission of *D. caudata* seen without cell separation from the previous cell division; D) Harvested cells of *D. caudata* after growth experiments, showing successful cultivation; E) Clumped *Mesodinium rubrum* cells, which may be caused by the release of some kind of allelopathic chemical from *D. caudata* cells; F) A *D. caudata* cell feeding on clumped *M. rubrum* cells. All scale bars = 30 μm.

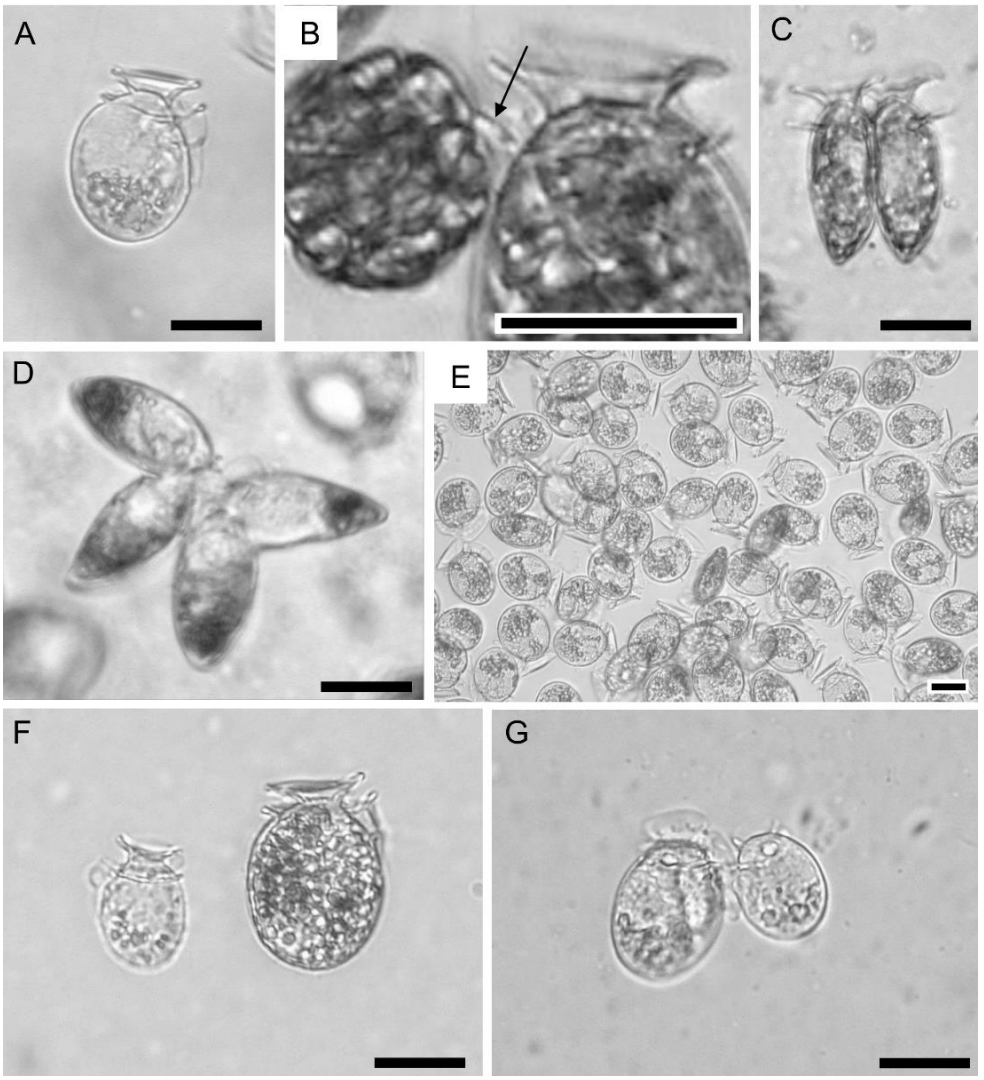

Figure 9. Observations of the feeding behavior and propagation in *Dinophysis infundibulum* (Nishitani et al. 2008a). Observations of feeding and propagation during maintenance culture or growth experiment. A) A natural cell collected from Hiroshima Bay, Japan; B) A cell actively ingesting prey, showing the round shape and loss of cilia in the prey. The arrow indicates the peduncle; C) Vegetative cell division by binary fission; D) Sequential binary fission observed without the separation of cells from the previous cell division; E) Harvested cells after growth experiments, showing their successful cultivation; F) A small cell (left) and a cell after feeding on *Mesodinium rubrum* (right); G) A couplet joined at the ventral side. All scale bars = 20 μm.

Toxins released by the haptophyte *Prymnesium parvum*, for instance, can immobilize their otherwise swimming microalgal prey, permitting their prompt capture (Skovgaard & Hansen, 2003; Tillmann, 2003). Free polyunsaturated fatty acids (PUFAs) produced by the dinoflagellate *Karenia mikimotoi* also exhibited allelopathic effects on the diatom *Chaetoceros gracile* (Arzul et al. 1995, 1998). In fact, the hemolytic/cytotoxic effects of several dinoflagellates and raphidophytes have been associated with high concentrations of free PUFAs (mainly eicosapentaenoic acid—EPA and docosahexaenoic acid—DHA),

especially in the presence of reactive oxygen species (ROS) (Marshall et al. 2003; Dorantes-Aranda et al. 2009; Mardones et al. 2015), suggesting that PUFA may also play a role in prey capture by mixotrophic microalgae. The release of a mucus trap, as reported for the dinoflagellate *Alexandrium pseudogonyaulax* (Blossom et al. 2012), enables the capture of fast swimming microalgal prey. In co-cultures of *Dinophysis* and its ciliate prey, cells of *D.* cf. *ovum* always succeeded in capturing their evasive prey, the jumping ciliate *M. rubrum*, even at a relatively low initial cell density of the predator (e.g., ~ 50 *Dinophysis* cells mL^{-1}) (Mafra et al. 2016) and despite the extremely high swimming velocity of up to 1.2 cm s^{-1} and initial acceleration of about 4 cm s^{-2} attained during *M. rubrum* jumps (Fenchel & Hansen, 2006). Hansen et al. (2013) advocated that *Dinophysis* cells would be able to blast an adhesive capture filament from a certain distance and then pull the tied prey closer to them. In addition, Nielsen & Kiørboe (2015) suggested that a reduced deformation in the flow fields around swimming *D. acuta* cells would allow them to intercept the ciliate without provoking its escape. Prey capture by *D. acuta*, *D. acuminata*, and *D.* cf. *ovum* cells has been witnessed (and documented) after *M. rubrum* had been caught in "mucus traps", eventually forming clumps of a few ciliate cells (Mafra et al. 2016; Ojamäe et al. 2016; Papiol, et al. 2016). After being attached to such adhesive, apparently filamentous/fibrous material, *M. rubrum* cilia were firmly tangled and the ciliate was not able to escape, even when only part of its cirri initially entangled within the mucus trap. At this moment, actively feeding *Dinophysis* cells would approach their prey, exhibiting an erratic, hunting swimming pattern, with their feeding peduncles already mounted. As hypothesized by Hansen et al. (2013), the capacity of *Dinophysis* to detect *M. rubrum* cells from a distance could be either due to chemical or hydromechanical sensing. Recently, the detection of *M. rubrum* by chemoreception and usage of mechanistic swimming features aiding to target resting *M. rubrum* cells by *D. acuminata* has been documented (Jiang, et al. 2018).

A large number of cells were harvested by sieving *Dinophysis* cultures with nylon mesh (10 μm, in pore diameter), showing the successful cultivation of these *Dinphysis* species (Figures. 7H, 8F, 9E). When establishing *D. fortii* clonal strains from a single cell, small and colorless cells became dominant in some isolates (Figure 7I) and we could not establish clonal strains in such cases. During the maintenance and growth experiments of *Dinophysis*, the formation of small cells was also sometimes observed, especially when cells entered their stationary phase (senescent culture) (Figures 7I-left, 9F-left, 9G-right, 11A-left). The shape of the smaller cells was similar to that of *D. acuminata* and was clearly different from the normal vegetative cells of *D. fortii* (Figure 7I-right) and *D. infundibulum* (Figure 9F-right), and thus may be a potential cause of species misidentification in natural samples. The appearances of small cells have also been reported in *D. acuta* (Reguera et al. 2004), *D. caudata* (Nishitani et al. 2003, 2008a; Reguera et al. 2007), *D. fortii* (Uchida et al. 1999), *D. pavillardi* (Giacobbe & Gangemi 1997) and *D. sacculus* (Delgado et al. 1996). Small cells are able to grow again to a large size in *D. acuminata* (Reguera & González-Gil 2001) and *D. fortii* (Nagai et al. 2008). The steps from gamete pairs, cell fusion, nuclear fusion and the fate of planozygotes were tracked in several *Dinophysis* species by Escalera & Reguera (2008) and others, especially cell fusion in *D. fortii* (Uchida et al. 1999; Koike et al. 2006), *D. infundibulum* (Figure 9. G), *D. pavillardi* (Giacobbe & Gangemi 1997), *D. acuminata*, *D.* cf. *ovum* and *D. acuta* (Escalera & Reguera 2008) and nuclear fusion in *D. fortii* (Koike et al. 2006) and *D.* cf. *ovum* (Escalera & Reguera 2008).

The general morphology of the cells, including a prominent nucleus, numerous chloroplasts with pyrenoids, several food vacuoles and the rhabdosomes (Vesk & Lucas 1986; Lucas & Vesk 1990) is shown in Figure 10. Three thylakoids were usually arranged parallel to the longitudinal axis of most chloroplasts observed in this study, but chloroplasts having two thylakoids were sometimes confirmed (Figure 10B, arrows). The pyrenoid apparently appeared as a homogeneous body with a diameter larger than 1 μm and was attached to one end of the chloroplast (Figure 10A-C). In *D. fortii* cells that heavily fed on the ciliate prey, an enormously developed food vacuole, with dimensions approximately the same as those of the nucleus, was observed (Figure 10A). Although the majority of food vacuoles were homogenous, membrane-like structures and/or mitochondria-like particles were also confirmed in some food vacuoles (Figure 10A, B, D), indicating active ingestion of the ciliate prey (Figure 10A, D). Large numbers of rhabdosomes were seen in all cells of *D. fortii* examined (data not shown) and they were mainly located in the cytoplasm (Figure 10C). Starch-like grains appeared either in the food vacuoles or in the cytoplasm (Figure 10A, D). Starved cells contained no food vacuoles, but a few numbers of small starch-like grains and a few small chloroplasts (0.5-2 μm) remained in the marginal regions. The number of chloroplasts in these starved cells was significantly decreased and, in particular, large chloroplasts were completely disappeared (Figure 11A-right). The disappearance of large chloroplasts was also seen in the small cells that were produced as a result of the depauperating division (Figure 11A-left). Interestingly, we observed typical dinoflagellate chromosome structures inside food vacuoles in a *D. fortii* cell, clearly showing their cannibal behavior (Figure 11B).

3.5. Observation of the Chloroplasts Sequestration Process by *Dinophysis*

TEM observation of *Dinophysis* cells that fed heavily on the ciliate prey revealed the majority of food vacuoles were homogenous, membrane-like structures and⁄or mitochondria-like particles, indicating active ingestion of the ciliate prey (Figure 12, A and D). Chloroplast-like particles, however, have not been confirmed in food vacuoles (Nagai et al. 2008), thus *Dinophysis* has a system to spare the stolen chloroplasts from digestion in food vacuoles for retaining those as kleptoplastids.

The sequestration process of the chloroplasts ingested from *M. rubrum* has been investigated in *D. fortii* (Nagai, et al. 2008) and *D. infundibulum* (Nishitani, et al. 2008a). After 40 days of starvation, chloroplasts in most *Dinophysis* cells were reduced in number and size, and the cells became colorless as shown in Figure 12A, B, Figure 13A, B. The color of chloroplasts, which had been kept by *Dinophysis* when the feeding started, was more orange than those ingested from *M. rubrum*, accordingly, they were distinguishable in most cases. After one minute, no ingestion of the chloroplasts by *Dinophysis* was started yet (Figure 12A, B; Figure 13A, B). After five minutes, ingestion of the chloroplasts via the peduncle had already started. Accumulation and retention of the ingested chloroplasts were seen in the center of the cell (Figure 12C, D; Figure 13C, D). After ten minutes, more than half of the chloroplasts were ingested and retained in the center of the cell (Figure 12E, F; Figure 13E, F). After 15 minutes, almost all the chloroplasts were ingested and the beginning of the dispersion of the chloroplasts was seen, while most other cell contents still remained in the *M. rubrum* cell (Figure 12G; H, Figure 13G, H). After 40 minutes, dispersion of the

chloroplasts throughout the cell was seen, but many more of them were located in the marginal region. By then, almost all cell contents of *M. rubrum* were ingested (Figure 12I, J; Figure 13I, J) (Nagai et al. 2008; Nishitani et al. 2008a).

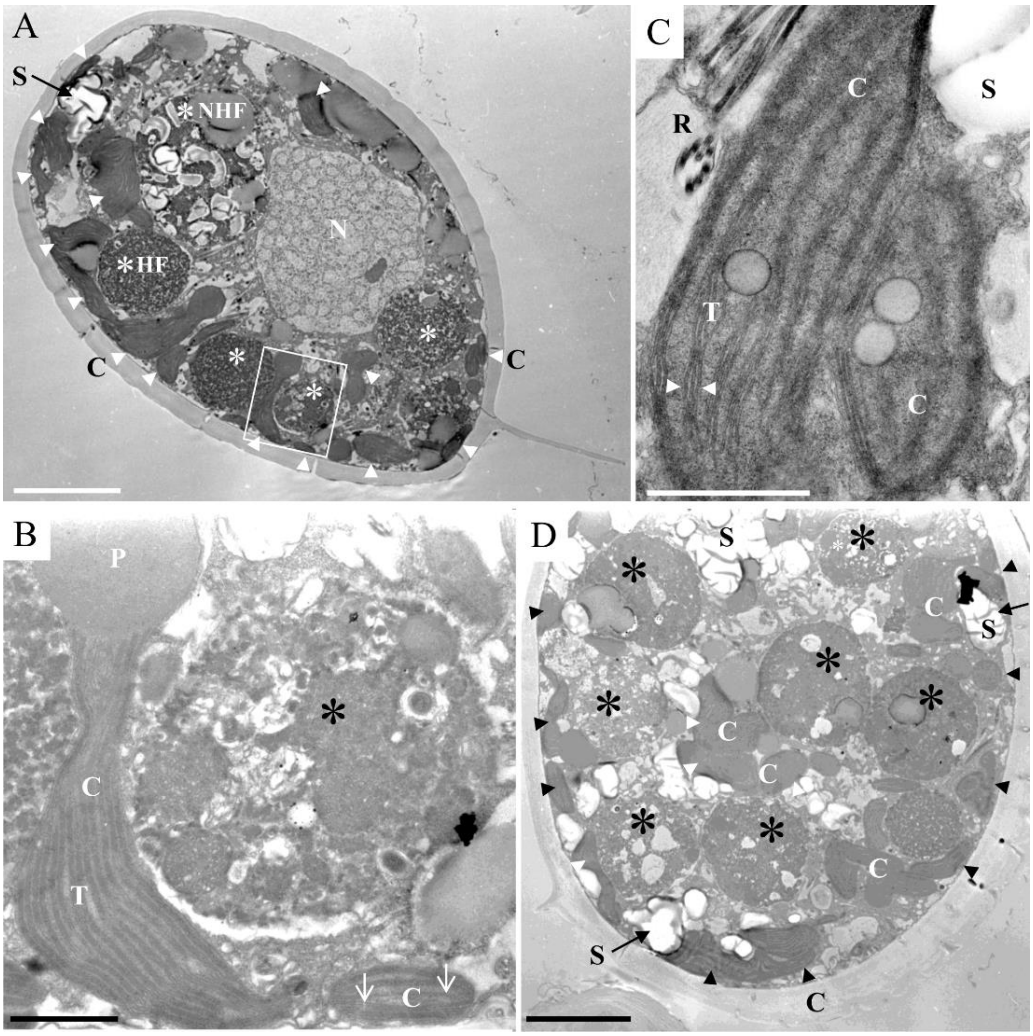

Figure 10. TEM observations of fully expanded *Dinophysis fortii* cells that had fed heavily on *Mesodinium rubrum* (Nagai et al. 2008). A, Lateral section of a cell showing a prominent nucleus, chloroplasts with pyrenoids (arrowheads), several food vacuoles, including homogeneous and non-homogeneous ones and starch-like grains. Scale bar = 10 µm; B, Enlargement of A, a homogenous/ round-shape pyrenoid and a chloroplast elongating vertically toward the cell wall and having three thylakoids oriented along the long axis of the organelle. A small chloroplast having a pair of thylakoids was also confirmed (arrows). Scale bar = 1 µm; C, a high magnification of chloroplasts clearly showing three thylakoids (arrowheads). Scale bar = 1 µm; D, another example of lateral section of a *D. fortii* cell fully expanded by feeding heavily on the ciliate prey. Numerous chloroplasts (arrowheads), food vacuoles and starch-like grains (arrows) are contained. Scale bar = 10 µm. Abbreviation; C, chloroplast; N, nucleus; P, pyrenoid; R, rhabdosome; S, starch-like grain; T, thylakoid; *, food vacuole (HF = homogeneous; NHF, non-homogeneous).

In the study by Nagai, et al. (2008), the ultrastructural observation by TEM showed several well-developed food vacuoles in *D. fortii* cells that had fully fed on the prey (Figure 10A), and membrane-like structures and/or mitochondria-like particles were confirmed in the vacuoles (Figure 10A, B, D). Chloroplast-like particles having thylakoid bands were, however, not contained in the vacuoles, although numerous sections of more than ten cells were observed. The epifluorescent microscopic observation of the chloroplast ingestion process showed that within 15 minutes after *D. fortii* captured *M. rubrum*, *D. fortii* had ingested almost all the chloroplasts and concentrated them in the center of their cells, while most of the other ciliate cell contents remained in the *M. rubrum* cell. After ingestion, the chloroplasts moved toward the marginal regions of the dinoflagellate cell (Figure 10, 12I), suggesting that chloroplasts are ingested from *M. rubrum* and dispersed in *D. fortii* cells in advance compared to the ingestion of other cell contents in order to spare the chloroplasts from digestion so that they utilized as kleptoplastids.

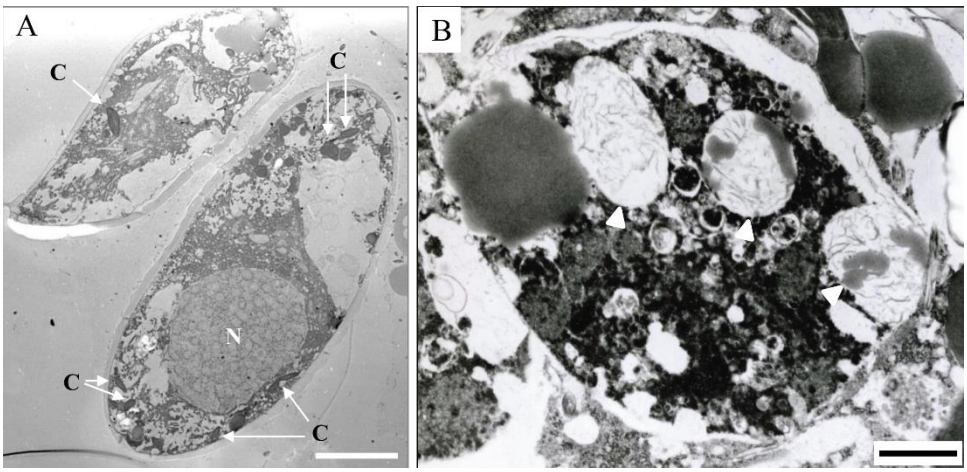

Figure 11. A TEM observation of *Dinophysis fortii* (modified from Nagai et al. 2008). *D. fortii* cells were incubated for 4 weeks under starvation conditions (after the growth experiment shown in Figure 6A). A); Longitudinal sections of a starved cell (right) and a small cell, produced as a result of depauperating division (left), showing no food vacuoles and only a few small chloroplasts (0.5-2 μm) in the marginal regions of the cell (arrows); B) A typical dinoflagellate chromosome structure observed in the food vacuoles in a *D. fortii* cell showing the cannibal behavior. Scale bar = 10 μm.

In both *D. acuminata* (Park et al. 2006) and *D. fortii* (Figure 10) chloroplast-like particles from *M. rubrum*, smaller than 5 μm in diameter, were ingested via peduncle. In contrast, much larger chloroplasts, > 10 μm in length, were observed in *D. fortii* using TEM observations (Figure 10A). These reported observations led us to question: Can *Dinophysis* control the size of stolen chloroplasts during retention? We observed the disappearance of relatively large chloroplasts (> 5 μm in length) from *D. fortii* after > 4 weeks of incubation without the ciliate prey, and only a few small chloroplasts (0.5-2 μm in length) remained in the marginal region of the cells, especially in small cells (Figure 11), although the dinoflagellates survived more than two months incubation without feeding on prey. For *D. caudata* decrease in plastid DNA $cell^{-1}$ d^{-1} closely followed culture growth rate (Pearson correlation, r = 0.91), indicating that existing plastids were diluted within the growing

population and that no new plastids were synthesized by the cells (Minnhagen et al. 2011). There are interesting studies in *D. acuminata* and *D. fortii* reporting that they harbor several genes encoding plastid-related proteins, which are thought to have originated from fucoxanthin dinoflagellates, haptophytes and cryptophytes via lateral gene transfer (LGT) (Wisecaver & Hackett 2010; Hongo et al. 2019). Expression of genes related to cryptophyte plastid function and maintenance is lower in *D. acuminata* than in completely phototrophic algae with permanent plastids (Wisecaver & Hackett 2010), suggesting that *D. acuminata* cannot retain kleptoplastids as the permanent plastids. In a recent study, however, 58 gene products involved in porphyrin, chlorophyll, isoprenoid, and carotenoid biosyntheses as well as in photosynthesis were identified in *D. fortii* (Hongo et al. 2019), showing the concrete evidence that *D. fortii* obtained these genes to utilize stolen plastids more efficiently in the evolutional process. One hypothesis is that *D. fortii* might ingest *M. rubrum* as merely a heterotrophic nutrient source and utilize the stolen chloroplasts for an ancillary function because the growth of *D. fortii* was observed only during the first 7 days after the removal of prey (Nagai et al. 2008). In our observations of *D. fortii* cells that were incubated without the prey ciliate for ca. two months still retain a few small chloroplasts, as shown in Figure 11A (by arrows). Are they permanent chloroplasts or retained kleptoplastids? Gene sequences of *Dinophysis* plastids showed striking similarities with those of the cryptophyte *Teleaulax*/*Geminigera* (Janson 2004, Minnhagen and Janson 2006), implying that more variable regions in the plastid genome may need to be analyzed to definitively answer this question. In a recent examination of *M. rubrum* based on ultrastructure by TEM and on the dynamics of photosynthesis, ingestion and growth rates in laboratory conditions, Hansen & Fenchel (2006) presented evidence that *M. rubrum* harbors a permanent cryptophyte endosymbiont that has different and larger chloroplasts than the *Teleaulax* added as prey. Three thylakoids were found in most of the chloroplasts of *D. fortii* cultured in this study, with a few exceptions (Figure 10B, C). This suggests that the chloroplasts observed in *D. fortii* originate from the permanent symbiont of *M. rubrum* because three thylakoid bands were clearly shown in TEM observation of chloroplasts in *M. rubrum* by Hibberd (1977) and Lindholm et al. (1988). In addition, *T. amphioxeia*, which we added to *M. rubrum* as prey, was observed to have pairs of thylakoid bands (Nagai et al. 2008). However, most of the TEM observations of naturally occurring photosynthetic *Dinophysis* species showed two thylakoid bands (Hallegraeff & Lucas 1988, Schnepf and Elbrächter 1988, Lucas & Vesk 1990, Jacobson & Andersen 1994, Carpenter et al. 1995, Vesk et al. 1996), except for one natural sample of *Dinophysis* sp., showing three thylakoid bands (Clement et al. 1988). In our TEM observations, we used *D. fortii* fed with *M. rubrum* that was pre-incubated for three weeks without addition of the cryptophyte prey. Thus, the difference in number of thylakoids observed in *Dinophysis* cells might be caused by the difference in abundance of the permanent cryptophyte symbiont and the cryptophyte prey inside the cells of *M. rubrum*. Otherwise, these contradictory results may show the instability of thylakoid bands formation. To trace the cryptophyte chloroplasts in cells of *Dinophysis* via *M. rubrum*, development of new technology such as chloroplast transformation with DNA encoding Green Fluorescent Protein (GFP) may be needed.

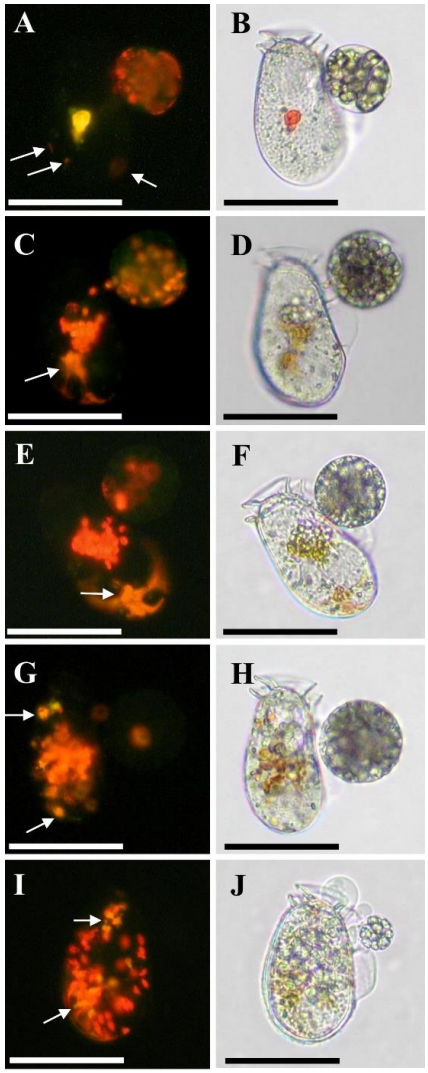

Figure 12. Observation of the sequestration process of the chloroplasts ingested from *Mesodinium rubrum* by *Dinophysis fortii* (Nagai et al. 2008). A and B, micrographs at one minute after the *D. fortii* cell captured *M. rubrum* showing that no ingestion of the chloroplasts by *D. fortii* had started. A, observation by an inverted epifluorescence microscope (EFM); B, observation by an inverted normal light microscope (LM). C (EFM) and D (LM), micrographs at five minutes after the *D. fortii* cell captured *M. rubrum*. Ingestion of the chloroplasts through the peduncle had already started. Accumulation and concentration of the ingested chloroplasts were seen in the center of the cell. E (EFM) and F (LM), micrographs at ten minutes after the *D. fortii* cell captured *M. rubrum*. More than half of the chloroplasts were ingested and retained in the center of the cell. G (EFM) and H (LM), micrographs at fifteen minutes after the *D. fortii* cell captured *M. rubrum*. Almost all of the chloroplasts were ingested and the beginning of the dispersion of the chloroplasts was observed, while most of the other cell contents still remained in the *M. rubrum* cell. I (EFM) and J (LM), micrographs at forty minutes after the *D. fortii* cell captured *M. rubrum*. Dispersion of the chloroplasts throughout the cell was observed, but many more of them were located in the marginal region. Most of the other cell contents were ingested. White arrows show chloroplasts, which had been retained by *D. fortii*, when the feeding experiment started. This is not a sequential series of observations and the cells shown in A, C, E, G and I are not the same. Scale bar = 50 μm (All at the same magnification).

4. THE SIMPLE FOOD CHAIN OF CRYPTOPHYTE/ *MESODINIUM*/*DINOPHYSIS*

It has become clear that plastid sequences of the 16S rRNA gene and *psbA* (encoding the photosystem II [PSII] reaction center protein D1) in natural *Dinophysis* cells (except *Dinophysis mitra*) (Koike et al. 2005) originate from cryptophyte *Teleaulax*/*Geminigera*/*Plagioselmis* clade (Janson 2004; Minnhagen & Johnson 2006; Takahashi et al. 2005; Takishita et al. 2002). Minnhagen & Janson (2006) also reported an exception to this finding in natural *D. acuminata* cells, isolated from the Greenland Sea, containing a plastid 16S rRNA gene sequence that was more closely related to *Geminigera cryophila*. These characteristics are probably caused by the cryptophyte prey preference of *M. rubrum* in natural environments. Under laboratory conditions, *M. rubrum* can be cultivated by providing the cryptophyte *T. amphioxeia* (Nagai et al. 2008, 2011, 2013; Nishitani et al. 2008a, b), *Teleaulax acuta* (Johnson & Stoecker 2005), or *G. cryophila* (Johnson et al. 2006). The first report of its prey preference in natural environments originated from Japanese coastal waters (Nishitani et al. 2010), motivated by unsuccessful culturing of some species, e.g., *D. norvegica* and the low percentages of the isolation success seen in *D. fortii* or *D. tripos* in the Okhotsk Sea (Nagai et al. 2008, 2013). The prey organisms of *M. rubrum* were determined by sequencing the cryptophyte nucleomorph 18S rRNA gene in natural *M. rubrum* cells; this genetic region has high substitution rates and facilitates species identification (Hoef-Emden et al. 2002). The origins of plastids in *Dinophysis* spp. were revealed by sequencing the plastid 16S rRNA gene and *psbA* in natural *Dinophysis* cells.

A total of 715 nucleomorph sequences from 114 PCR products of 134 *M. rubrum* cells were determined. Of these, 713 sequences were identical to the sequence of *T. amphioxeia* (99.7%), while the other 2 sequences (representing 0.3%), which were detected from the Harima-Nada station *M. rubrum* cells (cell identifier [ID] HAR12 and HAR76), were closely related to those of *G. cryophila* and *T. acuta* (Table 3 and Figure 14). These two *M. rubrum* cells contained two different cryptophyte sequences in each cell; in each of these cells, the sequences detected occurred in the proportion of seven sequences of *T. amphioxeia* to one other sequence. Knowledge of the availability of suitable prey for *M. rubrum* is very limited, but up to now, *M. rubrum* has been successfully cultured using *P. prolonga* (Hernández-Urcera, et al. 2018), *T. amphioxeia* (Nagai et al. 2008, Nishitani et al. 2008a, b), *T. acuta* (Johnson & Stoecker 2005), and *G. cryophila* (Johnson et al. 2006) as prey. In addition, Park et al. (2007) have isolated several *T. amphioxeia* like cryptophyte species inferred from the nuclear 18S rRNA gene phylogeny and have cultured *M. rubrum* by addition of those cryptophytes as prey. Considering these facts, the genera *Teleaulax* and *Geminigera* seem to be preferred prey for *M. rubrum.* In the study, however, the nucleomorph sequences of *T. acuta* and *G. cryophila* were never detected from natural cells of *M. rubrum*, suggesting that the two species are unlikely to become the prey of *M. rubrum*, at least in Japanese coastal waters. Accordingly, this study found that *T. amphioxeia* is probably the most important species for *M. rubrum* as a prey source (but see Rial, et al. 2015).

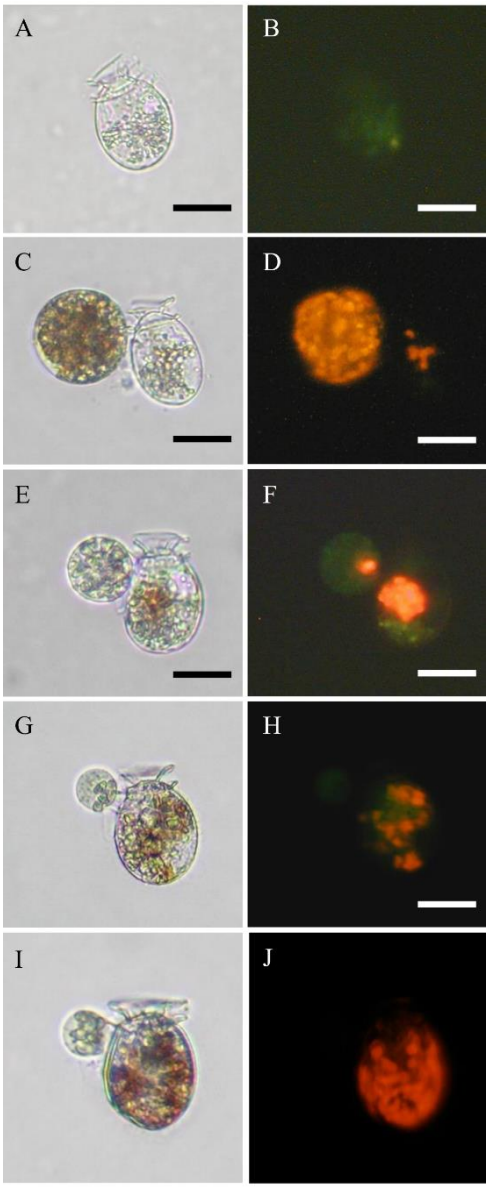

Figure 13. *Dinophysis infundibulum* (Nishitani et al. 2008a). Observations of the sequestration process of the chloroplasts ingested from *Mesodinium rubrum*. The left- and right-hand columns show micrographs by an inverted normal light microscope and an inverted epifluorescence microscope, respectively. This is not a series of sequential observations, and the *D. infundibulum* cells shown in the left-hand micrographs are not the same cell. (A, B) A cell cultivated for 51 days without the addition of *M. rubrum* and *Teleaulax amphioxeia*. (C, D) 2 min after the cell captured *M. rubrum*. Ingestion of the chloroplasts through the peduncle had not started. (E, F) 15 min after the cell captured *M. rubrum*. Most of the chloroplasts were ingested. Accumulation and concentration of the ingested chloroplasts were observed in the center of the cell. (G, H) 30 min after the cell captured *M. rubrum*. Apparently, all the chloroplasts were ingested, and the beginning of the dispersion of the chloroplasts was observed. (I, J) The *D. infundibulum* cell 2 days after *M. rubrum* was provided; the cell has certainly ingested some ciliates and shows accumulation of the ingested chloroplasts, possibly functioning as kleptoplastids. All scale bars = 20 μm (all at the same magnification).

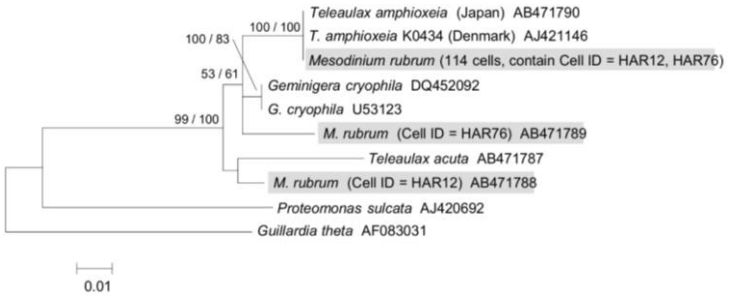

Figure 14. A maximum-likelihood tree inferred from the cryptophyte nucleomorph 18S rRNA gene (Nishitani et al. 2008a). Cryptophyte sequences detected from natural cells of *M. rubrum* are indicated by gray boxes. Bootstrap support values (left) and posterior probabilities (right) are provided. For further information on Cell ID, refer to Nishitani et al. (2010).

Plastid genes in natural *Dinophysis* cells were analyzed and 564 sequences of 16S from the successfully amplified PCR products (n = 68) were determined. Of these, 527 sequences were identical to the sequence of *T. amphioxeia* (93.4%). No variation among the sequences was confirmed (Figure 15), although Hackett et al. (2003) previously detected several variations (less than 16 bp out of ca. 1,200 bp) in the plastid 16S rRNA gene sequences in *Dinophysis* spp. isolated from the East Coast of North America. The remaining 37 sequences obtained in this study (5.6%), which were all identical and were detected only from the Funka Bay sample (*D. acuminata*, cell ID FUN03; *D. norvegica*, cell ID FUN07), showed a close phylogenetic affiliation and a divergence of 0.7% and 0.9% compared with sequences of *G. cryophila* and *T. acuta*, respectively (Table 3 and Figure 15). These *Dinophysis* cells contained two different cryptophyte sequences in a single cell. Four sequences of *T. amphioxeia* and 31 other sequences were detected from the *D. acuminata* cell (cell ID FUN03), and two sequences of *T. amphioxeia* and six other sequences were detected from the *D. norvegica* cell (cell ID FUN07). Interestingly, the other sequence was identical to that of a *D. acuminata* cell isolated from the Greenland Sea (Minnhagen & Janson 2006) although the cryptophyte species was not identified.

The result of the plastid *psbA* analysis was similar to that of the plastid 16S rRNA gene (Table 3). The partial plastid *psbA* sequences determined in this study were 890 bp, and 355 *psbA* sequences were determined from the successfully amplified PCR products. Of these, 352 sequences were identical to the sequence of *T. amphioxeia* (99.4%), and no variation was observed. As in the analysis of the plastid 16S rRNA gene, another three sequences, which were all identical and detected only from the Funka Bay sample (*D. acuminata*, cell ID FUN03; *D. infundibulum*, cell ID FUN04) (0.6%), showed a close phylogenetic affiliation and a 2.6% divergence from that of *G. cryophila* (Table 3 and Figure 16). Six sequences of *T. amphioxeia* and one other sequence were detected from the *D. acuminata* cell (cell ID FUN03), and five sequences of *T. amphioxeia* and two other sequences were detected from the *D. infundibulum* cell (cell ID FUN04). The high congruence of *M. rubrum* prey (cryptophyte *T. amphioxeia*) and *Dinophysis* plastid identities obtained in Japanese coastal waters are all identical with those collected from the Baltic Sea and the North Sea (Janson & Granéli 2003; Minnhagen & Johnson 2006), the North Atlantic Ocean (Janson & Granéli 2003), a Norwegian fjord (Minnhagen & Johnson 2006), and the North American East Coast (Hackett et al. 2003), showing the simple food web among *Teleaulax*/*Mesodinium*/*Dinophysis* species.

Table 3. Summary of the sequence analyses of the cryptophyte nucleomorph in natural *Mesodinium rubrum* cells and cryptophyte plastid in natural *Dinophysis* cells detected by single-cell PCR (modified from Nishitani et al. 2010)

Species	Site	Isolated cell number	nucleomorph			p16S			*psb*A		
			PCR success[*1]	*T. amp.*[*2]	others[*3]	PCR success[*1]	*T. amp.*[*2]	others[*3]	PCR success[*1]	*T. amp.*[*2]	others[*3]
M. rubrum	MUT (Mutsu Bay)	3	1 (33 %)	45	0	-	-	-	-	-	-
M. rubrum	HAR (Harima-Nada)	95	95 (100 %)	604	2	-	-	-	-	-	-
M. rubrum	INO (Inokushi Bay)	20	11 (55 %)	37	0	-	-	-	-	-	-
M. rubrum	YAT (Yatsushiro Sea)	16	7 (44 %)	27	0	-	-	-	-	-	-
D. fortii	NOT (Notoro lake)	5	-	-	-	5 (100 %)	75	0	5 (100 %)	88	0
D. acuminata	FUN (Funka Bay)	8	-	-	-	8 (100 %)	64	31	8 (100 %)	38	1
D. infundibulum	〃	8	-	-	-	7 (88 %)	43	0	8 (100 %)	45	2
D. norvegica	〃	10	-	-	-	10 (100 %)	75	6	10 (100 %)	36	0
D. tripos	〃	5	-	-	-	5 (100 %)	26	0	5 (100 %)	18	0
D. fortii	HIR (Hiroshima Bay)	20	-	-	-	19 (95 %)	135	0	17 (85 %)	53	0
D. acuminata	YAT (Yatsushiro Sea)	5	-	-	-	5 (100 %)	39	0	5 (100 %)	25	0
D. caudata	〃	5	-	-	-	5 (100 %)	38	0	5 (100 %)	26	0
D. fortii	〃	5	-	-	-	4 (80 %)	32	0	4 (80 %)	23	0
	total	M:134, D:71	114 (85 %)	713	2	68 (96 %)	527	37	67 (94 %)	352	3

[*1] Successfully PCR amplified cell number and (%).

[*2] Number of the sequence identical to that of *Teleaulax amphioxeia*.

[*3] Number of sequences differ from *T. amphioxeia*.

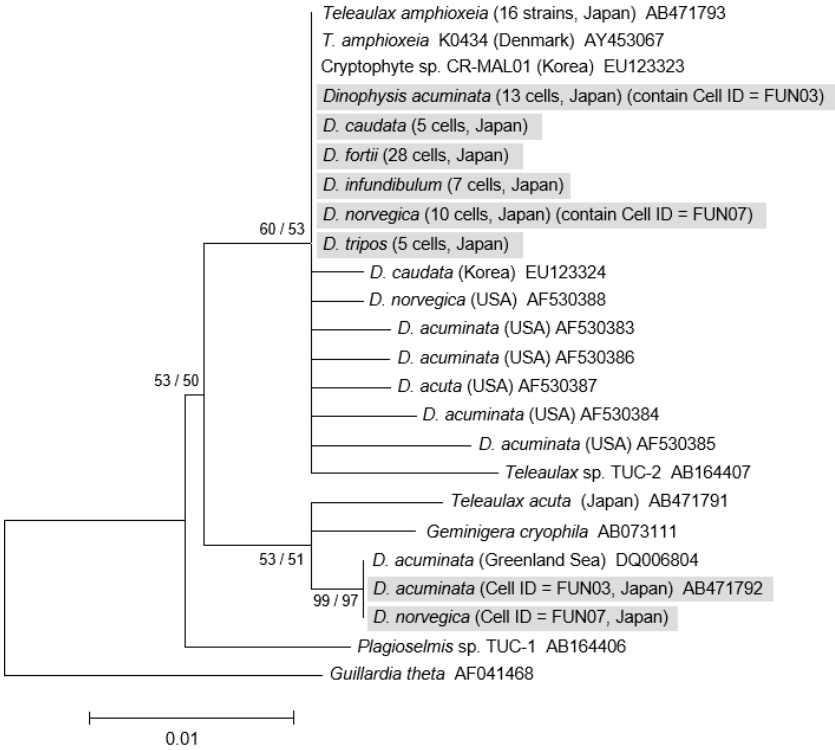

Figure 15. A maximum-likelihood tree inferred from the chloroplast 16S rRNA gene (Nishitani et al. 2008a). Cryptophyte sequences detected from the natural cells of *Dinophysis* are indicated by gray boxes. Bootstrap support values (left) and posterior probabilities (right) are provided.

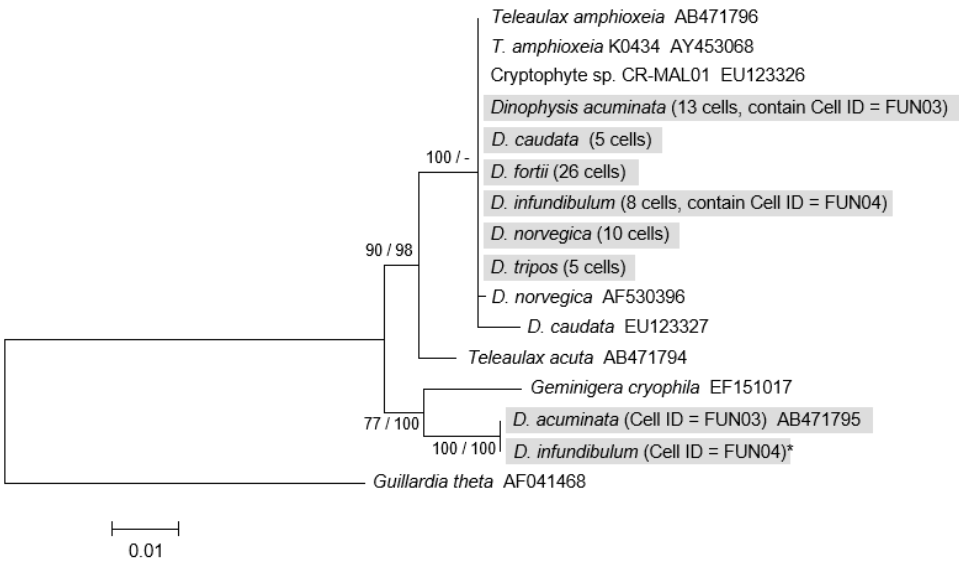

Figure 16. A maximum-likelihood tree inferred from the chloroplast *psbA* (Nishitani et al. 2008a). Cryptophytes sequences detected from the natural cells of *Dinophysis* are indicated by gray boxes. Bootstrap support values (left) and posterior probabilities (right) are provided.

Species belonging to the genus *Mesodinium* are among the most widely distributed and abundant marine ciliates in the coastal and estuarine ecosystems (Leppanen & Bruun 1986; Sanders 1995; Bell & Laybourn-Parry 1999). Phylogenetic analysis of rRNA genes (ITS region) has demonstrated that *M. rubrum* is actually a species complex, composed of at least six major clades (Herfort et al. 2011; Garcia-Cuetos et al. 2012; Johnson et al. 2016). One of these clades was described as a new species, *M. major*, based on molecular and ultrastructural characteristics, and is larger and has more plastids than *M. rubrum* (Garcia-Cuetos et al. 2012). The growth of *Dinophysis acuminata* and *D. acuta* fed on two *M. rubrum* strains isolated from different geographic origins, i.e., Spain and Denmark, was significantly different (García-Portela et al. 2018). This suggests that local *Mesodinium* populations may have different nutritional value, which may affect the metabolic functions for the growth of *Dinophysis*, causing differences in the success rate of establishing *Dinophysis* cultures. Otherwise, the difference may simply be caused by food preferences of *Dinophysis* that consume different *Mesodinium* species. Nishitani et al. (2018) developed a molecular technique to identify prey species consumed by *Dinophysis*, but it was applied only to *Phalacroma mitra* (previously named *Dinophysis mitra*). This method revealed the presence of several other prey species (several ciliate species, cryptophytes, dinoflagellate, and radiolaria) in addition to *M. rubrum*. This method may solve the mystery of *Dinophysis* food webs and allow a deeper understanding of *Dinophysis* ecology.

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Chapter 141

CARBON ASSIMILATION: DINOFLAGELLATES

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ABSTRACT

Nearly 50% of the estimated 2377 dinoflagellate species are photosynthetic while a few are mixotrophic; these make an important contribution to the global biological carbon pump. We reviewed the involvement of pigments in the photosynthetic carbon assimilation and photoprotection in the athecate, thecate and symbiotic groups of dinoflagellates. We collated the much scattered and limited data on carbon assimilation in dinoflagellates. A variety of units for photosynthesis have been reported based on biomass expressed as cell numbers, chlorophyll *a*, cell carbon and cell volume which makes intercomparison of results difficult. The impacts of temperature, irradiance, nutrients in the carbon assimilation are discussed. Intra and inter specific variations in carbon assimilation are observed. Carbon assimilation was higher in heterotrophic dinoflagellates compared to autotrophic cultures. Mixotrophy provides a competitive advantage for long-term survival. Photosynthetic rates in dinoflagellate groups are low, even lower than the senescent cells of diatoms and microflagellates. Perhaps in the dinoflagellates, the channeling of energy for toxin production together with their need to support a disproportionately large DNA requires a heavy metabolic requirement and may explain their lower carbon assimilation rates.

Keywords: photosynthesis, carbon assimilation, autotrophs, mixotrophs, comparison

INTRODUCTION

Dinoflagellates live in marine and freshwater habitats. Of the estimated 2377 dinoflagellate species, 1555 are described species of which 220 are from freshwater [1].

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Nutritionally the dinoflagellates present a great diversity including photosynthesis, heterotrophy, phagotrophy, mixotrophy, parasitism and symbiosis. Nearly 50% of the dinoflagellates are photosynthetic [2] and make an important contribution to the global biological carbon pump [3]. Some species cause red tides which are ephemeral but contribute to algal biomass, for example off Kuwait (~2 billion cells l^{-1} and ~4525 Chl *a* l^{-1} [4]. Winter blooms of *Heterocapsa triquetra* in Patagonian fjord ecosystem contribute 0.6 -1.6 gCm^{-2} d^{-1} to gross primary production. In the West Florida Shelf, blooms last one month and photosynthetic production during the blooms is higher than that during non-bloom conditions; on the average blooms contribute to 40% of the annual non-bloom production [5]. Recurrent seasonal occurrence as in "Florida red tide" caused by extensive blooms of *Karenia*, or *Alexandrium* along the northeastern U.S. coastline [6, 7] suggest their potential impact on the carbon footprint in the marine environment.

Nearly 75-80% of toxigenic red-tide algae are dinoflagellates [8], produce phycotoxins and impact the commercially important fisheries and human health. The saxitoxin produced by *Alexandrium* is 1000 times more potent than cyanide [9]. Red tides being ephemeral, studies are carried out in a sense of urgency, and are limited in duration. Such studies are focused more on the qualitative and quantitative abundance of the organisms, their origin, growth, distribution and production of phycotoxins rather than their ecophysiology. Difficulty in establishing dinoflagellate cultures stymied their ecophysiological studies. In contrast, more comprehensive studies have been carried out on the benign microalgae such as the diatoms and microflagellates because of their utility in aquaculture and their amenability for culturing. This explains why more "Cinderellas" or "weed species" are present amongst diatoms (*Thalassiosira, Nitzschia, Cylindrotheca, and Phaeodactylum*) and microflagellates (*Dunaliella, Monochrysis*) than the dinoflagellates.

In this essay we collated the much scattered and limited data on carbon assimilation in dinoflagellates. We reviewed the involvement of pigments in the photosynthetic carbon assimilation and photoprotection in the athecate, thecate and symbiotic groups of dinoflagellates. Photosynthetic rates in dinoflagellate groups are low compared to other planktonic microalgae. The need to emphasize systematic concerted studies on the dinoflagellate photophysiology is pointed out.

GENERAL METHODOLOGY

Usually red tides are constituted by a few species; these natural assemblages are fractionated using suitable sieves [10-12] and used for biomass and carbon assimilation determinations [13]. Cultured cells of dinoflagellates are used to express their biomass as cell abundance, chlorophyll and carbon assimilation rates. Symbiotic dinoflagellates from anthozoans are studied on isolates either by stripping coral tissues using Water-Pik method [14], manual fractionation [15] or treatment with NaOH solution [16].

The process of carbon assimilation in dinoflagellates is similar to that carried out by other microalgae, and is dependent on chlorophyll *a*, carbon dioxide, and light energy. Details on quantification of natural assemblages of phytoplankton cells and pigments are sufficiently documented which could be suitably modified for dinoflagellates. Cells are enumerated under a microscope using a haemocytometer, Sedgwick Rafter slides, or sedimentation chambers with Utermohl's inverted microscope [17]. Details on the microscopic and molecular methods

for phytoplankton quantitative analysis are discussed [18]. Flow cytometry has been increasingly used to obtain data on cell abundance, volumes of microalgae and their fluorescence [19].

Chlorophyll *a* is the main photosynthetic pigment. Pigments are determined by spectrophotometry and fluorometry [20-22]. For determining the phytoplankton composition and for estimating the biomass of the different algal groups High Pressure Liquid Chromatography (HPLC) offers a rapid, very sensitive and objective method [23, 24].

Photosynthesis is determined using the dark and light bottle oxygen exchange method originally described by Gaarder and Gran [25] and used for dinoflagellate [26] or by oxygen electrodes [27-29]. Using oxygen exchange method, respiration was measured in darkened cells [30-32]. Alternatively, a more sensitive method, the tracer technique using carbon-14, originally described by Steemann Nielsen [33] is utilized to determine photosynthetic activities of cultured dinoflagellates [34, 35]. Details of the oxygen exchange method and carbon-14 method and sources of error are discussed [17, 36].

Chlorophyll *a* specific photosynthesis (*P*)–irradiance (*I* µmol m^{-2} s^{-1}) in microalgae are shown in Figure 1 [37].

Measurements include determination of the initial slope of the light limited portion of the *P-I* relationship: the photosynthetic efficiency (α µg C µg Chl α^{-1} h^{-1}µmol m^{-2} s^{-1}), irradiance at which photosynthesis gets light saturated E_k (µmol m^{-2} s^{-1}), light saturated photosynthesis P^{B}_{max} and photoinhibitory decrease β (µmol m^{-2} s^{-1}) [36, 38] expressed as

$$P_m^{Chl} = P_s^{Chl}\left(\frac{\alpha}{\alpha+\beta}\right)\left(\frac{\beta}{\alpha+\beta}\right)(\beta/\alpha)$$

Usually log phase algal cultures are utilized and the initial slope, *Pmax* and *Ek* are used as indicators of the physiological functioning of the alga.

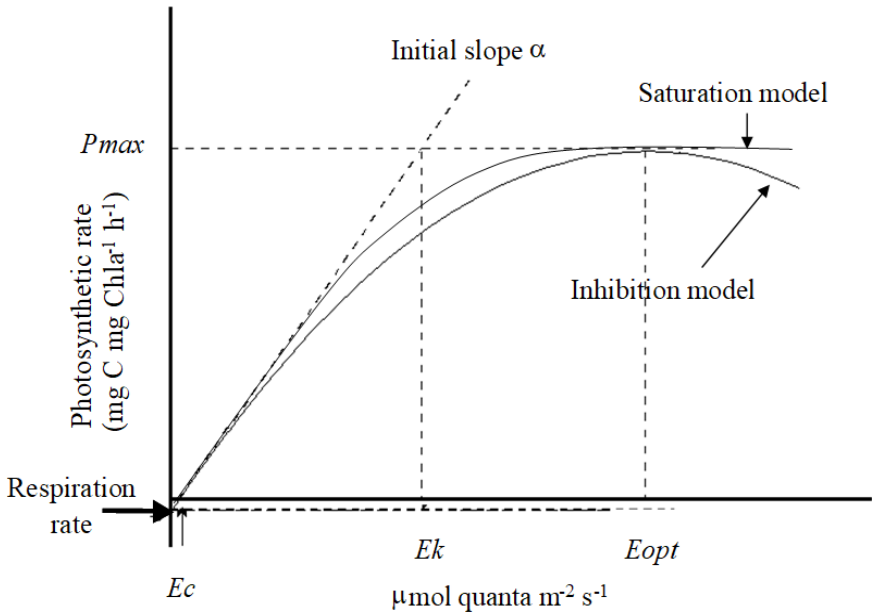

Figure 1. Photosynthesis-Irradiance model.

Photosynthetic Rates

A variety of units for photosynthesis have been reported based on biomass expressed as cell numbers, chlorophyll *a*, cell carbon and cell volume as given below.

Units reported include:

*P*max (Cell) = (10^{-6}) X μmol O_2 μg $Cell^{-1}$ h^{-1},
Total 14C fixed ((DPM x 10^{-3}).10^6 $cells^{-1}$)
μmol O_2 per min per 10^9 cells
μmol O^2 per cell h^{-1} (x10^7)
μmol O_2 per μmol chl *a* h^{-1} (x10)
μmol O_2 μg Chl *a* -1 h^{-1}oxygen evolved/cm3/h^{-1}
*P*max (Chl) = (10^{-1}) X μmol O_2 μg Chl a^{-1} h^{-1}
*P*max (C) = (10^{-3}) X μmol O_2 μg C^{-1} h^{-1}
Oxygen production (micromoles O_2 h^{-1} (10^6 $cells^{-1}$)
nmol O_2 (μg Chl *a*)-1 min^{-1}
mol oxygen evolved/cm3/h
Photosynthetic oxygen evolution (nmol/cell x 10^5 h^{-1})
pg O_2 $zooxanthella^{-1}$ h^{-1}, μg O_2 $polyp^{-1}$
mg C m^{-3} h^{-1}
pgC. $Cell^{-1}$, DPM $cell^{-1}$
μgC $Cell^{-1}h^{-1}$ (x10^{-5})
mg C mg^{-1} Chl *a* h^{-1}

As the cell constituents vary with the physiological state of the alga, comparison of the physiological rates should be based on organisms in the same growth phase. Values normalized per cell could be a single largest source of uncertainty because cell volumes of big dinoflagellates are 1500 times larger than small dinoflagellate species [39]. This is also the case with diatoms; larger diatoms have cell volumes 20,000 times more than the smaller species [40]. For example the dinoflagellate *Pyrocystis noctiluca* with a cell volume of 22x 10^6 μm^3 has the lowest growth rate of 0.1 d^{-1}, compared to *Gonyaulax tamarensis* with a cell volume of 22x 10^3 μm^3 and a rate of 0.5 d^{-1} [41]. Even in the cultures of *Karlodionium veneficum* cell volumes varied with the growth phase; in the exponential phase cell volumes varied between 416.6 and 646.8 μm^3 and in the stationary phase between 389.8 and 610.3 μm^3 [42]. Finkel and Irwin [43] noted that often the allometric ¾ rule deviates in scaling phytoplankton and does not account for the size dependence of light absorption and photosynthesis; they suggested a composite allometric and bio-optic model to provide a mechanistic explanation for microalgal photosynthesis and growth. Banse [44] summarized that in microalgae with increasing cell size growth rate decreased and proposed cell carbon as a scaling factor in comparative algal physiology but determination of cell carbon has analytical issues such as contamination due to organic matter and dead cells. Although cellular pigment varies with the growth phase, chlorophyll *a* –is the photosynthetic pigment and serves as a better index for normalizing photosynthesis. More comparable data utilizing dinoflagellate cultures are needed to address this issue.

The ratio of the energy stored (organic products) to the light energy (photons or formally Einsteins) absorbed is defined as photosynthetic efficiency. This is determined by whole cell fluorescence on controls (without DCMU) and those treated with DCMU (F_{+DCMU}/F_{-DCMU}) in cultures of *Symbiodinium microadriaticum* [45] or F_v/F_m in the peridinin-containing dinoflagellates *Akashiwo sanguinea*, *Prorocentrum micans* and *Scrippsiella trochoidea* [46] or *Fq'/Fm'* the effective quantum yield of PS II under actinic irradiance [47]. Maximum Quantum Yield of Photosystem II is expressed as:

$$Fv/Fm = \left(\frac{Fm - F0}{Fm}\right)$$

where *Fv* is the Chl *a* fluorescence, *F0* and *Fm* correspond to the minimum and maximum *in vivo* Chl *a* fluorescence yield (relative) in a dark-adapted state [48].

Overall the process of carbon assimilation in dinoflagellates is essentially the same as photosynthesis carried out by other microalgae and is dependent on chlorophyll *a*, carbon dioxide, and light energy.

Photosynthetic Pigments

Dinoflagellates contain five pigment types: chlorophylls *a* and c_2 as well as peridinin- the carotenoid only found in dinoflagellates- and small amounts of the xanthophylls, diadinoxanthin and dinoxanthin [49, 50]. More information on the pigments is presented in Chapter 2. [51].

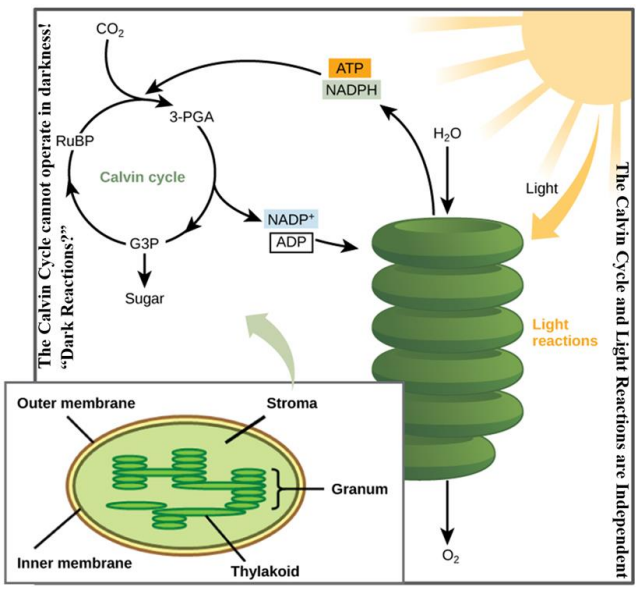

Figure 2. Calvin-Benson cycle of photosynthesis, adapted from https://courses.lumenlearning.com/biology1/chapter/the-calvin-cycle/.

The dinoflagellate chloroplasts contain membranes enclosing 3 thylakoids (lipids, chlorophyll, and proteins, electron carriers). Chloroplasts are the active sites of

photosynthesis which is initiated by absorption of light energy by antennae of light harvesting pigments or photochemically reactive proteins and passed on to the photosynthetic reaction centers via the flow of electrons from one molecular donor to another acceptor known as the Calvin cycle (Figure 2).

The electron carriers capture energy for reducing $NADPH_2$ and ATP, and ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) enzymes to fix CO_2. When light energy is too high, carotenoids quench superfluous light and prevent build up of toxic oxygen radicals.

High performance liquid chromatography (HPLC) data of 40 species using 45 strains of dinoflagellates including three habitat types- the sand-dwelling benthic forms, tidal pool inhabitants and planktonic species- showed that dinoflagellates seem to produce a part of their pigments in response to their habitats [52]. A greater diversity of pigments is found in the sand dwelling dinoflagellates than in the planktonic and tidal pool forms. Twelve pigments including Enol-13(2), 17(3)-cyclopheophorbide a enol (cPPB-aE) occurred only in benthic sand-dwelling species of red alga-derived type. An additional sixteen pigments including Chl *c* 3 (diatom-derived chloroplasts) were found only in sand-dwelling forms.

Peridinin and Photoprotection

Unique to photosynthetic dinoflagellates are the peridinin-plastids, a class of light-harvesting proteins known as peridinin-chlorophyll *a*-proteins (PCP) which consists of a central chlorophyll molecule and four peridinin molecules – the peridinin quartet. From the PCP, Schulte et al. [53] identified one specific carotenoid as a type of integrated lightning rod which operates on a nanosecond scale with the photosystems of chlorophyll where energy transformation and oxygen production take place.

These plastids contain less than 16 genes [54, 55] compared to 165-185 found in cryptomonads and diatoms. Unlike in other algae, the dinoflgellate plastids have an alpha-proteo bacterial type of rubisco (type II) with much lower specificity for CO_2 over O_2 [56, 57]. Of interest is the presence of the oxygen-sensitive homomeric type-II form of the enzyme in the dinoflagellate *Amphidinium carterae*, usually found in heterotrophic anaerobic proteobacteria and cyanobacteria [58]. Peridinin molecules while transferring the incoming light energy in the blue-green wavelengths (470 to 550 nm) to chlorophyll molecules with extremely high efficiency [59] also prevent the organism from building toxic oxygen radicals when solar radiation is too high. In experiments of >30 min duration utilizing *Glenodinium* sp. grown in the range 250--3000 $\mu W/cm^2$ irradiance, cellular chlorophyll *a* and peridinin increased under decreasing light but the photosynthetic rates remained unchanged which is considered significant in maintaining cellular photosynthesis even when irradiance decreases [30]. However, Schulte et al. [53] using femtosecond (10^{-5}seconds) resolution absorption spectroscopy investigated this carotenoid-chlorophyll interaction and identified a single peridinin molecule within the peridinin quartet which efficiently senses Chl *a* excitation. Studies of Kvíčalová et al. [60] showed that in *A. carterae* chlorophyll *a*–chlorophyll c2–peridinin protein complex quenches light with 100% efficiency with faster transfer times of 0.1 nS.

In two peridinin-containing red tide dinoflagellates, *Heterocapsa pygmaea* and *Prorocentrum minimum,* spectral dependency of the maximum quantum yield (φmax) for carbon fixation was reported [61]. This spectral dependency was most significant in *H.*

pygmaea under nearly all growth conditions but not detectable for *P. minimum* under any growth condition attributed to differences in the structure of their photosynthetic machinery and their cellular absorption properties.

Besides peridinin, intracellular lipid may play a protective role. In *A. sanguinea*, *Prorocentrum micans* and *Scripsiella trochoidea* grown both under low light (LL, 87–90 μmol photons m^{-2} s^{-1}) and high (HL, 450–490 μmol photons m^{-2} s^{-1}) peridinin was low but varied [46]. However, *S. trochoidea* and *P. micans* showed higher photosynthetic efficiency (average: F_v/F_m > 0.5) compared to *A. anguinea* (F_v/F_m ~ 0.2). Intracellular lipid concentration increased at HL in these species, which may serve as a protective mechanism for the cells. The *Chatonella marina* strain isolated from Australia had 110 fg $cell^{-1}$ UV-absorbing mycosporine amino acids (MAAs), absent in the Japanese strain which accounts for its tolerance to high intensities of visible light. In cultures of *Akashiwo sanguinea (=Gymnodinium sanguineum*) and *Gymnodinium (= Gyrodinium)* cf. *instriatum* established from the Chesapeake Bay, sensitivity to UV was less efficient particularly in N-limited cells but was elevated by a decrease in cell volume (10^4 μm^3); in *A. sanguinea* it was 4.46 in nutrient replete medium vs 1.53 and 1.39 in media with 25 and 5 μM NO_3 [62]. Corresponding cell volumes for *G.* cf. *instriatum* were 3.19 and 2.39. The photosynthetic inhibition (P^B_S g C g Chl a^{-1} h^{-1}) in *A. sanguine* nutrient replete cultures (25 μM NO_3) ranged between 4.57 and 4.66, while in cultures grown at 5 μM NO_3, it was 3.63. For *G.* cf. *instriatum* corresponding values were 31.3 and 8.06. In nutrient limited cultures the UV-B inhibition was 1.5 times greater which would affect the sensitivity and productivity of phytoplankton and in the concentration of photoprotective mycosporine –like aminoacids [62].

Carbon Assimilation

During photosynthesis carbon dioxide is fixed in light summarized as:

$$2H_2O + 2\ NADP+ + 3\ ADP + 3\ Pi >> 8h\nu >>> O2 + 2\ NADPH_2 + 3\ ATP$$

and in dark via the Calvin-Benson cycles as:

$$CO_2 + 2\ NADPH_2 + 3\ ATP >>>>> H_2O + (CH_2O) + 3\ ADP + 3Pi + 2\ NADP+$$

Ribulose biphosphate carboxylase/oxygenase (RuBisCO) is a 550-kDa enzyme and carries out the photosynthetic fixation of carbon dioxide in the chloroplast [63]. In vivo activities of extracellular and intracellular carbonic anhydrase (CA), photosynthetic O_2 evolution, CO_2 and HCO_3^- uptake rates were measured in bloom-forming dinoflagellates, *Prorocentrum minimum*, *Heterocapsa triquetra* and *Ceratium lineatum,* by membrane inlet mass spectrometry (MIMS) in cells acclimated to low pH (8.0) and high pH (8.5 or 9.1) [12]. Results showed the extracellular carbonic anhydrase (eCA) activity depended on the pH; at low pH it was negligible compared to that at high pH. Half-saturation concentrations ($K_{1/2}$) for photosynthetic O_2 evolution were low compared to RUBISCO kinetics. This affinity for HCO3- and increased maximum uptake rates under higher pH suggest the operation of an efficient CO_2 concentration mechanism (CCM) in these dinoflagellates. Results obtained on

Protoceratium reticulatum, a potentially toxic dinoflagellate that has a form II RUBISCO, confirmed that cells grown in low CO_2 (370 pCO ppm) are more efficient in using dissolved inorganic carbon than cells grown in high CO_2 (5000 pCO ppm); the $K_{1/2}$ (CO_2, DIC) corresponded to 1.66 and 11.5. Photosynthesis (pmol O_2 h^{-1} $cell^{-1}$) for cells at 370 pCO ppm was 3.14 compared to 2.70 for cells at 5000 pCO ppm.

The usual storage products in dinoflagellates are starch and oil. Buck et al. [64] reported a dinoflagellate cyst with thickening of its wall from 0.2 to 0.8 μm over 2 months and an increase in storage bodies. *Crypthecodinium cohnii*, a heterotrophic dinoflagellate produces neutral lipids (NL), glycolipids (GL) and phospholipids (PL) in the form of triacylglycerol, Docosahexaenoic Acid (DHA) and oils [65] that have commercial value.

Uptake in Dark

Not all dinoflagellates are totally photosynthetic [66]; but some carry on a mix of autotrophy and mixotrophy devouring diatoms or other protists, a few dinoflagellates, and even fish eggs as in the genus *Noctiluca*. *Noctiluca* also harbors a phototrophic prasinophycean endosymbiont, *Pedinomonas noctilucae* [67]. Because of their nutritional strategies these phagotrophs acquire plastids from their prey. In phagotrophic dinoflagellates, the acquired plastids, also known as kleptochloroplasts ('stolen chloroplasts') retain their own genome and function as permanent intracellular metabolite transporters [68]. Plastid-derived gene minicircles exist in the nucleus of the host, as for example in *Heterocapsa triquetra*, and become intimately connected with the biology of their host. Although understanding the functioning of such plastid physiology is fundamental and fascinating, it is beyond the scope of this chapter and the reader is referred to a review by Dorrell and Howe [68] on the plastids of the dinoflagellate *Karenia mikimotoi*. It is possible the plastids are integrated into their host's photosynthesis. Waller and Kořený [69] discussed the plastid complexity in dinoflagellates and pointed out that peridinin uses unusual protein trafficking routes and therefore there is a need to revise notions of the origin of the peridinin plastid.

Uptake of carbon in the dark varied in dinoflagellates from the Gullmar Fjord, and from the Koster Strait, the Swedish west coast [11]. In *Ceratium lineatum* and *C. furca* the uptake was in the light as well as in the dark. In the heterotrophic *Dinophysis rotundata*, *Protoperidinium granii* and *P. divergens* ^{14}C uptake (pg C $cell^{-1}$ h^{-1}) ranged from 0.07 to 13.7, in the autotrophic *C. furca*, *C. lineatum* and *C. tripos*, 21.69 and 148.31, and in the mixotrophic *Dinophysis acuminata*, *D. acuta*, *D. norvegica*, 18.22-107.61. Of interest, the uptake in dark was significantly higher than in light in *D. norvegica*, and *D, acuta* which suggests the importance of mixotrophy i.e., direct uptake of ^{14}C-labeled dissolved or particulate organic matter.

Uptake in Light

Photosynthetic carbon assimilation rates are presented for the athecate (Table 1), thecate dinoflagellates (Table 2) and for the symbiotic zooxanthellae that reside in corals (Table 3).

Table 1. Carbon assimilation characteristics of select athecate dinoflagellates

Taxa	Nature Toxic (T), Benign (B)	Isolates (I), Culture (C)	Temp 0ºC	Irradiance µmol m^{-2} s^{1-}	Ps µg C 10^6 cells h^{-1}	Ps µg C µg Chl α^{-1} h^{-1}	I_k µmol m^{-2} s^{-1}	α µg C µg Chl α^{-1} h^{-1} µmol m^{-2} s^{-1}	Remarks	Ref
Gymnodinium chlorophorum			15	100						Hader et al. [115]
			20							
			25							
			15	250						
			20							
			25							
Akashio sanguinea	Red tide B	C		354		4.6	274-444		f/2 nutrient	Litchman et al. [62]
						4.7	95.19		25 µm NO_3	
						3.6	68.51		5µm NO_3	
Gyrodinium Instriatum						4.3	58.84		f/2	
						1.7	59.77		5µm NO_3	
Gyrodinium galatheanum			10	60	20-46.7	3.3				Li et al. [70]
Gymnodinium aureolum	Bloom, nontoxic	C	20	<120 235-380		1.4-1.8	109-195	0.788-1.125 x10^{-3}		Garcia and Purdie [26]
				35	1.7	1.4	148	0.9		
				80	1.6	1.2	109	1.125		
				120	2.1	1.8	174	1.013		
				235	1.3	1.6	195	0.788		
				380	1.3	1.6	158	1.013		
Gyrodinium galatheanum			20	200	19.4-46.70		212.6-286.1	0.091-0.225		Li et al. [70]
Pyrocystis noctiluca			23	210	2950	2.5 -3.0				Rivkin and Seliger [41]
Pyrocystis fusiformis			23	210	935	3.3				Rivkin and Seliger [41]
Gymnodinium stellatum			23	210	104	1.8				Rivkin and Seliger [41]
G.nelsoni			15	80	138 -189	4.9				Rivkin and Seliger [41]
Pyrocystis noctiluca					1700-1900	2.5-3.0	225			Rivkin et al. [127]
Pyrocystis fusiformis					2200	3.3	340			Rivkin et al. [127]
Gymnodinium sp.						0.5				Subba Rao 1988
Cachonina niei		C	10	65		2.3-2.9				Subba Rao 1980
G. splendens			21	500		1.84-3.20				Cullen and Horrigan [71]

Units: I Irradiance (I) µmol m^{-2} s^{-1} ; Ps 10^6 cells h^{-1} ; (= pg C cell-1 h^{-1}); Ps (Z) µg C µg Chl α^{-1} h^{-1} ; I_k µmol m^{-2} s^{-1} ; α µg C µg Chl α^{-1} h^{-1}µmol m^{-2} s^{-1}.

Table 2. Carbon assimilation characteristics of select thecate dinoflagellates

Taxa	Temp °C	Irradiance (I) µmol m^{-2} s^{-1}	Ps µg C 10^6 cells h^{-1}	Ps µg C µg Chl α^{-1} h^{-1}	Ik µmol m^{-2} s^{-1}	α µg C µg Chl α^{-1} h^{-1} µmol m^{-2} s^{-1}	Ref
Alexandrium fundyense	6	75		1.6	133	0.012	Schofield et al. [86]
	15			2.0	128	0.016	
	24			4.9	190	0.026	
Prorocentrum mariae- lebouriae			15-34.9	2.7-5.5	40-251	0.024-0.038	Harding and Coats [75]
		5.4E m-2 d-1	6.1-28.6	2.4-11.2		0.074-0.263	Harding et al. [73]
		0.94	1. 8-2.8	3.5-8.1		0.102-0.189	
		2.8	0.9-1.7			0.074-0.151	
P. micans	15	500	7.4- 7.7				Nimer et al. [131]
D. norwegica				0.9-1.3			Carpenter et al. [29]
D. norwegica			16-25	0.5-1.1	14-69	0.0473	Subba Rao and Pan [10]
D. accuminata	20	100	6.5-38				Riisgaard and Hansen [76]
			38 (3days) 11(6d), 8(9d) 8(20d)				
D. acuta		100	138-177	2.2 -3.5			Hansen et al. [79]
			23.7 16.8	1.1			
D.acuminata			21	16	300	0.053	Berland et al. [77]
Gyrodinium galatheanum		60	~15-40				Li et al. [70]
Peridimium. cinctum westii		105	56.25		60-160		Lindstrom [31]
Alexandrium tamerense		250		0.1			Eberlein et al. [85]
Scripsiella trochoidea				0.2			
D.acuminata		45-231*	160-925		72-190	1.90-9.74	Setala et al. [78]
D. acuminata	16	100	31.2-40.1				Graneli et al. [11]
D.acuta			50.8-61.8				Graneli et al. [11]
D.norwegica			18.2-107.6				Graneli et al. [11]
Ceatium furca			21.7-148.3				Graneli et al. [11]
C.lineatum			26.5-28.7				Graneli et al. [11]
C. tripos			34.3				Graneli et al. [11]
Dinophysis rotundata			0.1-13.7				Graneli et al. [11]
Protoperidinium. Granii			7.7				Rivkin and Voytek [74]
P. divergense			5.2				Rivkin and Voytek [74]
Ceratium lineatum			55-78	1.2-3.6			Rivkin and Voytek [74]
C.. fusus			130	1.8-3.7			Rivkin and Voytek [74]

Taxa	Temp °C	Irradiance (I) µmol m^{-2} s^{-1}	Ps µg C 10^6 cells h^{-1}	Ps µg C µg Chl α^{-1} h^{-1}	Ik µmol m^{-2} s^{-1}	α µg C µg Chl α^{-1} h^{-1} µmol m^{-2} s^{-1}	Ref
C. tripos			190-390	2.1-5.2			Rivkin and Voytek [74]
Prorcentrum mariae lebouriae	22		12-47	4.3-6.0			Rivkin 1985
Gonyaulax tamarens	15		136	5.2			Rivkin and Seliger [41]
G. polyedra	23		144	3.0			Rivkin and Seliger [41]
P.mariae-lebouriae	23		17	4.3			Rivkin and Seliger [41]
Gonyaulax polyedra		60-210	54 phase 1 and 134 after adaptation				Rivkin et al [136]
Gonyaulax digitale	10-12		44				Subba Rao and Pan [10]
Ceratium tripos			40-109				Subba Rao and Pan [10]
Proro. micans	10			5.7-6.2			Subba Rao 1980
Gonyaulax polyedra	23	330 (=31µ mol*)	5.3-20.4	4.4-9.0			Prézelin and Matlick [12]
G. polyedra	21	400 (37)		1.9		0.052	Prézelin and Sweeney [111]
		800 (75)		1.9		0.047	
		1250 (117)		2.0		0.045	
		1700 (158)		2.5		0.048	
		2500 (234)		3.2		0.064	
		4000 (373)		2.1		0.046	
Glenodinium sp.	20	1400 (130)		1.0			Prézelin [30]
		2500 (234)		1.6			
Glenodinium sp.	21	5000 (466)	0.14	8.6			Prézelin et al. [81]
G. polyedra	21	“ 466	6.6- 8.4	8.6-11.3	0.23-0.31		Prézelin et al. [81]
		5000 (466*)	1.5	3	0.08		Prézelin et al. [81]
C. furca	21	5000 (466*)	11.3	22.5	0.62		Prézelin et al. [81]
C. tripos			51-71				Boulding and Platt [80]
Protoceratium reticulatum	20	470	1.2	1.0-1.2			Ratti and Giordano [137]
C. longipes				1.4			Angelidis 1987
				5.7			Taguchi 1981
C. lineatum				1.2-3.6			
C. longipes				1.4			Angelidis 1987
				5.7			Taguchi 1981

Units: I Irradiance µmol m^{-2} s^{-1} ; (* converted 1Wcm^2 = 4.66 µmol m^{-2} s^{-1}) ;Ps µg C 10^6 cells h^{-1} ; Ps (Z) µg C µg Chl α^{-1} h^{-1} ; I_k µmol m^{-2} s^{-1} ; α µg C µg Chl α^{-1} h^{-1}µmol m^{-2} s^{-1}.

Table 3. Carbon assimilation characteristics of zooxanthellae

Taxa	Nature Toxic (T), Benign (B)	Isolates (I), Culture (C)	Temp 0ºC	Irradiance (I) µmol m^{-2} s^{-1}	Ps µg C 10^9 cells h^{-1}	Ps µg C µg Chl α^{-1} h^{-1}	I_k µmol m^{-2} s^{-1}	α µg C µg Chl α^{-1} h^{-1} µmol m^{-2} s^{-1}	Remarks	Ref
Symbiodinium microadriaticum	B	C	20	80	11.3- 33.75				Ps bet 20-30 C only Pmax impaired at 34-36C	Iglesias-Prieto et al. [45]
			25							
			30							
			35		0					
Galaxea fascicularis	B	I	26	500		0.03 0.17	42-80	0.0116 FIZ 0.036 CZ	P:R -0.30 FIZ 2.11 CZ	Goiran et al. [27]
Zooxanthellae		I	26-27	Shade 300		0.01 FIZ	65	0.00006	low in low lt, high in high lt	Fitt and Cook [28]
		I		High lt 1500-1900		0.01 FIZ	71	0.0001	Isolated cells P:R 2.38-4.3	
Symbiodinium microadriaticum			20	80	69.8				Ps increased between 20-30 ºC and zero at 35ºC	Iglesias-Prieto et al. [45]
			25		96.8					
			30		135.0					
			35		0					

FIZ Freshly isolated, CZ Cultured zooxanthellae; Conversion factors: mols oxygen x 0.375 = grams carbon; mol 0_2/µmol chl *a* h^{-1}= X 0.010316 µg C µg Chl *a* h^{-1}.

Values are extracted from the various publications; they are not absolute due to methodological considerations in the C^{14} measurements: a) total carbon dioxide in the medium b) isotopic discrimination c) respiratory losses d) dark fixation and e) utilization of newly labelled products or respiration [17]. It should be noted that carbon assimilation values are often reported for a discrete light energy level but not on a photosynthesis-irradiance curve; so the P^B values may be either light limited or at inhibitory irradiance. The physiological state of the cells that affects the photosynthetic rates is also unknown.

Athecate Dinoflagellates

Photosynthetic measurements were made on cultured athecate dinoflagellates (Table 1) under a wide range of conditions i.e., temperature 10-25^{0}C and irradiance 35-500μmol m^{-2} $s^{-1.}$ On a cellular basis, carbon assimilation (μg C 10^6 cells h^{-1} = pg C $cell^{-1}$ h^{-1}) varied between ~20-46.7 in *Gyrodinium galatheanum* [70], 104 in *Gymnodinium stellatum* [41], 138 in *Gymnodinium nelsoni,* 935 in *Pyrocystis fusiformis* and 2950 in *Pyrocystis noctiluca* [71].

In *Gyrodinium galatheanum,* a photosynthetic, mixotrophic dinoflagellate, carbon assimilation was 20-46.7 μg C 10^6 cells h^{-1} and growth rates were 2- to 3 times higher than under strictly phototrophic conditions at the same irradiances [70]. In the cells grown under low light and/or nutrient-limited conditions, phagotrophy also enhanced photosynthetic capacity (P_{max}^{cell}), and/or by increased photosynthetic efficiency.

On chlorophyll basis, photosynthetic rates (μg C μg Chl α^{-1} h^{-1}) were generally low and ranged from 1.8 to 3.2 in *Gymnodinium splendens* from natural assemblages [72] and 4.9 in cultures of *Gymnodinium nelson* [41]. In *G. galatheanum* the rates were 3.3 [70], *in Akashiow sanguinae* 3.63-4.66 [62] and 4.3 in *G. instriatum* [62]. In *Gymnodinium aureolum* cells (Table 1) from a bloom in Denmark at 20ºC, photosynthesis (μg C μg Chl α^{-1} $h^{-1)}$ increased with irradiance 35-380 Irradiance (μmol m^{-2} s^{-1}) and ranged between 1.4 and 1.8 [26]. The irradiance at which photosynthesis is saturated (I_k μmol m^{-2} s^{-1}) varied between 59 and 361; in *Gymnodinium aureolum* the photosynthetic efficiency (α μg C μg Chl α^{-1} h^{-1}μmol m^{-2} s^{-1}) ranged between 0.788-1.125 x10^{-3} and cells grown at lower irradiance (I) <100 μmol m^{-2} s^{-1} had higher efficiency [26] (Table 1). The saturating irradiance (I) 274- 444 in *Akashiow sanguinea* is comparable to other athecate species *Gyrodinium galatheanum*, *Pyrocystis noctiluca* and *P. fusiformis* (Table 1).

Thecate Dinoflagellates

A wide range of carbon assimilation rates (μg C 10^6 cells h^{-1}) were observed in the thecate dinoflagellates (Table 2). The highest range 0.9-28.6 was in *Prorocentrum mariae-lebouriae* [73] but in *Dinophysis accuminata, D. acuta, Ceratium tripos, C. furca, Gonyaulax polyedra, G. tamarense* and *G. digitale* they ranged 2 to 6 times (Table 4). *Prorocentrum mariae-lebouriae* adapts rapidly to changes in irradiance. Within 72 h of a shift to low light chlorophyll *a* increased from 2.6 to 5.1 pg $cell^{-1}$, chl c from 1.8 to 3.0 pg $cell^{-1}$, and peridinin from 2.2 to 3.1 pg $cell^{-1}$ but the rate of carbon fixation remained the the same which according to Harding et al. [73] is a strategy of this dinoflagellate to reduced light.

In *Dinophysis acuta* carbon assimilation in holozoic fed cells was 23.7 and decreased to 16.8 pg cell^{-1} when starved [78]. In *Dinophysis acuminata* photosynthetic rates (pg cell^{-1}) decreased with the age of cells, thus 38 for 3 day cultures, 11 for 6 days, 8 for 9 days, 8 for 20 days growth [75], similar to what has been observed in batch cultures of other microalgae.

In the thecate dinoflagellates, carbon assimilation rates (Table 2) normalized to chlorophyll *a* (µg C µg Chl α^{-1} h^{-1}) also showed a wide range. The minimum 0.1 was in *Alexandrium tamarense* [85] but Schofield et al. [86] reported higher rates 1.6 to 4.9. Higher rates (Table 5) comparable to those reported in diatoms and microflagellates were reported in *Glenodinium* sp. (8.6), *Gonyaulax polyedra* (9.0), *Prorocentrum mariae-lebouriae* (11.2).

Table 4. Range of carbon assimilation rates (µg C 10^6 cells h^{-1}) in thecate dinoflagellates

Taxa	µg C 10^6 cells h^{-1}	Reference
Prorocentrum mariae-lebouriae	0.9-28.6	73 Harding et al. 1983
	12,17	41 Rivkin and Seliger 1981
	12-47	74 Rivkin 1985
Dinophysis acumimata	6.5-38	76 Riisgaard, Hansen, 2009
	21-32	77 Berland et al. 1995
	31.2-40.1	11 Granéli et al. 1997
	160-925	78 Setala et al. 2005
Dinophysis acuta	18.2-61.8	76 Riisgaard, Hansen, 2009
	138-177	79 Hansen et al. 2016
Ceratium tripos	34.3	11 Granéli et al. 1997
	51-71	80 Boulding, and Platt 1986
	390	71 Rivkin et al. 1982
Ceratium furca	11.25	81 Prézelin 1977
	148.31	31 lindstrom 1984
Gonyaulax polyedra	5.3-20.4	82 Prézelin and Matlick 1983
	6.56-8.4	81 Prézelin 1977
	144	41 Rivkin and Seliger 1981
Gonyaulax tamarensis	136	41 Rivkin and Seliger 1981
Gonyaulax digitale	44	83 Wall 1965,84
		84 Subba Rao and Pan 1993

Table 5. Range of carbon assimilation rates (µg C µg Chl α^{-1} h^{-1}) in thecate dinoflagellates

Taxa	µg C µg Chl α^{-1} h^{-1}	Reference
Alexandium tamarense	1.6 -4.9	86 Schiofield et al. 1991
Prorocentrum mariae-lebouriae	2.4-5.5	74 Rivkin 1985
Prorocentrum mariae- lebouriae	5.5.-11.2	73 Harding et al. 1983
Gonyaulax polyedra	3.0	41 Rivkin and Seliger
Gonyaulax polyedra	4.4-9.0	82 Prézelin, and Matlick, 1983).
G. tamarense	5.2	41 Rivkin and Seliger 1981
Prorocentrum micans	5.7-6.2	35 Subba rao 1980
Ceratium lineatum	1.2-3.6	71 Rivkin et al. 1982
C. fusus	1.8-3.7	71 Rivkin et al. 1982
C. lomgipes	5.7	87 Taguchi 1981
Glenodinium sp	8.6	81 Prézelin et al. 1977

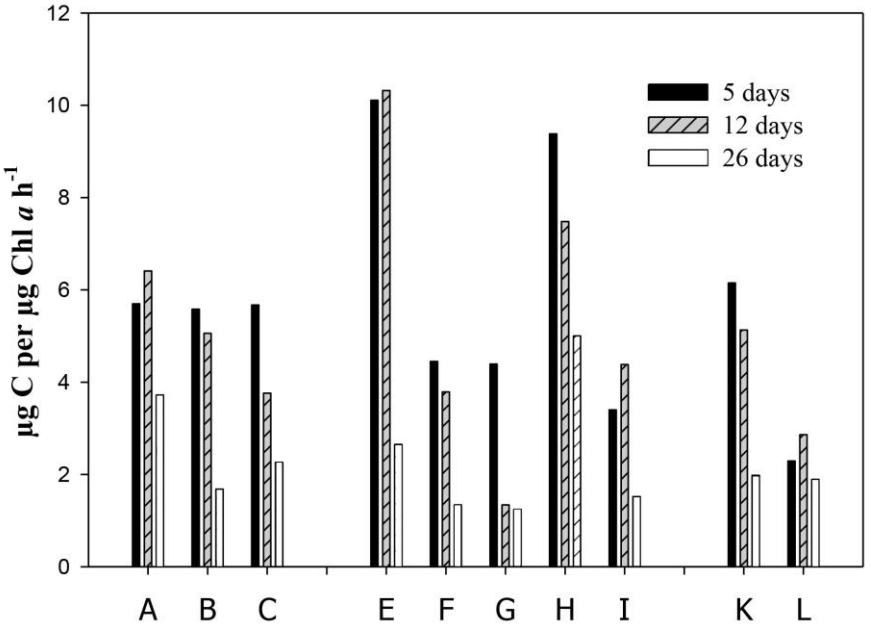

Figure 3. Photosynthesis in phytoflagellates, diatoms and dinoflagellates grown for 5, 12 and 26 days. Where A to C – Phytoflagellates: A-*Monmochrysis lutheri*, B-*Dunaliella tertiolecta*, C- Chrysomonad A; E to I- Diatoms: E-*Skeletonema costatum*, F-*Phaeodactylum tricornutum*, G- *Nitzschia* sp , H-Centric diatom 42, and I- T*halassiosira nordenskioldii. and* K to L- Dinoflagellates: K- *Prorocentrum micans* and L- *Cachonina niei.*

The photosynthetic rates (μg C μg Chl α^{-1} h^{-1}) in most of the athecate and thecate dinoflagellates (Table 5) are low (<6.2) compared to the photosynthetic rates of 5 day old log phase cultures (Figure 3) of diatoms *Skeletonema costatum* (9.0), *Thalassiosira rotula* (9.1-13.9), for centric diatom 42 (9.4), flagellates *Monochrysis lutheri* (5.7), *Dunaliella tertiolecta* (5.6) and *chrysomonad* (5.7) [13]. The highest photosynthetic rates, 22.8 and 18.1, were in the green flagellate Chlamydomonas *plethora* and the diatom *Nitzschia frustule*, respectively [88], and in *Nitzschia*, *Thalassiosira*, *Amphiprora* and *Prorocentrum* [89]. These are lower even compared to 26 day senescent cells (Figure 3) that yielded photosynthetic rates for centric diatom 42 (5.0), flagellates *Monochrysis lutheri* (3.7), *Dunaliella tertiolecta* (1.7) and a chrysomonad (2.3).

The few data on the irradiance at which photosynthesis is saturated (I_k μmol m^{-2} s^{-1}) in the thecate dinoflagellates varied between 60-300 (Table 2), comparable to the results on athecate species (Table 1). In *Alexandrium fundyense* the range was 128-190 [86], 40-251 in *Prorocentrum mariae-lebouriae* [75], 72-190 for *D. acuminata* [78], 60-160 *Peridimium cinctum* fa *westii* [31] and 300 in *D. acuminata* [77].

The thecate marine dinoflagellates *Gonyaulax polyedra*, *Glenodinium* sp. and *Ceratium furca* exhibit circadian rhythms in photosynthesis, usually with a maximum by about midday and show a 3 to 5 fold change in the photosynthetic magnitude over the day [81]. Observations of Prézelin et al. [81] on the dinoflagellates are similar to the rhythms in metabolic pathways in another dinoflagellate, *Lingulodinium polyedrum*, or in photosynthesis reported for the diatoms, *Phaeodactylum tricornutum*, *Acetabularia crenulata*, *Chlorella pyrenoidosa* and *Scenedesmus obliqus*.

Zooxanthellae

Some corals host symbiotic photosynthetic dinoflagellates – zooxanthellae belonging to the genus *Symbiodinium*- which awaits formal taxonomic description [90, 91]. Corals cover 6 × 10^5 km^2 of the world's oceans, from 10-50% of the reef surface, and zooxanthellae are about 10^6 cells per cm^{-2} and contribute to carbon fixation in the reef [92]. Loss of photosynthetic activity of zooxanthellae due to global warming, acidification, anthropogenic activities, and pollution results in coral "bleaching" that has been reported world-wide [93]. Expulsion of zooxanthellae, likely under sub aerial exposure and high water temperature, causes disorganization of thylakoid membranes of the zooxanthellae resulting in bleaching of corals [94, 95].

Because of their close-knit symbiotic relationship and integration with the life of their host, studies on the photosynthesis (*P*) *vs.* irradiation (*I*) (*P-I*) studies of cultured or isolated zooxanthellae, determined either by oxygen exchange using an electrode or by C^{14} tracer uptake, or by photosynthetic efficiency measured on whole cell fluorescence (F_{+DCMU}/F_{-DCMU}) would be instructive.

Photosynthesis determined by oxygen electrode ranged between 0.5 and 1.5 μgC per 10^9 cells per h^{-1} and was extremely low compared to the athecate dinoflagellates given in Table 1. On chlorophyll *a* basis, photosynthesis (μg C μg Chl α^{-1} h^{-1}) and photosynthetic efficiency (α μg C μg Chl α^{-1} h^{-1}μmol m^{-2} s^{-1}) were also low. The irradiance for saturated photosynthesis (I_k μmol m^{-2} s^{-1}) varied between 42 and 181, low compared to the athecate dinoflagellates (Table 1). Photosynthetic efficiency measured on whole cell fluorescence (F_{+DCMU}/F_{-DCMU}) of *Symbodinium microadriaticum* cultures was maximum (~2.2) at 24^0C and decreased steadily as a "stress"response at temperatures higher than 30^0C and ceased at 34-36^0C [45].

In the scleratinian corals, *Favia* sp. and *Acropora* sp., the *P-I* curves demonstrated an adaptation of zooxanthellae to low light; the initial slope was low at 0.05, P_{max} 13.65 nmol O_2 $cm^{-2}s^{-1}$, and the I_K 273 μE $m^{-2}s^{-1}$ [96]. *Symbiodinium*, living in the tropical hydroid, *Myrionema amboinense*, acclimates to low photon flux by increasing the amount of photosynthetic pigments per algal cell [28], which protects algae from photobleaching of pigments and or photoinhibition of photosynthesis at high light intensities present in shallow water habitats.

Zooxanthellae associated with the hydroid host tissue are " shade" adapted and have low maximum photosynthesis (P_{max}) compared to symbionts removed from the host, suggesting that the availability of carbon dioxide for photosynthesis may be limited in the intact hydroid [87]. Photosynthetic rates (pg C per μg chl α h^{-1}) of intact zooxanthellae adapted to low light usually available in the tissue were low (3.64) compared to 6.6 in cells isolated from the tissue [28]. In *Symbiodinium*, sustained photosynthetic alternative electron flows (AEF) were reported under high light intensities [98] which act as a photoprotective mechanism and leads to an increase of the ATP/NADPH ratio.

Host Factor

Gates et al. [15] investigated the impact of a homogenate of *Aiptasia pulchella*, a tropical sea anemone, designated it as a "host factor" or "synthetic host factor" (HRF or SHF), on the photosynthetic physiology of a symbiotic dinoflagellate. Compared to dinoflagellates

incubated in sea water in vitro, the host factor resulted in release of fixed carbon, higher photosynthetic carbon fixation, heterotrophic carbon fixation, photosynthetic oxygen production, respiration rates, and cell specific concentrations of chlorophyll *a*. Gates et al. [15] suggested that in nature, symbiotic dinoflagellates released from anthozoan hosts may survive in amino acid rich environments such as fish guts and "intermediate hosts." Interestingly, zooxanthellae that were excreted through feces of the host had similar photosynthetic rates similar to regular zooxanthellae that did not pass through a digestive tract [99]. There is a constant nutrient shuffling between the host and the zooxanthellae, for example ammonium retained by the anemone is utilized by the zooxanthellae in the light [100]. Together they can synthesize 20 aminoacids [101]. Photosynthetic activity enhanced the calcification rate of the coral *Plesiastrea urvillei* from 0.35 to 1.80 $g.m^{-2}$ d^{-1}[102]. *Symbiodinium* produced higher amounts of carbohydrates when living inside a host rather than when free living [103].

Extracts (HRF) from fed and starved sea anemone *Aiptasia pallida* released different proportions of photosynthetic products [104]. In filtered seawater alone it was about 5% of photosynthate, 14% with addition of 10-kDa ultrafiltrate of *A. pallida* and increased to over 25% from anemones starved for 29 days or more. Carbon fixation by zooxanthellae from *A.pallida* was dependent on temperature; it increased exponentially in the range 12-32^{0}C and decreased at 34.5^{O}C [105]. Translocation of the photosynthate was 82% at 12°C compared to 63% at 27°C. The marine coelenterates, *Zoanthus flos marinus (*Zoantharia*), Stoichactis helianthus* (Actinaria) and *Scolymia lacera* (Madreporaria), incorporated C^{14} after 3h photosynthesis; between 24 and 40 per cent of the total photosynthate was in the tissues of the anemones [106].

Investigations on the mechanisms of dissolved inorganic carbon (DIC) uptake by the scleractinian coral, *Galaxea fascicularis*, and its delivery to the photosynthetic endosymbionts showed that zooxanthellae inside the host had lower photosynthetic rates than isolated cells [27]. Further, intact cells had higher light saturation (I_k) and were able to utilize DIC. The maximum rate of photosynthetic production μg C μg Chl α^1 h^{-1} (Table 3) was 0.3 in the coral, 0.30 in freshly isolated zooxanthellae (FIZ) and 2.0 in cultured zooxanthellae (CZ) , and the half-maximal rate of photosynthetic rate is achieved at 408, 71 and 178 μM HCO, for coral, FIZ , and (CZ), respectively [27]. Between FIZ and CZ marked differences existed in the photosynthetic characteristics; in the FIZ, I_K was 42 and α was 0.031; for CZ they were higher and corresponded to 80 and 0.095 [27].

Additional studies [107] showed bicarbonate uptake depended on the 4, 4'-diisothiocyanato-stilbene-2, 2'-disulfonic acid – DIDS sensitive carriers. Carbonic anhydrase located in the zooxanthellae participates in the carbon supply while in cultured cells bicarbonate uptake appears to be strictly Na+ dependent.

The Photosynthesis: respiration ratio (P:R) in *Symbiodinium microadriaticum* is about 2 [45]. The zooxanthellae provide enough oxygen for their host as evident from the P:R ratios, in *Favia* it was 77.7% [96], in *A. pulchella* 95% [15], it ranged from 1.26 to 1.66 in *P. urvillei* [102], *Anthopleura elegantissima* exhibited net photosynthesis twice that of zoochlorellae [108]. The P:R ratios in *Anthopleura elegantissima* ranged between 2 and 3 for starved anemones but not over 1 for fed anemones [109]. Of interest is the P:R ratios of the dinoflagellate with a phototrophic endosymbiont, *Pedimonas noctilucae* [67]. Gross photosynthesis: respiration was 1.6 in the non-feeding *Noctiluca* without a food vacuole, compared to 0.8 in those with a food vacuole [67]. The concentration of humic, fulvic and

hydrophilic acids affect P:R; in *Prorocentrum minimum* cells treated with >25 μg ml^{-1} , fulvic acid only produced more oxygen in excess of the respired [32] unlike the humic, and hydrophilic acids fractions. There appears to be a difference in the response of the symbiotic *Symbiodinium* and free-living dinoflagellate *Prorocentrum minimum* in their response to ammonium limited conditions; in the former there was no enhancement of photosynthetic efficiency (F_v/F_m) whereas it recovered within 2 days in the latter [110].

Although data on photosynthesis of dinoflagellates are insufficient and not systematic (Tables 1, 2 and 3), we summarize briefly the general processes such as irradiance, temperature, nutrients and their carbon assimilation.

Irradiance

Generally, photosynthesis (μgC μg Chl α^{-1} h^{-1}) is usually low at low light and attains a maximum at higher light. In *Gymnodinium aureolum* (Table 1), it increased from 1.4 at 35 μmol photons m^{-2} s^{-1} to 1.8 at 120 photons and decreased to 1.6 at 380 photons [26]. In the thecate *Gonyaulax polyedra* (Table 2) at 37 photons it was 1.9 and steadily increased to a maximum of 3.2 at 233 μmol m^{-2} $s^{-1}$and at 373 μmol m^{-2} s^{-1}(Table 2) [111]. It should be noted that inhibition of photosynthesis was not observed in *Glenodinium* sp. cultures at 560 μmol m^{-2} s^{-1}[30], in *G. polyedra* at 746 μmol m^{-2} s^{-1} [111], in natural concentrates of *Dinophysis norwegica* at 1600 μmol m^{-2} s^{-1} [10], and in *Prorocentrum mariae-lebouriae* at 400 μmol m^{-2} s^{-1} [75]. However, freshly isolated zooxanthellae and cultured zooxanthellae showd ~20% inhibition of the maximum [27]. In zooxanthelle living in shade (300μmol m^{-2} s^{-1}) it was 9.7pg O_2 h^{-1} chl^{-1} compared to 17.6 pg O_2 h^{-1} chl^{-1} at 1500-1900 μmol m^{-2} s^{-1} [28]. This is mainly because cells grown at high light (HL) have less chlorophyll, smaller antenna and use high light more effectively than those grown at low light. Photosynthetic efficiency (α), Initial Slope of P vs I, is lower for HL grown cells. Fitt and Cook suggested that the host cell environment reduced the available light to zooxanthellae by >50% which prevents photobleaching of the algae. The photosynthetic efficiencies ngC [ng Chl *a*] $^{-1}$ h^{-1} [μ mol m^{-2} s^{-1}] $^{-1}$ in dinoflagellates (Tables 1, 2, 3) are low compared to 39.6 and 79.5 reported for microalgae *C. plethora* and *N. frustula* [88].

In *Peridinium cinctum* fa.*westii* cultures established form Lake Kinneret, Israel Ik varied between ~60 and >160 (μmol m^{-2} $s^{-1)}$ with the quality of light [31], so did the compensation point and saturation light. The reduction in antenna size is regarded as a strategy to increase productivity; under continuous yellow light production of *Chlamydomonas reinhardtii* was 54 g m^{-2} d^{-1} , twice that obtained in blue and red light [112]. *P*max in three out of four temperate dinoflagellates i.e., *Gonyaulax hyalina, Gymnodinium splendens, Dinophysis caudata*, and *Glenodinium* sp. daylength did not influence photosynthesis unlike in polar diatoms; the *P*max: *P*min was similar for polar diatoms and temperate dinoflagellates [113]. Observations on the maximum quantum yield for photosystem II (determined by differences in oxygen production rates and other biochemical parameters), showed minimal alterations (FV/F_M = variable fluorescence/maximum fluorescence) in *Gymnodinium breve* (*Karenia brevis*) cultures possess an inherent resistance to UV and a robust photosynthetic activity [114]. Response to light seems to be species specific. For example, in a high light-adapted species *Alexandrium fundyense* results with Chl *a* inversely proportional to growth irradiance, FV/F_M

data suggested a trade-off between photoprotection and carbon fixation [139]. In contrast, *Heterocapsa rotundata*, a low light-adapted species with high photosynthetic efficiencies, the trade-offs were in the form of substantial photoinhibition and a lack of plasticity in Chl *a* content [139]. Previous light history of cultured *Gymnodinium chlorophorum* cells affected the photochemical quantum yield [115]; yield was more in cultures raised at 100 μmol m^{-2} s^{-1} than at 250 μmol m^{-2} s^{-1} between 15-20 ^{0}C but at 25^{0}C it was the opposite. In the toxigenic *Gambierdiscus carolinianus* and *G. silvae* exposure to increasing photon flux density up to 1464 μmol photons m^{-2} s^{-1}, cell size and chlorophyll content increased but photosynthetic efficiency was reduced [116]. Net photosynthesis (pgC $cell^{-1}$ h^{-1}) in cultures of *Gonyaulax polyedra* increased, from 54 to 130 during phase 1 (light limiting) and from 58 to 134 after light adaptation phase, as the irradiance increased from 60 to 210 μE m^{-2} s^{-1}.

Rates of carbon incorporation per $cell^{-1}$ h^{-1} (Table 6) ranged widely from 12 in *Prorocentrum mariae-lebouriae* to 2950 in *Pyrocystis noctiluca.* The data show that the lowest rates were in cells with smaller cell - volume *Prorocentrum mariae-lebouriae and Heterocapsa triquetra;* they increased with the cell volume.

Table 6. Range of carbon assimilation (μg C 10^6 cells $h^{-1)}$ in dinoflagellates (Rivkin and Seliger 1981)

Taxa	μg C 10^6 cells h^{-1}
Gymnodinium stellatum	104 ± 4 n = 84
G. nelson	42-189 ±13 n = 32
	189 ± 14 n = 29
Gonyaulax polyedra	144 ± 8 n = 40
G. tamarensis	136 ± 11 n = 44
Prorocentrum mariae-lebouriae	17 ± 1 n = 77
	12 ± 1 n = 66
Heterocapsa triquetra	16 ± 2 n = 22
Pyrocystis noctiluca	2950 ± 85 n = 49
P.fusiformis	935 ± 27 n = 53

Temperature

With an increase in temperature, photosynthesis increased (μg C μg Chl α^{-1} h^{-1}); in *Alexandrium fundyense* (Table 2) it was 1.58 at 6^{0}C, 2.04 at 15 ^{0}C and 4.96 at 24^{0}C; their I_k values corresponded to 133,128 and 190 and a photosynthetic efficiency (α μg C μg Chl α^{-1} h^{-1}μmol m^{-2} s^{-1}) of 0.012, 0.016 and 0.026 [86]. In the zooxanthellae *Symbiodinium microadriaticum* (Table 3) between 20ºC and 30ºC, photosynthesis increased with temperature but was impaired at 34-36ºC; thus Ps (μmol O_2 10^9 cells) 3 at 20ºC, 4 at 25ºC, 5.5 at 30ºC and 0 at 35ºC [45]. Results on *Prorocentrum mariae-lebouriae* at photon flux densities (PFD) from 1.7 to 170 μmol of quanta s^{-1} m^{-2} showed a 50% increase in photosynthesis from 10ºC to 30 ºC at saturating photon flux density [138]. Such an increase in magnitude with increased PFD is transient and known as CO_2-'burst' and CO_2-'gulp' [138]. From Lake Kinneret, Israel the bloom forming dinoflagellate *Peridinium cinctum* fa. *westii,* cultures established at irradiances 9 to 105 (μE.m^{-2}.s^{-1}), the photosynthetic Q_{10} increased with irradiance but decreased as the temperature increased [31]. The photosynthetic Q_{10} ranged from 1.6 to 2.3 in the temperature range 10-20ºC, thus 1.2-1.7 for 15-25ºC, and

1.0-1.2 for 20-30ºC [31]. Photosynthetic performance of *Prorocentrum donghaiense* and *Karenia mikimotoi* was temperature dependent [117]; in the former the *Fq'/Fm'* was 0.517 at 16ºC and decreased to 0.490 at 28ºC. In these dinoflagellates the α also decreased and corresponded 0.248 and 0.239 and their I_k 775 and 970. In *K. mikimotoi Fq'/Fm' was* 0.260 at 16ºC and 0.353 at 28ºC, their α corresponded to 0.154 and 0.193 and the I_k 699 and 900.

Nutrients

Dinoflagellate cultures are raised in media based on enrichments and are utilized to study their division rates to elucidate consequences of eutrophication [6, 118-121]. However, only a few studies exist on the impact of nutrients on photosynthetic functioning of the dinoflagellates.

Studies by Summons et al. [122] with $^{15}NH^{+}_{4}$ on the symbiotic *Gymnodinium microadriaticum,* from tridacnid clams and corals, demonstrated light stimulated ammonium uptake but not photosynthesis; they concluded that the glutamine synthase-glutamate synthetase pathway of ammonium incorporation is light-driven.

Encouraged by observations of others on neretic and oceanic phytoplankton, Doucette and Harrison [123] tested iron limitation on the vivo fluorescence (F) and DCMU-enhanced F (FD) in the dinoflagellate *Gymnodinium sanguineum.* In nitrogen depleted medium, chlorophyll *a* quota (Qchl) decreased while chlorophyll fluorescence (F/chl *a*) and fluorescence DCMU (FD/chl *a*) increased. But severe iron stress modified F/chl *a* and adversely affected the utilization of harvested light energy. Phosphate deprivation significantly affected fluorescence in *Karenia mikimotoi* [124].

The toxigenic *Alexandrium tamarense* was able to take up substantial amounts of nitrate, ammonium and urea in the dark and changes in the C: N ratios were dependent on the N supply mode rather than the nutrient status of the cell [125]. This implies that in the absence of continuous supply of nutrients, growth will be uninterrupted to the advantage of the alga [125]. Burkholder et al. [126] discussed the linkage of growth of 31 species of dinoflagellates to nutrient enrichment in eutrophic or marine coastal waters. In mixotrophic mode, growth was significantly higher in harmful algal species (HAS) than in phototrophic mode; detailed investigations are suggested on phototrophy, osmotrophy and phagotrophy in the nutritional ecology of HAS utilizing assemblages from various phases of the bloom from eutrophic waters.

A few studies were carried out on the same species by different investigators (Table 2) which facilitates comparison of intra and inter species specific carbon assimilation. These are summarized in Table 5. Data presented in Table 7 permit comparison of photosynthetic rates (μg C 10^6 cells h^{-1} and μg C μg Chl α^{-1} h^{-1}) within and between the species. The lowest rates (μg C 10^6 cells h^{-1}) were in *Alexandrium tamarense* (0.1–4.9) and the highest (1700-2950) in *Pyrocystis noctiluca*. Within the species the range (max:min) was <1.7x in *Pyrocystis noctiluca*, 3.5-3.8x *Dinophysis acuta*, *Gonyaulax polyedra*, 36.4x in *Prorocentrum mariae-lebouriae*, 44x in *Dinophysis acuminata*, 49x in *Alexandrium tamarense* and 120x in *Dinophysis norwegica*.

The range of photosynthetic rates (μg C μg Chl α^{-1} h^{-1}) within the species was however was low 1.2–3.8 times. Between the species their rates ranged between 0.5 and 11.3, the highest was in *Gonyaulax polyedra* (Prézelin, and Matlick 1983). While this wide latitude of

values could be due to differences in experimental variables, light, temperature, growth phase of cells, production of toxin, presence of plastids acquired through phagotrophy, differences between strains and genomic differences all point out the need for the usage of standard methodology.

Table 7. Comparison of carbon assimilation rates (µg C 10^6 cells h^{-1} and µg C µg Chl α^{-1} h^{-1}) within and between eight dinoflagellate species

Taxa	µg C 10^6 cells h^-	µg C µg Chl α^{-1} h^{-1}	Reference
Pyrocystis noctiluca	2950		41 Rivkin and Seliger 1981
	1700-1900	2.5-3.0	127 Rivkin et al. 1984
Pyrocystis fusiformis	935		41 Rivkin and Seliger 1981
	200		127 Rivkin et al. 1984
Prorocentrum mariae-lebouriae	15-34.9	2.4-5.5	75Harding and Coats 1988
	12-437	4.3-6.0	74 Rivkin 1985
	17	4.3	41 Rivkin and Seliger 1981
	12		87 Taguchi 1981
Gonyaulax polyedra	144	3	41 Rivkin and Seliger 1981
	5.3-20.4	4.4-9.0	82 Prézelin, and Matlick, 1983
	6.6-8.4	8.6-11.3	82 Prézelin, and Matlick, 1983
Dinophysis acuta	138-177	2.2-3.5	79 Hansen et al. 2016
	50-61.8		11 Granéli et al. 1997
D. acuminata	21		77 Berland et al. 1995
	160-925		78 Setala et al. 2005
	31-41.1		11 Granéli et al. 1997
D. norwegica	16-25	0.5-1.1	10 Subba Rao and Pan 1993
	18.2-107.6		11 Granéli et al. 1997
	0.9-1.3		29 Carpenter et al. 1995
Alexandrium tamarense	0.1		85 Eberlin et al. 2014
	1.6-4.9		86 Schofield et al. 1991

In 7 strains of *Dinophysis acuminata* isolated from different locations in Denmark the photosynthetic rate increased from about 7 to 38 pg C $cell^{-1}$ h^{-1} in the presence of the prey (>1000 cells of *Mesodinium rubrum*) within the first 2 days, but subsequent starvation decreased rapidly to a pre-feeding level [76]. Intraspecific variability in pectenotoxin -2 (PTX-2) in the range 12.7 to 35.6 pg $cell^{-1}$ existed in 7 strains of *Dinophysis acuminata* and are related more likely to the growth phase and genetic differences [128]. In these strains when the prey was in abundance, photosynthetic rate at 130 µmol photons m^{-2} s^{-1} was 51.7 but the role of kleptoplasty in this is not known [128]. Intraspecific variability in the growth rates, mixotrophy and lipid composition of *Karlodinium veneficum* existed in isolates from Aklfacs Bay, in the NW Mediterranean coastal waters [42]. Growth and feeding responses also varied in two strains. More investigations utilizing cells of the same growth phase (continuous cultures) are needed to establish intra and interspecific differences in their carbon assimilation rates.

Response to Chemical Perturbation

Copper sulfate treatment inhibited photosynthesis in *Prorocentrum minimum* and is attributed to 515 genes of which 8.6 % are related to chloroplast and mitochondria. This

suggests the possibility of $CuSO_4$ caused cellular oxidative stress to photo system [129]. Photosynthetic activity of toxigenic *Alexandrium catenella* and the benthic *Ostreopsis* cf. *ovata* exposed to increasing concentrations of Cu^{2+} (10^{-4} to 31 nM) or Butylin (0.084 to 84 nM) for seven days decreased [130]. In *Prorocentrum minimum* 609 genes were identified of which 10.2% responded to PCB treatment [131]. While these were related with cell cycle, apoptosis, signal transduction, and transport pathway, photosynthesis was not inhibited.

Survival Strategies

The dinoflagellates are characterized by low division rates, low carbon assimilation rates (μg $C10^6$ cells h^{-1} , μg C μg Chl α^{-1} h^{-1}) and low α μg C μg Chl α^{-1} h^{-1}μmol m^{-2} s^{-1} which suggest they are photosynthetically inefficient. To overcome this disadvantage, the dinoflagellates have to develop a survival strategy to supplement their energy requirements. Although the supporting data are dire, we comment on how the dinoflagellates use a diverse range of strategies for long-term survival. Of the strategies, the most important one is mixotrophy as it provides a competitive advantage. Mixotrophy (phototrophy and phagotrophy) appears to be common amongst dinoflagellates. As stated earlier, Stoecker lists 46 mixotrophic dinoflagellate species belonging to Prorocentrales (4), Dinophysiales (2), Gymnodiniales (13), Noctilucales (1) Gonyaulacales (15), Peridiniales (2), Blastodiniales (5), Phytodiniales (3) and Dinamoebales (1). Jeong et al. [132] reported *Takayama helix* (family Kareniaceae) injested large dinoflagellates *Alexandrium minutum, A. lusitanicum, A. tamarense, A. pacificum, A. insuetum, Cochlodinium polykrikoides, Coolia canariensis, Coolia malayensis, Gambierdiscus caribaeus, Gymnodinium aureolum, Gymnodinium catenatum, Gymnodinium instriatum, Heterocapsa triquetra, Lingulodinium polyedrum,* and *Scrippsiella trochoidea* but not small flagellates and dinoflagellates ≥13 μm in size. The newly described *Yihiella yeosuensis* grows mixotrophically only and feeds on 2 bacillariophytes, 3 Prymnesiophytes, 2 Prasinophytes, 4 cryptophytes, 1 taphidophyte, and 6 dinoflagellates [131]. The usual storage products of dinoflagellates are starch and oil and supply the necessary energy. *Crypthecodinium cohnii*, a heterotrophic dinoflagellate produces neutral lipids (NL), glycolipids (GL) and phospholipids (PL) in the form of triacylglycerol, Docosahexaenoic Acids (DHA) and oils [65].

Other survival strategies include: a) rapid adaptation to changing conditions and with the phase of cell division, with photosynthetic rates of 54-130 μg C 10^6 cells h^{-1}) in light limiting phase 1 (60 μE m^{-2} s^{-1}) and 134 after adaptation to 210 μE m^{-2} s^{-1} in *Gonyaulax polyedra* [71], b) a 3-5 fold increase in their photosynthetic magnitude over the day through circadian rhythms in photosynthesis as in the thecate marine dinoflagellates, *Gonyaulax polyedra, Glenodinium* sp. and *Ceratium furca*, which allows them to survive even in the dark [81], c) reduction of metabolic rates by encystment and d) adjustments in carbon concentrating mechanisms, as in the dinoflagellates *Scripsiella trochoidea* and *Alexandrium tamarense* [85]. In *Gyrodinium galatheanum,* a photosynthetic, mixotrophic dinoflagellate, growth rates were 2 to 3 times higher than under strictly phototrophic conditions at the same irradiances [70]. In the cells grown under low light and/or nutrient-limited conditions, phagotrophy also enhanced photosynthetic capacity (P_{max}^{cell}), and/or by increased photosynthetic efficiency. Photosynthesis and respiration remained unaltered in *S. trochoidea* whereas respiration

increased in *A. tamarense.* In *S. trochoidea* it was facilitated by exceptionally high and CO_2-independent carbonic anhydrase activity. This strategy enables both species to maintain growth over a wide range of ecologically relevant pCO_2.

CONCLUSION

Dinoflagellates are the main constituents of toxigenic red tides and consequently impact human health and cause economic losses. Difficulties in culturing dinoflgellates hindered studies aimed at understanding their physiological ecology. Majority of dinoflagellates assimilate carbon via photosynthesis while a few heterotrophs and mixotrophs assimilate carbon in dark as well. Analyses of photosynthetic carbon assimilation patterns in the athecate, thecate and symbiotic dinoflagellates (zooxanthellae) showed that in general their photosynthesis – irradiance relationships conform to the patterns established for diatoms and microflagellates. Peridinin, an exclusive pigment to dinoflagellates, prevents photoinhibition.

Intraspecific variability in photosynthesis existed amongst strains. In general, carbon assimilation rates (μg $C10^6$ cells h^{-1}, μg C μg Chl α^{-1} h^{-1} and ngC [ng Chl *a*] $^{-1}$ h^{-1} [μ mol m^{-2} s^{-1}] $^{-1}$) in dinoflagellates were low, even lower than the scenescent cells of diatoms and microflagellates. This is perhaps due to channeling of energy for toxin production and synthesis of cellulose skeleton, suggested by the differences in the carbon: volume (pgC $cell^{-1}$ 0.288 x volume 0.811) relationships in phylogenetic groups [134]. In photosynthetic and thecate dinoflagellates carbon density was higher than in heterotrophic and athecate dinoflagellates; chlorophytes, chrysophytes, prasinophytes and prymnesiophytes have similar carbon to volume relationships. Dinoflagellates use a diverse range of strategies for long-term survival of which the most common and important one is mixotrophy which occurs in nearly 50 species and it provides a competitive advantage.

Also, dinoflagelaltes have large amount of DNA (3000–215000 Mb), which is orders of magnitude larger than other eukaryotic cells, or compared to 3180 Mb for the haploid human genome [135]. Supporting this disproportionately large DNA results in heavy metabolic requirements which may explain the lower carbon assimilation rates in dinoflagellates compared to other unicellular algae.

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Chapter 142

MIXOTROPHY IN DINOFLAGELLATES: PREY SELECTION, PHYSIOLOGY AND ECOLOGICAL IMPORTANCE

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ABSTRACT

This chapter reviews the current knowledge on "mixotrophy" among freshwater and marine dinoflagellates. The term "mixotrophy" is here used for the combination of phototrophy and phagotrophy in the same organism. Among the dinoflagellates it includes species with their own permanent chloroplasts (called constitutive mixotrophs, CMs) and species which lack their own chloroplasts and instead sequester chloroplasts from their prey (called non-constitutive mixotrophs, NCMs). We document here that mixotrophy is widespread among dinoflagellates with species representatives of both groups. Feeding may not always be expressed among the CM dinoflagellates, especially as light and nutrients impact feeding for the majority of dinoflagellates. Mixotrophic dinoflagellates primarily eat other protists, but some species can exploit large prey and metazoans as part of their diet. Some mixotrophic dinoflagellates are highly selective in which prey types they ingest, while others are quite omnivorous. Especially the NCM dinoflagellates seem to be quite restricted in which types of prey they can utilize as donors of chloroplasts and other cell organelles. Few data are available on *in situ* grazing rates of mixotrophic dinoflagellates, and there is a strong need to develop new techniques to measure *in situ* grazing rates. Development of reliable *in situ* techniques to measure feeding is not only important to assess the significance of phagotrophy as a way for dinoflagellates to harvest nutrients in inorganic nutrient limited waters, but also to assess the impact dinoflagellate mixotrophy on the food web.

Keywords: mixotrophy, phototrophy, phagotrophy, chloroplasts, grazing rates, symbionts

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INTRODUCTION

Recently, mixotrophy has been recognized as a traditionally unappreciated, but important and widespread trait of photosynthetic protists (Flynn et al., 2013; Mitra et al., 2014; Mitra et al., 2016; Stoecker et al., 2017). The term "mixotrophy" is largely self-explanatory as a mix or combination of autotrophy and heterotrophy in a single organism. Despite its seemingly straightforward meaning, a few more definitions are needed. Mixotophy is sometimes (e.g., Pringsheim, 1958; Gaines and Elbrächter, 1987; Schnepf and Elbrächter, 1992), but not here, used in a strict sense only when an organism cannot thrive by a single trophic mode, whereas the term amphitrophy refers to organisms that facultatively combine, but do not rely on, different trophic modes. Moreover, mixotrophy may include any combination of trophic modes. Heterotrophy may refer to phagotrophy and/or uptake of dissolved organic substrates (often termed osmotrophy, although osmotic pressure processes are not involved). In the protistan realm, however, the uptake of organic molecules (e.g., amino acids) is ubiquitous so using osmotrophy as a discriminating feature of mixotrophy in protists appears weak, as de facto all protist would be mixotrophs by such a definition (Flynn et al., 2013; Mitra et al., 2014; Mitra et al., 2016; Stoecker et al., 2017). We thus use here the definition of Mitra et al. (2016) with mixotrophy as the dual capability of engaging phototrophy and phagotrophy by a single cell, regardless of being obligate (i.e., mixotrophy sensu stricto) or facultative (i.e., amphitrophy).

Dinoflagellates as a group are well known for their high diversity of lifestyles and trophic modes, and have been traditionally divided into autotrophic and heterotrophic species. However, we are now aware that such a classification is not appropriate (Jeong et al., 2010b; Hansen, 2011), as many species belonging to either group may actually combine phototrophy and prey uptake, and thus be mixotrophic. Studies of geographic distribution of mixotrophic protists on the global scale have shown that mixotrophic dinoflagellates are ubiquitous. They are common in polar, temperate, subtropical and tropical freshwater and seawater (Leles et al., 2017; 2019). Very few studies have dealt with *in situ* grazing rates of mixotrophic dinoflagellates, simply because rates are difficult to measure due to prey selectivity and low feeding frequency.

Dinoflagellate mixotrophy has been the subject of a number of reviews in the past. Most of the earlier reported cases of mixotrophy in dinoflagellates were limited to observations of what was interpreted as "food vacuoles" (reviewed by Hansen, 1998; Hansen and Calado, 1999; Stoecker, 1999). At that time, very little was known about feeding mechanisms, prey preferences, or the role of food uptake. Since then, many more protists have been studied, especially the dinoflagellates, and some of this knowledge has been presented briefly in recent broad review papers (Burkholder et al., 2008; Stoecker et al., 2009) or in reviews specifically on dinoflagellates (Jeong et al., 2010b; Hansen, 2011). The review by Jeong et al. (2010) treated current knowledge of prey preferences and scaling of ingestion and growth rates among heterotrophic and mixotrophic dinoflagellates (Jeong et al., 2010b), while the review by Hansen (2011) treated the importance of photosynthesis and food uptake for growth of marine planktonic dinoflagellates, dealing with different functional groups within the dinoflagellates. Since then knowledge on dinoflagellate mixotrophy has increased considerably, especially for species that sequester chloroplasts and other cell organelles from their prey.

This review will give a short historical survey of dinoflagellate mixotrophy research and discuss how common mixotrophy is among the dinoflagellates. To do this, we have compiled literature we could find on dinoflagellates in which mixotrophy has been indicated and provide comprehensive tables summarizing all the published information. We then provide some details concerning feeding mechanisms, prey size and prey specificity, and discuss the role of environmental controls on the physiology of mixotrophic dinoflagellates. We specifically focus on how light and inorganic nutrients affect feeding and growth among the different types of mixotrophic dinoflagellates. Finally, we discuss the influence of mixotrophic dinoflagellates as grazers in natural waters.

A SHORT HISTORICAL SURVEY OF DINOFLAGELLATE MIXOTROPHY RESEARCH

Since the dawn of dinoflagellate research, the phylogenetic placement and the trophic nature of these peculiar unicellular organisms have been a matter of debate and controversy. The plant/animal dichotomy of macroscopic life in the 18th century understandably was translated to all the newly discovered microorganisms. In analogy to the macro-world, many researchers considered motility of microscopic organisms as a clear sign for their animal nature, and consequently the search for evidence of food uptake was in focus early on. So, records and observations of ingested food (or particles) in dinoflagellates can be found in the very early literature.

The earliest observation probably comes from Christian Gotthilf Ehrenberg (1795-1876), who reported his use of carmine and/or indigo suspensions to systematically study particle uptake by protistan species (Ehrenberg, 1830). For the dinoflagellates, which he at the time considered as "Kranzthierchen," Ehrenberg reported uptake of indigo particles by two freshwater species, *Peridinium pulvisculus* and *P. cinctum*. While Ehrenberg was wrong with some of his ideas about unicellular organisation (e.g., he considered chloroplasts to be the female ovaries), he was right that particle uptake may be regarded as an "animal character" in dinoflagellates. *Peridinium cinctum* doubtlessly has chloroplasts (see Figure 1 A), and original drawings of Ehrenberg for *P. pulvisculus* imply chloroplasts for that species as well (Ehrenberg, 1838). Thus, this seems to be the first documented cases of dinoflagellate mixotrophy, although not recognized as such by Ehrenberg. Subsequent observations of ingested food inside of dinoflagellates by e.g., Schmarda (1854), Stein (1878), Bergh (1881), and Schilling (1891) mainly refer to heterotrophic species. However, Friedrich Ritter von Stein (1818-1885), who -like Ehrenberg- considered all dinoflagellates as animals, described food particles ("große grüne gefressene Körner" [large, green ingested grains]) inside the freshwater species *Hemidinium nasutum* (Stein, 1878), which is a phototrophic species with its own chloroplasts. Stein explicitly mentioned the yellowish colour of that species. Another early hint for dinoflagellate mixotrophy comes from Gourret (1883), who described the presence of an algal fragment inside of the phototrophic species *Ceratium tripos* (=*Tripos muelleri*). Likewise, Kaarlo Levander (1867-1943) in 1894 observed inclusions inside of the phototrophic species *Gymnodinium fissum* (now regarded conspecific with *Levanderian fissa*, see Moestrup et al., 2014), which he described and depicted as a diatom and a long spiny particle of unknown origin (Levander, 1894).

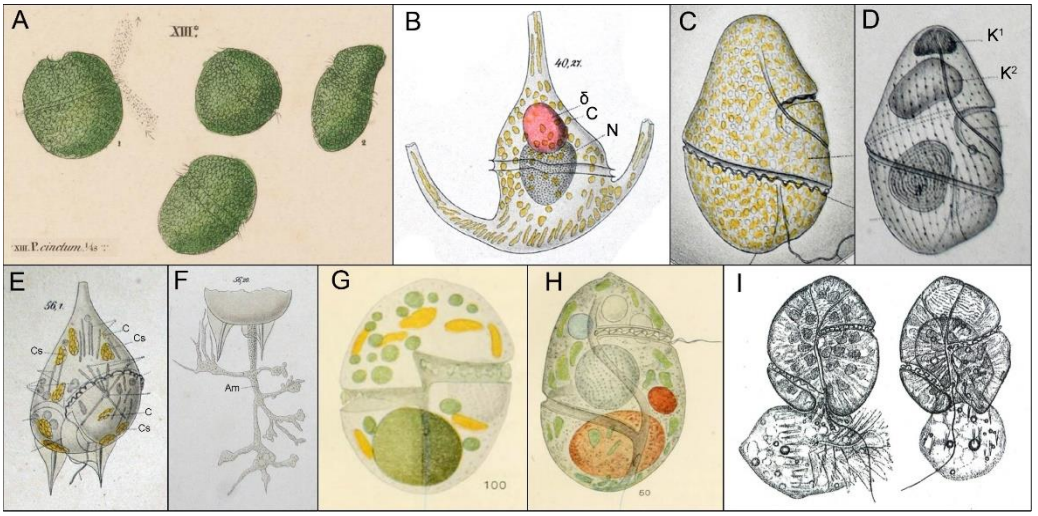

Figure 1. Some historic drawings of mixotrophic dinoflagellates. A: *Peridinium cinctum*, (redrawn from Ehrenberg 1838, Tafel XXII, Figure XIII). This species was described by Ehrenberg (1830) to take up indigo particles. B–F: Various drawings of dinoflagellate species with inclusions, redrawn from Schütt (1895). B: *Tripos muelleri* (as *Ceratium tripos*, Tafel 10, 40.27). Schütt´s abbreviations: δ = δ-oil, C = chromatophore, N = nucleus. C–D: *Cochlodinium pirum* (as *Gymnodinium pirum*, Tafel 23, 76.1 (D) and 76.1 (C)) Note the many chloroplast in C, and the presence of two different inclusions K^1 and K^2 in D. E–F: *Podolampas bipes* (Tafel 19, 56.1 (E) and 56.20 (F). Schütt´s abbreviations: C = chromatophore, Cs = chromatosphere (clumps of chromatophores), Am = amoeboid plasma. G, H: Drawings of two dinoflagellate species with inclusions, redrawn from Kofoid & Swezy (1921). G: *Gymnodinium flavum* (Plate 9, Figure 100), original legend: "…Note the presence of food body and yellow chromatophores….".H: *Gyrodinium melo* (Plate 5, Figure 50), original legend: "…Note the presence of both food bodies and green chromatophores…".I: *Levanderia fissa* (as *Gyrodinium pavilardii*), redrawn from Biecheler (1952), Figure LXIX. Two different stages *of G. pavilardii* ingesting a *Strombidium* cell.

In contrast to Ehrenberg and Stein (among others), Franz Schütt (1859-1921) considered dinoflagellates strictly as plants. Consequently, in all his descriptions and illustrations (e.g., Schütt, 1895), he interpreted any unusual inclusion inside dinoflagellate cells as either conglomerates of plastids ("chromosperes") or as "Klumpen" [clumps], which he thought were endogenous storage products (either oil or starch) (see Figure 1 B–F). Schütt not only described such clumps inside naked heterotrophic species known at his time as phagotrophs (e.g., *Gyrodinium spirale*), but also in a number of species with chloroplasts, and even for some thecate species (e.g., *Dinophysis*, *Podolampas*). Other researchers (Dogiel, 1906; Entz, 1907) were thus prompted to consider Schütt´s observation as evidence that phagotrophy was in fact quite widespread in dinoflagellates. The presence of foreign bodies inside phototrophic species was also noted by Entz (1907) for *Ceratium hirudinella*, *Gonyaulax spinifera*, and *G. polygramma* based on LM observations using thin sections.

Charles Atwood Kofoid (1865-1947) and Olive Swezy (1878-??) compiled and illustrated all available information on naked dinoflagellates (Kofoid and Sweezy, 1921). They put much emphasis on including all information on the presence of chloroplasts and ingested food particles visible inside the cells, and explicitly identified nine species with chloroplasts as also being phagotrophs (i.e., mixotrophic) (Table 1, see also Figure 1 G, H). Other researchers more specifically worked on the nutrition of individual species. For example, Hofeneder

(1930) extensively worked on the animal nutrition of *Ceratium hirudinella,* a freshwater phototrophic dinoflagellate. While some of his observations (e.g., on extruding plasma networks) may be artefacts, his descriptions of inclusion bodies of different sizes identifiable as "Cyclotellen" (i.e., diatoms) and *Vorticella* (a ciliate) is convincing evidence that *Ceratium hirudinella* indeed is mixotrophic. So, all these observations led Schiller (1933) in his encyclopedic compilation on dinoflagellates to shortly summarize dinoflagellate nutrition as: "Trophic mode variable, at times even within the same species; either holophytic (autotroph), holozoic or mixotroph, often also saprophytic..." He was thus probably the first to use the term "mixotroph,"which originally was coined by Pfeffer (1897) for higher plants, in a dinoflagellate context.

The actual ingestion process of mixotrophic dinoflagellates probably was observed first and described in detail in the fascinating work of Berte Biecheler (Biecheler, 1936b, a), which was also published posthumously with detailed drawings 20 years later (Biecheler, 1952). She described *Gyrodinium pavillardii* (now considered to be identical to *Levanderina fissa*, see Moestrup et al., 2014) ingesting other small dinoflagellates and ciliates (see Figure 1 I). Likewise, for her newly described phototrophic species *Gyrodinum vorax* (note the name vorax means voracious), Biecheler observed ingestion of a large dinoflagellate (and, ironically, here *Gyrodinium pavillardii* was the prey) using a peduncle (a tube, see the section on feeding mechanisms). Among the early workers on freshwater dinoflagellates, the pioneering early work of Harris (1940) has to be highlighted. He systematically included observations on nutritional mode in his compilation of freshwater dinoflagellates of the British Isles, listing six species with chloroplasts and ingested food (Table 1).

During the last decades of the 20th century, ultrastructural studies of a number of dinoflagellate species (largely motivated by theories of an endosymbiotic origin of chloroplasts) documented for the first time that what was originally described as chloroplasts were in fact symbionts, or suggested them to be "endosymbiont-like phagocytotic vacuoles" (Wilcox and Wedemayer, 1984, 1985; Larsen, 1988; Schnepf et al., 1989). Schnepf et al. (1989) suggested for the first time the term "cleptochloroplasts," in analogy to the "cleptocnides" of some molluscs. At roughly the same time, new conceptual and methodological developments in the area of microbial ecology in the 1980th (e.g., the "microbial loop": Azam et al., 1983) led to an increased interest among marine ecologists in prostistan phagotrophy, including mixotrophic phytoflagellates (Baird and Kalff, 1986; Boraas et al., 1988; Porter, 1988; Sanders and Porter, 1988). For dinoflagellates, this interest in mixotrophic species coincided with a general interest in dinoflagellate food uptake mechanisms (Schnepf and Deichgräber, 1983; Gaines and Taylor, 1984; Jacobson and Anderson, 1986), which led to a rapid progress in revealing the great trophic diversity of dinoflagellates (Gaines and Elbrächter, 1987; Schnepf and Elbrächter, 1992; Jacobson and Andersen, 1994; Jacobson and Anderson, 1996; Jacobson, 1999). In the last few decades, with advanced cell isolation-and culture techniques, more and more detailed studies and observations of mixotrophy of many dinophyte species emerged (e.g., Jeong et al., 2010b). Dinoflagellate research on mixotrophy thus ultimately developed from a descriptive to an experimental and quantitative stage, now providing many data on e.g. growth, ingestion, and food spectrum for a variety of species (Tables 1, 2).

MIXOTROPHY IS A WIDESPREAD PHENOMENON AMONG DINOFLAGELLATES

Mixotrophy can be displayed in different ways among the dinoflagellates. The dinoflagellates that have permanently built-in chloroplasts do not acquire chloroplasts or any other cell organelles from their prey. Instead, these utilize the ingested material for improved growth. Such organisms have recently been referred to as constitutive mixotrophs, CMs (Mitra et al., 2016) (Figures 2, 3).

Figure 2. Light microscopy images of some important (mostly bloom-forming) constitutive-mixotrophic dinoflagellate species. A: *Akashiwo sanguinea*. B: *Karlodinium veneficum*. C: *Prorocentrum micans*. D: *Karenia mikimotoi*. E. *Tripos fusus*. F: *Prorcentrum cordatum*. G. *Heterocapsa steinii*. H. *Gymnodinium catenatum*. I. *Tripos furca*. J: *Protoceratium reticulatum*. K: *Heterocapsa rotundata*. L: *Scrippsiella acuminata*. M: *Dissodinium pseudolunula*. (a mixotrophic parasite of copepod eggs). N: *Alexandrium catenella*. O: *Levanderina fissa* (picture courtesy: Niels Daugbjerg). Scale bars = 5 µm (B, F–G, J–L) or 10 µm (A, C–E, H–I, M–O).

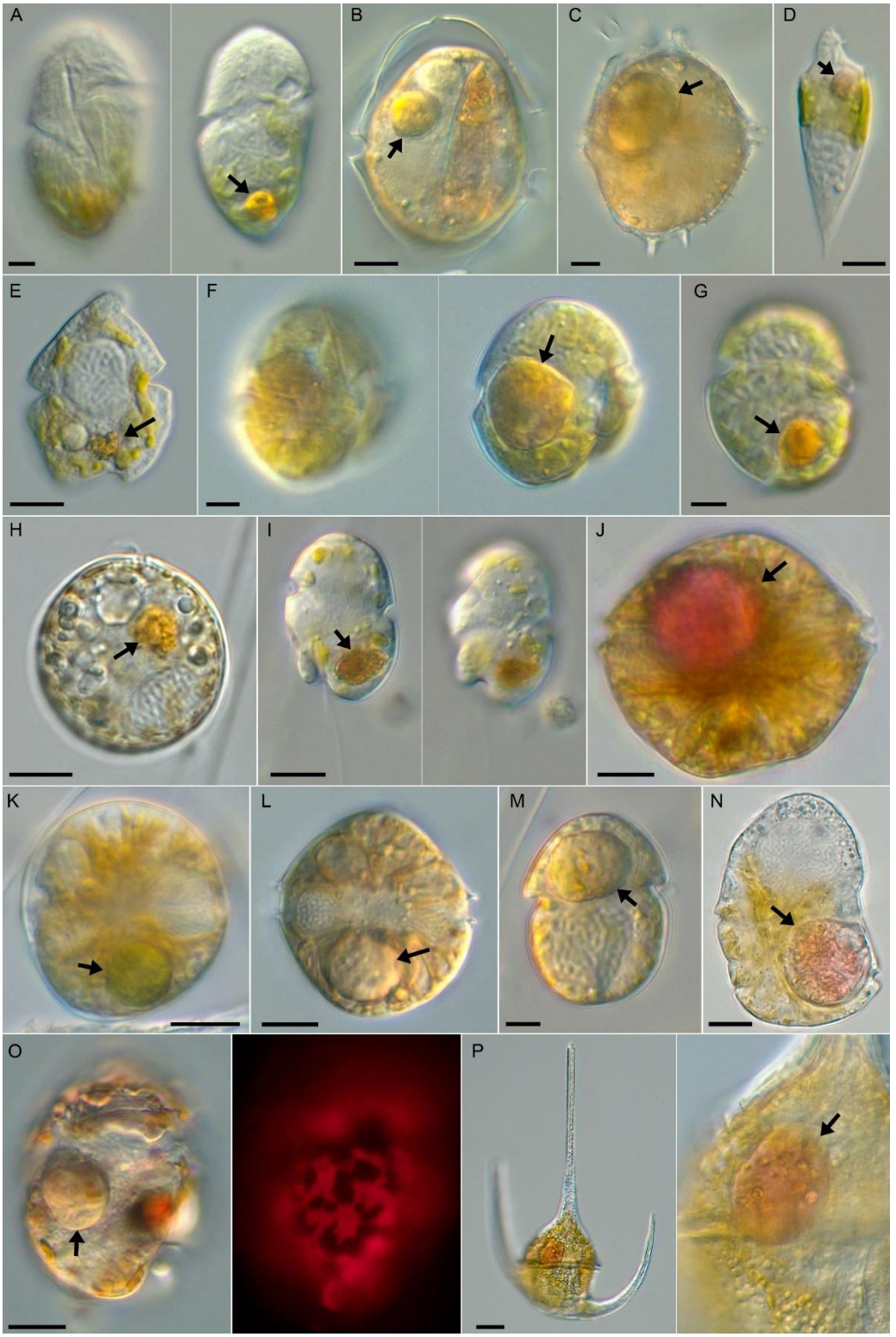

Figure 3. (Continued).

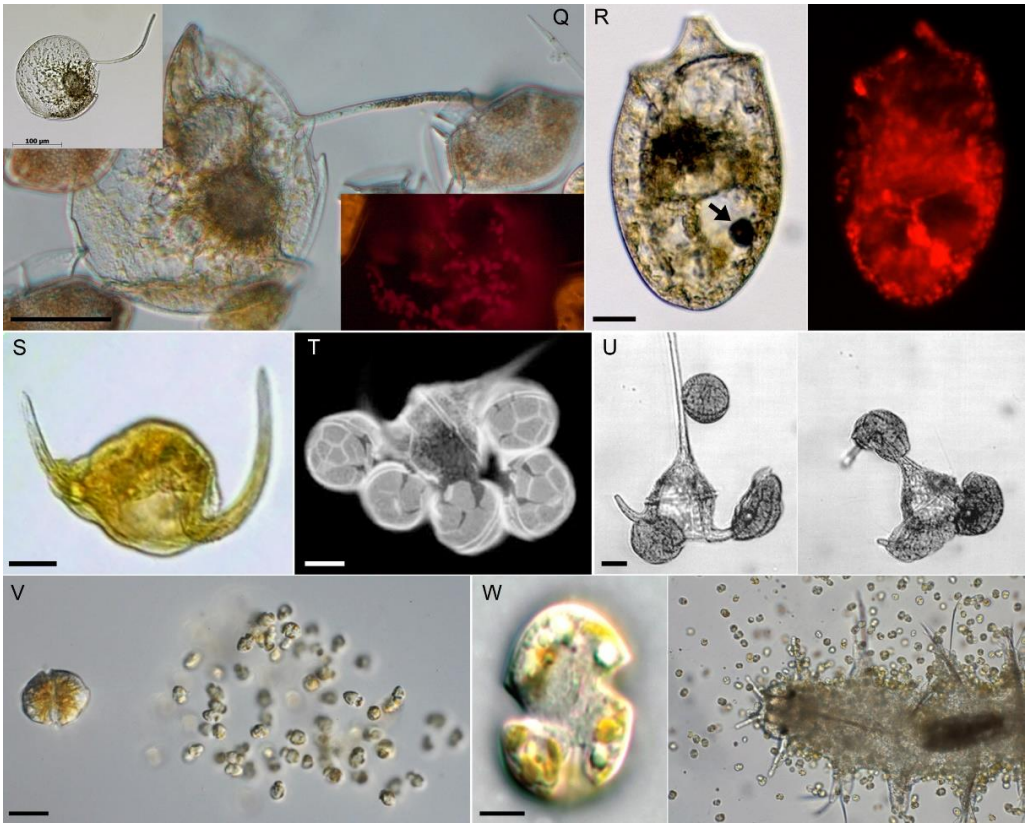

Figure 3. Collection of dinoflagellate field sample specimens with (food?) inclusions (arrows) (**A–Q**), or from algal cultures (**S–W**). **A**: Two focal planes of an unidentified gymnodiniod species. **B**: Unidentified thecate species (likely to be *Fragilidium* sp?). **C**: Unidentified species of *Gonyaulax*. **D**: *Oxytoxum* cf. *caudatum*. **E**: Unidentified gymnodinioid species. **F**: Two focal planes of an unidentified gymnodinioid species. **G**: Unidentified gymnodinioid species. **H**: Unidentified species of a planktic *Prorocentrum.* **I:** Two focal planes of an unidentified gymnodinioid species (likely to be a *Margalefidinium*?). **J**: *Alexandrium pseudogonyaulax*. **K**: *Alexandrium* sp. **L**: *Alexandrium ostenfeldii*. **M**: Unidentified gymnodinioid species. **N**: Unidentified cell of *Margalefidinium*. **O**: an unidentified species of *Nematodinium* in bright field (left) and epifluorescence (right) to indicate presence of chloroplasts. **P**: Two views of the same cell of *Tripos muelleri*. **Q**: Mature cell of *Spatulodinium pseudonoctiluca*. The upper left insert shows a total view of this large tentacle-bearing species. The lower left insert shows a fluorescent view of the cell to indicate the presence of chloroplast. **R:** An immature stage of *Spatulodinium pseudonoctiluca* in bright field (left) and epifluorescence (right) to indicate presence of chloroplasts. **S–U**: *Fragilidium subglobsoum* ingesting *Tripos muelleri*. **S**: Late stage of ingestion, note that the two short horns of *T. muelleri* are not yet ingested. **T**: Epifluorescence view of calcofluor stained cells of five *Fragilidium* during ingestion. Note how thecal plates of *Fragilidium* separate to allow for increase in size. **U**: Two different stages of three cells of *F. subglobosum* attacking and ingesting a *T. muelleri* cell. **V**: *Alexandrium pseudogonyaulax* attached to a mucus trap with many immobilizes cells of *Heterocapsa rotundata*. **W**: *Karlodinium armiger*, whole cell (left) and low magnification view (right) of *K. armiger* cells feeding on an immobilized polychaete larva. Scale bars = 5 µm (A, D, F, G, M, W (left)), 10 µm (B, C, H, E, I, K, L, N, O, J), 20 µm (P, R, S–U, V), or 50 µm (Q).

Other dinoflagellates lack chloroplasts of their own, but instead acquire/sequester chloroplasts from their prey. Such mixotrophic organisms are now referred to as Non-Constitutive Mixotrophs (NCMs; Mitra et al., 2016) (Figure 4). This NCM group has been

subdivided into prey specialist (SNCM) and prey generalist (GNCM). Some of the SNCM dinoflagellates harbor intact symbionts, either as endosymbionts (i.e., the green *Noctiluca* and *Amphisolenia* spp.) or as ectosymbionts (i.e., *Histioneis*, *Ornithocercus*, *Amphisolenia*) (Hansen et al., 2004; Tarangkoon et al., 2010), and are labelled eSNCMs. Other species take up algal prey and digest parts of the ingested cells, whereafter they keep these "reduced" endosymbionts for some time (days to a month) (i.e., *Gymnodinium gracilentum* and *Nusuttodinium* spp.) (Skovgaard, 1998; Drumm et al., 2017), or just sequester the chloroplasts, while remaining parts of the ingested cells are digested (i.e., *Dinophysis* spp.) (Hansen et al., 2013). These two groups have previously been referred to as pSNCMs (Mitra et al., 2016), but here we use the term reSNCMS to separate out the species that have reduced endosymbionts.

A thorough literature search indicates mixotrophic capabilities for ~ 249 chloroplast-bearing species belonging to 91 genera (Tables 1–3), of which approx. 202 species are marine, while the rest are freshwater species. Thus, mixotrophy among dinoflagellates is quite widespread. Of these species, 116 can be considered CMs (Table 1), while 109 are NCMs (Table 2), and 24 are parasites (Table 3). Of the NCMs, 75 species have entire endo/ectosymbionts (eSNCMs), 26 species have reduced endosymbionts (reSNCMS) and 8 only harbour the chloroplasts from their prey (pSNCMs) (Table 2.)

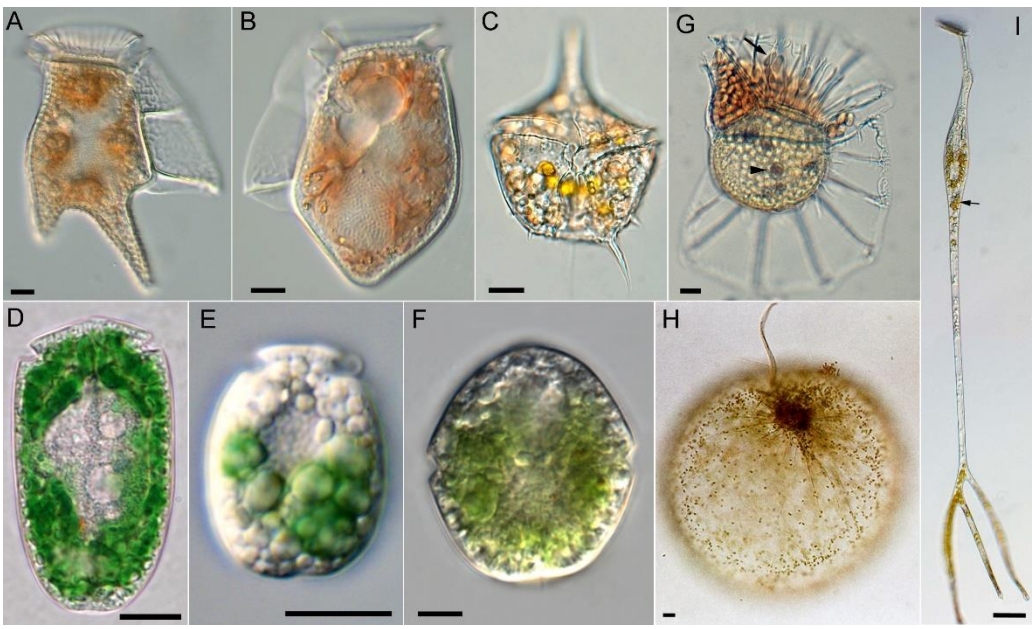

Figure 4. Light microscopy images of some selected non-constitutive mixotrophic dinoflagellate species. A: *Dinophysis tripos*. B: *Dinophysis acuta*. C: *Amylax triacantha*. D: *Gymnodinium myriopyrenoides* (picture courtesy: Myung Gil Park). E: *Amphidinium poecilochroum* (picture courtesy: Myung Gil Park). F: *Nusuttodinium aeruginosum* (picture courtesy: Niels Daugbjerg. G: *Ornitocercus steinii* (picture courtesy: Gert Hansen). H: green *Noctiluca scintillans*. I: *Amphisolenia thrinax* (picture courtesy: Gert Hansen). Scale bars = 10 µm (A–G) or 50 µm (H, I).

Howewever, it is important to note that prey uptake has not yet been documented for some NCMs listed in Table 2, which host endosymbionts (e.g., *Amphisolenia*/*Triposolenia*). Some of the CMs, e.g., the dinotoms (dinoflagellates harboring reduced diatom

endosymbionts), can be grown in monocultures without the supply of algal prey. To which extent these can still ingest prey is unknown. Recently, it has been shown that at least one of the dinotom species cannot maintain the endosymbiont, but relies on continual ingestion of a diatom (Yamada et al., 2019), making this species belonging to the NCMs.

In quantitative terms, the list of course is heavily biased for example by a large number of parasitic CM species (and thereby are easily classified as mixotrophs), or by the large number of warm-water species with ecto-and endosymbionts (eNCMs), which are over-represented because species determination with light microscopy is possible in this case. Likewise, restrictions in the list of mixotrophic species are likely due to lack of cultures for many species and difficulties in species determination for field collected phototrophic dinoflagellates with food inclusions (e.g., Figure 3 A–N). It is important to note that the evidence in the literature for phagotropic capabilities for the different species is of course quite varied. For a large number of species, LM studies of field samples report the presence of "food vacuoles," "ingested food," or vague descriptions of other unusual structure such as "inclusion bodies" or "accumulation bodies" inside of cells. However, the presence of "particles" inside a dinoflagellate cell does not necessary prove phagotrophy, as other explanations such as internal storage products or endosymbiotic bacteria have to be taken into account. Some inclusions of dinoflagellates may involve endoparasitic infections (Jacobson, 1999) such as early or late trophocyte stages of *Parvilucifera* (Alacid et al., 2016) or *Amoebophrya*. Moreover, autolytic accumulation bodies may also be present in non-phagotrophic dinoflagellates (Lee, 1977; Schnepf and Elbrächter, 1992), and orange-fluorescent PAS bodies (named because these are stained by the periodic acid-Schiff reaction) e.g., in *Alexandrium* spp., which resemble e.g., ingested cryptophytes because of their orange autofluorescence, have been linked to autophagy or to a digestive use of stored metabolites (Schmitter and Jurkiewicz, 1981).

Thus, many cases of limited evidence listed in Table 1 are in need of confirmation by more detailed direct observations and/or methods that are more specific. For example, clear LM identification of intracellular inclusions as ingested prey is possible using protargol-staining (Bockstahler and Coats, 1993a), or thin sections and transmission electron microscopy (TEM) can give information of the presence of food vacuoles and even the type of ingested prey cells (Jacobson and Anderson, 1996). Other methods that can be applied to field samples include the use of adding labelled prey (i.e. using either fluorescent or radioactive labelled prey) that subsequently can be detected in target cells (Nygaard and Tobiesen, 1993).

For a number of species included in Table 1, phagotrophy based on ingestion of food has not yet been observed directly, or indirectly. In those cases, phagotrophy was suggested based on ultrastructural evidence for the presence of a microtubular basket (e.g., Schnepf and Winter, 1990), which dinoflagellates that use a feeding tube always have. Here it has to be kept in mind that we currently do not know if such a structure may serve also for other cellular functions, e.g., sexuality. With a few exceptions (Biecheler, 1952), older studies indicating mixotrophy in dinoflagellates provide little information on the actual ingestion process. In the last couple of years, however, increased interest in mixotrophy and improved algal culture techniques have led to a significant number of culture-based studies providing detailed new observations and experimental data for many dinoflagellate species, including identification and documentation (sometimes with videos, see Table 1) of the actual feeding process. The majority of these are on marine species and comparable studies for freshwater

species (e.g., *Bernardinium*: Fawcett and Parrow, 2014; and *Nussotodinium*: Drumm et al., 2017) seem to be exceptions.

The compilation of data on dinoflagellates revealed that evidence for mixotrophy is available for most of the higher taxonomic groups (orders, see Table 1–3), with a lack of records only for a few poorly known orders having a limited number of genera/species (e.g., Ptychodiscales, Desmomastigales, Gloeodiniales, Lophodiniales, Haplozoodiniales). Almost all of the basal dinoflagellates are heterotrophic, but mixotrophy can be found here as well: within Noctilucales, the green form of *Noctiluca scintillans* harbours endosymbiotic phototrophs (Sweeney, 1976). Moreover, life stages of *Spatulodinium pseudonoctiluca*, including the gymnodinoid stage also described as a separate species (*Gymnodinium lebourae*) and the large round *Noctiluca*-like stage that feeds with a large tentacle, contain plastids (see Figure 3 O, P), and thus are probably the deepest branching CM-dinoflagellates (Gómez et al., 2010). Although most of the parasitic dinoflagellates are heterotrophic, 24 species from 10 genera are known to contain plastids (Table 3). While heterotrophy in these mixotrophic parasites is obvious, the role of photosynthesis is less well known, but may be important for survival during their dispersal stage. There are of course dinoflagellate genera without known mixotrophs, such as most of the parasitic forms that lack chloroplasts. Pure heterotrophy in pallium feeding genera (see section 3.2) such as *Protoperidinium, Oblea*, *Diplopsalis* etc. may indicate that this feeding mechanism is not compatible with mixotrophy. However, the minute drawings and descriptions of Schütt for *Podolampas bipes* (Schütt, 1895, see Figure 1 E, F) present substantial evidence that this species is photosynthetic and performs pallium feeding. Phototrophic abilities of *P. bipes* (and of *P. reticulata* as well) were later shown to be based on the presence of eukaryotic dictyochophycean endocytobionts (Schweikert and Elbrächter, 2004). Of course, there are also genera of phototrophic species where mixotrophy is yet undescribed and unknown (e.g., *Azadinium*, *Amphidoma*), but this is likely because detailed studies for these genera have not yet been performed.

PREY TYPE, FEEDING MECHANISMS, PREY SIZE AND PREY SELECTIVITY

Prey Types

The most common prey for mixotrophic dinoflagellates are other protists, but some reports also show uptake of small prokaryotic prey (single cells of bacteria and cyanobacteria; 0.5-2 µm) and very large prey, including metazoans like copepods and larvae of benthic invertebrates (see reviews by Jeong et al., 2010 and Hansen, 2011). In the large majority of studied cases, however, other protists are the ecologically relevant prey types. The role of heterotrophic bacterial uptake in dinoflagellates is still controversial, and data demonstrating increased growth at natural heterotrophic bacterial concentrations and sizes of bacteria are lacking. However, significant uptake rates of small cyanobacteria (*Synechoccoccus* sp.) and elevated growth rates have been reported for *Karenia brevis* (CM) cultures that have been starved of major nutrients (Glibert et al., 2009).

Feeding Mechanisms, Prey Capture and Prey Size

Three types of feeding mechanisms occur among dinoflagellates: direct engulfment, tube feeding, and pallium feeding. The pallium-feeders have almost exclusively been found among heterotrophic dinoflagellates (see the discussion in the previous paragraph), but the two other feeding mechanisms are common among mixotrophic dinoflagellates (see Tables 1, 2). Prey size matters for dinoflagellates (Hansen et al., 1994; Jeong et al., 2010b). However, entire prey size spectra have so far only been investigated for heterotrophic dinoflagellates (Hansen, 1992; Naustvoll, 2000a, b). Dinoflagellates typically detect their prey before ingestion. When encountering a prey item, dinoflagellates will typically change swimming behavior (Jacobson and Anderson, 1986; Hansen and Calado, 1999). In some cases, it has been shown that dinoflagellates will respond to chemical cues (e.g., Hansen and Calado, 1999; Martel, 2006; Berge et al., 2012), but to what extent they are dependent on a chemical cue is presently unknown. Following the detection of a given prey, many dinoflagellates use a capture or tow filament (similar to a harpoon) to attach to the prey cells. In most cases, we do not know the nature of these capture filaments; however, they have been demonstrated to be related to nematocysts/trichocysts in some cases (Gavelis et al., 2017). The use of a capture filament allows dinoflagellates to catch motile and in some case fast swimming prey, like ciliates for instance. Some dinoflagellates that eat large prey cells do not seem to require a capture filament or any other attachment techniques at all (i.e., *Fragilidium* spp., Skovgaard, 1996b; Jeong et al., 1997).

Dinoflagellates generally ingest single particles and thus localization/detection of prey items is essential. Consequently, lower prey size limit exist (Hansen, 1992; Hansen et al., 1994; Jeong et al., 2010b). This is why uptake of bacteria in mixotrophic dinoflagellates is generally of minor importance, at least in terms of carbon supply. Uptake of bacteria might play a role in acquisition of nutrients (N, P), vitamins or trace metals, but this needs to be explored further in the future. An upper prey size limit is found among species that take up prey by direct engulfment. The upper prey size limit for mixotrophic species seems to be somewhat lower than for purely heterotrophic species (see review by Jeong et al., 2010), which have been reported to be able to ingest particles five times their initial cell volume (Hansen, 1992). Nevertheless, mixotrophic *Fragilidium* can ingest prey items that are double their own initial cell volume (Hansen and Nielsen, 1997; Park and Kim, 2010). An upper size limit does not seem to exist for at least some species that use a feeding tube to suck out the contents of the prey. Examples of this have been found in the case of *Karlodinium* species, like *Karlodinium armiger*, which have been shown to kill and ingest copepods and invertebrate larvae that are >100 times larger in linear dimensions (Berge et al., 2012) (see Figure 3 W).

Some species of mixotrophic dinoflagellates are very selective, and will only ingest certain prey items, even if other available prey is within the right size range. Examples of this include *Fragilidium* species (all CMs). It has even been shown that the prey items fed upon differ among *Fragilidium* species (Hansen and Nielsen, 1997; Jeong et al., 1997; Jeong et al., 1999; Park and Kim, 2010). Among the NCMs, *Dinophysis* spp. have been shown to feed only on ciliates of the genus *Mesodinium* (Hansen et al., 2013). However, many species have proven to be much more versatile and to ingest a wide range of different prey items within a certain size range (Jeong et al., 2010, Berge et al., 2012). Examples of omnivorous species include common bloom-forming species like *Akashiwo sanguinea*, *Lingulodinium polyedra*

and *Prorocentrum micans* (Jeong et al., 2010). The most extreme species is *Karlodinium armiger*, which utilizes a huge prey size spectrum, from small 4 µm flagellates to mm-cm large metazoans, like copepods and nematodes (Berge et al., 2010, 2012).

Toxin or Mucus Assisted Prey Capture

Some dinoflagellates appear to use toxins to immobilize their prey cells. An example of this is *Karlodinium armiger*, which produce a toxin, karmitoxin (Rasmussen et al., 2017). The chemical structure of this toxin resembles the karlotoxins (from *K. veneficum*) and amphidinols (from *Amphidinium carterae*). *Karlodinium armiger* is an excellent prey for planktonic grazers like copepods (Berge et al., 2012) when it occurs in low cell concentrations. Adult female copepods produce viable eggs when fed this alga, and the copepod nauplii are able to exploit it as prey as well. However, if the *K. armiger* exceeds a certain cell concentration, the food chain is reversed, and the dinoflagellate becomes the predator of copepods. The dinoflagellate not only immobilizes and feeds on adult copepods (using a feeding tube), it also feeds on the eggs, nauplii and even the fecal pellets of the copepods (Berge et al., 2012). Karmitoxin leaks to the surrounding water. However, relatively high concentrations of karmitoxin are required to observe lysis of target cells and organisms (EC_{50}: 100-400 nM; Andersen et al., 2017). Videos indicate potential transfer/application of the toxin to the prey either by the close contact of the *K. armiger* cells or through the feeding tube (Berge et al., 2012). *Karlodinium armiger* has recently also been shown to kill adult mussels (*Mytilus edulis*) as well as mussel embryos and larvae (Binzer et al., 2018). Lytic extracellular compounds of yet unknown chemical configuration that immobilize and kill other protists are also known for a number of gonyaulacoid species, *Alexandrium* spp. (Tillmann and Hansen, 2009), *Protoceratium reticulatum* (Sala-Peréz et al., 2016), *Fragilidium* spp. (Tillmann et al., 2008; Park and Kim, 2010), and thus are probably linked to phagotrophy of these species. This has in fact been shown experimentally for the photosynthetic *Alexandrium pohangense*, which chemically immobilizes the fast swimming *Margalefidinium polykrikoides* prey prior to ingestion (Lim et al., 2015).

Other dinoflagellates utilize mucus to catch their prey, which then may include relatively small cells that otherwise the dinoflagellates are unable to catch efficiently. This phenomenon is expressed in different ways. In *Noctiluca scintillans* mucus is secreted on the tip of its tentacle, to which prey cells get caught prior to ingestion (Kiorboe and Titelman, 1998). Other species, i.e., *Dinophysis* spp., secrete clumps of mucus that float freely around in the surrounding water. Prey cells, in this case the fast jumping ciliate *Mesodinium rubrum*, are caught and immobilized in the mucus (Mafra et al., 2016; Ojamäe et al., 2016; Papiol et al., 2016). This allows *Dinophysis* spp. to localize prey cells and to start feeding directly on the ciliates using a feeding tube and thus not requiring a capture filament.

Another way of utilizing mucus to capture preys is to form a "mucus trap" to which the dinoflagellate is attached. In *Alexandrium pseudogonyaulax*, mucus is secreted at the tip of the longitudinal flagellum and the mucus trap enlarges as prey cells are caught in the trap (Blossom et al., 2012; Blossom et al., 2017) (see Figure 3 V). The dinoflagellate can then ingest trapped single cells individually, while still carrying the other prey cells caught in the mucus trap. When the trap has reached a certain size it is abandoned, and the dinoflagellate

starts to make another one. Since the prey cells are immobilized, it has been speculated that the dinoflagellate may have excreted toxins in the mucus.

ENVIRONMENTAL CONTROLS OF FEEDING, GROWTH AND PHOTOSYNTHESIS

The utilization of food uptake may serve many different roles in CM and NCM dinoflagellates. While some dinoflagellates will always ingest prey, others will only take up prey if limited by environmental factors (e.g., nutrient limitation). In the following sections, the roles of irradiance, prey concentration, inorganic nutrients, and strain differences for CM and NCM dinoflagellates are discussed.

Constitutive Mixotrophs (CMs)

The CM dinoflagellates almost all have the standard dinoflagellate "peridinin-type" chloroplasts. Exceptions are a few dinoflagellates that have permanent aberrant chloroplasts and photo-pigments originating from green algae (*Lepidodinium* spp.) and diatoms (the "dinotoms", i.e., species of *Kryptoperidinium, Durinskia, Unruhdinium, Dinotrix, Galaedinium* and *Blixea*) (Hansen, 2011; Gottschling, 2017). While the green chloroplasts of *Lepidodinium* spp. are the only prey-derived organelles retained, the dinoflagellates that harbour diatom chloroplasts also have cytoplasm and cell organelles like mitochondria and nuclei derived from diatoms. These species can generally be grown as monocultures using standard algal growth medium (like the f/2 or L medium) without the supply of algal prey. To which extent these species can ingest prey is unknown. Recently, however, it has been shown that one of the dinotom, *Durinskia capensis*, sequester the symbionts from the ingestion of a diatom making this dinotom species a member of the NCMs (Yamada et al., 2019). Generally, the CM dinoflagellates tend to split into two "types" with regard to their feeding responses and growth as a function of irradiance and food concentration (Hansen, 2011): *Type 1 and Type 2.*

The type 1 CM group is comprised of primarily phototrophic species that only marginally increase their growth rates even at low irradiances and food in excess (Hansen, 2011; Lim et al., 2018), when nutrients are plentiful (Figure 5). This means that carbon uptake via food uptake cannot replace carbon uptake from photosynthesis, and food uptake plays only a little role for these dinoflagellates for their carbon budget. However, the small growth increases observed are achieved at relatively low prey abundances (often ~100–200 µg C l^{-1}). This group includes common "red tide" species, such as *Heterocapsa*, *Prorocentrum*, *Takayma* and *Tripos* (=*Ceratium*) (Hansen, 2011; Lim et al., 2018). If such species (shown for *Tripos furca* and *Prorocentrum cordatum*) are subjected to nutrient limitation (N and P), food uptake will increase dramatically (Smalley et al., 2003, 2012; Johnson, 2014). At present, very limited data are available on this topic, besides the documentation of increased feeding rates. Thus, we know very little about what extent growth rates will change under different levels of inorganic nutrient limitation.

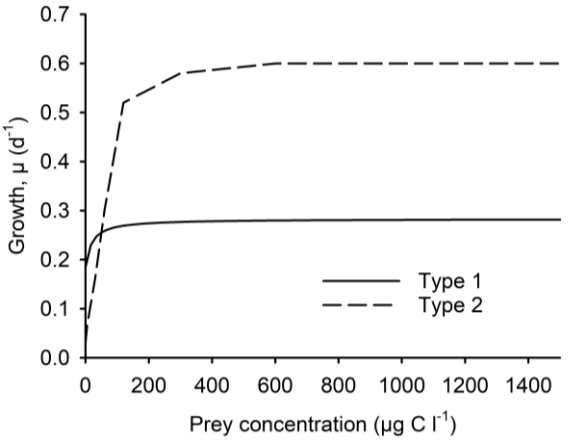

Figure 5. The effects of prey concentration on the growth rates of type 1 and type 2 constitutive mixotrophic dinoflagellates when grown at low irradiance (20 µmol photons photons m^{-2} s^{-1}) and in nutrient rich culture media.

The type 2 CM group is comprised of species that are able to increase their growth rates to a high degree at low irradiances even when inorganic nutrients are plentiful (Figure 5). Prey concentrations required for maximum growth rates of these dinoflagellates are quite high (240–400 µg C l^{-1}). Representatives of this group include *Fragilidium* spp., *Karlodinium armiger*, *Alexandrium pohangense*, and *Paragymnodinium shiwhaense* (Hansen and Nielsen, 1997; Jeong et al., 1999; Berge et al., 2008a; Yoo et al., 2010; Lim et al., 2015; Jeong et al., 2018). Most of these species can grow in standard phytoplankton growth media based on nitrate as the nitrogen source. However, as is known for *K. armiger*, some species may not grow in such media unless low concentrations of ammonium (50 µM) are added, suggesting lack of the main enzyme, nitrate reductase, necessary for the conversion of nitrate to ammonium (Binzer et al., 2018). The dependence on ammonium among phototrophic dinoflagellates may be more widespread than previously thought. Species like *Gymnodinium resplendens* (Skovgaard, 2000), and *Yihiella yeosuensis* (Jang et al., 2017) have been shown to depend upon food for growth, as they will not grow in standard nitrate based growth media, and their growth potential after ammonium addition need to be explored. Responses to light differ slightly among species within the group. The majority of the species studied cannot grow in complete darkness. An exception is *Fragilidium subglobosum*, which can grow in complete darkness, if fed (Skovgaard, 1996a). Among the many species within this group that require light for growth, *Paragymnodinium shiwhaense* exhibits high growth rates at low irradiance, with growth rates only slightly influenced at irradiances > 10 µmol photons $m^{-2}s^{-1}$ (Jeong et al., 2018). While growth rates increased slightly with increasing irradiance, ingestion rates (in carbon units) were not significantly affected. In other species, ingestion rates and growth rates depend much more on irradiance. For example, *Karlodinium veneficum*, does not ingest prey at all (Li et al., 1999) or at reduced rates (Calbet et al., 2011) in darkness, but ingestion rates increase dramatically with irradiance (Li et al., 1999). The strain of *K. veneficum* studied by Li et al., increased its growth rate from a maximum of 0.25 d^{-1} in monoculture to 0.7 d^{-1} when grown mixotrophically at irradiances > 100 µmol photons $m^{-2}s^{-1}$.

Non Constitutive Mixotrophs (NCMs)

The NCM dinoflagellates are a very diverse group of organisms, which handle ingested prey very differently. At one extreme, we have organisms that retain entire algal cells either intracellular or extracellular. At the other extreme organisms are found that only sequester chloroplasts. In between exist organisms that retain only parts of the ingested prey, typically prey cytoplasm, prey mitochondria and prey nuclei. These different groups of NCMs are treated separately here because they have very different physiologies.

Species That Harbour Intact Symbionts

This group (eNCMs) includes species that harbour intact prey cells as endosymbionts, like the green *Noctiluca scintillans* (see Figure 4 H) and several species of *Amphisolenia* (see Figure 4 I) and *Triposolenia*, as well as species that harbour intact ectosymbionts, which can be found among the genera *Cistharistes*, *Histioneis* and *Ornithocercus* (see Figure 4 G) (Table 2). All these species are found in the euphotic zone of tropical waters. However, while the green *Noctiluca scintillans* is found in eutrophic tropical waters (Harrison et al., 2011), the other species are exclusively found in oligotrophic waters (Tarangkoon et al., 2010). The green *Noctiluca scintillans* is unfortunately the only one where data on physiology are available (Hansen et al., 2004; Gomes et al., 2018). For material collected from Manila Bay, it was shown that the growth rate of *N. scintillans* was dependent upon both light and prey concentration. A comparison of the contribution of photosynthesis and phagotrophy to carbon metabolism revealed that phagotrophy only contributed significantly (30%) to direct growth of green *N. scintillans* at very high prey concentrations. At natural prey concentrations (<500 µg C l^{-1}) contribution of prey ingestion to the carbon metabolism was < 10% at high light, 150 µmol photons m^{-2} s^{-1} (Hansen et al., 2004).

Species That Harbour "Reduced Endosymbionts"

Some species of NCM dinoflagellates ingest whole cells but retain only parts of prey cells, including chloroplasts, mitochondria, prey nuclei and prey cytoplasm. Such species thus retain "reduced endosymbionts" (reNCMS). The taxonomy of this group of dinoflagellates is still unresolved, but it is clear that they are not all closely related. The group includes freshwater as well as marine species (Table 2). Information on the physiology of species within this group is sparse. The dependency of feeding and growth on irradiance has so far only been studied in three marine species *Gymnodinium gracilentum*, *Nusuttodinium poecilochroum* and *Amylax triacantha* (Skovgaard, 1998; Jakobsen et al., 2000; Park et al., 2013; Kim et al., 2014), and in one freshwater species (*Nusuttodinium aeruginosum*) (Drumm et al., 2017).

Gymnodinium gracilentum and *N. poecilochroum* are the only species that can grow in complete darkness if supplied with fresh prey. However, their growth rates depend on irradiance, and rates increase by a factor of 2–3 at irradiances > 50 µmol photons m^{-2} s^{-1} compared to in darkness. Ingestion rates are also light dependent, and the rates increase by the same factor. *Amylax triacantha* is much more dependent upon light. In complete darkness,

this species does not feed at all, and although short-term growth rates have been reported (Park et al., 2013), these rates are most likely due to residual cell divisions. Feeding in this species is quite dependent upon irradiance, and ingestion rates are quite high at high irradiances (~4 ciliates d^{-1}). Growth rates as high as 0.8 d^{-1} have been reported at high food concentrations and irradiances. In many ways, *N. aeruginosum* functions similar to *A. triacantha*. Experiments have not been carried out with this species in complete darkness, but the light compensation point, where the dinoflagellate are able to sustain itself, was achieved at an irradiance of ~10 µmol photons m^{-2} s^{-1}. Maximum growth rates of 0.3 (d) were obtained at irradiances of ~40 µmol photons m^{-2} s^{-1} (Drumm et al., 2017).

To what extent can measured rates of photosynthesis explain increased growth rates in these dinoflagellates? This question has so far only been addressed for *N. aeruginosum*. In this species, highest rates of photosynthesis only corresponded to ~26% of cell carbon content, with estimated biomass production and measured rates of photosynthesis of fed cells unable to explain observed increased growth rates at high irradiances (Drumm et al., 2017). A hypothesis to explain this phenomenon could be that high irradiance induces and aids digestion of phytoplankton. Some studies have shown that prey, especially phototrophic prey, are digested faster in the light (Klein Breteler et al., 1986; Strom, 2001). This is supported by a recent study demonstrating that the oxidation of carbohydrates by enzymes increased substantially in light (Cannella et al., 2016). Another pathway to utilize solar energy could be through the protein, rhodopsin, which was recently shown in the heterotrophic dinoflagellate *Oxyrrhis marina* (Guo et al., 2014). Thus, three explanations for the observed positive effect of light have been put forward (Drumm et al., 2017): (1) Algal pigments are highly labile in the presence of light and oxygen, making ingested material more readily utilizable for growth (Porra et al., 1997); (2) Enzymes or other proteins are expressed to a greater extent in light; (3) ATP produced by photosynthetic activity aids ingestion and digestion of prey, rather than glucose production. In the latter, photosynthesis increases with increasing irradiance resulting in higher productions of ATP, allowing faster digestion and hence greater ingestion rates. More research is definitely required to explore this further.

Prey starvation responses within this group have also been studied in some detail in a few species. In *G. gracilentum*, photosynthetic activity decreases rapidly when starved for prey. Hardly any photosynthesis was measured in prey-starved cultures after 48h. Populations of *G. gracilentum* that were subjected to sudden prey starvation divided only once at maximum, and cells did not survive more than seven days. Similar population starvation response has been shown for *A. poecilochroum*. Thus, these species rely heavily on the continuous ingestion of prey. In general, these species did not survive much better in the light compared to in the dark when starved for prey (Skovgaard, 1998; Jakobsen et al., 2000). Other species, *A. triacantha* and *N. aeruginosum*, maintained their functionally active kleptochloroplasts for a much longer time (Park et al., 2013; Drumm et al., 2017). In *A. triacantha* the acquired plastids were retained for up to one month, and they have been shown to be functionally active for ~ 18 d, when measured by a pulse-amplitude modulation fluorometer. Likewise, *N. aeruginosum* can be starved for up to a month. The kleptochloroplasts were photosynthetically active for at least 27 days in prey-starved cultures (^{14}C measurements), and *N. aeruginosum* maintained a high photosynthetic rate for the first nine days, despite going through three cell divisions. These observations indicate that *N. aeruginosum*, unlike the other species in this group, is able to divide their sequestered chloroplasts, but details need to be investigated further.

In conclusion, the species within this group that are able to grow at a fairly high rates in darkness (*G. gracilentum* and *A. poecilochroum*) are not able to maintain high rates of photosynthesis when prey-starved. The two other species, *A. triacantha* and *N. aeruginosum* cannot grow in the dark even if supplied with food in excess. However, they can maintain their acquired chloroplasts for a much longer time than *G. gracilentum* and *A. poechilochroum*. This indicates that the ability to maintain functional chloroplasts for a long time comes at the expense of not being able to grow in complete darkness. It will be interesting to explore gene expression in these dinoflagellates to discover the mechanisms underlying host control of retained reduced endosymbionts.

Species That Only Sequester the Chloroplasts

The only dinoflagellates that exclusively sequester chloroplasts from their prey (pNCMs) are found in the genera *Dinophysis and Phalacroma*. While many *Dinophysis* species have been shown to harbour cryptophyte chloroplasts, only one species of *Phalacroma, P. mitra*, has been reported to retain chloroplasts, and their origin seem to be of mainly haptophyte origin (Koike et al., 2005; Nishitani et al., 2012; 2018). While *Dinophysis* species have been cultured since 2006 (Park et al., 2006), *P. mitra* has not yet been brought into culture and nothing is known of its physiology. Six species out of the existing ~100 described *Dinophysis* species have been successfully cultured (Hansen et al., 2013); all cultured on *Mesodinium rubrum* as prey. To what extent *Dinophysi*s relies on sequestered chloroplasts and/or whether *Dinophysis* species permanently maintain chloroplasts was debated for some time (see Hansen et al., 2013 for a discussion). The final proof that the chloroplasts are continuously sequestered from its prey was provided by Kim et al. (2012). They were able to show the movement of sequestered chloroplasts to the different chloroplast centers inside the *Dinophysis* cell using video recordings. They were also able to show morphological changes of the chloroplasts following their incorporation in the *Dinophysis* cell.

Dinophysis species cannot grow in complete darkness, even if they are fed prey in excess (Kim et al., 2008). Both food uptake and growth are highly dependent upon irradiance (Kim et al., 2008) and of course prey concentration (Kim et al., 2008; Riisgaard and Hansen, 2009). In addition, prey quality (growth rate of the prey ciliate) affects apparent prey ingestion (Riisgaard and Hansen, 2009). However, grazing rates published by Kim et al. (2008) and Riisgaard and Hansen (2009) should be interpreted with caution. Estimations of grazing rates of *Dinophysis* on its prey have been based on the reduction of prey in grazing bottles (with *Dinophysis* and *M. rubrum*) as compared to control bottles (with *M. rubrum* alone) using standard grazing equations. Thus, the grazing rates are calculated assuming that the differences in *M. rubrum* cell concentration in the grazing and control bottles are due to cells that have been ingested by *Dinophysis*. Recent papers, however, have shown that *Dinophysis* species exudate/excrete mucus in which the prey, *M. rubrum*, is caught (Mafra et al., 2016; Ojamäe et al., 2016; Papiol et al., 2016). Some of the ciliates that are caught in the mucus lyse and disintegrate and thus are no longer available to *Dinophysis* for ingestion. Since the ability of the mucus clumps to catch prey is dependent on prey and predator concentrations (Ojamäe et al., 2016), the "lysis problem" will mostly be a problem at high *Dinophysis* cell concentrations, and therefore this phenomenon will especially impact calculated grazing rates at high prey concentrations (where you need high *Dinophysis* concentrations to detect the

grazing response). The implication of this is that grazing rates of *Dinophysis* on its prey most probably have been overestimated. Thus, it is most likely that *Dinophysis* species get most of their carbon from photosynthesis (Ojamäe et al., 2016).

Dinophysis species can starve for months without food, and the cells may divide up to ~ four times before cell divisions stops (Park et al., 2008; Riisgaard and Hansen, 2009). It has been shown that *D. acuminata* and *D. acuta* can maintain functionally active chloroplasts for many weeks, up to months without access to prey (Park et al., 2008; Hansen et al., 2016). Prey-starved *D. acuta* cells cannot only maintain photosynthetic activity for an extended period, they also are able to produce photosynthetic and photoprotective pigments (Hansen et al., 2016). Also, both *D. acuminata* and *D. acuta* have been shown to be able to divide their ingested chloroplasts, at least once, sometimes twice (Rusterholz et al., 2017). Thus, *Dinophysis* species seem to have considerable control of their ingested cryptophyte chloroplasts compared to species that also sequester prey nuclei (i.e., red *Mesodinium* spp. and *A. triacantha*). The photosynthetic performances of kleptochloroplasts in these latter species are coupled to the sequestration of prey nuclei and nucleomorph material. This is not the case in *Dinophysis* spp. Thus, it seems that the function of chloroplasts in *Dinophysis* may depend upon genes that in the past have been transferred from prey nuclei and nucleomorphs to the dinoflagellate genome.

Very little is known about such a transfer of genes from the cryptophyte genome to the genome of *Dinophysis* spp. Five proteins, complete with plastid-targeting peptides that may function in photosystem stabilization and metabolite transport, have been found encoded in the nuclear genome of *D. acuminata* (Wisecaver and Hackett, 2010). However, four of the five proteins appear to have been derived from haptophytes and/or fucoxanthin dinoflagellates, while only the fifth protein appear to derive from cryptophytes via lateral gene transfer. Recently, Hongo et al., (2019) investigated the origin of plastid proteins in *Dinophysis fortii* via RNA sequencing, and identified 58 gene products involved in porphyrin, chlorophyll, isoprenoid and carotenoid biosyntheses as well as in photosynthesis. Their phylogenetic analysis revealed that the genes associated with chlorophyll and carotenoid biosyntheses and photosynthesis originated from fucoxanthin dinoflagellates, haptophytes, chlorarachniophytes, cyanobacteria and cryptophytes. Thus, it appears that *Dinophysis* spp. have maintained genes from an earlier event and only later incorporated cryptophyte chloroplasts. All together the experimental and molecular findings suggest that *Dinophysis* spp. have some functional control of their plastids, but that these species rely on feeding to sustain growth.

IMPORTANCE OF MIXOTROPHIC DINOFLAGELLATES AS GRAZERS IN NATURAL WATERS

Mixotrophic dinoflagellates are common in freshwater, brackish and marine environments (Tables 1–3) and are found in aquatic systems at all latitudes (Leles et al., 2017; Leles et al., 2019). Also, mixotrophic dinoflagellates are found in eutrophic as well as in oligotrophic waters. Thus, they are indeed ubiquitous. Despite the growing awareness of mixotrophic dinoflagellates as grazers in pelagic waters, estimations of their grazing impacts are quite limited. There are many reasons related to prey preference and physiology for why

this is the case. First, some dinoflagellates ingest only specific prey items, while others are quite omnivorous within a certain prey size range. The prey items are often of the same size as the dinoflagellates themselves, and prey items may include heterotrophic protists and metazoans. Second, a number of physical and chemical factors like irradiance, temperature and nutrients influence feeding rates. Lastly, different types of dinoflagellates feed for various reasons like acquiring carbon and/or nutrients and vitamins for enhanced growth (the CMs). Others, the NCMs, also ingest prey to sequester functional chloroplasts.

The prevalence of CMs with food vacuoles has been measured in field samples in a number of cases, and those studies provide valuable information on spatial and temporal variations in the importance of food uptake in CMs in red tide species such as *Akashiwa sanguinea*, *Tripos furca* and *Levandering fissa* (Bockstahler and Coats, 1993a; Bockstahler and Coats, 1993b; Li et al., 1996), *Karlodinium veneficum* (Li et al., 1996; Adolf et al., 2008) and *Prorocentrum cordatum* (Li et al., 1996; Stoecker et al., 1997; Johnson et al., 2018). The published data indicate that 0–20% of the CM dinoflagellates have easily identifiable food vacuoles. However, values as high as 50% have been reported in *P. cordatum* (Stoecker et al., 1997). The percentage of dinoflagellate cells with food vacuoles depend on irradiance, time of day, food availability, and nutrient (N, P) concentration (Stoecker et al., 1997; Li et al., 2001).

The transformation of data on the prevalence of cells with food vacuoles to ingestion rates is not trivial as digestion rates are affected by a number of factors like light, nutritional quality and quantity of prey cells and water temperature (Bockstahler and Coats, 1993b; Li et al., 1996; Stoecker et al., 1997; Li et al., 2001). Nevertheless, Bockstahler and Coats (1993b) using the "gut clearance/gut fullness" approach, reported estimates of *in situ* ingestion and clearance rates of *Akashiwo sanguinea* that ranged from 0–0.06 prey dinoflagellate^{-1} h^{-1} and 0–5.8 µl dinoflagellate^{-1} h^{-1}, respectively, with daily removal of ciliate biomass representing 6 to 67% of the < 20 µm oligotrich ciliate standing stock. Daily consumption of ciliate biomass by *A. sanguinea* averaged 2.5% of body carbon and 4.0% of body nitrogen, with maximal values of 11.6 and 18.5 %, respectively (Bockstahler and Coats, 1993b). Likewise, for *K. veneficum* feeding on cryptophytes, Li et al., (2001) reported that ingestion rates ranged from 0–0.26 prey dinoflagellate^{-1} d^{-1}, corresponding to an estimated daily ingestion of 0–12% body carbon, 0-13 % of body nitrogen and 0-21% of body phosphorous d^{-1} and leading to a daily removal of 0–4 % of the cryptophyte standing stock in that specific study.

An approach that has been used to document the uptake of specific prey cells in CM dinoflagellates involves studying the changes in prevalence of ingested prey cells over time following the addition of live, untreated, prey organisms (e.g., cryptophytes) to field samples. This approach has been used to indicate the willingness of natural *K. veneficum* populations to feed, but has not been able to provide *in situ* grazing rates (Stoecker et al., 1997; Adolf et al., 2008). Nevertheless, it was shown that feeding rates depend on time of the day, with highest prey uptake during night (Stoecker et al., 1997). Addition of live stained-prey to natural plankton communities has been attempted in the past, with some success in tracking prey uptake in heterotrophic (Martinez et al., 2014) and CM dinoflagellates (Stoecker et al., 1997; Johnson et al., 2018). Some of the more successful dyes (e.g., CellTracker, LysoSensor) do not seem to affect prey or the predator in the concentrations used and as such seems promising. However, the tested stains generally stain only certain algal species, not others. Thus, further research is needed to obtain more universal dyes.

Addition of live prey to natural populations is problematic, since nutrients may be added along with the algal prey. Thus, the use of fluorescent beads or fluorescently labelled heat-killed prey have been attempted instead to measure grazing in protists (Sherr et al., 1987; Rublee and Gallegos, 1989; Beisner et al., 2019). Such methods have provided valuable data for filter feeding heterotrophic ciliates and flagellates. However, these methods may lead to reduced feeding rates for raptorial predators (Stoecker, 1988; Nygaard and Hessen, 1990; Putt, 1991; Verity, 1991; Landry, 1994; Li et al., 1996; Beisner et al., 2019), and the approach does not seem to work very well for CM dinoflagellates.

Finally, since *in situ* grazing rates are so difficult to obtain, laboratory-generated grazing rates have been used to infer potential impacts of specific CM dinoflagellates on specific prey items. Using the prey density depended grazing responses (functional responses) of the dinoflagellates on specific preys and applying them to *in situ* cell concentrations of dinoflagellates and their prey, the potential grazing impacts have been estimated. Such approaches have been used for evaluating grazing impacts of red tide species, like *Heterocapsa steinii*, *Gonyaulax polygramma*, *Lingulodinium polyedra*, *Prorocentrum donghaiense*, *P. micans* and *Scrippsiella acuminata* (Jeong et al., 2005a; 2005b). Measured grazing rates were temperature corrected using a standard Q_{10} value (of 2.8). Reported grazing rates lie in the range 0–98% of prey populations removed per h. The highest estimated removal rates of prey were during blooms of the specific dinoflagellates, while the lowest rates were observed when the dinoflagellates were less abundant and/or the prey items were very abundant.

CONCLUSION

Mixotrophy among dinoflagellates is quite common among freshwater and marine dinoflagellates, including species with their own permanent chloroplasts (CM dinoflagellates) and species that sequester chloroplasts from their prey (NCM dinoflagellates). In fact, mixotrophy among dinoflagellates may now be considered more the rule than the exception. Feeding may not always be expressed among the CM dinoflagellates, especially as light and nutrients impact feeding for the majority of species. Mixotrophic dinoflagellates primarily eat other protists, but some species can exploit much larger metazoans as part of their diet. Some mixotrophic dinoflagellates are highly selective in which prey types they ingest, while others are quite omnivorous. Especially the NCM dinoflagellates seem to be quite restricted in which types of prey they can utilize as donors of chloroplasts and other cell organelles. Few data are available on *in situ* grazing rates of mixotrophic dinoflagellates, and there is a strong need to develop new techniques to measure *in situ* grazing rates. Development of reliable *in situ* techniques to measure feeding is not only important to assess the significance of phagotrophy as a way for dinoflagellates to harvest nutrients in inorganic nutrient limited waters, but also to assess the impact dinoflagellate mixotrophy on the food web.

Table 1. Literature compilation of constitutive mixotrophic dinoflagellate species. Study type: F = field samples, C = culture study. Methods: LM = light microscopy, SEM = scanning electron micsroscopy, TEM = transmission electron microscopy, Fl = fluorescence; FLP = fluorescently labelled prey, FLB = fluorescently labelled bacteria. GGE = gross growth efficiency. Question mark (?) express uncertainty

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Gymnodiniales Gymnodiniaceae	*Akashiwo sanguinea*	marine plankton	CM	Bockstahler and Coats, 1993b	*Gymnodinium sanguineum*	F	LM (protargol-staining) ingested ciliates	–	ingestion
				Li et al., 1996	*Gymnodinium sanguineum*	F	LM (FL), FLP ingested FLP	–	% feeding cells
				Jeong et al., 2005b	*Akashiwo sanguinea*	C	LM ingested food	engulfment (sulcus)	prey spectrum
Gonyaulacales Ostreopsidaceae	*Alexandrium andersonii*	marine plankton	CM	Lee et al., 2016	*Alexandrium andersonii*	C	LM, TEM, video[1] ingested food	engulfment (after immobilization!)	prey spectrum, growth, ingestion, swimming speed, lytic capacity
Gonyaulacales Ostreopsidaceae	*Alexandrium catenella*	marine plankton	CM	Nygaard and Tobiesen, 1993	*Alexandrium tamarense*	C	RLB uptake of RLB	–	ingestion
				Jeong et al., 2005a	*Alexandrium tamarense*	C	LM (FL) ingested *Synechococcus*	–	ingestion
				Jeong et al., 2005b	*Alexandrium tamarense*	C	LM ingested food	engulfment (sulcus), no tow filament	prey spectrum
Gonyaulacales Ostreopsidaceae	*Alexandrium minutum*	marine plankton	CM	Jeong et al., 2005b	*Alexandrium minutum*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
Gonyaulacales Ostreopsidaceae	*Alexandrium ostenfeldii*	marine plankton	CM	Jacobson and Anderson, 1996	*Alexandrium ostenfeldii*	F	LM, TEM presence of food vacuoles	–	–
				Tillmann et al., 2014	*Alexndrium ostenfeldii*	F	LM ingested food	–	–
Gonyaulacales Ostreopsidaceae	*Alexandrium pacificum*	marine plankton	CM	Jeong et al., 2005a	*Alexandrium catenella*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
Gonyaulacales Ostreopsidaceae	*Alexandrium pohangense*	marine plankton	CM	Lim et al., 2015	*Alexandrium pohangense*	C	LM, video[1] ingested food	engulfment (after immobilization!)	prey spectrum, growth, ingestion

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Gonyaulacales Ostreopsidaceae	*Alexandrium pseudogonyaulax*	marine plankton	CM	Jacobson, 1999	*Alexandrium pseudogonyaulax*	F	LM, description of half-ingested *Mesodinium*	engulfment (sulcus) with no apparent protrusive structures	–
				Blossom et al., 2012	*Alexandrium pseudogonyaulax*	C	LM (Fl), video[1] ingested food	engulfment (sulcus), immobilization of prey	prey spectrum, growth
				Blossom et al., 2017	*Alexandrium pseudogonyaulax*	C	LM	–	trap formation, number of cells trapped
incertae sedis incertae sedis	*Amphidiniella sedentaria*	marine benthos	CM	Horiguchi, 1995	*Amphidiniella sedentaria*	C	LM accumulation bodies	–	–
Amphidiniales Amphidiniaceae	*Amphidinium carterae*	marine plankton	CM	Yoo et al., 2009	*Amphidinium carterae*	C	LM (description) ingested *Skeletonema*	–	–
Amphidiniales Amphidiniaceae	*Amphidinium scissum*	marine benthos	CM (?)	Kofoid and Sweezy, 1921	*Amphidinium scissum*	F	LM (drawing) ingested food	–	–
Amphidiniales Amphidiniaceae	*Amphidinium steinii*	marine plankton	CM (?)	Kofoid and Sweezy, 1921	*Amphidinium steinii*	F	LM (drawing) ingested food	–	–
Suessiales Suessiaceae	*Ansanella granifera*	marine plankton	CM	Lee et al., 2014b	*Ansanella granifera*	C	LM, TEM ingested prey, food vacuoles	engulfment	prey spectrum, growth, ingestion, swimming speed
incertae sedis incertae sedis	*Apicoporus glaber*	marine benthos	CM (?)	Sparmann et al., 2008	*Apicoporus glaber*	F	LM (Fl), TEM chloropl. and food vacuoles	–	–
incertae sedis incertae sedis	*Apicoporus parvidiaboli*	marine benthos	CM (?)	Sparmann et al., 2008	*Apicoporus parvidiaboli*	F	LM (Fl), TEM chloropl. and food vacuoles	–	–
Gymnodiniales Gymnodiniaceae	*Barrufeta bravensis*	marine plankton	CM	Sampedro et al., 2011	*Barrufeta bravensis*	C	TEM food vacuoles	–	–
Gymnodiniales Gymnodiniaceae	*Barrufeta resplendens*	marine plankton	CM	Hulburt, 1957	*Gyrodinium resplendens*	F	LM (drawing) ingested food	–	–
				Skovgaard, 2000	*Gyrodinium resplendens*	C	LM ingested food, feeding tube	feeding tube	growth, ingestion
Suessiales Suessiaceae	*Biecheleria baltica*	marine plankton	CM	Moestrup et al., 2009a	new combination from *Woloszynskia halophila*	C	TEM presence of peducle	peduncle?	–
Suessiales Suessiaceae	*Biecheleria cincta*	marine plankton	CM	Kang et al., 2011	*Woloszynskia cincta*	C	LM, TEM feeding process, food vacuoles	peduncle, tow filament	prey spectrum, growth, ingestion, swimming speed

Table 1. (Continued)

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Suessiales Suessiaceae	*Biecheleriopsis adriatica*	marine plankton	CM (?)	Moestrup et al., 2009b	*Biecheleriopsis adriatica*	C	TEM food vacuoles	–	–
Gymnodiniales Gymnodiniaceae	*Bispinodinium angelaceum*	marine benthos	CM	Yamada et al., 2013	*Bispinodinium angelaceum*	C	LM, TEM accumulation bodies	–	–
Gymnodiniales Brachidiniaceae	*Brachidinium capitatum*	marine plankton	CM (?)	Gómez, 2005	*Brachidinium capitatum*	F	LM accumulation bodies	–	–
Peridiniales incertae sedis	*Bysmatrum austrafum*	marine benthos	CM	Dawut et al., 2018	*Bysmatrum austrafum*	C	LM accumulation bodies	–	–
Gonyaulacales Ostreopsidaceae	*Coolia* sp.	marine benthos	CM	Almada et al., 2017	*Coolia* sp.	F	LM (Fl) Fl stained inclusions	–	% cells with inclusion
Suessiales incertae sedis	*Dactylodinium pterobelotum*	marine plankton	CM	Takahashi et al., 2017	*Dactylodinium pterobelotum*	C	LM, TEM presence of peduncle	peduncle?	–
Gonyaulacales Pyrophacaceae	*Fragilidium duplo-campanae-forme*	marine plankton	CM	Nézan and Chomérat, 2009	*F. duplo-campanae-forme*	F	LM ingested *Dinophysis*	–	–
				Park and Kim, 2010	*F.duplo-campanae-forme*	C	LM (Fl), video[1] ingested food	engulfment (sulcus), immobilisation of prey	prey spectrum
				Rodriguez et al., 2014	*F.duplo-campanae-forme*	C	LM, video[1] ingested *Dinophysis*	–	growth, ingestion
Gonyaulacales Pyrophacaceae	*Fragilidium heterolobum*	marine planton	CM	Balech, 1988	*Fragilidium heterolobum*	F	LM (description) described as predatory	–	–
Gonyaulacales Pyrophacaceae	*Fragilidium mexicanum*	marine plankton	CM	Balech, 1988	*Fragulidium mexicanum*	C	LM (description) described as predatory	–	–
				Jeong et al., 1997	*Fragilidium* cf. *mexicanum*	C	LM ingested *Protoperidinium*	engulfment	growth

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Gonyaulacales Pyrophacaceae	*Fragilidium subglobosum*	marine plankton	CM	Balech, 1988	*Fragilidinum subglobosum*	C	LM (description) described as predatory	–	–
				Skovgaard, 1996a	*Fragilidium subglobosum*	C	–	–	growth, ingestion, light-response
				Skovgaard, 1996b	*Fragilidium subglobosum*	C	LM, TEM ingested *Tripos*	engulfment	prey specificity
				Hansen and Nielsen, 1997	*Fragilidium subglobosum*	C	–	–	functional and numerical responses; effects of light intensity on feeding and growth
				Hansen et al., 2000	*Fragilidium subglobosum*	C	–	–	effects of time scale and prey concentration on photosynthetic performance
				Skovgaard et al., 2000	*Fragilidium subglobosum*	C	–	–	photosynthesis, ingestion
Gonyaulacales Ostreopsidaceae	*Gambierdiscus toxicus*	marine benthos	CM	Faust, 1998	*Gambierdiscus toxicus*	F	LM ingested food	–	% cells with prey
Gonyaulacales Gonyaulacaceae	*Gonyaulax alaskensis*[2]	marine plankton	CM	Jacobson and Anderson, 1996	*Gonyaulax alaskensis*	F	LM, TEM ingested food, food vacuoles	–	–
Gonyaulacales Gonyaulacaceae	*Gonyaulax diegensis*	marine plankton	CM	Jacobson and Anderson, 1996	*Gonyaulax diegensis*	F	LM, TEM ingested food, food vacuoles	–	–
Gonyaulacales Gonyaulacaceae	*Gonyaulax polygramma*	marine plankton	CM	Jeong et al., 2005a	*Gonyaulax polygramma*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
				Jeong et al., 2005c	*Gonyaulax polygramma*	C	LM ingested food	engulfment[3]	prey spectrum, growth, ingestion
				Yoo et al. 2009	*Gonyaulax polygramma*	C	LM, TEM ingested *Skeletonema*	–	ingestion, clearance, swimming speed
Gonyaulacales Gonyaulacaceae	*Gonyaulax spinifera*	marine plankton	CM	Jacobson and Anderson, 1996	*Gonyaulax spinifera*	F	TEM (description) presence of food vacuole	–	–
				Jeong et al., 2005a	*Gonyaulax spinifera*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
Gonyaulacales incertae sedis	*Grammatodinium tongyeonginum*	marine plankton	CM	Li et al., 2017	*Grammatodinium tongyeonginum*	C	LM accumulation bodies	–	–

Table 1. (Continued)

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Gymnodiniales Gymnodiniaceae	*Gymnodinium aureolum*	marine plankton	CM	Jeong et al., 2010a	*Gymnodinium aureolum*	C	LM, TEM ingested food	peduncle	growth, ingestion, clearance
Gymnodiniales Gymnodiniaceae	*Gymnodinium catenatum*	marine plankton	CM	Jeong et al., 2005a	*Gymnodinium catenatum*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
				Jeong et al., 2005b	*Gymnodinium catenatum*	C	LM ingested food, direct observation	engulfment (sulcus)	prey spectrum
Gymnodiniales Gymnodiniaceae	*Gymnodinium flavum*	marine benthos (?)	CM (?)	Kofoid and Sweezy, 1921	*Gymnodinium flavum*	F	LM (drawing) ingested food	–	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium fulgens*	marine plankton	CM (?)	Kofoid and Sweezy, 1921	*Gymnodinium fulgens*	F	LM (drawing) ingested food	–	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium herbaceum*	marine plankton	CM (?)	Kofoid and Sweezy, 1921	*Gymnodinium herbaceum*	F	LM (description) ingested food	–	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium ravenescens*	marine plankton	CM (?)	Kofoid and Sweezy, 1921	*Gymnodinium ravenescens*	F	LM (drawing) ingested food	–	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium impudicum*	marine plankton	CM	Jeong et al., 2005a	*Gymnodinium impudicum*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
				Jeong et al., 2005b	*Gymnodinium impudicum*	C	LM ingested food	engulfment (sulcus)	prey spectrum
Gymnodiniales Gymnodiniaceae	*Gymnodinium smaydae*	marine plankton	CM	Lee et al., 2014a	*Gymnodinium smaydae*	C	LM ingested food, direct observation	peduncle	prey spectrum, growth, ingestion, swimming speed
Gymnodiniales Gyrodiniaceae	*Gyrodinium flavescens*	marine plankton	CM (?)	Kofoid and Sweezy, 1921	*Gyrodinium flavescens*	F	LM (drawing) ingested food	–	–
Gymnodiniales Gyrodiniaceae	*Gyrodinium melo*	marine plankton	CM (?)	Kofoid and Sweezy, 1921	*Gyrodinium melo*	F	LM (drawing) ingsted food	–	–
Gymnodiniales Gyrodiniaceae	*Gyrodinium vorax*	marine plankton	CM (?)	Biecheler, 1952	*Gyrodinium vorax*	F	LM (drawing) engulfment	engulfment	–
Peridiniales Protoperidiniaceae	*Herdmania litoralis*	marine plankton	CM (?)	Kofoid and Sweezy, 1921	*Gymnodinium agile*	F	LM (description) ingested food	–	–

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Peridiniales Heterocapsaceae	*Heterocapsa rotundata*	marine plankton	CM	Jeong et al., 2005a	*Heterocapsa rotundata*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
				Yoo et al., 2009	*Heterocapsa rotundata*	C	LM (description) ingested *Skeletonema*	–	ingestion
				Millette et al., 2017	*Heterocapsa rotundata*	C, F	LM (Fl), FLB ingested FLB	–	ingestion
Peridiniales Heterocapsaceae	*Heterocapsa steinii*	marine plankton	CM	Legrand et al., 1998	*Heterocapsa triquetra*	C	LM (Fl), FLP ingested *Thalassiosira*	–	prey spectrum, ingestion (light/dark)
				Jeong et al., 2005a	*Heterocapsa triquetra*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
				Jeong et al., 2005b	*Heterocapsa triquetra*	C	LM ingested food	engulfment (sulcus)	prey spectrum, growth, ingestion
Gymnodiniales Brachidiniaceae	*Karenia brevis*	marine plankton	CM	Jeong et al., 2005a	*Karenia brevis*	C	LM (FL) ingested *Synechococcus*	–	ingestion
				Glibert et al., 2009	*Karenia brevis*	C	LM (confocal) ingested *Synechococcus*	–	growth, ingestion
Gymnodiniales Brachidiniaceae	*Karenia mikimotoi*	marine plankton	CM	Zhang et al., 2011	*Karenia mikimotoi*	C	LM (Fl) ingested food	–	growth, ingestion
Gymnodiniales Brachidiniaceae	*Karenia papilionacea*	marine plankton	CM	Haywood et al., 2004	*Karenia papilionaceae*	C	LM accumulation bodies	–	–
Gymnodiniales Brachidiniaceae	*Karlodinium armiger*	marine plankton	CM	Berge et al., 2008a	*Karlodinium armiger*	C	LM, TEM, video[1] ingested food, direct observation	peduncle (inconspicuous…), extract prey content OR ingest whole algal prey	prey spectrum, growth (light/dark)
				Berge et al., 2008b	*Karlodinium armiger*	C	LM, experiments	–	prey size spectrum, functional and numerical responses
				Berge et al., 2012	*Karlodinium armiger*	C	LM, experiments	–	ingestion and growth experiments when fed adult copepods, nauplii, eggs and fecal pellets

Table 1. (Continued)

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Gymnodiniales Brachidiniaceae	*Karlodinium armiger*	marine plankton	CM	Berge et al., 2017	*Karlodinium armiger*	C	experiments	–	photosynthesis, growth, ingestion, nutrients
Gymnodiniales Brachidiniaceae	*Karlodinium veneficum*	marine plankton	CM	Nygaard and Tobiesen, 1993	*Gyrodinium galatheanum*	C	RLB uptake of RLB	–	ingestion
				Li et al., 1996	*Gyrodinium estuariale*	F	LM (Fl), FLB and FLP ingested food	–	% feeding cells
				Li et al., 1999	*Gyrodinium galatheanum*	C	LM (Fl) ingested food	–	growth, ingestion, photosynthesis
				Li et al., 2001	*Gyrodinium galatheanum*	C, F	LM (Fl) ingested food	–	ingestion, digestion
incertae sedis incertae sedis	*Levanderina fissa*	marine plankton	CM	Levander, 1894	*Gymnodinium fissum*	F	LM (drawing) ingested food	–	–
				Biecheler, 1952	*Gyrodinium pavillardi*	F	LM (drawing) ingested food	engulfment (?)	–
				Bockstahler and Coats, 1993b	*Gymnodinium uncatenum*	F	LM (protargol-staining) ingested ciliates	–	ingestion
				Uchida et al., 1997	*Gyrodinium instriatum*	C	LM, direct observation of peduncle feeding	engulfment through the posterior end of the sulcus	–
				Moestrup et al., 2014	*Levanderina fissa*	C	TEM presence of peduncle	peduncle (?)	–
Gonyaulacales Gonyaulacaceae	*Lingulodinium polyedra*	marine plankton	CM	Jeong et al., 2005a	*Lingulodinium polyedrum*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
				Jeong et al., 2005b	*Lingulodinium polyedrum*	C	LM ingested food	engulfment (sulcus)	prey spectrum, growth, ingestion
				Yoo et al., 2009	*Lingulodinium polyedrum*	C	LM (description) ingested *Skeletonema*	??	ingeston

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Gymnodiniales Gymnodiniaceae	*Margalefidinium polykrikoides*	marine plankton	CM	Jeong et al., 2004	*Cochlodinium polykrikoides*	C	LM (Fl) ingested food	engulfment (sulcus)	prey spectrum, growth, ingestion
				Jeong et al., 2005a	*Cochlodinium polykrikoides*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
Gymnodiniales Gymnodiniaceae	*Moestrupia oblonga*	marine plankton	CM	Hansen and Daugbjerg, 2011	*Moestrupia oblonga*	C	TEM evidence for peduncle	peduncle (?)	–
Gymnodiniales Warnowiaceae	*Nematodinium* sp.	marine, plankton	CM	Elbrächter, 1991	*Nematodinium* sp.	F	LM observation of defecation	–	–
				Gavelis et al., 2015	*Nematodinium* sp.	F	LM ingested food	–	–
Gonyaulacales Ostreopsidaceae	*Ostreopsis labens*	marine benthos	CM	Faust, 1998	*Ostreopsis labens*	F	LM (Fl) ingested food	proposed: expandable feeding canal	% feeding cells
Gonyaulacales Ostreopsidaceae	*Ostreopsis lenticularis*	marine plankton	CM	Faust, 1998	*Ostreopsis lenticularis*	F	LM (Fl) ingested food	proposed: expandable feeding canal	% cells with prey
Gonyaulacales Ostreopsidaceae	*Ostreopsis ovata*	marine plankton	CM	Faust, 1998	*Ostreopsis ovata*	F	LM (Fl) ingested food	proposed: expandable feeding canal	% cells with prey
Gonyaulacales Ostreopsidaceae	*Ostreopsis siamensis*	marine plankton	CM	Faust, 1998	*Ostreopsis siamensis*	F	LM (Fl) ingested food	proposed: expandable feeding canal	% cells with prey
Gonyaulacales Ostreopsidaceae	*Ostreopsis heptagona*	marine benthos	CM	Faust et al., 1996	*Ostreopsis heptagona*	F	LM (description) ingested food	–	–
Gonyaulacales Ostreopsidaceae	*Ostreopsis mascarenensis*	marine benthos	CM	Faust et al., 1996	*Ostreopsis mascarenensis*	F	LM (description)	–	–
Gymnodiniales Gymnodiniaceae	*Paragymno-dinium shiwhaense*	marine plankton	CM	Kang et al., 2010	*Paragymno-dinium shiwhaense*	C	LM, TEM ingested food, direct observation	peduncle, tow filament	prey spectrum, growth, ingestion, swimming speed
Gmnodiniales Gymnodiniaceae	*Paragymno-dinium stigmaticum*	marine benthos	CM	Yokouchi et al., 2018	*Paragymno-dinium stigmaticum*	C	LM (Fl), SEM, TEM ingested food, food vacuoles, peduncle-like structure	engulfment through the antapical area	-
Gymnodiniales Gymnodiniaceae	*Polykrikos hartmanii*	marine plankton	CM	Lee et al., 2015	*Polykrikos hartmanii*	C	LM, video[1] ingested food	engulfment, after anchoring prey using nematocysts	prey spectrum, growth, ingestion, swimming speed

Table 1. (Continued)

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Gymnodiniales Gymnodiniaceae	*Polykrikos lebourae*[4]	marine plankton	CM	Kim et al., 2015	*Polykrikos lebourae*	C	LM ingested food	engulfment, tow filament	prey type, growth, ingestion, GGE
Prorocentrales Prorocentraceae	*Prorocentrum belizeanum*	marine benthos	CM	Faust, 1998	*Prorocentrum belizeanum*	F	LM (Fl) presence of food vacuoles	–	% cells with prey
Prorocentrales Prorocentraceae	*Prorocentrum cordatum*	marine plankton	CM	Li et al., 1996	*Prorocentrum minimum*	F	LM (Fl), FLB and FLP ingested food	–	% feeding cells
				Stoecker et al., 1997	*Prorocentrum minimum*	F	LM (Fl) ingested food	–	% of cells with inclusions
				Jeong et al., 2005a	*Prorocentrum minimum*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
				Jeong et al., 2005b	*Prorocentrum minimum*	C	LM ingested food	engulfment (along sutures)	prey spectrum
Prorocentrales Prorocentraceae	*Prorocentrum donghaiense*	marine plankton	CM	Jeong et al., 2005a	*Prorocentrum donghaiense*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
				Jeong et al., 2005b	*Prorocentrum donghaiense*	C	LM ingested food	engulfment (along sutures)	prey spectrum, growth, ingestion
Prorocentrales Prorocentraceae	*Prorocentrum hoffmanianum*[5]	marine benthos	CM	Faust, 1998	*Prorocentrum hoffmanianum*	F	LM (Fl) ingested food	–	% feeding cells
Prorocentrales Prorocentraceae	*Prorocentrum lima*	marine benthos	CM	Faust, 1998	*Prorocentrum arenarium*	F	LM (Fl) ingested food		% cells with prey
Prorocentrales Prorocentraceae	*Prorocentrum micans*[6]	marine plankton	CM	Jacobson and Anderson, 1996	*Prorocentrum micans*	F	LM, TEM food vacuoles	–	–
				Jeong et al., 2005a	*Prorocentrum micans*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
				Jeong et al., 2005b	*Prorocentrum micans*	C	LM ingested food	engulfment (along sutures)	food spectrum, growth, ingestion
				Yoo et al. 2009	*Prorocentrum micans*	C	LM, TEM ingested *Skeletonema*	–	ingestion, clearance
Prorocentrales Prorocentraceae	*Prorocentrum triestinum*[7]	marine plankton	CM	Jeong et al., 2005b	*Prorocentrum triestinum*	C	LM ingested food	engulfment (along sutures)	prey spectrum

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Gonyaulacales Protoceratiaceae	*Protoceratium reticulatum*	marine plankton	CM	Jacobson and Anderson, 1996	*Gonyaulax grindleyi*	F	LM food vacuole	–	–
Thoracosphaerales Thoracosphaeraceae	*Scrippsiella acuminata*	marine plankton	CM	Jeong et al., 2005a	*Scrippsiella trochoidea*	C	LM (Fl) ingested *Synechococcus*	–	ingestion
				Jeong et al., 2005b	*Scrippsiella trochoidea*	C	LM ingested food	engulfment (sulcus, apical horn!)	prey spectrum
Thoracosphaerales Thoracosphaeraceae	*Scrippsiella* sp.	marine plankton	CM	Jacobson and Anderson, 1996	*Scrippsiella* sp.	F	LM, TEM food vacuoles	–	–
				Coats et al., submitted	*Scrippsiella* sp.[8]	F, C	LM, video[1] phototrophic *Scripsiella* consuming tintinnids	–	–
Noctilucales Noctilucaceae	*Spatulodinium pseudonoctiluca*	marine plankton	CM (?)	Gómez and Furuya, 2007 [9]	*Spatulodinium pseudonoctiluca*	F	LM chloropl. and ingested food	–	–
Gymnodiniales Gymnodiniaceae	*Spiniferodinium galeiforme*	marine benthos	CM	Horiguchi and Chihara, 1987	*Spiniferodinium galeiforme*	C	LM, TEM vacuoles (TEM) and red droplets (LM)	–	–
Suessiales Symbiodiniaceae	*Symbiodinium* sp.	marine plankton	CM	Jeong et al., 2012	*Symbiodinium* sp.	C	LM, TEM ingested food	peduncle, tow filament	prey spectrum, growth, ingestion
Gymnodiniales Brachidiniaceae	*Takayama helix*	marine plankton	CM	Jeong et al., 2016	*Takayama helix*	C	LM, video[1] ingested food, direct observation	two types: (1) direct engulfment or (2) prey material sucked into the intercalary region of the sulcus	prey spectrum, growth, ingestion, swimming speed
Gymnodiniales Brachidiniaceae	*Takayama tasmanica*	marine plankton	CM	Lim et al., 2018	*Takayama tasmanica*	C	LM, video[1] ingested food, direct observation	engulfment (sulcus), tow filament	prey spectrum, growth, ingestion, swimming speed
Gymnodiniales Gymnodiniaceae	*Testudodinium magnum*	marine benthos	CM	Pinto et al., 2016	*Testudodinium magnum*	C	TEM accumulation bodies	–	–
Peridiniales Thecadiniaceae	*Thecadinium kofoidii*	marine benthos	CM	Yoo et al., 2018	*Thecadinium kofoidii*	C	LM, TEM ingested food, food vacuoles	peduncle or myzocytosis, prey was anchored	prey spectrum, growth, ingestion

Table 1. (Continued)

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Gymnodiniales Brachidiniaceae	*Torodinium teredo*	marine plankton	CM	Gómez, 2009	*Torodinium teredo*	F	LM peduncle, and accumulation bodies	peduncle (?)	–
				Elbrächter, 1991	*Torodinium teredo*	F	LM defecation	–	–
Gonyaulacales Ceratiaceae	*Tripos declinatum* f. *normale*	marine plankton	CM	Chang and Carpenter, 1994	*Ceratium declinatum* f. *normale*	F	LM (Fl) inclusion bodies	–	–
Gonyaulacales Ceratiaceae	*Tripos furca*	marine plankton	CM	Bockstahler and Coats, 1993a	*Ceratium furca*	F	LM (protargol-staining) ingested ciliates	–	ingestion
				Chang and Carpenter, 1994	*Ceratium furca*	F	LM (Fl) inclusion bodies	–	–
				Li et al., 1996	*Ceratium furca*	F	LM (Fl), FLP ingested FLP		% feeding cells
				Smalley and Coats, 2002	*Ceratium furca*	F	LM (Fl) uptake of FLP	–	number of food vacuoles, ingestion
				Smalley et al., 2003	*Ceratium furca*	C	LM (Fl) uptake of FLP	–	growth, ingestion, photosynthesis, nutrient effects
				Smalley et al., 2012	*Neoceratium furca*	F	LM (Fl) uptake of FLP	–	ingestion
Gonyaulacales Ceratiaceae	*Tripos muelleri*	marine plankton	CM	Schütt, 1895 [10]	*Ceratium tripos*	F	LM (drawing) inclusions[11]	–	–
Gonyaulacales Ceratiaceae	*Tripos fusus*	marine plankton	CM	Chang and Carpenter, 1994	*Ceratium fusus*	F	LM (Fl) inclusion bodies	–	–
Gonyaulacales Ceratiaceae	*Tripos longipes*	marine plankton	CM	Jacobson and Anderson, 1996	*Ceratium longipes*	F	LM, TEM food vacuoles	–	–
Gonyaulacales Ceratiaceae	*Tripos lunula*	marine plankton	CM	Norris, 1969	*Ceratium lunula*	F	LM ingested *Protoperidinium*	–	–
Gonyaulacales Ceratiaceae	*Tripos teres*	marine plankton	CM	Chang and Carpenter, 1994	*Ceratium teres*	F	LM (Fl) inclusion bodies	–	% cells with inclusions

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Suessiales Suessiaceae	*Yihiella yeosuensis*	marine plankton	CM	Jang et al., 2017	*Yihiella yeosuensis*	C	LM, TEM ingested food	–	prey spectrum, growth, ingestion swimming speed
Suessiales Borghiellaceae	*Baldinia anauensis*	freshwater plankton	CM	Hansen et al., 2007b	*Baldinia anauensis*	C	LM, TEM peduncle and accumulation bodies	peduncle (?)	–
Tovelliales Tovelliaceae	*Bernardinium bernardiense*[4]	freshwater plankton	CM (?)	Hansen and Calado, 1999	*Bernardinium bernardiense*	F	LM prey uptake	direct engulfment, driven by pseudopod with short peduncle	–
				Fawcett and Parrow, 2014	*Esoptrodinium/Bernardinium*	C	LM ingested food, prey uptake	peducle (whole prey cells)	prey spectrum, growth, ingestion
Suessiales Suessiaceae	*Biecheleria pseudopalustris*	freshwater plankton	CM	Moestrup et al., 2009a	new combination from *Woloszynskia pseudopalustris*	F	TEM evidence for peduncle	peducle (?)	–
Gonyaulacales Ceratiaceae	*Ceratium hirudinella*	freshwater plankton	CM	Hofeneder, 1930	*Ceratium hirudinella*	F	LM (description) ingested food	–	–
				Dodge and Crawford, 1970	*Ceratium hirudinella*	F, C	TEM food vacuoles	–	–
Thoracosphaerales Thoracosphaeraceae	*Chimonodinium lomnickii*	freshwater plankton	CM	Craveiro et al., 2011	*Chimonodinium lomnickii*	F	TEM evidence for peduncle	peduncle (?)	–
Peridiniales incertae sedis	*Glenodinium eurystomum*	freshwater plankton (?)	CM (?)	Harris, 1940	*Glenodinium eurystomum*	F	LM (drawing) ingested food	–	–
Peridiniales incertae sedis	*Glenodinium helicozoster*	freshwater plankton (?)	CM (?)	Harris, 1940	*Glenodinium helicozoster*	F	LM ingested food	–	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium cnecoides*	freshwater plankton	CM (?)	Harris, 1940	*Gymnodinium cnecoides*	F	LM (drawing) ingested food	–	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium discoidale*	freshwater plankton	CM (?)	Harris, 1940	*Gymnodinium discoidale*	F	LM (drawing) ingested food	–	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium hyperxanthoides*	freshwater plankton	CM (?)	Harris, 1940	*Massartia hyperxanthoides*	F	LM (drawing) ingested food	–	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium hyperxanthum*	freshwater plankton	CM (?)	Harris, 1940	*Massartia hyperxantha*	F	LM (drawing) ingested food	–	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium obesum*	freshwater plankton	CM (?)	Hansen and Flaim, 2007	*Gymnodinium obesum*	F	LM (description) accumulation bodies	–	–

Table 1. (Continued)

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/evidence	Feeding mode	Quantitative data
Gymnodiniales Gymnodiniaceae	*Gymnodinium posthiemale*	freshwater plankton (?)	CM (?)	Moestrup and Calado, 2018	*Gymnodinium posthiemale*	F	LM, numerous grains in cytoplasma indicating mixotrophy	–	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium triceratium*	freshwater plankton (?)	CM (?)	Moestrup and Calado, 2018	*Gymnodinium triceratium*	F	LM (description) emanated peduncle	peduncle (?)	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium bohemicum*	freshwater plankton (?)	CM (?)	Moestrup and Calado, 2018	*Gymnodinium bohemicum*	F	LM (description) extended pseudopodia	pseudopodia (?)	–
Suessiales Hemidiniaceae	*Hemidinium nasutum*	freshwater plankton	CM	Stein, 1878	*Hemidinium nasutum*	F	LM (description) ingested food	–	–
				Calvacante et al., 2017	*Hemidinium nasutum*	F	LM reddish accumulation bodies	–	–
Thoracosphaerales Thoracosphaeraceae	*Naiadinium polonicum*	freshwater plankton	CM	Craveiro et al., 2015	*Peridinium polonicum*	F	TEM evidence for peducnle	peduncle (?)	–
Peridiniales Peridiniaceae	*Parvodinium inconspicuum*	freshwater plankton	CM	Porter, 1988	*Peridinium inconspicuum*	F	LM (Fl) ingestion of FLB	–	ingestion
Peridiniales Peridiniopsidiaceae	*Peridiniopsis borgei*	freshwater plankton	CM (?)	Moestrup and Calado, 2018	*Peridiniopsis borgei*	F	LM (description) peduncle present	peduncle (?)	–
Thoracosphaerales Thoracosphaeraceae	*Theleodinium calcisporum*	freshwater plankton	CM	Craveiro et al., 2013	*Theleodinium calcisporum*	C	SEM, TEM peduncle present	peduncle (?)	–
Tovelliales Tovelliaceae	*Tovellia sanguinea*	freshwater plankton	CM	Moestrup et al., 2006	*Tovellia sanguinea*	C	LM, TEM, peduncle, food vacuoles	peduncle (?)	–

[1] Video available as suppl. material in the referenced paper.
[2] In the paper the species is described as “non-photosynthetic”?
[3] Engulfment described in the paper either through the apical horn or through the sulcus.
[4] This species is an obligate mixotroph.
[5] *P. hoffmanianum* has been proposed to be conspecific with *P. belizeanum* (Herrera-Sepúlveda et al., 2015).
[6] After that paper, a *Prorocentrum* species very similar to *P. micans* was described from Korea (*Prorocentrum koreanum*), so the species designation of the strain used in this study need to be confirmed.
[7] There is a taxonomic issue here because the species usually (and in this paper) referred to as *P. triestinum* does not correspond to *P. triestinum* as described by Schiller.
[8] This paper report at least two different species, which are likely to be new to science but which are not yet formally described.
[9] See also Figure 3 Q–R.
[10] See also Figure 3 P.
[11] Red inclusion, described by Schütt as oil inclusion, but likely to represent a food vacuole. See Figure 1 B.

Table 2. Literature compilation of non-constitutive (NCM) dinoflagellate species. GNCM = Generalist NCMs, reSNCM = Specialist NCMs with reduced endosymbionts; pSNCM = Specialist NCMs, which just sequester the chloroplasts; eSNCM = Specialist NCMs with symbionts (a = ectosymbionts, b = endosymbionts). For abbreviations of Study type and Method see Table 1. Question mark (?) express uncertainty

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/ evidence	Feeding mode	Quantitative data
Gonyaulacales Gonyaulacaceae	*Amylax* sp.	marine plankton	reSNCM	Jacobson and Anderson, 1996	*Amylax* cf. *longiconnu*	F	LM food vacuole	–	–
Gonyaulacales Gonyaulacaceae	*Amylax triacantha*	marine plankton	reSNCM	Koike and Takishita, 2008	*Amylax triacantha*	F	LM, TEM cryptophyte vestiges	–	–
				Park et al., 2013	*Amylax triacantha*	C	LM, video[1] prey capture, engulfment	engulfment, tow filament, immobilization	growth, ingestion, GGE
Gonyaulacales Gonyaulacaceae	*Amylax triacantha* var. *buxus*	marine plankton	reSNCM	Koike and Takishita, 2008	*Amylax buxus*	F	LM, TEM cryptophyte vestiges	–	–
Thoracosphaerales Thoracosphaeraceae	*Cryptoperidiniopsis* sp.	marine plankton	reSNCM	Eriksen et al., 2002	*Cryptoperi-diniopsis* sp.	C	LM (Fl) ingested food, direct observation	peduncle	growth, grazing
Dinophysales Dinophysaceae	*Dinophysis acuminata*	marine plankton	pSNCM	Jacobson and Andersen, 1994	*Dinophysis acuminata*	F	LM, TEM food vacuoles	–	–
				Park et al., 2006	*Dinophysis acuminata*	C	experimental	peduncle	ingestion, growth
				Kim et al., 2008	*Dinophysis acuminata*	C	experimental	–	ingestion, growth (effects of light and prey concentration)
				Riisgaard and Hansen, 2009	*Dinophysis acuminata*	C	experimental	–	growth, ingestion, starvation (effect of prey concentration)
				Garcia-Cuetos et al., 2010	*Dinophysis acuminata*	C	LM, TEM ultrastructure of chloroplasts and chloroplast centres	–	–

Table 2. (Continued)

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/ evidence	Feeding mode	Quantitative data
Dinophysales Dinophyceae	*Dinophysis acuminata*	marine plankton	pSNCM	Nielsen et al., 2012	*Dinophysis acuminata*	C	experimental	–	growth and toxin production (light effects)
				Hansen et al., 2016	*Dinophysis acuminata*	C	experimental	–	photoregulation during starvation
				Mafra et al., 2016; Ojamäe et al., 2016	*Dinophysis acuminata*	C	LM, videos[1], experimental capture of prey using secreted mucus	peduncle	–
				Rusterholz et al., 2017	*Dinophysis acuminata*	C	experimental	–	division of kleptochloroplasts, starvation responses
				Garcia-Portela et al., 2018	*Dinophysis acuminata*	C	experimental	–	metabolomic profiles: effect of nutritional status and prey
Dinophysales Dinophysaceae	*Dinophysis acuta*	marine plankton	pSNCM	Nielsen et al., 2013	*Dinophysis acuta*	C	LM, experimental	peduncle	growth, toxin prod. (light effects)
				Hansen et al., 2016	*Dinophysis acuta*	C	LM, experimental	peduncle	photoregulation during starvation
				Rusterholz et al., 2017	*Dinophysis acuta*	C	LM (confocal), experimental	–	division of kleptochloroplasts, starvation responses
				Garcia-Portela et al., 2018	*Dinophysis acuta*	C	experimental	–	metabolomic profiles: effect of nutritional status and prey
Dinophysales Dinophysaceae	*Dinophysis norvegica*	marine plankton	pSNCM	Jacobson and Andersen, 1994	*Dinophysis norvegica*	F	LM, TEM food vacuoles	–	–

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/ evidence	Feeding mode	Quantitative data
Dinophysales Dinophysaceae	*Dinophysis caudata*	marine plankton	pSNCM	Nishitani et al., 2008a	*Dinophysis caudata*	C	LM, TEM	peduncle	growth and disappearance of prey
				Kim et al., 2012	*Dinophysis caudata*	C	LM, TEM, video[1]	peduncle	growth and disappearance of prey
Dinophysales Dinophysaceae	*Dinophysis fortii*	marine plankton	pSNCM	Nagai et al., 2008	*Dinophysis fortii*	C	LM, TEM	peduncle	growth and disappearance of prey
Dinophysales Dinophysaceae	*Dinophysis infundibulus*	marine plankton	pSNCM	Nishitani et al., 2008b	*Dinophysis infundibulus*	C	LM, TEM	–	growth and disappearance of prey
Dinophysales Dinophysaceae	*Dinophysis tripos*	marine plankton	pSNCM	Rodriguez et al., 2012	*Dinophysis tripos*	C	LM	–	growth and disappearance of prey
Gymnodiniales Gymnodiniaceae	*Gymnodinium gracilentum*	marine plankton	reSNCM	Skovgaard, 1998	*Gymnodinium ´gracilentum´*	C	LM, food vacuoles, kleptochloroplasts	–	growth, ingestion
				Jakobsen et al., 2000	*Gymnodinium gracilentum*	C	LM (description) kleptochloroplasts	–	prey spectrum, growth, ingestion, irradiance
Gymnodiniales Gymnodiniaceae	*Nusuttodinium acidotum*	freshwater plankton	reSNCM	Fields and Rhodes, 1991	*Gymnodinium acidotum*	C	LM (Fl) long-term growth in co-culture with *Chroomonas*	–	–
Gymnodiniales Gymnodiniaceae	*Nusuttodinium latum*	marine benthos	reSNCM	Horiguchi and Pienaar, 1992	*Amphidinium latum*	F	LM, TEM chryptophyte kleptochloroplasts	ungulfment without peduncle?	–
Thoracosphaerales Pfiesteriaceae	*Pfiesteria piscicida*	marine plankton	reSNCM (?) or GMCN (?)	Lewitus et al., 1999	*Pfiesteria piscicida*	C	LM, TEM, bicarbonate uptake activity of ingested chloroplasts	–	time series of chloroplast presence
				Feinstein et al., 2002	*Pfiesteria piscicida*	C	physiology kleptoplastidic activity	–	growth, grazing light effects
				Kim et al., 2013	*Pfiesteria piscicida*	C	molecular genes assoc. with plastids and photosynthetic functions	–	–

Table 2. (Continued)

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/ evidence	Feeding mode	Quantitative data
undetermined undetermined	undetermined undetermined	marine plankton	reSNCM	Gast et al., 2007	undescribed Antarctic dino	C	LM, TEM (klepto?)chloroplast of *Phaeocystis* origin; no long-term maintenance, only in co-culture	–	ingestion
Gymnodiniales Gymnodiniaceae	*Nusuttodinium myriopyrenoides*	marine benthos (?)	reSNCM (?)	Yamaguchi et al., 2011	*Gymnodinium myriopyrenoides*	F	LM, TEM, molecular evidence for kleptochloroplasts	–	–
Gymnodiniales Gymnodiniaceae	*Nusuttodinium poecilochroum*	marine benthos	reSNCM	Larsen, 1988	*Amphidinium poecilochroum*	F	LM, TEM ingestion, food vacuoles	peducle	–
				Jakobsen et al., 2000	*Amphidinium poecilochroum*	C	LM	–	ingestion, growth, survival (light effects)
Dinophysales Oxyphysaceae	*Phalacroma cuneus*	marine plankton	reSNCM (?)	Hallegraeff and Lucas, 1988	*Phalacroma cuneus*	F	LM (Fl) chlorophyll fluorescence	–	–
Dinophysales Oxyphysaceae	*Phalacroma favus*[2]	marine plankton	reSNCM (?)	Hallegraeff and Lucas, 1988	*Phalacroma favus*	F	TEM vacuole containing algal cells	–	–
Dinophysales Oxyphysaceae	*Phalacroma mitra*	marine plankton	pSNCM (?)	Hallegraeff and Lucas, 1988	*Phalacroma mitra*	F	LM (Fl) chlorophyll fluorescence	–	–
				Koike et al., 2005	*Dinophysis mitra*	F	LM, TEM	–	–
				Nishitani et al., 2012	*Dinophysis mitra*	F	molecular	–	–
Dinophysales Oxyphysaceae	*Phalacroma rapa*	marine plankton	reSNCM (?)	Hallegraeff and Lucas, 1988	*Phalacroma rapa*	F	LM (Fl), TEM chlorophyll fluorescence	–	–
Dinophysales Oxyhysaceae	*Phalacroma rapa*	marine plankton	reSNCM (?)	Hallegraeff and Jeffrey, 1984	*Dinophysis rapa*	F	LM (Fl) symbiont identification	–	–

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/ evidence	Feeding mode	Quantitative data
Dinophysales Dinophysaceae	*Citharistes* **2 spec** *C. apsteinii* *C. regius*	marine plankton	eSNCM a	Hallegraeff and Jeffrey, 1984; Foster et al., 2006; Tarangkoon et al., 2010		F	LM (Fl), molecular symbiont identification	–	–
Dinophysales Dinophysaceae	*Histioneis* **27 spec**. *H. pulchra* *H. highleyi* and 25 species as listed in Tanrangkoon et al. 2000	marine plankton	eSNCM a	Hallegraeff and Jeffrey, 1984; Lucas, 1991; Foster et al., 2006; Tarangkoon et al., 2010		F	LM (Fl), molecular symbiont identification	–	–
Dinophysales Dinophysaceae	*Ornithocercus* **8 spec.** *O. francescae* and 7 species as listed in Tarangkoon et al. 2000	marine plankton	eSNCM a	Hallegraeff and Jeffrey, 1984; Lucas, 1991; Foster et al., 2006; Tarangkoon et al., 2010		F	LM (Fl), molecular symbiont identification	–	–
Dinophysales Amphisoleniaceae	*Amphisolenia* **10 spec**.[3] as listed in Tarangkoon et al. 2000	marine plankton	eSNCM b	Hallegraeff and Jeffrey, 1984; Lucas, 1991; Foster et al., 2006; Tarangkoon et al., 2010; Daugbjerg et al., 2013		F	LM (FL), molecular symbiont identification	–	–
Dinophysales Amphisoleniaceae	*Triposolenia* **2 spec.**[4] *T. bicornis* *T. truncata*	marine plankton	eSNCM b	Tarangkoon et al., 2010		F	LM (Fl) symbiont identification	–	–
Noctilucales Noctilucaceae	*Noctiluca scintillans*	marine plankton	eSNCM b	Sweeney, 1976	*Noctiluca miliaris*	F	LM, TEM symbiont identification	–	–
Dinophysales Dinophysaceae	*Dinophysis miles*	marine plankton	eSNCM b (?)	Hallegraeff and Jeffrey, 1984	*Dinophysis miles*	F	LM (Fl) symbiont identification	–	–
Noctilucales Kodoidiniaceae	*Kofoidinium splendens*[5]	marine plankton	eSNCM b (?)	Gómez and Furuya, 2007	*Kofoidinium splendens*	F	LM red inclusion bodies	–	–
Peridiniales Kofoidiniaceae	*"kofoidiniaceans"*	marine plankton	eSNCMb (?)	Gómez and Furuya, 2007	immature kofoidiniaceans	F	LM presence of symbionts (?)	–	–
Peridiniales Podolampaceae	*Podolampas bipes*	marine plankton	eSNCM b (?)	Schweikert and Elbrächter, 2004	*Podolampas bipes*	F	LM (Fl), TEM symbiont identification	–	–

Table 2. (Continued)

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/ evidence	Feeding mode	Quantitative data
Peridiniales Podolampaceae	*Podolampas reticulata*	marine plankton	eSNCM b	Schweikert and Elbrächter, 2004	*Podolampas reticulata*	F	LM (Fl) autofluorescent endocytobionts	–	–
Dinophysales incertae sedis	*Sinophysis canaliculata*[6]	marine benthos	eSNCM b	Escalera et al., 2011	*Sinophysis canaliculata*	F	LM (Fl), TEM, molecular evidence for endosymbiont-like microorganisms	–	–
				Garcia-Portela et al., 2017	*Sinophysis canaliculata*	F	molecular characteristics of symbionts - cyanobacteria		
Peridiniales Kryptoperidiniaceae	*Unruhdinium* **7 species**	freshwater plankton	eSNCM b	Species list of the „dinotoms“ according to Moestrup & Calado 2018 and AlgaeBase (Guiry and Guiry, 2019)		C	These are the “dinotoms” genera /species characterised as hosting tertiary endosymbionts derived from diatoms (Gottschling, 2017). Although included here in the mixotroph table, the phagotrophic capabilities for most of the species are unknown and are in need of investigation. Recently, one dinotom (*Durinskia capensis*) was shown to be a kleptoplastic protist keeping its diatom only temporarily and thus to rely on phagotrophy (Yamada et al., 2019)		
Peridiniales Kryptoperidiniaceae	*Kryptoperidinium foliaceum*	marine plankton	eSNCM b						
Peridiniales Kryptoperidiniaceae	*Dinotrix paradoxa*	marine plankton	eSNCM b						
Peridiniales Kryptoperidiniaceae	*Durinskia* **6 species**	marine/ freshwater plankton	eSNCM b						
Peridiniales Kryptoperidiniaceae	*Galeidinium rugatum*	marine plankton	eSNCM b						
Peridiniales Kryptoperidiniaceae	*Blixaea quincecorne*	marine plankton	eSNCM b						
Gymnodiniales Gymnodiniaceae	*Lepidodinium* two species: *L. viride* and *L. chlorophorum*	marine plankton	eSNCM b	Elbrächter and Schnepf, 1996; Hansen et al., 2007a		C	These are two “green” dinoflagellates with endosymbionts of probable prasiophyte origin. Although included here in the mixotroph table, the phagotrophic capabilities for both species are unknown and are in need of investigation. Note that for *L. viride* there is SEM and TEM evidence for the presence of a peduncle (Hansen et al. 2007a)		
Amphidiniales Amphidiniaceae	*Amphidinium wigrense*[7]	freshwater?	reSNCM	Wilcox and Wedemayer, 1985	*Amphidinium wigrense*	F	TEM "endosymbiotic chloroplasts"	–	–
Thoracosphaerales Thoracosphaeraceae	*Apocalathium aciculiferum*	freshwater plankton (?)	(?)	Moestrup and Calado, 2018	*Apocalathium aciculiferum*	F	LM[8]	–	–

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/ evidence	Feeding mode	Quantitative data
Dinamoebidiales Dinamoebidiaceae	*Dinamoebidium coloradense*	freshwater benthos	(?)	Bursa, 1970	*Dinamoebidium coloradense*	F	LM presence of chloropl.	engulfment (amoeboid)	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium cryophilum*	freshwater plankton	reSNCM	Wilkox and Wedemayer, 1991	*Amphidinium cryophilum*	F	LM, TEM phagotrophy, food vacuoles	phagopod (NOT peduncle)	–
				Moestrup and Calado, 2018	*Gymnodinium cryophilum*	F	LM description of peduncle	–	–
Gymnodiniales Gymnodiniaceae	*Gymnodinium eucyaneum*	freshwater plankton	reSNCM	Xia et al., 2013	*Gymnodinium eucyaneum*	C	LM evidence for kleptochloroplasts	–	–
Gymnodiniales Gymnodiniaceae	*Nusuttodinium acidotum*[9]	freshwater plankton	reSNCM	Wilcox and Wedemayer, 1984	*Gymnodinium acidotum*	F	LM, TEM symbiont identification, peduncle	peduncle (?)	–
				Farmer and Roberts, 1990	*Gymnodinium acidotum*	F	LM, TEM evidence for kleptochloroplasts	–	–
Gymnodiniales Gymnodiniaceae	*Nusuttodinium amphidinoides*	freshwater plankton	reSNCM (?)	Geitler, 1924	*Gymnodinium amphidinoides*	F	LM (description) blue-green chromatophores, food vacuoles	–	–
Gymnodiniales Gymnodiniaceae	*Nusuttodinium aeruginosum*	freshwater plankton	reSNCM	Schnepf et al., 1989	*Gymnodinium aeruginosum*	F	LM, TEM evidence for symbionts	–	–
				Onuma and Horiguchi, 2015	*Nusuttodinium aeruginosum*	C	LM, TEM description of kleptochloroplasts	–	–
				Drumm et al., 2017	*Nusuttodinium aeruginosum*	C	LM	–	photosynthesis, growth and grazing
Gymnodiniales Gymnodiniaceae	*Nusuttodinium montanum*	freshwater plankton	reSNCM (?)	Moestrup and Calado, 2018	*Nusuttodinium montanum*	F	LM vacuoles containing blue bodies, *Chlorella* also observed inside	–	–

Table 2. (Continued)

Order Family	Genus species	Habitat	Type	Reference	Species name in the paper	Study type	Method/ evidence	Feeding mode	Quantitative data
Gymnodiniales Gymnodiniaceae	*Gymnodinium albulum*	freshwater plankton	reSNCM (?)	Moestrup and Calado, 2018	*Gymnodinium albulum*	F	literature[10] coloured globules	–	–
Suessiales Suessiaceae	*Prosoaulax lacustris*	freshwater plankton	reSNCM (?)	Calado and Moestrup, 2005	*Prosoaulax lacustris*	F	literature field sample evidence for kleptochloroplasts, presence of peduncle	peduncle (?)	–

[1] Video available as suppl. material in the referenced paper.

[2] This species is included in the review of NCMs by Park et al. (2014). In Hallegraeff & Lucas (1988) it is listed as heterotrophic species, and they just give a TEM "showing large vacuoles containing algal cells".

[3] Although included here in the mixotroph table, the phagotrophic capabilities for all *Amphisolenia* species are unknown and are in need of investigation.

[4] Although included here in the mixotroph table, the phagotrophic capabilities for both *Triposolenia* species are unknown and are in need of investigation.

[5] Described as "tentatively identified" in the reference.

[6] Is described to keep symbionts up to five month in culture. Phagotrophic capability for *S. canaliculata* is unknown and in need of investigation.

[7] Calado & Moestrup (2005) list more freshwater "blue-grey" or "blue-green" "*Amphidinium*" of uncertain phylogenetic affinities that likely also have symbionts: *Am. amphidinoides* (now in *Nusotodinium*), *Am. bidentatum* (now in *Nusottodinium*); *Am. bourrellyi*; *Am. caerulescens* (now in *Nusottodinium*); *Am. glaucum*; *Am. lacunarum* (now in *Nusottodinium*); *Am. oculatum* (now in *Nusottodinium*); *Am. phtartatum* (now in *Nusottodinium*).

[8] From Moestrup & Calado (2018): Reported to be mixotrophic, sucking out the contents of other dinoflagellates through a tube (Entz & Sebestyén in Huber-Pestalozzi, 1950). However, a peduncular system is absent (Craveiro et al. 2016).

[9] The genus *Nusottodinium* now has 18 freshwater species (Moestrup an Calado, 2018) which all have green to bluish or blue green plastids (which probably are all kleptochloroplasts). Listed in the table here are only species where additional evidence is available.

[10] Moestrup & Calado (2018) concluded from their literature survey: "The absence or presence of chloroplasts indicates a mixotrophic species".

Table 3. Mixotrophic parasitic dinoflagellate

Order Family	Genus species	Habitat	Type	Reference	Method/evidence	Feeding mode/comments
Peridiniales Blastodiniaceae	*Blastodinium* spp. (**12 species** as listed in Skovgaard et al. 2012)[1]	marine parasite of copepods	CM	Schiller, 1935; Sewell, 1951; Skovgaard et al., 2012	LM, TEM presence of chloroplasts	heterotrophic nutrition via osmotrophy?[2]
incertae sedis Oodiniaceae	*Crepidoodinium* *cyprinodontum*	marine parasite of fish	CM	Lom and Lawler, 1973; Cachon and Cachon, 1987; Coats, 1999	TEM presence of chloroplasts	attachment with finger-like rhizoids, suggested to be a symphoriont rather than an ectoparasite?
incertae sedis Oodiniaceae	*Crepioodinium* *australe*	marine parasite of fish	CM	Lom et al., 1993	LM, TEM presence of chloroplasts	might be considered to be an ectocommensal rather than a parasite?
Pyrocystales Pyrocystaceae	*Dissodinium* *pseudolunula*	marine parasite of copeod eggs	CM	Drebes, 1978; Elbrächter and Drebes, 1978; Gomez et al., 2009	LM (Fl) presence of chloroplasts	peduncle penerating the eggs
Thoracosphaerales Thoracosphaeraceae	*Pentapharsodinium* *tyrrhenicum*[3]	marine parasite of ctenophores	CM	Smith et al., 2007; Smith, 2011	LM, molecular parasite mode of photrophic species	peduncle that penetrates host epithelial cells
incertae sedis Oodiniaceae	*Protoodinium* *chattoni*	marine parasite of jellyfish	CM	Hovasse, 1935; Cachon and Cachon, 1971; Coats, 1999	LM, TEM yellow pigmentation, presence of chloroplasts	peduncle
incertae sedis Oodiniaceae	*Protoodinium* *hovassei*[4]	marine parasite of siphonophore	CM	Cachon and Cachon, 1971, 1987	LM presence of chloroplasts	–
Phytodiniales Phytodiniaceae	*Cystodinedria*[5] *inermis*	freshwater parasite of filamentous green alage	CM	Pfiester and Popovsky, 1979; Popovsky and Pfiester, 1982; Cachon and Cachon, 1987; Coats, 1999	LM, presence of chloroplast; amoeboid stages eat filamentous algae	–
Phytodiniales Phytodiniaceae	*Cystodinium*[6] *bataviense*	freshwater parasite of filamentous green algae	CM	Pfiester and Lynch, 1980; Timpano and Pfiester, 1985; Cachon and Cachon, 1987; Coats, 1999	LM, TEM presence of chloroplasts, feeding on filamentous algae	–
Suessiales Suessiaceae	*Piscinoodinium* *limneticum*	freshwater parasite of fish	CM	Jacobs, 1946	LM chloroplasts in swarmers	–
Suessiales Suessiaceae	*Piscinoodinium* *pillulare*	freshwater parasite of fish	CM	Lom, 1981; Cachon and Cachon, 1987; Coats, 1999	LM presence of plastids	peduncle with rhizoids

Table 3. (Continued)

Order Family	Genus species	Habitat	Type	Reference	Method/evidence	Feeding mode/comments
Phytodiniales Phytodiniaceae	*Haidadinium ichtyophilum*	freshwater parasite of fish	CM	Buckland-Nicks et al., 1990; Buckland-Nicks and Reimchen, 1995; Buckland-Nicks et al., 1997	LM presence of chloroplasts in vegetative cysts and dinospores	amoeboid form feeds on bacteria
Phytodiniales Phytodiniaceae	*Stylodinium sphaera*[7]	freshwater parasite of filamentous green algae	CM	Pfiester and Popovsky, 1979; Popovsky and Pfiester, 1982; Cachon and Cachon, 1987; Coats, 1999	LM presence of chloroplast	amoeboid stages eat filamentous algae

[1] Skovgaard et al. (2012) listed six more yet undetermined *Blastodinium* species.
[2] Note that heterotrophic nutrition of *Blastodinium* is suggested to be via osmotrophy and not phagotrophy.
[3] This species is mainly known as a phototrophic plankton species. Con-specificity of planktonic and parasitic taxa (despite their 100% SSU identity) needs confirmation?
[4] The nomenclature of *P. hovasse* is quite unclear. The name was provided by Cachon (1964) but without any description and thus probably is not validly described.
[5] Reported as parasites by Pfiester & Popovsky (1979) or as facultative predator-autotrophs by Popovsky & Pfiester (1982). The genus *Cystodinedria* has 19 species (Moestrup & Calado 2018) that lives as phototrophs attached to filamentous algae, macrophyte leaves, *Lemna* roots, etc. It is unknown if species other than *C. inermis* are phagotrophic.
[6] Likely to be the only species of the genus *Cystodinium* (19 species, Moestrup & Calado 2018) reported to have a parasitic amoeboid stage. For other *Cystodinium* species phagotrophy is unknown.
[7] Reported as parasites by Pfiester & Popovsky (1979), as facultative predator-autotrophs by Popovsky & Pfiester (1982). The genus *Stylodinium* has 13 phototrophic species (Moestrup & Calado 2018) attached to other algae or macrophytes. It is unknown if species other than *S. sphaera* can be phagotrophic.

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Chapter 143

ECOPHYSIOLOGY AND BLOOM DYNAMICS OF *KARENIA* WITH EMPHASIS ON *KARENIA BREVIS* IN FLORIDA WATERS

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ABSTRACT

A *harmful algal bloom* or HAB can refer to any number of different "harmful" attributes related to the proliferation of a particular species or consortium of species of microscopic algae. The vast majority of HABs are caused by dinoflagellates. The family Kareniaceae is a primarily photosynthetic, unarmored lineage of dinoflagellates that includes the marine genus, *Karenia.* The majority of the ten validly described *Karenia* species can be taxonomically differentiated by morphology and molecular phylogeny. Phycotoxins have been characterized in six species. Brevetoxins, produced largely by *K. brevis*, can cause extensive wildlife mortalities during and after a bloom as well as human health impacts when aerosolized via sea spray or consumed via contaminated shellfish. Due to the substantial impacts related to *K. brevis* and *K. mikimotoi* blooms in particular, progress towards understanding the drivers of sequential phases of blooms – initiation, growth, maintenance, and termination – has necessitated an interdisciplinary approach to best consider linkages between offshore and nearshore processes. Laboratory and field studies demonstrate the versatility of *K. brevis* and *K. mikimotoi* necessary to exist across ecological niches. Although measured growth rates are moderate, strategies such as diel vertical migration, allelopathy, and mixotrophy further broaden the potential niche of these species. Yet, in spite of these similarities, there are intriguing differences between *K. brevis* and *K. mikimotoi* and among the other *Karenia* species in terms of the toxins they produce, the frequency and magnitude of impacts related to blooms, and their biogeographic distribution. High frequency ocean observing technology is needed to complement routine monitoring in the development and validation of more

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comprehensive forecasting and hindcasting systems. Future approaches need to be flexible in assimilating new discoveries into bloom dynamics gleaned from contemplating the variable and shared characteristics of *Karenia* species and other ecologically similar – or different – HABs.

Keywords: Kareniaceae, *Karenia brevis*, *Karenia mikimotoi*, bloom, brevetoxin, Gulf of Mexico, Florida

1. INTRODUCTION

1.1. *Karenia* and Algal Blooms

"Harmful Algal Blooms" (HABs) occur world-wide in freshwater, brackish, coastal and open marine waters, and in both the pelagic and benthic realms. Over 60% of toxic marine microalgae are dinoflagellates (Moestrup et al., 2009). The marine dinoflagellate genus *Karenia* is currently represented by ten species, six of which are confirmed to produce toxins, three suspected of producing toxins because of associated effects, and one undocumented to produce toxins (Tables 1–2). In 2000, when Danish scientists Daugbjerg, Hansen, Larsen and Moestrup described the genus *Karenia*, there were only three species; now there are ten with several morphs still awaiting morphologic and genetic characterization. *Karenia* is now recognized to be a genus with representation world-wide, even though the most documented species, *K. brevis* (C.C. Davis) Gert Hansen & Moestrup, is only verified from the Gulf of Mexico, adjacent Caribbean, and US Atlantic waters influenced by the Gulf Stream (Figure 1). A bloom of *K. brevis*, for example, is often defined by its effects, which may vary, that is, cell concentrations that can cause respiratory irritation and shellfish toxicity, cell concentrations that reach high enough numbers to kill fish or concentrations that may discolor the water and even be detected by satellite (Table 3).

All HABs, regardless of final biomass, go through sequential development phases: initiation, growth, maintenance, and dispersal/termination of blooms (Steidinger et al., 1998). However, the entire development cycle may not be in the same area or habitat, and life cycle history varies across species. Depending on species and/or the event, initiation can originate from a planktonic population or a benthic population (cyst or resting stage) and, in the marine environment, can occur in oceanic or coastal waters, or in the estuarine environment, in areas that tend to have longer residence times such as bays. After initiation comes growth (or bloom development), when the influence of environmental parameters such as temperature, light, salinity, nitrogen, phosphorus, silicates, humic substances, grazing, etc. determine if growth will exceed loss and result in increases in biomass. Bloom maintenance requires that population growth be sustained for some period of time, and that may also include retention in a gyre, a current, thin layer, or some other physical dimension. Termination of a bloom may be physical (transported out of an area), chemical (change in nutrients, salinity, temperature, etc.), or biological (grazing by predators, viral infection, parasites, life cycle transitions).

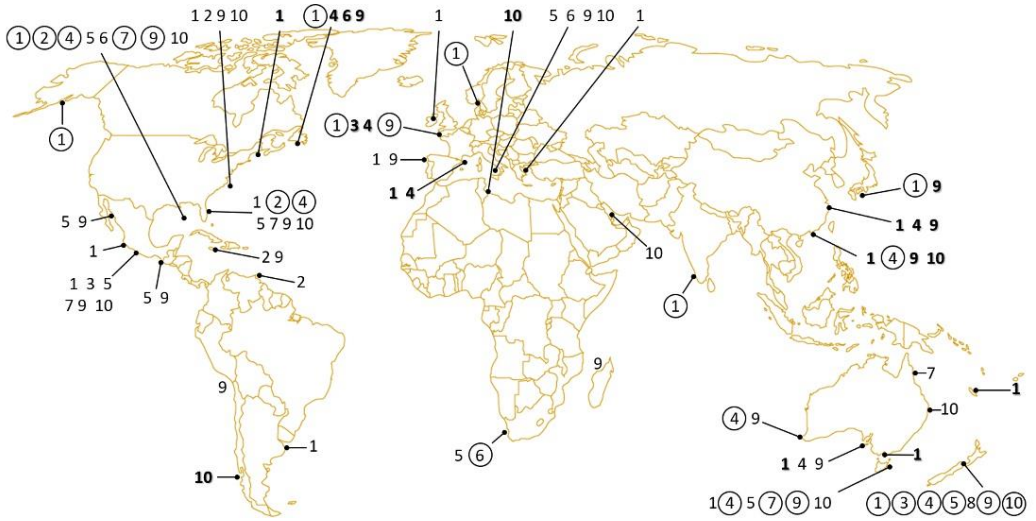


Figure 1. World distribution of *Karenia* spp. Numbers correspond to species in chronological order in which they were described: 1 – *mikimotoi*, 2 – *brevis*, 3 – *brevisulcata*, 4 – *longicanalis*, 5 – *bicuneiformis*, 6 – *cristata*, 7 – *asterichroma*, 8 – *concordia*, 9 – *papilionacea*, and 10 – *selliformis*. Plain text is used for morphological information only and bold for genetic information only; circles are used when both types of information are available. Genetic information is based on *Karenia* sequences available in NCBI's Genbank database as of April 2019 that provide location of origin (see Table 1), except for the following species: 1 in India (Godhe et al., 2001); 10 in Chile (Guillou et al. 2002, GenBank AF318247 as *Gymnodinium* sp., J. Mardones pers. comm.); 1, 2, 4, 7, and 9 in the Gulf of Mexico (FWC, unpublished), 2 and 4 on the Florida East Coast (FWC, unpublished); and 1 in New Hampshire, USA (FWC, unpublished). Source of morphological information is as follows: 9 in Pacific South America (Gómez, 2006); 1, 3, 5, 7, 9, 10 in Pacific Mexico (del Castillo et al., 2013); 1 in Alaska (NCCOS, 2014); 1 in Argentina (Negri et al., 1992); 2 in Trinidad (Lackley, 1956); 2 and 9 in Jamaica (Ranston, 2008); Gulf of Mexico (Steidinger, 2018); Florida East Coast (FWC, 2019); 1 in Delaware, USA (www.dnrec.delaware.gov/Pages/RedTideINformation.aspx, accessed on May 2019); 2, 9, and 10 in Delaware, USA (ARC, Algal Resources Collection http://www.algalresourcescollection.com, accessed on May 2019); 1 in eastern Canada (Blasco et al., 1996); 5 an 6 in South Africa (Botes et al., 2003); 1 in northwestern Europe (Hansen et al., 2000); 9 in northwestern Europe (Fraga & Sanchez, 1985; Nézan, 1998); 5, 6, 9, and 10 in eastern Italy (Zingone et al., 2006); 1 in the Aegian Sea (Bizsel & Bizsel, 2002); 10 in the Arabian Sea (Heil et al., 2001); 1 in India (Robin et al., 2013); 9 in Madagascar (Sournia, 1972); 1 in Japan (Oda, 1935); 4 in China (Chang, 1999); 1, 4, 5, 7, 9, 10 in Australia (Hallegraeff et al., 2010); and 1, 3, 4, 5, 8, 9, 10 in New Zealand (Haywood et al., 2004; Chang & Ryan, 2004).

In this chapter, we discuss the ecophysiology and bloom dynamics of *K. brevis*. To this end, we have set the stage by summarizing our current understanding of *Karenia* taxonomy, their worldwide distribution, and the effects of toxin production. This is followed by some basic information on life cycle, behavioral, and physiological aspects that are key to better understand bloom dynamics. As much as possible, we have traced contrasts and parallels with what is known for *K. mikimotoi* (Miyake & Kominami ex Oda) Gert Hansen & Moestrup, another extensively studied species in the genus. Although there is not a large body of work directly comparing *K. brevis* with *K. mikimotoi*, there are a number of viable comparisons of interest that can be made from independent research efforts.

1.2. Taxonomy and Distribution

The family Kareniaceae Bergholts, Daugbjerg, Moestrup & Fernández-Tejedor was established to accommodate *Karenia* G. Hansen & Moestrup, *Karlodinium* J. Larsen, and *Takayama* M.F. Salas, Bolch, Botes & Hallegraeff (Bergholtz et al., 2005). This is a well-supported evolutionary lineage, as inferred from partial LSU rDNA sequences of unarmored, primarily photosynthetic dinoflagellates with straight or "s" shaped apical grooves. Pigment composition is also typical for this family, that is, lack of peridinin and the presence of gyroxanthin-diester and a host of fucoxanthin-type accessory pigments (Steidinger et al., 2008 and references therein). Refinement of our understanding of phylogenetic relationships indicates a close association between Kareniaceae and *Brachidinium capitatum* F.J.R. Taylor (e.g., Henrichs et al. (2011); Kretschmann et al. (2015)). If this branch is recognized as a valid grouping at the family level, then Brachinidiaceae (Sournia, 1972) would take priority over Kareniaceae, in agreement with the International Code of Nomenclature for algae, fungi and plants. In addition, phylogeny based on nucleus-encoded rDNAs suggests that the recently described *Gertia stigmatica* K. Takahashi & Iwataki may be also positioned within Kareniaceae (Takahashi et al., 2019); the presence of peridinin in this new taxon, however, is not recognized as a taxonomic character for this family.

Understanding past and current morphological diagnostic characters used to discriminate the taxa in question is important to help navigate recent and future updates in the taxonomy and nomenclature of *Karenia* species. Until recently, morphological differentiation of *Karlodinium* from *Karenia*, both with straight apical grooves, was mostly based on the presence of a ventral pore to the left of the groove on the former. However, as the presence of a ventral pore has proved to be a variable character (e.g., de Salas et al. (2008)), a better way to distinguish these two genera morphologically has been suggested based on pyrenoid shape: an internal lenticular pyrenoid in *Karlodinium* vs. a triangular, multi-stalked pyrenoid in *Karenia* (Lou et al., 2018 and references therein).

All ten validly published *Karenia* species are marine and most were originally described from bloom events, except for *K. papilionacea* A.J. Haywood & K.A. Steidinger that was isolated from a water sample of < 500 cells L^{-1}. All taxa have been studied using morphological and molecular data combined, except for *K. concordia*, for which only morphological information is available (Table 1). The taxa listed in the literature as *Karenia umbella* Salas, Bolch & Hallegraeff and *Karenia digitata* Yang, Takayama, Matsuoka & Hodgkiss have undergone recent taxonomic revision. *Karenia umbella* is now established as a junior synonym of *K. longicanalis* (Wang et al., 2018). In the second case, *K. digitata* has been transferred to *Karlodinium digitatum* (Yang, Takayama, Matsuoka & Hodgkiss) Gu, Chan & Lu, based on its genetic make-up, lack of ventral pore, and the presence of lenticular pyrenoids (Lou et al., 2018).

Diagnostic characters helpful in discriminating between *Karenia* species using light microscopy (LM) are cell outline and nucleus shape and position, especially when stained with Lugol's solution (see illustrations in Table 2). Apical groove and sulcal design are key taxonomic features discernable under LM optimal settings and with electron microscopy (EM). Except for *K. mikimotoi*, named after Mikimoto Koukichi who was involved with red tides while establishing the famous Akoya pearl oyster hatchery in Japan in the early 1900s, all other *Karenia* species have very descriptive names (see etymology in Table 2). That said,

unarmored dinoflagellates are pleomorphic because of their flexible amphiesma or theca, and *Karenia* species can also be polymorphic due to sexual stages (see section 2.1; see Figure 2).

Eight of the ten *Karenia* species have been implicated in wildlife and/or human health impacts, and only six of them have had toxin(s) characterized: *K. brevis*, *K. brevisulcata*, *K. concordia*, *K. mikimotoi*, *K. papilionacea*, and *K. selliformis* (Table 2). Only *K. asterichroma* and *K. bicuneiformis* are not known (or confirmed) to produce toxins. Due to the difficulties in the identification of unarmored dinoflagellates from field preserved samples, one may expect that as of yet unnamed taxa could fit in the genus *Karenia* once their genetics and description of other characters are properly assessed. Some *Karenia* morphotypes have actually already been established, based on field material from the Gulf of Mexico (Steidinger et al., 2008). Ideally, species descriptions would be based on a combination of morphological and genetic characters, in association with biochemical (pigment composition, toxin profiles) and behavioral features such as swimming pattern and modes/stages in sexual reproduction. Biogeographic distribution patterns vary among species – and potentially intraspecific variants – and can be another important factor to consider as part of taxonomic evaluation.

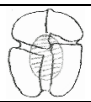
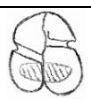
Table 1. List of *Karenia* species, synonyms, and genetic information available

Karenia species	Synonym	(‡) Genbank representation
(1) *K. mikimotoi* (Miyake & Kominami ex Oda) Gert Hansen & Moestrup	*Gymnodinium mikimotoi* Miyake & Kominami ex Oda *Gyrodinium nagasakiense* Takayama & Adachi *Gymnodinium nagasakiense* H. Takayama & M. Adachi	386 entries, 50+ genes (Including: 5.8S, 18S, 28S, *cox1*, ITS1, ITS2, *rbcL*)
(2) *K. brevis* (C.C. Davis) Gert Hansen & Moestrup in Daugbjerg *et al.*	*Gymnodinium brevis* C.C. Davis *Gymnodinium breve* C.C. Davis *Ptychodiscus brevis* (C.C. Davis) K.A. Steidinger	375 entries, 50+ genes (Including: 5.8S, 18S, 28S, *cox1*, ITS1, ITS2, *rbcL*)
(3) *K. brevisulcata* (F.H. Chang) Gert Hansen & Moestrup	*Gymnodinium brevisulcatum* F.H.Chang	2 entries, 1 gene (28S)
(4) *K. longicanalis* Z.B. Yang, I.J. Hodgkiss & G.Hansen	*K. umbella* Salas, Bolch & Hallegraeff	20 entries, 4 genes (5.8S, 28S, ITS1, ITS2)
(5) *K. bicuneiformis* Botes, Sym & Pitcher	*Karenia bidigitata* Haywood & Steidinger	11 entries, 7 genes (5.8S, 18S, 28S, *cox1*, ITS1, ITS2, *rbcL*)
(6) *K. cristata* L.Botes, S.D.Sym & G.C.Pitcher		2 entries, 1 gene (28S)
(7) *K. asterichroma* Salas, Bolch & Hallegraeff		1 entry, 1 gene (28S)
(8) *K. concordia* F.H. Chang & K.G. Ryan		no submissions
(9) *K. papilionacea* A.J.Haywood & K.A.Steidinger		52 entries, 6 genes (5.8S, 18S, 28S, *cox1*, ITS1, ITS2)
(10) *K. selliformis* A.J. Haywood, K.A. Steidinger & L. MacKenzie		14 entries, 7 genes (5.8S, 18S, 28S, *cox1*, ITS1, ITS2, *rbcL*)

Original descriptions and subsequent selected taxonomic information is found in: (1) Oda (1935), Takayama & Matsuoka (1991), and Hansen et al. (2000); (2) Davis (1948); (3) Chang (1999); (4) Yang et al. (2001), de Salas et al. (2004a), and Wang et al. (2018); (5) (6) Botes et al. (2003); (7) de Salas et al. (2004b); (8) Chang & Ryan (2004); (9) (10) Haywood et al. (2004).

(‡) https://www.ncbi.nlm.nih.gov/genbank/ (accessed on April 2019). Genes included if either a partial or full sequence available. Abbreviations 18S, 5.8S, ITS1, ITS2, 28S refer to genes within the rRNA operon, and do not discriminate between sequences originating from RNA or DNA. Although all entries are reported, a number of sequences did not have associated journal publications; these numbers are as follows: *K. brevis* (114), *K. mikimotoi* (40), *K. papilionacea* (7), and *K. selliformis* (6).

Table 2. Cell outline of *Karenia* species in ventral view (not to scale, original drawings by K. A. Steidinger), depicting typical shape and position of the nucleus (dashed lines); meaning of the epithet; location of original description; and impacts to wildlife and human health cause by their respective toxins

	Karenia	date described	etymology [1]	described from [1]	wildlife impacts [2]	human impacts [2]	toxins [2]
	mikimotoi	1935	After Mikimoto Koukichi, (Akoya pearls)	Japan	mortality of fish and invertebrates	none reported	haemolytic and cytotoxic compounds, brevetoxins (?)
	brevis	1948	Latin brevis – short: shorter apical length	Florida, USA	mortality of fish, invertebrates, birds, turtles, mammals	NSP, respiratory distress	brevetoxins and open-ring oxidized derivatives
	brevisulcata	1999	Latin brevis – short, sulcatum – groove: short apical groove	New Zealand	mortality of invertebrates, vertebrates, and flora	respiratory distress and skin irritations	brevisulcenals (KBTs) and brevisulcatic acids (BSXs)
	longicanalis	2001	Latin longi – long, canalis – canal: long apical groove	Hong Kong	fish kill (aquaculture) [3]	none reported [3]	not confirmed [3]
	bicuneiformis	2003	Latin bi – two, cuneiformis – wedge-shape: form of the hypocone	South Africa	none reported	none reported	not confirmed [4]
	*cristata*	2003	Latin cristata – crest: apical crest	South Africa	mortality of benthos, especially abalone	respiratory distress and skin irritations	not confirmed
	asterichroma	2004	Greek astēr – star, chroma – color: radiating chloroplast	Tasmania	fish kill (?)	none reported	not confirmed
	concordia	2004	Lateral view resembles Concord aircraft	New Zealand	mortality of fish and abalone	none reported	brevetoxins [5]
	papilionacea	2004	Latin papilionaceus – butterfly-like: swimming pattern	New Zealand	none reported	none reported	brevetoxins [6]
	*selliformis*	2004	Latin selliforme – saddle-shaped: form of the hypocone	New Zealand	fish kills	none reported	gymnodimines

? Toxic compound not detected or direct cause of fish kill not confirmed (species part of mixed *Karenia* assemblage).

[1] References in Table 1. See broader geographic distribution in Figure 1.

[2] Based on Lassus et al. (2016 and references therein), except otherwise noted.

[3] Fish kill observed in Tasmania (de Salas et al., 2004a) but toxin not confirmed (Yang et al. 2001; de Salas et al., 2004a).

[4] Brevetoxins from preliminary ELISA in culture (as *K. bidigitata* in Haywood et al., 2004) have not been confirmed.

[5] Toxin results presented in conference abstract (Chang et al., 2006) without further details or corroboration.

[6] Fowler et al. (2015).

The perception that *Karenia* are typically neritic species, though not incorrect, is likely biased by the many reports of bloom manifestations in coastal waters. As will be discussed in sections 2 and 3, multiple sources of evidence suggest that at least *K. brevis* and *K. mikimotoi* can also thrive in more oligotrophic, offshore waters. Likewise, *K. papilionacea* and *K. asterichroma* reports indicate their distributions may extend offshore the Florida west coast (respectively, Steidinger et al., 2008 and FWC, 2019). Geographic locations where the highest numbers (≥ 4) of *Karenia* species have been reported are, not unexpectedly, where more intensive and targeted research (and bloom-related impacts) have taken place (Figure 1): the Gulf of Mexico, North America Atlantic coast (Florida, Delaware, and Newfoundland), the Pacific coast of Mexico, the northeast coast of Europe, the west coast of Italy, New Zealand, Tasmania, and China (Hong Kong region). Admittedly, there may be species check-lists that cite a *Karenia* species not included in our assessment that only considered morphological records with illustrations and/or a minimal description of identifiable taxonomic characters and/or a corresponding sequence in NCBI's Genbank database. Gaps in our understanding of *Karenia* diversity worldwide are more critical in the southeast Pacific, the south Atlantic, and the Indian Ocean.

The range of *Karenia brevis* is restricted to the region encompassing the Gulf of Mexico and the Caribbean (Jamaica, and possibly Trinidad), the eastern Atlantic coast of the US from the Florida Keys up to Delaware, as well as the Gulf Stream (Figure 1). The Gulf Stream has been a transport mechanism of surface waters from the Gulf of Mexico and Caribbean to northern latitudes (Tester et al., 1989). *Karenia mikimotoi*, *K. papilionacea*, and *K. seliformis* records, on the other hand, are the most widespread of all *Karenia* species (Figure 1). One should be aware that, prior to its description in 2004, some *K. papilionacea* records were published as an unidentified dinoflagellate (Sournia, 1972) and as *Gymnodinium* breve-like cells (Fraga & Sanchez, 1985; Nézan, 1998). Illustrations in those cases, however, are unequivocal: cell wider than long; hypotheca deeply excavated posteriorly forming an inverted V indentation; the left hypothecal lobe rounded and the right hypothecal lobe truncate; and round nucleus in left hypothecal lobe (see line drawing in Table 2).

Reconciling past and current information on *K. mikimotoi* (the first *Karenia* ever to be described in Oda (1935)) requires following the trail of not only its synonyms (Table 1) but also three decades of its misidentification as *Gymnodinium aureolum* (Hulburt) G. Hansen (=*Gyrodinium aureolum* Hulburt) in European waters (Hansen et al. 2000). The identity and distribution of this taxon is actually still unfolding. At least two distinct populations have been suggested based on sequences from ribosomal RNA (28S and ITS) and the *rbc*L genes. Isolates from Europe and New Zealand are more closely related to each other than to isolates from Japan (Al-Kandari et al., 2011). Similarly, our knowledge of the degree of intraspecific genotypic diversity within *K. brevis* is limited. To date, 18S rRNA sequencing analyses by Loret et al. (2002) identified little intra-specific diversity within this gene, whereas finer scale microsatellite population markers developed by Renshaw et al. (2006) and Henrichs et al. (2013) identified a larger degree of genotypic diversity among *K. brevis*.

Not surprisingly, *K. brevis* and *K. mikimotoi* have the highest number of sequences submitted to Genbank, approximately seven times as many sequences as the next highest species, *K. papilionacea* (Table 1). Three *Karenia* species, *K. asterichroma*, *K. breviculcata*, and *K. cristata* have less than 10 Genbank submissions and *K. concordia* has no sequences submitted to Genbank. The seven most represented regions among *Karenia* species are: 5.8S, 18S, and 28S and ITS1/ITS2 (ribosomal RNA genes and associated intergenic spacers,

respectively), *cox1* (cytochrome c oxidase subunit I), and *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit). *K. brevis*, *K. mikimotoi*, *K. bicuneformis*, and *K. selliformis* have sequences submitted for all seven of these genes and *K. papilionacea* is only missing one (*rbcL*) of the seven genes. Overall, 28S is the best represented gene as all *Karenia* species that have submitted Genbank sequences have at least one 28S sequence.

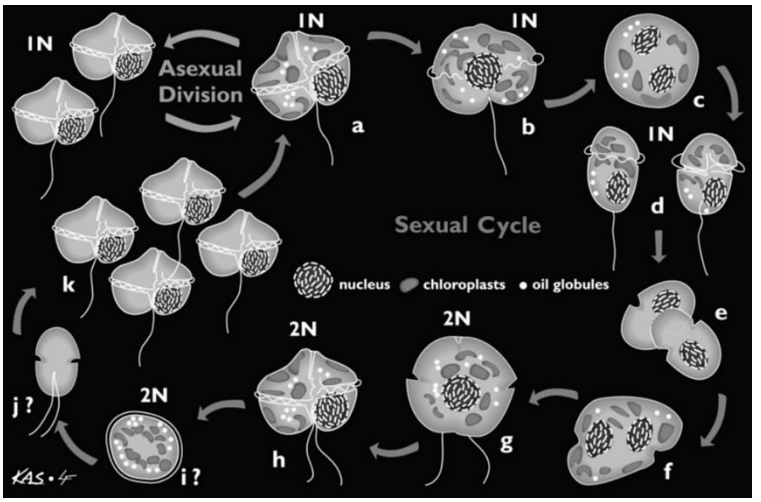


Figure 2. *Karenia brevis* life cycle. (a) Vegetative 1N cell that is capable of mitotic division or producing gametes. (b) First stage in gamete formation with movement of nucleus to center. (c) Second stage of gamete formation with division of nucleus. (d) Third stage with production of isogametes. In heterothallic strains, there are + and – mating types that fuse. In homothallic strains, gametes are the same mating type. (e) (f) Cytoplasmic fusion precedes nuclear fusion. (g) Planozygote with 2N nucleus and 2 longitudinal flagella. (h) Motile planozygote. (i) Unconfirmed presumed hypnozygote. (j) Unconfirmed meiocyte. (k) Resultant 4 1N cells from meiosis II. In Steidinger et al. (1998), copyright permission from K. Steidinger.

Large scale sequencing efforts for *Karenia* began with sequencing of EST (expressed sequence tag) libraries of *K. brevis* in 2005, which was subsequently used to evaluate the transcriptome by microarray (Liddie et al., 2005; Morey et al., 2011; Johnson et al., 2012; Morey & Van Dolah, 2013). This data has led to valuable insight into the molecular underpinnings of *K. brevis* and of its chimeric proteome, which has been acquired over the course of multiple plastid replacements (Nosenko et al., 2006). Since then, numerous high throughput sequencing efforts have been conducted targeting transcriptomes of *K. brevis* (Keeling et al., 2014; Ryan et al., 2014) and *K. mikimotoi* (Kimura et al., 2015; Dorrell et al., 2016; Luo et al., 2017), which promise even finer phylogenetic analyses of the Kareniaceae (e.g., Janouškovec et al. (2017)). Nuclear genome size is approximately four times larger in *K. brevis* as compared with *K. mikimotoi* (Cuadrado et al., 2019); this large genome has numerous implications, including having different genetic stocks available for evolutionary adaptation, altering the nutritional requirements due to greater/lesser nucleic acid biosynthesis, and possibly thereafter affecting the time required for DNA synthesis and gating thresholds for S-phase initiation (see Section 2.2).

To date, there are no draft genomes for any *Karenia* species, although these and other new approaches will continue to yield further important genomic insights into these and other

toxic dinoflagellates in spite of challenges associated with the large genome sizes of dinoflagellates.

1.3. Toxins and Their Effects

Karenia species may produce a host of bioactive compounds (Caruana & Amzil, 2018 and references therein), some cytotoxic whose effects have been tested solely in bioassay-model cells (e.g., Tanaka et al. (2013)). Phycotoxins that have been demonstrated to negatively affect wildlife and/or human health are brevetoxins, gymnodimine, brevisulcenals (KBTs), and brevisulcatic acids (BSXs) (Table 2). There are also reports on the production by some *Karenia* species of allelochemical compounds that suppress the growth of other microalgae (e.g., Freeburg et al. (1979); Kubanek et al. (2005); Prince et al. (2008); Chang (2011)), although stimulation of diatom growth has also been observed under certain experimental conditions (e.g., Poulson et al. (2010)). Here we focus on the effects of brevetoxins, and the reader is referred to Miles et al. (2000) for information on gymnodimine and Holland et al. (2012) for KBTs and BSXs. Interestingly, *K. brevis* from laboratory cultures and natural samples also produces brevenal, which is a natural inhibitor of brevetoxin action in sodium channel receptor binding assays thus antagonistic to brevetoxin toxicity (Bourdelais et al., 2004; Errera et al., 2010).

Brevetoxins are neurotoxins produced by at least three *Karenia* species (Table 2). These toxins are a suite of at least nine structurally related congener compounds from which numerous derivatives and metabolites of varying toxicity may be produced (Baden et al., 2005; Abraham et al., 2006; Caruana & Amzil, 2018). Brevetoxins may cause indiscriminate wildlife mortalities, affecting hundreds of fish species, as well as birds, turtles, and marine mammals. Examples of the diversity of toxin molecule structures, the toxicokinetics at the cellular level, and the species affected and their symptoms are detailed in Landsberg (2002). Although the most common perception of harmful effects to wildlife is that of massive mortality during intense blooms (e.g., Gunter et al. (1947); Forrester et al. (1977); Bossart et al. (1998); Flewelling et al. (2010); Fauquier et al. (2013); Fire et al. (2015); Foley et al. (2019)), there is mounting evidence for effects on wildlife resulting from lower level, chronic exposures to brevetoxins (e.g., Walsh et al. (2015); Perrault et al. (2016)). The complexity of assessing chronic exposure rests in a combination of factors, such as the *K. brevis* strain/population in question, the brevetoxin type and its extracellular stability, the exposure routes, and the susceptibility of the affected species (Landsberg, 2002; Landsberg et al., 2014). Toxins transferred through the food web is an additional layer of complexity given that brevetoxins may be present in different forms and compartments, such as inside the cell, dissolved in the water (once cells lyse or die), aerosolized in sea spray, adsorbed to particles in the water or in the sediments, and stored in biota tissue (Landsberg et al., 2009; Landsberg et al., 2014). For instance, the space-time decoupling between *K. brevis* blooms and marine mammals found sick or dead on the Gulf coast of Florida has been traced to fish and seagrass acting as toxin vectors for, respectively, bottlenose dolphins (*Tursioples truncatus*) and manatees (*Trichecus manatus latirostris*) (Flewelling et al., 2005). Likewise, evidence suggests that brevetoxins may cause negative effects to nesting loggerhead sea turtles (*Caretta caretta*) long after a bloom has dissipated, with signs of impairment to their immune and reproductive systems, including hatching success (Perrault et al., 2016). Brevetoxin-

contaminated fish that wash on beaches may also represent a risk factor for terrestrial mammals (Castle et al., 2013) and scavenging shorebirds (van Deventer et al., 2012), with potential consequences to resident or migratory populations not fully understood.

Table 3. *Karenia brevis* concentrations and corresponding expected effects, based on criteria established by the Florida Fish and Wildlife Conservation Commission for the statewide status report on red tide monitoring and prediction

Concentrations	Possible effects
BACKGROUND: not detected to 1,000 cells L^{-1}	no effects anticipated
VERY LOW: >1,000 - 10,000 cells L^{-1}	possible respiratory irritation and shellfish harvesting closures
LOW: > 10,000 - 100,000 cells L^{-1}	respiratory irritation; shellfish harvesting closures; possible fish kills; probable detection of surface chlorophyll by satellites at upper range of cell abundance
MEDIUM: > 100,000 - 1,000,000 cells L^{-1}	respiratory irritation; shellfish harvesting closures; possible fish kills; detection of surface chlorophyll by satellites
HIGH: > 1,000,000 cells L^{-1}	as above, plus water discoloration

(https://myfwc.com/research/redtide/statewide/, accessed on April 2019). This discussion considers "medium" and "high" concentrations as bloom level, even though effects may be observed and management procedures start at "very low" concentrations due to brevetoxins' high toxicity.

Brevetoxins can threaten human health in two primary ways:

- *Aerosolized brevetoxins.* The toxins from ruptured *K. brevis* cells can be aerosolized in sea spray so that beachgoers and nearby communities can experience respiratory irritation such as coughing, sneezing, tearing, and itchy throat. Because the toxins are aerosolized as waves break, the severity of respiratory symptoms depends on both *K. brevis* cell concentration in the water (e.g., Table 3), on wind speed and direction, as well as vulnerability of the person exposed.
- *Brevetoxin contaminated shellfish.* Bivalve and gastropod molluscan shellfish accumulate the toxins in their tissues and can cause neurotoxic shellfish poisoning (NSP) in human and animal consumers. NSP symptoms can include few to several of the following: headache, nausea, diarrhea, abdominal pain, tingling of the mouth, reversal of hot-cold temperature sensation, vertigo, loss of coordination, slurred speech, slow heart rate, dilated pupils, muscle pain, and respiratory distress (Landsberg, 2002; Watkins et al., 2008). No human fatalities have been attributed to NSP, and no outbreaks have occurred in Florida from legally harvested bivalve molluscs since regulatory guidelines were put in place in the 1970s based on cell abundance as a proxy for brevetoxins. In a continued effort to prevent NSP, systematic monitoring of *K. brevis* concentrations in Florida waters has informed timely closures of commercial shellfish beds (Table 3) in association with toxin testing in the tissues of the harvestable molluscs (NSSP, 2017). Communication loopholes still need to be addressed to guarantee the same level of public safety when it comes to recreational harvest of bivalve shellfish and gastropods (Reich et al., 2015).

2. LIFE CYCLE, BEHAVIOR AND PHYSIOLOGY OF *K. BREVIS* (WITH CONTRASTS AND PARALLELS FOR *K. MIKIMOTOI*)

Understanding the life cycle and ecophysiology of the target organism(s) sets the stage to investigate and interpret the biological and oceanographic connections involved in bloom dynamics. *Karenia brevis* is by far the most intensively studied species within the genus, seconded by *K. mikimotoi*. Among many other publications, information on the basic biology of the former has been discussed as it pertains to bloom dynamics in Steidinger et al. (1998) and in special issues edited by Walsh & Kirkpatrick (2008) and O'Neil & Heil (2014); the latter has been the focus of reviews by Partensky et al. (1991) and Gentien (1998) for European waters, and by Honjo (2004) for Japanese waters. The review by Brand et al. (2012) also provides extensive material on many aspects relating to *Karenia* species.

2.1. Life History, Diel Cycle and Vertical Migration

Vegetative cells of *K. brevis* reproduce asexually by oblique binary division, and its sexual cycle has been described only through the planozygote stage (Walker, 1982; Steidinger et al., 1998) (Figure 2). Gamete production has been observed in both homothallic and heterothallic strains. Gametes are identifiable by their smaller size, which is typically 1/3 to 1/2 the size of the vegetative cell, which also differs morphologically and cytologically (Figure 2d, e). Gamete conjugation is the same for homothallic and heterothallic strains, that is, cytoplasmic fusion precedes nuclear fusion (Figure 2f) and a planozygote with two longitudinal flagella is generated (Figure 2g, h). Production of hypnozygotes (resting cysts, diploid) have not been observed in culture, although thick-walled round cells that form within the cell membrane have been seen in nature (not to be mistaken for the single layered, rounded stressed or dying cells also observed in nature, often during blooms). The thick-walled palmelloid (non-motile) stage has not been confirmed as a resting cyst or a stressed cell (see alternative interpretation for such forms in Persson et al. (2013)), and this remains to be an important and challenging question to address. A life cycle with a benthic resting phase would have important implications for bloom dynamics (Steidinger, 1975) as has been shown with other dinoflagellates. According to Walker (1982), gametes and planozygotes have been detected only in the fall to late winter in both cultures and field samples from Florida waters, which suggests that transitions in *K. brevis* life cycle phases may be endogenously controlled or possibly seasonally mediated through exogenous cues. This information must be taken with caution, however, since field data in those days were skewed towards event response only, thus not providing the best representation of year around *in situ* dynamics.

Further characterizing *K. brevis* cell behavior, that is, diel phased cell division and diel vertical migration (DVM), brings additional insights into bloom dynamics. Efforts to understand these processes at the molecular level are reviewed in Van Dolah et al. (2009). *Karenia brevis* cell cycle has been demonstrated to be under circadian clock control (Brunelle et al., 2007). One five-year study of cell growth in batch cultures, followed by a mesocosm column experiment and measurements of *in situ* populations yielded the following findings (Van Dolah et al., 2008): (1) *K. brevis* displays diel phased cell division with S-phase beginning a minimum of 6 h after the onset of light and continuing for 12–14 h; (2) mitosis

takes place during the dark period and is generally completed by the beginning of the following day; and (3) the timing of cell cycle phases within the circadian cycle does not differ substantially with temperature, day length, or in bloom populations displaying distinct growth rates (μ_{min} 0.17–0.55). Likewise, both experimental and *in situ* observations of the migratory behavior of *K. brevis* have described a pattern of cell aggregation in a narrow band (~ 1-5cm) at the surface during daylight hours and dispersion through the water column at night. These behaviors are interpreted, in part, as phototactic and geotactic responses that realize their full potential in nature in the absence of a thermocline (Heil, 1986; Kamykowski et al., 1998a; Heil et al., 2014b; Garrett, 2015).

The rhythm of cell cycle progression in *K. brevis* was found to be independent from the rhythm controlling DVM (Van Dolah et al., 2008); nevertheless, biochemical changes occurring over the cell cycle seem to also play a role in cell displacement in the water column. For instance, a pattern of cell segregation between surface and mid-water populations has been identified during the daylight period, in which a larger percentage of the surface population is composed of cells that have entered the cell cycle (S-phase) whereas the mid-water population is composed of cells with higher carbohydrate, lipid, protein, and chlorophyll *a* concentrations (Heil, 1986; Kamykowski et al., 1998b; Van Dolah et al., 2008). There is evidence that daughter cells resulting from asexual division receive unequal shares of the parental resources so that nutrient reduced cells were more strongly influenced by phototaxis for carbon production through photosynthesis, thus resulting in behavior inequality within population during DVM (Schaeffer et al., 2009). Once photosynthate accumulation/utilization reaches a certain threshold, it is challenging to tease apart how much of the cell orientation is triggered by a sensory response to environmental cues or from a displacement mechanism due to the ballast effect of mass distribution in the cells.

Karenia brevis nutritional requirements (discussed in section 2.2), as well as nutrient sources in the predominantly oligotrophic waters of the Gulf of Mexico (discussed in section 3), are quite complex (Figure 3). DVM is a strategy that greatly expands *K. brevis* niches for nutrient exploitation. On one hand, it has been postulated that migration toward and aggregation in the surface microlayer may provide access to additional nutrient sources since this was shown to be an area of rapid nutrient cycling (Heil et al., 2014b). On the other end of the spectrum, it has also been suggested that *K. brevis* populations may be adapted to a coupled planktonic-benthic habitat. Accumulations of *K. brevis*, observed during DVM in the bottom half of a 22 m-deep water column, have been assumed to be able to either access nutrient flux at the sediment/water interface or to migrate into the sediment pores where higher nutrient concentrations exist (Sinclair et al., 2006). Indeed, there is laboratory evidence that migration does occur from the overlying water through thin layers of sediment, and that this behavior increases in response to elevated nutrient concentrations (Sinclair & Kamykovski, 2008).

The potential relationship between DVM and a purported immotile benthic life stage in *K. brevis* is, of course, hypothetical. That said, if cysts and/or newly excysted cells were demonstrated to be present at a particular time and location, the possibility of asynchronous division occurring under pre-bloom conditions would have to be taken into account when interpreting bloom initiation (i.e., implying more rapid growth rates than synchronous population division allows). Meanwhile, studies of *K. brevis* bloom dynamics from seeding through maintenance, currently rely on our understanding of the synchronous division of vegetative cells within the nighttime.

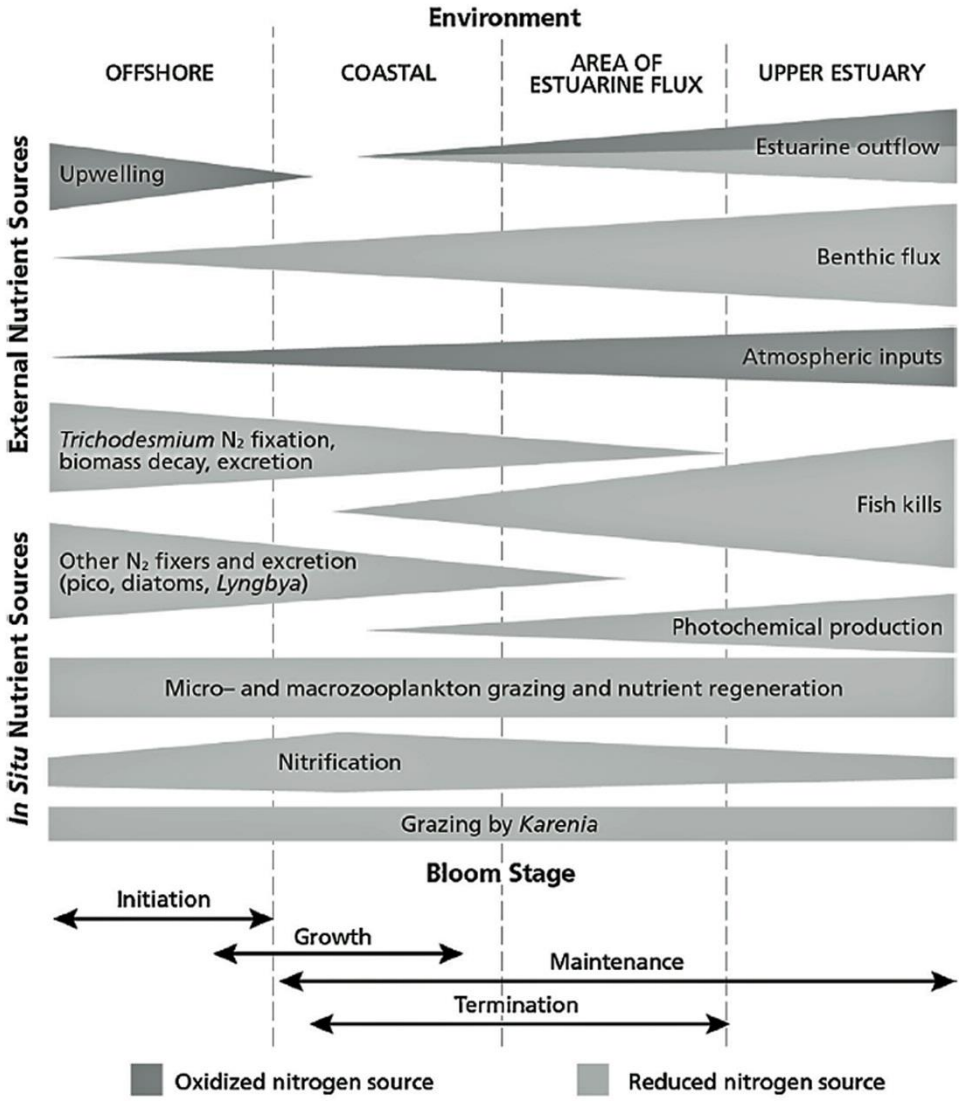


Figure 3. Schematic of nitrogen and phosphorus sources supporting *Karenia brevis* blooms over the four bloom environments and bloom stages on the west Florida coast and shelf. Length and width of bars indicate relative importance of specific nutrient source in each environment and bloom stage. Reproduced from Heil et al. (2014a), with copyright permission.

DVM in *K. mikimotoi* follows the same pattern described for *K. brevis*, with patches forming at the surface during daytime and downward movement in the dark hours when synchronous mitotic division is observed (Honjo et al., 1990; Koizumi et al., 1996; Partensky et al., 1991). In nature, swimming speeds of *K. mikimotoi* are estimated to vary between 0.3–1.3 (2.2) m/h in Japanese waters (Honjo, 2004 and references therein) while the range for *K. brevis* is 0.5–1 m/h (Hu et al., 2016). Field observations for *K. mikimotoi* vertical distribution in NW European waters, a region marked by oceanographic frontal zones, expose an interesting pattern reliant on the degree of water column stratification: migration takes place within a range of up to 15m in mixed or slightly stratified waters whereas a non-migrating,

population maximum is observed in the pycnocline when stratification is greater (Gentien, 1998 and references therein).

Swimming speeds estimated in laboratory experiments are between 0.01–0.8 m/h in *K. mikimotoi* (Throndsen, 1973, Bauerfeind et al., 1986) and 0.4–1.4 m/h in *K. brevis* (respectively, at 13°C and 21°C, McKay et al., 2006). These laboratory assays indicate that swimming pattern in *K. brevis* can be quite complex, suggesting a dependence on environmental conditions such as temperature, nutrient availability, and light regime (faster swimming at higher light, though variable according to light:dark cycles). Indeed, the interactive effects of high light and nutrient availability have also been shown to be important determinants of migration and cell death in *K. mikimotoi* (Yuasa et al., 2018). A layer of complexity is added when one compares swimming capabilities of *Karenia* cells at different stages of the cell cycle, that is, swimming speeds in fully grown cells of *K. brevis* are significantly higher than recently divided, doublet cells (Heil, 1986).

Details of *K. mikimotoi* life cycle are also scarce. Pairing gametes transitioning into a nonmotile globular zygote and then into a zygote with the same shape as the vegetative cells were observed in non-clonal cultures from Japanese waters (Ouchi et al., 1994). Similarly to *K. brevis*, an idealized model for the life cycle of *K. mikimotoi* with possible transitions between life stages has been proposed by Liu et al. (2019) after observing delicate diploid resting cells in cultures, and detecting morphologically similar cells (confirmed to be *K. mikimotoi* by molecular methods) in aged sediment samples from known bloom areas in the East China Sea.

2.2. Cell Growth and Its Requisites

Specific growth rates for *K. brevis* have been shown to be between 0–1 divisions d^{-1}, based primarily on circadian cell cycle gating which is expected to limit the maximum rate. The maximum growth rates in laboratory cultures generally approach 0.5–0.6 divisions d^{-1} (e.g., Hardison et al. (2012); Sunda et al. (2013); Hardison et al. (2013); Hardison et al. (2014)), though there is also substantial intra-specific variability (Errera et al., 2010). Particularly, cultures such as the 'Wilson' clone, which is nearing 70 years, may no longer yield reliable growth rates or other physiological information as *in vitro* selection has proceeded (Berge et al., 2012). And, although cell growth information is of interest, the relatively low rates of growth of *K. brevis* have led to hypotheses that physical accumulations may be key to bloom dynamics in certain areas in the Gulf of Mexico (see section 3.3). It is not clear, however, what the relative importance of cell growth vs. cell accumulation is in bloom formation/persistence. Few growth rates have been measured *in situ*, in part due to the technical nature of making these measurements without disturbing the cells. *In situ* measures by flow cytometry line up well with average laboratory culture growth rates (Van Dolah et al. 2008; Redalje et al. 2008). Another approach applied *in situ*, that of using C^{14} radiolabeling of gyroxanthin-diesters, confirmed rates between 0.1–0.4 divisions d^{-1}, with a maximal rate of 0.7 d^{-1} (Richardson et al., 2006). Imaging flow cytometry has shown promise as a technique for measuring *Karenia* growth rates *in situ* (Campbell et al., 2013), and has already been used to estimate growth rates of a bloom of *B. capitatum*, a sister genus to *Karenia* (Henrichs et al., 2011).

Specific growth rates for *K. mikimotoi* also range between 0–1 divisions d^{-1} (Gentien, 1998 and references therein). Efforts to estimate *K. mikimotoi* growth rates *in situ* also suffer from the same difficulties as those for *K. brevis* and phytoplankton more generally (e.g., undersampling, Barnes et al. (2015)). There appear to be fewer estimates of field growth rates published for *K. mikimotoi*, however Davidson et al. (2009) estimated a realistic value of 0.44 divisions d^{-1}, similar in magnitude to rates estimated for *K. brevis in situ*. Fortunately, non-microscopic techniques have been developed to measure cell abundance of both *K. brevis* and *K. mikimotoi* (e.g., gyroxanthin diester quantification (Örnólfsdóttir et al., 2003; Staehr & Cullen, 2003) and nucleic acid sequence based amplification - NASBA (Casper et al., 2004; Ulrich et al., 2010)), which may help in the generation of new/improved estimates of growth rates *in situ* due to the higher sample throughput potential.

In terms of phosphorus nutrient uptake, both *K. brevis* and *K. mikimotoi* show similar usage/preferences for organic and inorganic phosphate compounds tested (Richardson & Corcoran, 2015). Moreover, half saturation constants for growth with phosphate are similar: 0.14 µM for *K. mikimotoi* (Yamaguchi, 1994) and 0.18 µM for *K. brevis* (Sinclair et al., 2009). In terms of nitrogen, *K. mikimotoi* exhibits an 80% reduction in growth rate when grown on urea compared to nitrate (Yamaguchi, 1994), whereas *K. brevis* exhibits a 20% faster growth rate on urea compared to nitrate under a subsaturating light regime (Sinclair et al., 2009). Both species seem to be capable of dark nitrate uptake, which in concert with DVM, may afford these species a competitive advantage over non-migrators.

The two species seem to share similar photophysiological attributes including, for example, capabilities to exploit low light levels with compensation irradiances between 10 to 30 µmol photons m^{-2} s^{-1} (Richardson & Kullenberg 1987; Shanley & Vargo 1993; Gentien, 1998; Magaña & Villareal 2006), saturation of photosynthesis at relatively high irradiances, and little inhibition upon exposure to high light (Gentien, 1998; Dixon & Holligan, 1989; McKay et al., 2006; Schaefer et al., 2007). Their last common ancestor, which exchanged a peridinin containing plastid for a fucoxanthin containing plastid, likely imparted some degree of photosynthetic similarities (Yoon et al., 2005).

Temperature range for *K. brevis* grown in laboratory cultures varies from about 9°C to 33°C with optimum growth between 22–28ºC (Kusek et al., 1999; Vargo, 2009 and references therein). However, the uppermost *in situ* temperature range (> 3°C) has not been achieved with certain cultures (Magaña & Villareal, 2006, Eng-Wilmot et al., 1977). The salinity range for *K. brevis* grown *in vitro* indicates optimum growth between 27–37 with decreased yields below 24 (Steidinger et al., 1998 and references therein). Japanese strains of *K. mikimotoi* have optimum growth at 25ºC, with a lower threshold at 15ºC, and are able to grow at salinities between 15–35 (Honjo, 2004 and references therein). *In situ* temperature and salinity tolerances for both *K. brevis* and *K. mikimotoi* can be illustrated by 30 years of data acquired from estuarine to offshore waters around Florida (Figure 4). It confirms that both species follow a very similar pattern of having fairly broad temperature and salinity tolerances (98% of the data points between, respectively, 14–33°C and 20–37), though geared towards warmer waters and a salinity more typical of coastal to offshore areas.

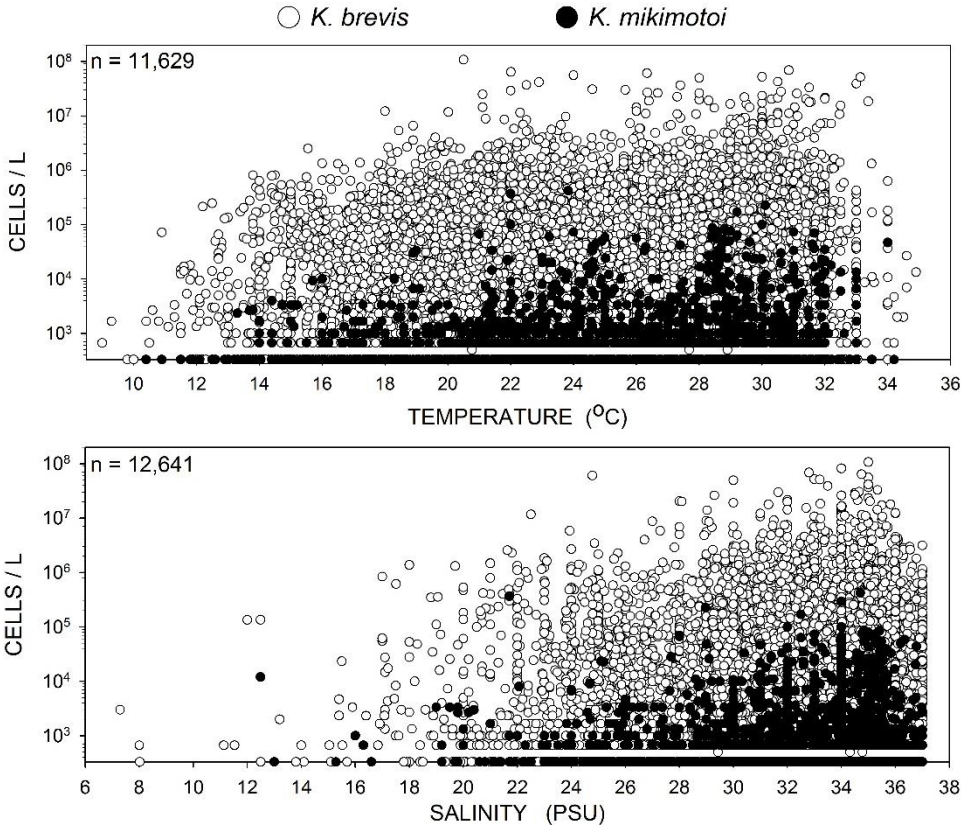


Figure 4. Abundance of *K. brevis* and *K. mikimotoi* in Florida coastal and offshore waters, according to temperature and salinity, from January 1995 to January 2019 (FWC, 2019). Cut-off values for salinity was 37, even though we acknowledge that higher values may be detected in some areas such as beach surf-zones and shallow embayments in the Florida Keys; however, those few data points could not be easily teased apart from likely spurious high values. The low cutoff value on the Y-axis (333 cells L^{-1}) corresponds to the detection limit established for routine cell counts.

Both *K. brevis* and *K. mikimotoi* exhibit allelopathic interactions in culture with other phytoplankton (e.g., Uchida et al. (1999); Kubanek et al. (2005); Gentien et al. (2007); Legrand et al. (2003)), and both can exhibit anti-grazer properties (e.g., Waggett et al. (2012); Dang et al. (2015)). Their potential to inhibit grazers does not preclude the importance of grazers at certain bloom stages (e.g., during initiation when chemical defenses may not be effective due to low cell concentrations). Few studies have assessed grazing rates on *K. brevis* in the field (but see Walsh & O'Neil (2014)). Local adaptation to *K. brevis* allelopathy has been proposed as a mechanism that might facilitate different phytoplankton dynamics over time (Kubanek et al., 2007). Notably, the allelopathic effects of *K. brevis* are unrelated to the NSP-causing brevetoxins (Kubanek et al., 2005), though brevetoxins themselves have been shown to affect bacterioplankton communities (Sipler et al., 2014). Allelopathy by *K. brevis* is clearly complex and, though new investigative approaches have improved our understanding of this phenomenon (Granéli & Hansen, 2006; Poulin et al., 2018), its role during blooms remains in its infancy.

Like most dinoflagellates, both *K. brevis* and *K. mikimotoi* exploit mixotrophic strategies (Glibert et al., 2009; Zhang et al., 2011), with some evidence for phagocytosis of cyanobacteria in *K. brevis* and phagocytosis of eukaryotic nanoplankton in *K. mikimotoi*. Collectively, these accounts highlight the multitude of strategies and mechanisms that can potentially play a role in the formation and duration of high biomass blooms as well as in cell upkeeping during non-bloom periods.

3. BLOOM DYNAMICS OF *K. BREVIS*

An operational definition of what constitutes a bloom is necessary for the function and advancement of HAB monitoring and management programs, yet this definition can vary for a given taxon and/or region. Long-term monitoring and observation efforts, coupled with modeling, have helped tighten the bounds of bloom classification for *K. brevis* in Florida waters (Tester et al., 2008; Heil & Steidinger, 2009; Weisberg et al., 2009; see section 1.1). Keeping in mind that a bloom can be characterized by different indicators such as cell abundance, water discoloration, associated impacts, etc. (Smayda, 1997), here we will use *K. brevis* cell concentrations as our main criteria in the discussion that follows. Our threshold for bloom level will be ≥ 100,000 cells L^{-1}, even though effects may be observed and management procedures start at lower cell concentrations due to brevetoxins' high toxicity (Table 3). Concentrations < 1,000 cells L^{-1} will be referred to as background levels. We will conclude this section by outlining a few parallels and contrasts between *K. brevis* and *K. mikimotoi* blooms that have caused serious harm to fisheries and wildlife in other regions of the world. For the latter, however, it was not possible to standardize an operational definition of bloom that was most often considered as such in the literature when cells exceeded *ca.* 10^6 cells L^{-1}, in which case it was frequently accompanied by water discoloration and fish kills.

3.1. The *Karenia* Assemblage

Karenia species are commonly found in coastal and offshore Florida waters, detected in about 31% of samples collected in the period between January 1995 and February 2019 (total n = 150,443 samples; FWC, (2019)). *K. brevis* is the dominant *Karenia* species in the region in terms of relative abundance and was present 69% of the time, followed in terms of frequency of occurrence by *K. mikimotoi*, *K. longicanalis*, *K. papilionacea*, *K. selliformis*, *K. asterichroma*, and *K. bicuneiformis* (Figure 5). This multi-species pattern is well illustrated by the severe, 17-month long bloom of 2004–2006 (Heil et al., 2006). While *K. brevis* was dominant and widespread over the entire bloom season, other species (namely, *K. mikimotoi*, *K. papilionacea*, *K. selliformis*) were also present, though temporally limited to particular times of the event or spatially restricted to specific areas within the region. Mixed *Karenia* assemblages have been detected in other regions (e.g., de Salas et al. (2004b)) and it would not be unexpected for a similar scenario to be found in areas where several *Karenia* species have been reported to occur (Figure 1). An extra challenge comes into play when other morphologically similar gymnodinioid dinoflagellates such as *Karlodinium* and *Takayama* co-occur with the *Karenia* assemblage (Steidinger et al., 2008).

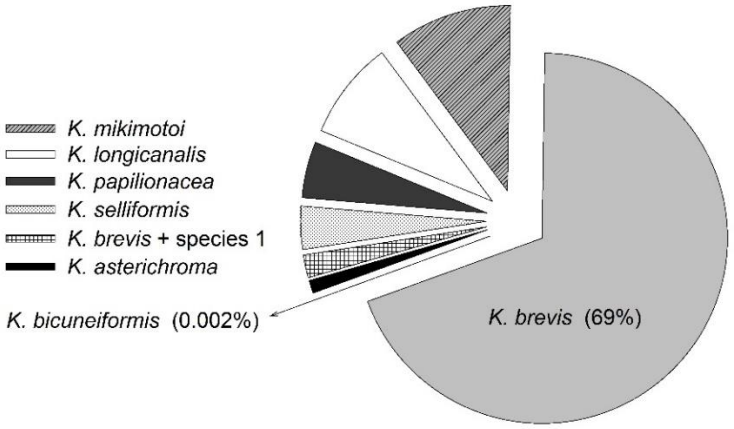


Figure 5. Percent occurrence of *Karenia* species from coastal and offshore Florida between January 1995 and February 2019 (FWC, 2019). *Karenia* sp. 1, a as of yet unidentified species, often co-occurs with *K. brevis*, and is thus here combined with the latter for sake of simplicity. See Steidinger et al. (2008) for further details on *Karenia* sp. 1 morphotype, aka "Mexican hat".

As expected, phytoplankton species richness is relatively low during bloom events. The possibility of *K. brevis* exerting allelopathy to drive phytoplankton exclusion, as suggested by laboratory experiments (see section 2.2), is very challenging to demonstrate in nature (e.g., West et al. (1996)). Moreover, the ability to detect less abundant taxa in bloom samples is a limitation intrinsic to some enumeration methods, although coupling routine microscopic examination with molecular and/or imaging flow cytometric analysis has helped improve detection limits. For example, a continuous automated imaging-in-flow time series of a 2011 bloom on the Texas coast revealed that *Karenia* cells comprised at most 60–75% of the total microplankton (Campbell et al., 2013).

3.2. Bloom Frequency, Duration, and Large-Scale Area Coverage

K. brevis blooms in Florida waters have a well-documented seasonality, typically occurring annually in late summer/early fall (Figure 6) and subsiding in the following winter, when observed concentrations decrease to background levels.

About 34% of the time, blooms initiate during the months of September-November and most often occur on the order of months (i.e., about 70% of the time <5 months), with highly variable intensities within (i.e., spatial extent and cell concentrations). However, as bloom initiation, development and termination all depend on a series of complex and synergistic factors not fully resolved (see section 3.3), bloom duration varies greatly from event to event, may span over the expected bloom season, and even persist into the next calendar year (e.g., 2002–2004, 2004–2006, 2017–2019) (Figure 7).

The longest ever recorded bloom event occurred from 1994 to 1997 and lasted 30 months (FWC, 2019). In such cases when blooms exceed one year, the following years' bloom may augment bloom patches lingering from the prior season (and perhaps seasons) during the typical initiation period (Weisberg et al. 2019). As the environment changes over the course of a single event (see section 3.3), bloom intensity may wax and wane. Observations have

shown advection of cells (both at surface and at depth) to different parts of the Gulf of Mexico and the Atlantic shores, where the bloom may proliferate and even intensify (see section 3.3). This may give the appearance of separate bloom phenomena when these are likely separate episodes of the same event. Not only regional expanse, but also timing of initiation, maintenance, and termination of a bloom event are all thought to be intertwined and tied to physical environmental forcings such as the degree of vertical stratification, the prevailing currents and advection mechanisms, as well as the dynamics of large-scale ocean features, i.e., the Gulf of Mexico Loop Current and shelf-break upwelling off of the west Florida shelf, the Texas-Louisiana coast, and the Yucatán Peninsula (Tester & Steidinger, 1997) (Figure 8a).

Looking at the recorded observations beginning in the 1950s, manifestations of *K. brevis* blooms most frequently affect the SW Florida shoreline from Pinellas to Lee counties, respectively, 28º08'N and 26º19'N (e.g., reviewed in Steidinger et al. (1998), in Walsh et al. (2006), and in Weisberg et al. (2019); FWC (2019)). While rarer, blooms can occur along the Panhandle region and the East Coast of Florida (Figure 9), and even less frequently along the coastlines of the remaining gulf U.S.A. states from Alabama to Texas (e.g., Henrichs et al. (2015); Soto et al. (2018)). Bloom events on Texas shores, however, seem to be part of a Western Gulf current transport mechanism associated with those blooms detected in northern Mexico waters (e.g., Henrichs et al. (2015)).

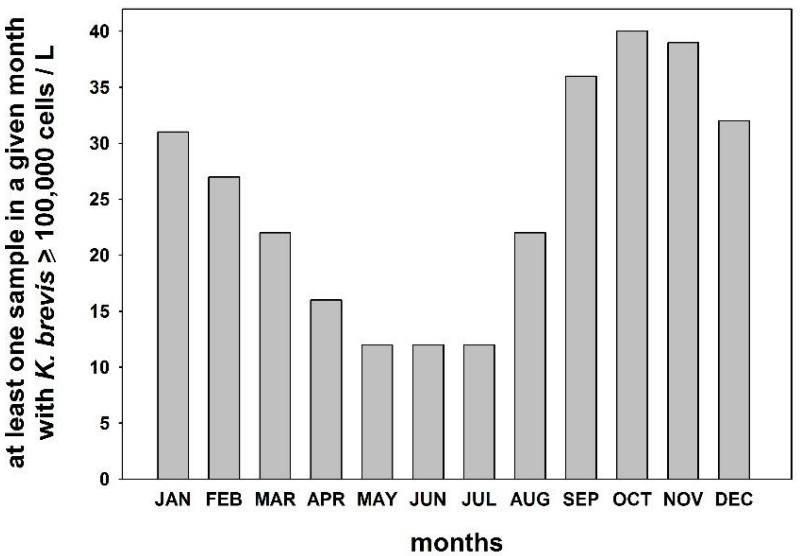


Figure 6. Seasonality of *K. brevis* blooms based on cell abundances in coastal and offshore Florida waters between August 1953 and February 2019 (total n = 799 months, *ca.* 38% of samples correspond to bloom condition) (FWC, 2019). See text and Table 3 for information on operational definition of bloom. Histogram bars show the number of months with at least one sample with *K. brevis* ≥ 100,000 cells L^{-1}.

In Florida, *K. brevis* blooms have reached the Atlantic coast in the following years: 1957, 1972, 1980, 1983, 1997, 1999, 2002, 2007, 2008, 2011, and 2018 (FWC, 2019). Since the mid-1990s, with expanded and more systematic research and monitoring efforts set in place, the highest cell concentrations have been confirmed in the Southwest region, followed by the

Panhandle and the East Coast (maximum values recorded, respectively: 2 x 10^8 cells L^{-1}, 2 x 10^7 cells L^{-1}, and 1.2 x 10^7 cells L^{-1}). There is an on-going debate centering on whether *K. brevis* blooms are becoming more frequent and intense or if this may be interpreted as an apparent increase instead, related to historic gaps in bloom records as the result of lower observational and monitoring efforts prior to 1995 (Steidinger et al., 2008).

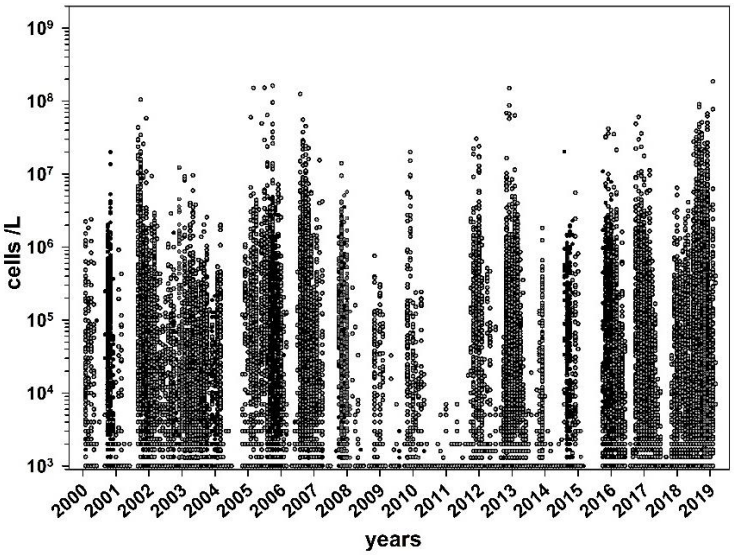


Figure 7. Time series of *K. brevis* concentrations in coastal and offshore Florida waters between 2000 and February 2019 (FWC, 2019). Note that blooms can span over the typical season (mostly fall) and persist for more than 12 months.

3.3. Bloom Initiation, Growth, Maintenance, and Termination

It may come as no surprise that blooms of *K. brevis* may occur across many oceanographic zones and environments, given their somewhat unique life history strategies and physiology (section 2.2); nevertheless, the success of this species in estuarine to offshore systems is still somewhat at odds with observed growth rates, which are relatively slow and suggest that *K. brevis* would likely be out-competed by other phytoplankton. Physical forcing is considered to be one of the primary drivers of bloom initiation, development, severity and termination; however, biological and chemical processes play key roles as well (discussed below).

The initiation zone for *K. brevis* blooms that occur in Southwest Florida (including those advected to the Panhandle region and East Coast) was first hypothesized to be located between 18 and 74 km offshore by Steidinger (1975). Augmented sampling coverage and tracking of cell concentrations suggested that potential initiation zones were positioned offshore and west of the most frequent manifestation area along the southwest Florida coast, and blooms and cells in southwest Florida were the source of less frequent *K. brevis* events on the Atlantic coast (Tester & Steidinger, 1997; Walsh et al., 2006) (Figure 8b). More recently, the use of remote sensing imagery, *in situ* data streams (collected by sensors on moorings and autonomous vehicles such as gliders), and hindcasted modeling have given further credence

to this theory and indicate the presence of a re-current initiation zone northwest from the coastal

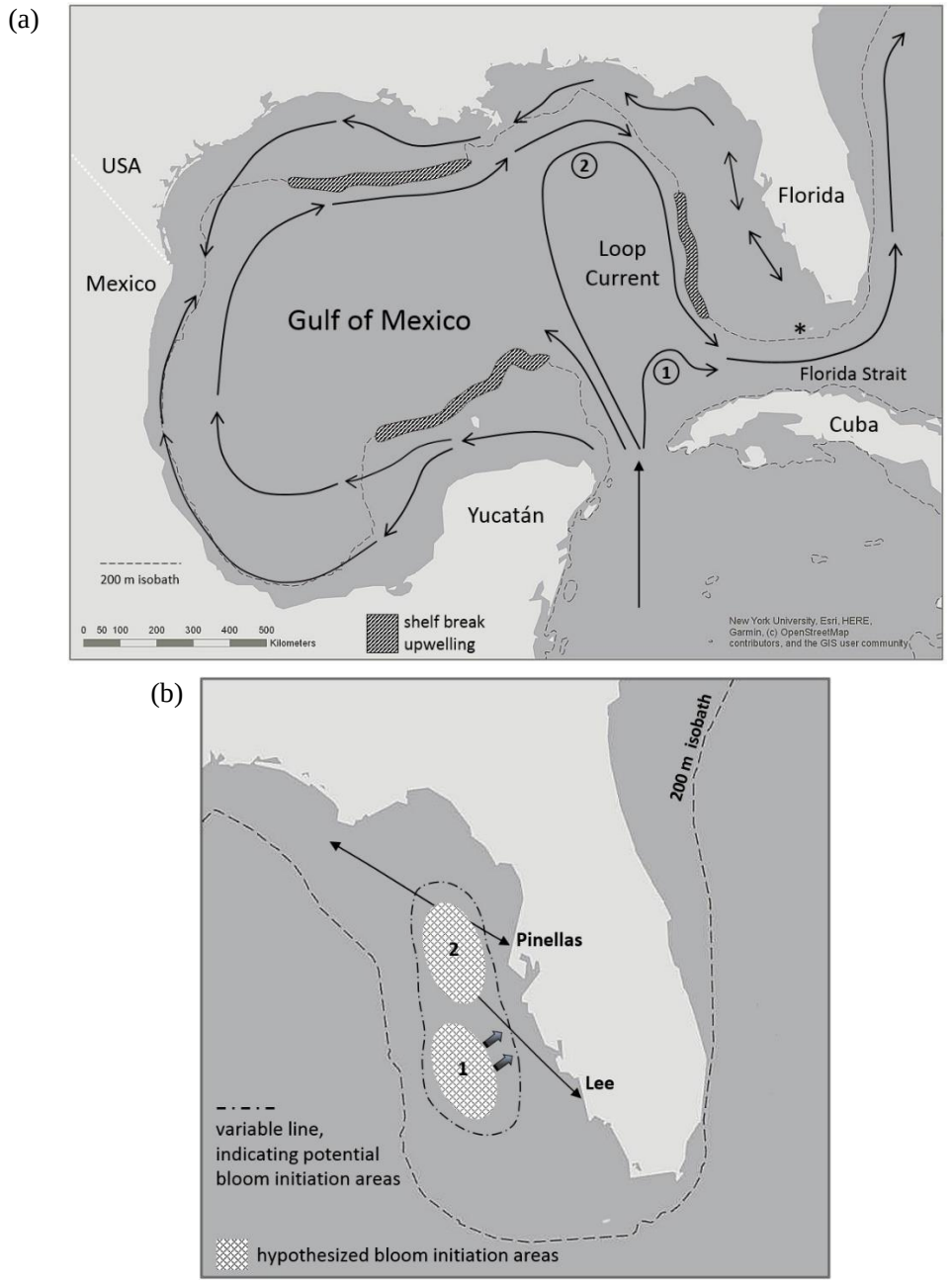


Figure 8. (a) Gulf of Mexico and its generalized surface circulation with emphasis on the Loop Current (thicker arrows): (1) sometimes, it barely enters the gulf before heading to the Atlantic; (2) sometimes, it penetrates deeper into the gulf before swinging back towards the Florida Strait. ***** asterisk indicates location of Dry Tortugas. (b) Detail of the southwest Florida coast depicting hypothesized bloom initiation areas according to (1) Tester & Steidinger (1997) and Walsh et al. (2006) and to (2) Weisberg et al. (2019).

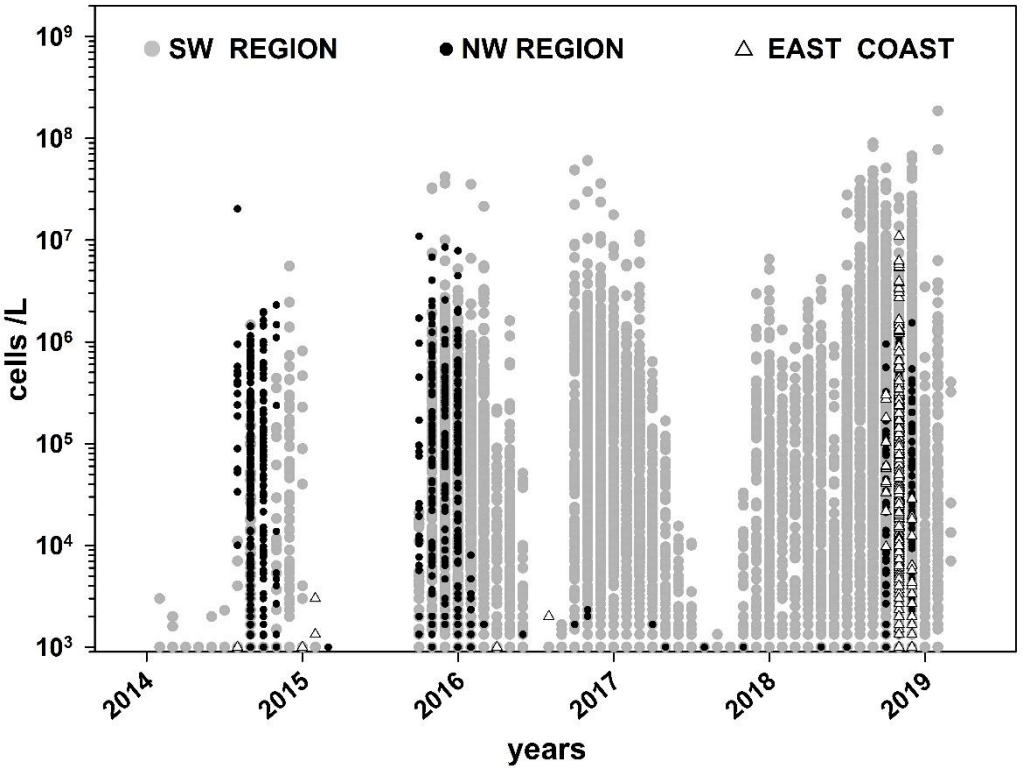


Figure 9. Time series of *K. brevis* concentrations in coastal and offshore Florida waters between 2014 and February 2019 (FWC, 2019). Note (1) there was no bloom in 2014 and a less intense one in 2015, though it extended towards Northwest Florida; (2) that seasonality was as expected in 2016 and 2017 with high concentrations in the panhandle in the former; and that (3) a long-lasting event started in the fall of 2017 and persisted through early 2019, which expanded to both the panhandle and the Atlantic in the fall of 2018.

manifestation areas on Florida's southwest and northwest coasts (Weisberg et al., 2019) (Figure 8b). The impingement of the major current in the Gulf of Mexico, the Loop Current, on the West Florida Shelf (WFS), often observed adjacent to the Dry Tortugas, FL, can cause a regime shift across the WFS whereby colder, nutrient richer water is upwelled on the shelf (Liu et al., 2016) (Figure 8a). The timing, intensity, and duration of this interaction can alter the physical and thereby chemical environment on the shelf. Intense and persistent summer and fall upwelling events on the WFS are associated with increased nutrient loading, which appear to favor faster growing phytoplankton (i.e., diatoms) and subsequently act to suppress *K. brevis* blooms (e.g., Weisberg et al. (2014); Weisberg et al. (2016)). The same studies found that, in the majority of years, more moderate upwelling conditions prevail and thus favor bloom development in Southwest Florida. Prior to blooms and during bloom initiation, cells may reside offshore in low concentrations throughout the water column, thus out of reach from the detection capability of remote sensing satellite imagery (see Table 3) and rendering the use of autonomous detection vehicles such as gliders ever more valuable (Robbins et al., 2006; Hu et al., 2016).

A similar overall strategy for studying bloom initiation applies for blooms that occur along the Texas and northern Mexico coastlines. With the limited data available, it has been hypothesized that the initiation zone in this case is the region west of the Yucatan Peninsula (Henrichs et al., 2015). The WFS and Campeche Bank (west of Yucatán) are similar environments in that both occur in a semi-enclosed back-arc basin (Gulf of Mexico), are broad and relatively shallow shelfs, and both interact with the dominating oceanographic feature in the Gulf, the Loop Current (Figure 8a).

Most years, in the summer/fall, cells that reside at depth are thought to be transported coastward via the bottom Ekman layer (Liu & Weisberg, 2012) (Figure 10). Blooms typically manifest in coastal waters where advection, accumulation, and/or cell growth are further impacted by physicochemical conditions occurring there and in associated estuaries (e.g., Walsh et al. (2003); Stumpf et al. (2008); Weisberg et al. (2009)). The offshore and nearshore environment of the WFS where blooms initiate and occur is also prone to vertical stratification, which can develop and persist during typical bloom periods, though varying in intensity throughout the event. This becomes increasingly important with respect to *K. brevis* DVM to access nutrients, trophic impacts, as well as bloom advection and the development, duration, and extent of hypoxic and anoxic zones that can cause wildlife mortality and damage benthic and reef habitats (e.g., Colella et al. (2008); Dupont et al. (2010)).

Research has suggested certain characteristics that may lend this species to out-competing other taxa across the same range of oceanographic zones, such as the aforementioned DVM behavior (Sinclair & Kamykowski, 2008; Hu et al., 2016; Garrett 2015); the broad distribution of seed populations and capacity to exploit nutrient sources at the surface, throughout the water column, and seafloor (Kamykowski et al., 1998ab; Walsh et al., 2006; Dixon et al., 2014; Heil et al., 2014b); and the ability to utilize a multitude of nutrients with respect to both form and source (Heil et al., 2014c) (Figure 3). There is continued debate around the purpose for ichthyotoxin production in *K. brevis*. The nutrients released from the decay of fish, primarily in the form of dissolved ammonia, can be quickly and readily used by *K. brevis* blooms, suggesting that fish kills could provide a continued source of nutrients as the bloom continues and/or intensifies (Killberg-Thoreson et al., 2014). In offshore oligotrophic waters, where dissolved inorganic nutrients are relatively limited and cells are typically less abundant, *K. brevis* nitrogen requirements can be met from exudates from the often co-occurring cyanobacteria *Trichodesmium* Ehrenberg ex Gomont (Mulholland et al., 2006), which similarly can change position in the water column throughout the day. Heil et al. (2014c) estimated that nutrient fixation and regeneration by *Trichodesimium* alone was capable of sustaining coastal and offshore *K. brevis* blooms based on estimated cellular nitrogen and phosphorus requirements. As mentioned earlier, blooms of *K. brevis* have allelopathic properties, and perhaps a combination of these properties, as well as species ability to thrive in multiple niches, is why persistent blooms occur and may become mono-specific as they intensify. Overall, *K. brevis* exhibits a "ecologically flexible" strategy which allows the species to exist across niches, and exploit a multitude of nutrient sources. However, the exclusion of certain conditions (as in the examples provided earlier) could temper and inhibit intense bloom events.

The DVM behavior in *K. brevis* has the potential to influence cell distribution offshore, along the coast, and in nearshore zones and contribute to localized bloom persistence and/or advection. For example, daytime cells entrained in surface water masses that are driven offshore during upwelling-favorable and/or offshore winds may be advected back to shore via

sub-surface currents during night hours, providing a mechanism for entrainment in the nearshore environment (Ruiz-de la Torre, 2013) (Figure 10). Studies have shown that changes in migratory behavior related to stratification intensity not only modulates access to nutrient pools and therefore growth, but can also alter distribution and advection of the population (as described above); thus, degree of vertical stratification plays a critical role in determining the occurrence and rate of DVM (Garrett, 2015). Further studies have found that growth of *K. brevis* under moderate to high light intensities is comparable to or greater than growth under low light (Tilney et al., 2019), so that DVM might be one way to achieve optimal growth. Less is known about if and how growth and termination are modulated by life cycle dynamics vs. environmental factors, in addition to physical concentration or dissipation.

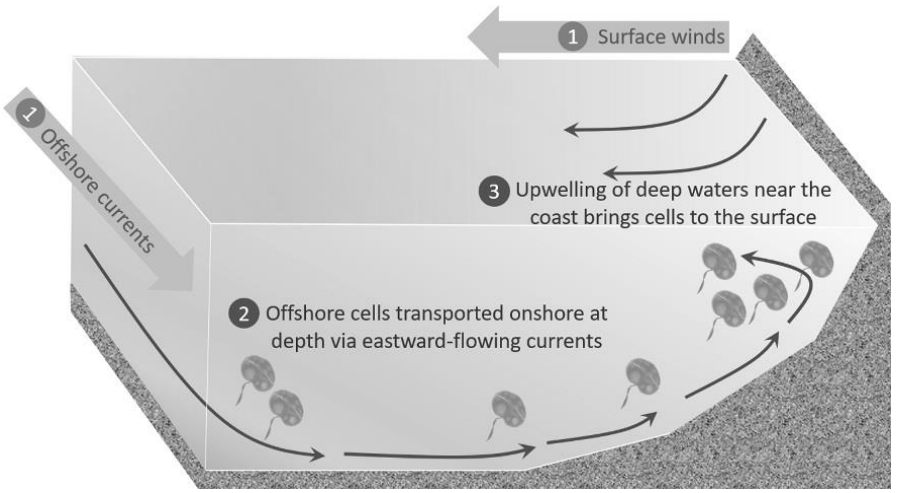


Figure 10. Offshore-inshore transport of *K. brevis* cells on the West Florida Shelf and mechanism for entrainment in the nearshore environment. Cells that reside at depth are thought to be transported coastward via the bottom Ekman layer. Once close to shore, cells accumulated closer to the surface (enhanced by upward migration during day time) may be driven offshore during periods of upwelling-favorable and/or offshore winds, only to be advected back to shore during night hours (downward vertical migration).

Although several blooms have lasted for more than one year, blooms may also dissipate within months. Background or even higher cell concentrations are not uncommon and appear occasionally in the absence of a larger-scale bloom. As alluded to, multiple interacting and complex factors contribute to bloom initiation and severity, although varied aspects of these dynamics as well as bloom termination (beyond physical dissipation) remain enigmatic. Nevertheless, as new tools and studies emerge, our understanding of the overall importance of drivers, and their importance in timing, underlying these key bloom dynamics will continue to evolve.

3.4. Comparison with *K. mikimotoi* Blooms

K. mikimotoi is a regular component of the phytoplankton assemblages of Florida waters, and it is often a co-dominant taxa during *K. brevis* blooms (see Figure 4) with whom it also

shares some ecophysiological attributes (see section 2.2). By exploring some of the similarities and contrasts between these two phylogenetically related taxa, we expect to broaden our understanding of likely trends observed under controlled experimental conditions and in nature or, at the least, set the stage for mindsets that may spark future, novel research questions.

Re-current *K. mikimotoi* blooms have been reported in temperate waters, most notably in the Setouchi region but more recently in Hokkaido in Japan (e.g., Oda (1935); Honjo (2004) and references therein; Shimada et al. (2016)), as well as in northwest Europe from Galicia to the Baltic Sea (e.g., Braarud & Heimdal (1970); Pingree et al. (1975); Gentien (1998) and references therein; Raine et al. (2001); Davidson et al. (2009)). Blooms have also been recorded in Atlantic Canada (Blasco et al., 1996), Alaska (NCCOS, 2014), Argentina (Negri et. al, 1992), the Aegean Sea on the Turkish coast (Bizsel & Bizsel, 2002), the Arabian Sea on the Indian coast (Robin et al., 2013), as well as in Hong Kong, South Korea, and New Zealand (Lassus et al. 2016 and some references therein). It was not possible to verify reports of *K. mikimotoi* blooms, then identified as *Gyrodinium aureolum*, in Maquoit Bay (Maine) and in Long Island Sound (New York) (respectively, Heinig & Campbell (1992) and Chang & Carpenter (1985)).

According to Gentien (1998)'s review, shoreline manifestations of *K. mikimotoi* blooms have been observed in semi-confined water bodies, buoyant plumes and estuaries, and tidal fronts. An apparently consistent feature of the blooms in European and Japanese waters is that these are summer events that may extend into the fall. Except for cases in which a population develops in shallow and weakly stratified embayments, bloom initiation, growth, and maintenance have been associated with offshore frontal zones, especially in connection with the establishment of the seasonal pycnocline; this may be followed or not by advection to the shoreline since the bloom tends to disappear rapidly as the thermocline is somehow disrupted (Holligan, 1987). In an earlier description as a "magic carpet" (Bjoersen & Nielsen, 1991), it was proposed that this *K. mikimotoi* thin layer may be transported as a result of wind-regimes that promote relaxation of upwelling and subsequent incursion of offshore waters shorewards. Recent modeling of large-scale blooms in the northwest European continental shelf corroborates this mechanism may be in place; it also pointed out to the need to take into account multiple spatially distinct seed populations at the shelf break to properly describe patterns of bloom transport (Gillibrand et al., 2016). Multiple seed population areas offshore may also need to be investigated to account for variability in bloom manifestation on the SW Florida shores (Figure 8).

As shown for *K. brevis*, the eco-physiological versatility of *K. mikimotoi* observed in culture also translates into successful strategies in nature. For example, its ability to sustain low growth rates and take advantage of subdued light levels (see section 2.2) may explain bloom reports in southern Norway (59°–60° N) as late as mid-November at much reduced water temperatures and surface irradiation (Tangen, 1977). Likewise, DVM capability has shown to be an asset not only in shallow waters by providing potential access to nutrient uptake from the sediment (Koizumi et al., 1996), but also offshore where populations may either be "free" for vertical displacement in weakly stratified water columns or exhibit a non-migrating maximum at the pycnocline/nutricline under stratified water column scenarios (Birrien et al., 1991).

Unravelling niche partitioning between *K. brevis* and *K. mikimotoi* may bring new light into understanding their bloom dynamics in Florida waters, where the latter often co-

dominates (Figure 4), and elsewhere. If it were not for *K. brevis*, would *K. mikimotoi* blooms be the main culprit of fish kills and wildlife mortalities in the Gulf of Mexico, as it is in several other regions? Indeed, high level of hemolytic activity among *K. mikimotoi* strains isolated from the Gulf of Mexico has been demonstrated, with one Texas strain inducing significantly higher hemolysis compared to Florida *K. brevis* isolates (Neely & Campbell, 2006).

3.5. *K. brevis* Bloom Dynamics and the Life-Form Selection Approach

The life-form selection approach provides a conceptual framework when seeking to identify similar and contrasting characteristics among phytoplankton taxa, dinoflagellate HAB species included (Smayda & Reynolds, 2001). In other words, how do *Karenia* species "fit in the dinoflagellate world"? This method stands on the following foundations: (1) literature support to the axiom that species assemblage-types characterize water masses and current systems; (2) the considerable influence of species size and shape on phytoplankton processes supports the axiom that form follows function, that is, habitat selection of a given morphotype is also favoring the selection of a given process, such as growth rate, suspension mode, etc. (Margalef, 1978; Sournia, 1982; Reynolds, 1988).

Dinoflagellate HAB species were thus classified by Smayda & Reynolds (2001) according to their size and shape, in association with some ecological attributes observed in nature, into nine categories ranging from coastal small, fast-growing, high surface-to-volume colonist (C) species, to offshore large, slow-growing but biomass conserving, nutrient stress-tolerant (S) species. A third category, of special interest to this discussion, was that of attuning, light-harvesting, disturbance-tolerant ruderal (R) species, represented by HAB dinoflagellates adapted to: increased velocities of shear/stress forces in physically-disturbed water masses such as frontal zones (Type IV); areas of reduced but still elevated vertical mixing during relaxations in coastal upwelling (Type V); perform while being entrained in and dispersed by coastal currents (Type VI). Examples given for Types IV–VI were, respectively, *K. mikimotoi*, *Gymnodinium catenatum* H.W. Graham, and *K. brevis*. Of course, there is overlapping and intergrading traits among Type IV–VI species and, according to the authors, these may reveal to encompass a single category as our understanding of bloom dynamics progresses. Indeed, we contend that, at least for *K. brevis*, Types IV–VI may actually represent our current understanding of sequential stages (or strategies) in bloom dynamics for this species: relaxation in coastal upwelling for initiation (Type V), frontal zone during transport and delivery shoreward (Type IV), and current entrained during further coastal transport, including alongshore and via the Gulf Stream (Type VI).

This analysis questioned the long-standing paradigm that dinoflagellates are best adapted to less turbulent, stratified environments (Margalef, 1978) and proposed that HAB dinoflagellate R-Type species and their habitats are intermediate between the diatom dominated domains and the other dinoflagellate life-form Types favored by more pronounced stratification (e.g., *Dinophysis*, *Ornithocercus*, *Pyrocystis*). Is *K. brevis* somehow better adapted to tolerating shear/stress forces? According to Smayda (2002, citing paleo-circulation hypothesis from Emslie & Morgan, 1994), this could be a trait inherited from a time when upwelling may have been more common in the Gulf of Mexico. Could behavior such as DVM help minimize exposure to unfavorable conditions while also contributing to maintenance of

cells in conditions associated with reduced loss and/or enhanced growth? The authors also advocate that Types IV–VI survival strategies rely heavily on light-harvesting mechanisms which is, actually, an important on-going line of investigation for *K. brevis* (Tilney et al., 2019). Furthermore, life-form selection acknowledges a stochastic aspect regarding growth opportunities for the best fit species "on site", that is, taxa with the most appropriate pre-adaptations and largest inoculum are likely to gain the greatest advantage. It is a matter of being the best candidate at the right place at the right time. This underscores the importance of high frequency, small-scale ocean observing technology as well as forecasting and hindcasting modeling as part of routine monitoring.

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Chapter 144

PFIESTERIA: A COMMON ESTUARINE DINOFLAGELLATE WITH A COLORFUL PAST

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ABSTRACT

In the 1990's the estuarine dinoflagellate *Pfiesteria piscicida* came under intense scientific scrutiny and garnered widespread media and public attention, as it was blamed for causing dramatic finfish kills and human health effects in coastal North Carolina and Chesapeake Bay. Universities, marine science institutions and state agencies in the region focused on environmental and human health, quickly turned their attention to research on, and monitoring of, this organism and its' perceived adverse health impacts. Federal and state funding quickly became available to support these efforts and resulted in rapid scientific advances, but also troubling controversies regarding interpretation of data. Researchers soon realized that morphologically similar dinoflagellates, now known as *Pfiesteria*-like dinoflagellates (PLDs), were initially counted as *Pfiesteria* cells, often co-occurred at fish kill events, and that this resulted in early *Pfiesteria* cell counts at fish kills likely being significantly inflated. Further, although *P. piscicida* might be observed in a water sample collected from a fish kill event, it was often found at low abundance or was absent. Other PLDs, most notably *Karlodinium veneficum*, were subsequently found at high abundance in samples from several mortality events in Maryland waters of Chesapeake Bay. Additionally, the complex multi-stage life cycle of *P. piscicida*, initially reported to involve several amoebae and chrysophyte-like cyst stages, came under intense

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scrutiny. The life cycle was ultimately shown to be simpler; lacking amoebae and chysophyte-like cyst stages and typical of other dinoflagellate species. However, the longest-standing and most troubling controversy surrounded the possible production of a toxin by *P. piscicida* and *Pseudopfiesteria shumwayae*, and the role that toxin played in adverse human health effects and as a cause of finfish mortalities and ulcerous lesions often observed on fish during kill events. The causes of the fish kill events in North Carolina estuaries during the late 1990's have never been identified, however, the deeply penetrating ulcers in menhaden, *Brevoortia tyrannus*, at the time attributed to *Pfiesteria* toxin, are now known to be caused by a highly pathogenic water mold called *Aphanomyces invadans*. *Pfiesteria* has been clearly shown capable of killing fish in a captive environment from which the fish cannot escape, however, the mechanism of pathogenicity has been shown not to be due to toxin but to the micropredatory feeding activity (myzocytosis), which at abnormally high cell concentrations results in disruption and loss of the fish epidermis and ultimately, loss of osmoregulatory ability and death. To date, the full structure of a *Pfiesteria* toxin remains unresolved and no adverse human health impacts from contact with coastal waters where *Pfiesteria* occurs have been confirmed, despite an extensive multistate study conducted by the Centers for Disease Control and Prevention. It also has been demonstrated that *Pfiesteria* has a simple dinoflagellate life cycle lacking amoeboid or chrysophyte stages and does not cause fish lesions or kill fish by means of a toxin. The history of the events, controversies, research and current status surrounding the *Pfiesteria* story will be reviewed in this chapter.

Keywords: *Pfiesteria piscicida*, *Pseudopfiesteria shumwayae*, *Pfiesteria*-like dinoflagellates, fish kills, fish lesions, life cycle, amoebae, dinospore, *Karlodinium veneficum*, *Aphanomyces invadans*

INTRODUCTION

Pfiesteria piscicida, a dinoflagellate whose name means "fish killer", was first identified in 1991 from fish tanks maintained by Dr. Edward Noga at North Carolina State University (Litaker et al. 2002). These tanks had been inoculated with water from the Pamlico River, North Carolina, USA. Subsequently, fish began to die as a bloom of a small heterotrophic dinoflagellate developed. By transferring these dinoflagellates to other tanks containing fish and observing similar mortality rates, a cause-effect relationship was made, with the dinoflagellates believed to produce potent ichthyotoxins that killed the fish. In 1991, shortly after this discovery, high densities of morphologically similar dinoflagellates, presumed to be *Pfiesteria piscicida*, were identified in water samples collected after massive fish kills in North Carolina estuaries, including samples from the Pamlico River (Burkholder et al. 1992). The assumption was made that the fish kills observed in the field were caused by the same organism that was killing fish in Dr. Noga's tanks, since the water used in the tanks was from the same general area where fish kills were occurring. However, no detailed morphological or molecular information was provided to confirm that the dinoflagellate species in the tanks was identical to those associated with the estuarine fish kill events. Many of the dead fish, primarily menhaden, at these sites had deep ulcerative lesions, which at the time were assumed to be caused by exposure to a *Pfiesteria* toxin (Burkholder and Glasgow 1995, 1997a; Burkholder et al. 1992).

Despite being first observed in 1991, *Pfiesteria piscicida* was not formally described as a new genus and species until 1996 (Steidinger et al. 1996). By that time there was growing controversy within the scientific community regarding the role, if any, of *P. piscicida* in the fish kills and in causing the deep ulcerative lesions that were frequently observed on menhaden, the fish species most often involved in those kill events. Over the next decade additional dinoflagellate species would be found in association with these fish kills in the mid-Atlantic, including several species that are morphologically similar to *P. piscicida* known as *Pfiesteria*-like dinoflagellates (PLDs). These include *Pseudopfiesteria shumwayae, Luciella* species, *Cryptoperidiniopsis* species and *Karlodinium veneficum*. The next decade also would find the intensification and expansion of scientific debates surrounding the roles of *P. piscicida* and *P. shumwayae* in fish kills, their life cycles and toxigenicity of *P. piscicida* and the PLDs and, perhaps most importantly, the hypothesized human health effects of these organisms. This chapter will attempt to present the data from the years of scientific research, as well as different views on the several controversial topics surrounding *P. piscicida* and the PLDs.

TAXONOMY

Pfiesteria piscicida and PLDs are small (~10-15 μm), heterotrophic dinoflagellates and, although often referred to as microalgae, they are not true algae. Although many dinoflagellates are photosynthetic, *Pfiesteria* and most of the PLDs lack chloroplasts (note: *K. veneficum* does contain chloroplasts and is mixotrophic) and obtain their energy through consumption of cells and tissues of other organisms (see below for more discussion on this topic). As noted above, by the time the genus *Pfiesteria* was formally described (Steidinger et al. 1996; Figure 1), a controversy had arisen regarding how to distinguish *P. piscicida* from morphologically similar co-occurring heterotrophic dinoflagellates (Steidinger et al. 2001). The controversy arose after other research groups funded to study *Pfiesteria* began isolating small heterotrophic dinoflagellates from "*Pfiesteria* fish kill" sites. They found convincing morphological and molecular evidence indicating that not all of the microorganisms at these sites were *P. piscicida* or *Pfiesteria shumwayae*, a second closely related species that was described in 2001 (Glasgow et al. 2001b; Figure 2) and now called *Pseudopfiesteria shumwayae* (Litaker et al. 2005) (see below). Prior to these findings, the prevailing practice was to count all morphologically similar heterotrophic dinoflagellates from the "fish kill" sites as *Pfiesteria* (Burkholder, Mallin and Glasgow 1992; Burkholder 1999; Burkholder and Marshall 2012). Groups finding these morphologically distinct isolates asserted that they should be described as new species where appropriate and their abundance relative to the *Pfiesteria* species determined. In response, the groups which had been counting all small heterotrophic cells as *Pfiesteria* argued that this practice should be continued.

While the controversy continued, several groups worked to characterize their environmental isolates in greater detail morphologically and molecularly. The morphological characters investigated included both the size and shape of the cell and the number and shape of the thecal plates covering the cells. These thecal cellulose plates are a key diagnostic feature used in identifying many dinoflagellate species and are organized into series according to a classification system first developed by Kofoid (1911; Figure 3). In this

system, a single difference in the number of plates in one of the major plate series is typically sufficient to place species in separate genera (Table 1). Using these criteria, it was possible to identify three new genera co-occurring with *Pfiesteria* – *Pseudopfiesteria* (Litaker et al. 2005), *Cryptoperidiniopsis* (Steidinger et al. 2006) and *Luciella* (Mason et al. 2007; Table 1), each containing one or more species. Additionally, rDNA region sequence data were obtained from the same isolates and analyzed phylogenetically. The results of these molecular studies were congruent with the morphologically described species and genera (Litaker et al. 2005; Mason et al. 2007; Steidinger et al. 2006). The resulting rDNA sequence data were also used to develop molecular assays capable of quantifying *Pfiesteria* abundance (i.e., quantitative polymerase chain reaction-qPCR assays) in the field. Results obtained using those assays indicated *Pfiesteria* densities in nature were low and comprised only a small portion of the heterotrophic dinoflagellates observed in the field. This argued against the generic classification of small heterotrophic dinoflagellates as *Pfiesteria*.

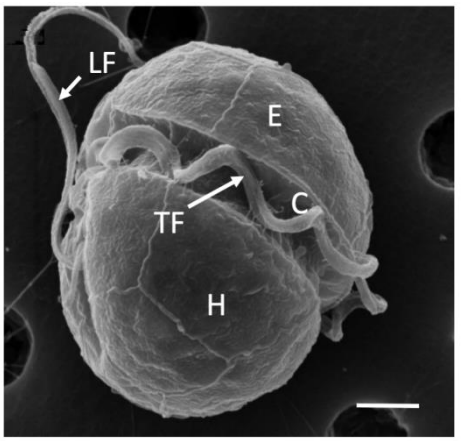

Figure 1. Scanning electron micrograph of *Pfiesteria piscicida* illustrating the basic morphology of the flagellated dinospore. E: epitheca, H: hypotheca, C: cingulum, TF: transverse flagellum, LF: longitudinal flagellum. Scale bar: 2.0 µm.

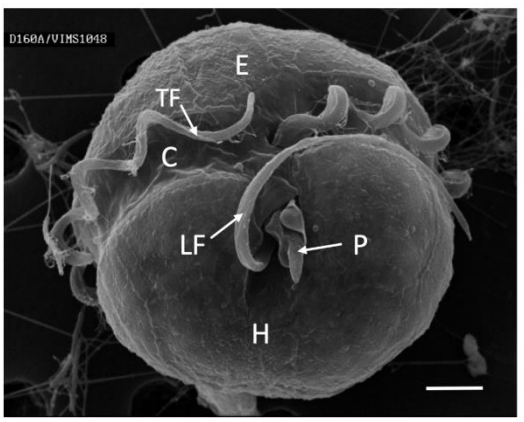

Figure 2. Scanning electron micrograph of *Pseudopfiesteria shumwayae* illustrating the basic morphology of the flagellated dinospore. E: epitheca, H: hypotheca, P: peduncle, C: cingulum, TF: transverse flagellum, LF: longitudinal flagellum. Scale bar: 2.0 µm.

Currently, the only remaining taxonomic controversy with regard to the genus and species described above is that regarding *Pseudopfiesteria shumwayae*. Originally this species was published as *Pfiesteria shumwayae* (Glasgow et al. 2001b). This new species was included in the genus *Pfiesteria* despite having a Kofoidian plate tabulation with 6 precingular (′′) plates compared to *P. piscicida* with 5 precingular (′′) plates. All the other closely related dinoflagellate genera described before and after *P. shumwayae*, including *Aduncodinium*, (Kang et al. 2015), *Speroidium* (Moestrup and Calado 2018), *Stoeckeria* (Jeong et al. 2005) and *Tyrannodinium* (Calado et al. 2009) were distinguished by at least one or more differences in the number of plates in the major Kofoidian plate series relative to the other described genera (Table 1). There are no instances where a genus in either the family Pfiesteriaceae or the closely related family, Thoracosphaeraceae, has been described as containing a variable number of plates in one of the major Kofoidian plate series. Accordingly, Litaker et al. (2005) formally transferred *P. shumwayae* to the genus *Pseudopfiesteria shumwayae* maintaining the convention that plate difference is sufficient to define a new genus.

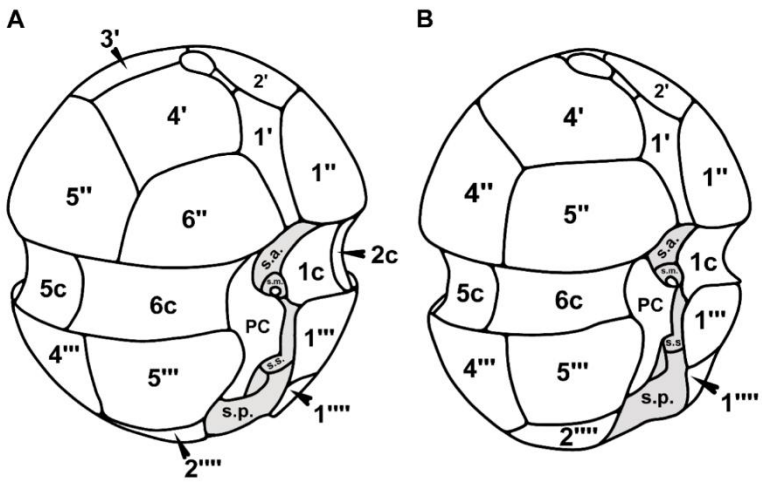

Figure 3. Line drawings from Litaker et al. (2005; fig. 3) showing the plate tabulations of (A) *Pseudopfiesteria shumwayae* and (B) *Pfiesteria piscicida* and providing illustration of the plate series referred to in Table 1. Reprinted with permission from the *Journal of Phycology*, from Litaker, R. Wayne, Karen A. Steidinger, Patrice L. Mason, Jan H. Landsberg, Jeffrey D. Shields, Kimberly S. Reece, Leonard W. Haas, Wolfgang K. Vogelbein, Mark W. Vandersea, Steven R. Kibler, and Patricia A. Tester. 2005. "The Reclassification of *Pfiesteria shumwayae* (Dinophyceae): Pseudopfiesteria, Gen. Nov.1." *Journal of Phycology* 41(3):643–51. https://doi.org/10.1111/j.1529-8817.2005.00075.x.; Figure 2.

Despite established convention, Marshall, Seaborn and Wolny (2006) proposed that *Pseudopfiesteria shumwayae* be reassigned back to *Pfiesteria*. The primary argument supporting this proposal was an rDNA phylogeny employing the 159 bp 5.8S gene that showed *Pfiesteria* and *Pseudopfiesteria* having the same DNA sequence in this region. This gene, however, is not typically used for phylogenetic analyses because it is often conserved among species. Other analyses conducted since that time have used more phylogenetically informative rDNA region sequences and have shown that *Pfiesteria* and *Pseudopfiesteria* are

not any more closely related to each other than *Pseudopfiesteria* is to other distinct genera such as *Cryptoperidiniopsis* or *Luciella* (Grzebyk et al. 2017; Kang et al. 2015). These phylogentic analyses along with the differences in a major plate tabulation series support the original establishment of *Pseudopfiesteria* as a distinct genus.

Table 1. Comparison of the plate formulae among the genera in the dinoflagellate genera Pfiesteriaceae and representative members of the closely related family Thoracosphaeraceae. Delineation of species is based on the following enhanced Kofoidian nomenclature: apical series (′), anterior intercalaries (a), precingular series (″), cingulars (c), peduncle cover (PC), sulcals (s), postcingular series (‴), posterior intercalaries (p), and antapical series (⁗). Additional plates supplement the Kofoidian series, i.e., Po (pore plate), cp (closing plate), X (canal plate), and PC (peduncle cover plate)

Family/Genus	Po	cp	X	′	a	″	c	PC[1]	s	‴	⁗
Pfiesteriaceae											
Pfiesteria	+	+	+	4	1	5	6	+	5+	5	2
Pseudopfiesteria	+	+	+	4	1	6	6	+	5+	5	2
Tyrannodinium	+	+	+	4	0	6	6	+	5+	5	2
Aduncodinium	+	-	+	4	2	6	6	+	3+	5	2
Speroidium	+	+	+	4	2	6	?	?	?	5	2
Representative Thoracosphaeraceae											
Luciella	+	+	+	4	2	6	6	+	5+	5	2
Stoeckeria	+	+	+	4	2	7	6	+	6	5	2
Cryptoperidiniopsis	+	+	+	5	0	6	6	+	5+	5	2

[1] The questionable presence of a PC plate in *Speroidium* may, upon further evaluation, be confirmed.

PFIESTERIA LIFE CYCLE

The original description of the *Pfiesteria piscicida* life cycle was very different from that published previously for any other dinoflagellate (Burkholder and Glasgow 1995; 1997a; 1997b). It contained over 35 separate stages including numerous amoeboid forms never before observed in dinoflagellate life cycles. It was reported that these amoebae, upon sensing fish or fish excreta, could readily transform into free-swimming dinospores that could track and kill fish. Later publications showed an even more complex version of the life cycle that included dinospore-, amoeboid-, and chrysophyte-like cyst forms with varying toxicity that were theorized to provide *Pfiesteria piscicida* access to diverse food resources (Burkholder and Glasgow 2002; Burkholder, Glasgow, and Deamer-Melia 2001; Burkholder et al. 2001a; Burkholder, Glasgow and Steidinger 1997; Parrow and Burkholder 2003; Parrow et al. 2002). Given the uniqueness of this life cycle, and the proposed role of this organism in causing massive fish kills (Burkholder et al. 1992), these findings generated considerable interest. Close examination of the original data, however, raised concerns. The photomicrographs of the amoebae showed morphologies and surface structures usually considered diagnostic of Gymnamoebia of the class Lobosea and not dinoflagellates (Page and Siemensma 1991). Both photosynthetic and non-photosynthetic life cycle stages were reported without any

explanation as to how photosynthesis could be lost and gained in the same life cycle (Burkholder and Glasgow 1997b). Later, the presence of chloroplasts was attributed to a process called kleptochloroplasty. Some heterotrophic dinoflagellates such as *P. piscicida* and all the PLDs feed on other plankton using a peduncle (Figure 4), a tube-like structure that attaches to a prey cell and withdraws contents for food through a process called myzocytosis (Steidinger et al. 2001; Figure 5). When feeding myzocytotically on photosynthetic microalgae, *P. piscicida* can capture chloroplasts from microalgal prey and then sequester them in food vacuoles (Burkholder and Glasgow 1995; 1997a; Burkholder and Marshall 2012; Steidinger et al. 1996). There was some evidence that these chloroplasts had limited function for a few days (Lewitus, Glasgow and Burkholder 1999). When exposed to prey cells, 25-54% of the *P. piscicida* cells contained intact chloroplasts on days 1-2. Retention dropped to < 15% after 3-4 days and ~ 1-2% thereafter. At the peak of chloroplast retention on day 2, carbon (C) fixation normalized to the total population was at most 8.16 ± 0.48 pg C•cell^{-1} day^{-1} (Lewitus, Glasgow and Burkholder 1999). Given the reported estimate of 180 pg total C•*Pfiesteria* cell^{-1}, kleptochloroplasty would have supplied no more than 9% of the total C demand needed for cell division over days 1-2 (i.e. 4.5% per day for the first two days). That contribution declined to 2.5% on days 3-4 and was negligible thereafter. These data indicate that the growth demand for C contributed by kleptochloroplasty was minimal compare to the C acquired through direct consumption of prey cells.

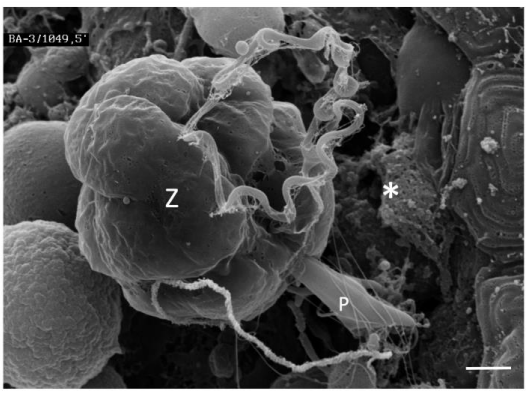

Figure 4. Scanning electron micrograph of a single *Pseudopfiesteria shumwayae* dinospore (Z) attached to the skin of a larval finfish via the peduncle (P) and actively feeding. Note the extensive damage to epidermal cells (*). Scale bar: 2.0 µm.

Further, Burkholder and Glasgow (1995; 1997a; 1997b) did not use single cell isolates to determine the life cycle and were unwilling to share cultures with other researchers so that their observations could be verified. Instead, their proposed life cycle was inferred by observing aliquots of water removed from laboratory tanks where abnormally dense cultures of *Pfiesteria* had been killing fish for months. Another striking feature of the original papers are the narratives describing how *Pfiesteria* could transform into the various forms (e.g., ameboid, dinospore or chrysophyte cyst) with no accompanying methods of how the observations were made (Burkholder and Glasgow, 1995, Burkholder et al. 1997a, Burkholder and Glasgow 1997b). This made it impossible for other investigators to set up controlled experiments that would allow confirmation of how the descriptive determinations of stage transformation or highly unusual behaviors being presented were determined.

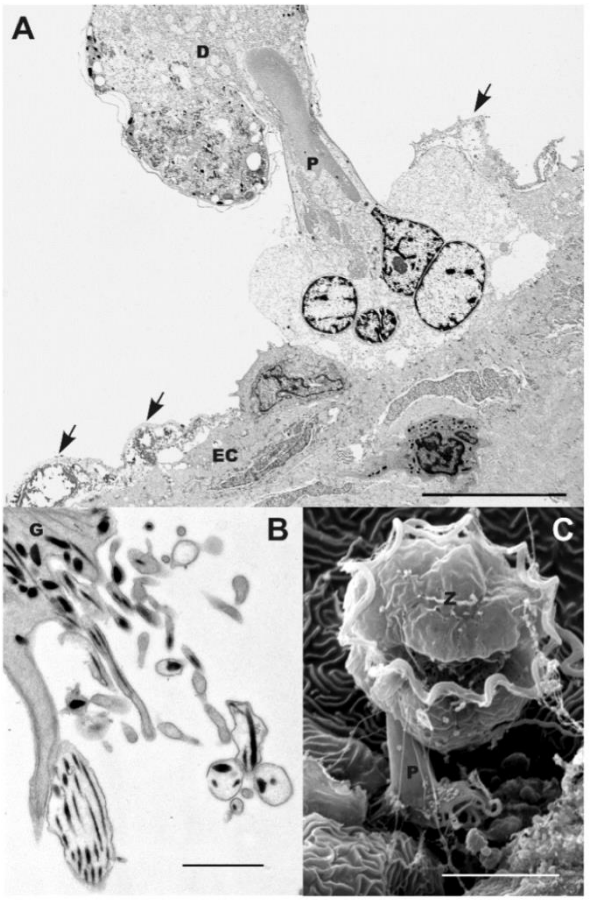

Figure 5. Scanning and transmission electron micrographs of the direct physical attachment of P*seudopfiesteria shumwayae* to the epidermis of a larval finfish. A. Dinoflagellate/fish skin interface and attachment site. Dinoflagellate (D) attaches to the fish epidermis (EC) by peduncle (P), seen here withdrawing the contents of several dead cells. Extensive cell damage (arrows) due to active feeding. B. An as yet unattached *P. shumwayae* peduncle exhibiting numerous electron-dense granules and rodlets (G), not evident once dinoflagellate is attached and feeding (See panel A). C. *Pseudopfiesteria shumwayae* dinospore (Z) attached to the fish skin via a peduncle (P) and actively feeding. Note damage to epidermal cells. Scale bars: A, C, 10μm; B, 5μm. Figure reprinted with permission from Nature from: Vogelbein, Wolfgang K., Vincent J. Lovko, Jeffrey D. Shields, Kimberly S. Reece, Patrice L. Mason, Leonard W. Haas, and Calvin C. Walker. 2002. "*Pseudofiesteria shumwayae* Kills Fish by Myzocytosis Not Exotoxin Secretion." *Nature* 418:967-970. https://doi.org/10.1038/nature01008.

Given these concerns and the lack of sufficient information to repeat the previous studies, Litaker et al. (2002) began systematically investigating the *P. piscicida* life cycle by first establishing single cell isolates. Initially, cells matching the morphology of *P. piscicida* were isolated from the estuarine system from which they had first been reported. Once cultures containing *P. piscicida* were identified, single cell isolates were used to establish secondary cultures (Litaker et al. 2002). This process was repeated multiple times with the initial subcultures often containing both *Pfiesteria* dinospores and amoebae. By the third or fourth isolation, however, subclones yielded only typical amoebae or dinospore (*P. piscicida*) life

cycle stages. After cell cultures were established, genomic DNA was extracted and the SSU, ITS and 5.8S ribosomal RNA (rDNA) genes were PCR amplified using dinoflagellate-specific primers, cloned and sequenced to determine their composition (Bowers et al. 2000; Litaker et al. 1999; Saito et al. 2002). These latter clonal cultures were maintained and observed for several years. Cultures containing typical dinoflagellate life cycle stages never produced amoebae and the amoebae cultures never produced dinospore life cycle stages. To verify the identity of the amoebae and dinoflagellate cultures, genomic DNA was sequenced as described above and compared with sequences in GenBank. The results confirmed the amoebae cultures were indeed true free-living amoebae and the sequences of the dinospore cultures completely matched *P. piscicida*.

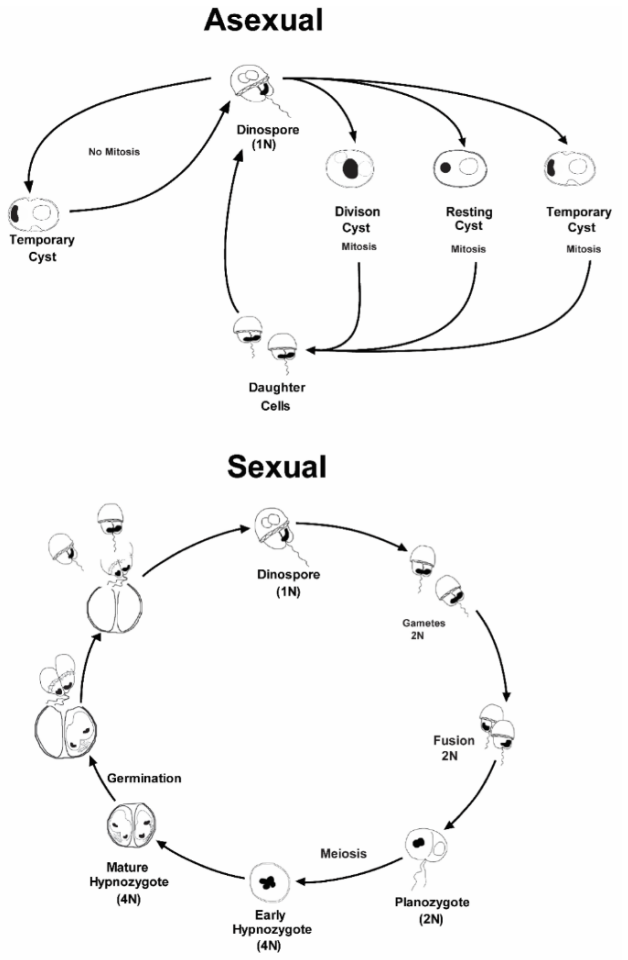

Figure 6. Life cycle of *Pfiesteria piscicida* typical for a heterotrophic dinoflagellate. During the asexual phase of the life cycle, mitosis occurs in resting cysts and not by binary fission of motile cells. Cells shed their flagellae, form thin walled cysts, their sulcus becomes indistinct, and over a period of minutes to hours they undergo mitosis. Once complete, a cyst germinates releasing a protoplast that almost instantly separates and transforms to produce two free-swimming dinospores (Litaker et al. 2002). The sexual phase consists of a fusion of gametes, formation of hypnozygote cysts, meiosis, and release of 4 daughter cells. The hypnozygotes remain viable for a period of months and perhaps longer. Redrawn from information provided in Litaker et al. (2002).

Another group of researchers obtained similar results with cultures established from water and sediment samples collected in Chesapeake Bay and from Noga's tanks at North Carolina State University where fish mortality had been attributed to *Pfiesteria* (Peglar et al. 2004). They followed eleven clonal PLD cultures from Chesapeake Bay over a three-year period and also observed typical dinoflagellate life cycles with no evidence of amoeboid forms. From those same Chesapeake Bay samples and from the Noga tank samples they also isolated several amoebae that had morphological characteristics consistent with various forms that had been described as *Pfiesteria* amoebae. They stated "Most lobosean amoebae have the ability to produce a rayed floating form and light microscopic observations cannot differentiate these amoebae from the "filose star amoeba" described in the life cycles of *Pfiesteria*-like dinoflagellates". These data were again consistent with the original amoeboid stages in the life cycle being simple contaminants introduced into the original fish tank system. Such amoebae are commonly found on fish epithelium (English et al. 2019).

To further test the hypothesis that amoebae were not part of the life cycle, two fluorescently labeled i*n situ* hybridization probes were developed. One targeted amoebae species and the other was specific for *P. piscicida* (Litaker et al. 2002). The process of selecting the fluorophore for the probes proved challenging as amoebae naturally fluoresce at commonly used fluorescent wavelengths. To avoid this problem, Cy5 fluorophores with far-red excitation wavelengths were coupled to the hybridization probes. *In situ* hybridization controls included universal eukaryotic and prokaryotic rDNA probes. The former control confirmed that all cells, including amoebae, contained intact RNA and the latter control helped identify non-specific probe hybridization. Samples from tanks where *Pfiesteria* were feeding on fish were preserved using standard methods and probed (Litaker et al. 2002). Only life cycle stages typical of dinoflagellates were positive with the *P. piscicida* probe. The amoebae probe only hybridized to amoebae and not to typical dinoflagellate life cycle stages.

Finally, the constant "stage transformations" where dinospores rapidly convert to amoebae, and back again is not possible. All alveolates, which include dinoflagellates, are characterized by the presence of cortical alveoli (sacs). flattened vesicles underlying the plasma membrane and supported by a single-layered sheet of microtubules forming a corset surrounding the entire algal cell. In dinoflagellates the alveoli can also form the characteristic "thecal plates". In contrast, no amoebae, whether they belong to the Amoebozoa, Rhizaria, Excavata, or Opisthokonta, have a cytoskeletal cell cortex under their plasma membrane. The amoebae described in the original *Pfiesteria* life cycle met the morphological criteria for true free-living amoebae in every respect. That the complex dinoflagellate cell cortex/alveolar system could somehow dissolve immediately upon transformation into an amoeba, and then instantly reform upon transformation from amoeba to dinospore is untenable based on everything known about the cellular structure of these organisms.

Litaker et al. (2002) used detailed light microcopy, including time-lapse photographic studies, in conjunction with *in situ* hybridization probes to document the life cycle of *P. piscicida*. This work was done using the single cell isolates established as described above. The results showed a typical marine dinoflagellate life cycle with relatively few asexual and sexual stages (Figure 6). A similar life cycle lacking amoeboid stages was reported for *P. piscidida*, *Pseudopfiesteria shumwayae* and the Cryptoperidiniopsis species from the Burkholder laboratory (Parrow and Burkholder 2003; Parrow and Burkholder 2004). It also should be noted that another set of *in situ* hybridization probes was developed prior to the Litaker et al. (2002) study and used to screen field samples, but results obtained are

questionable (Burkholder, Glasgow, and Deamer-Melia 2001; Rublee et al. 1999; Rublee et al. 2005). The reason is that those probes were labeled with fluorophores that fluoresced at the wavelengths at which amoebae naturally autofluoresce. Consequently, the observed signals likely represented false positives. Furthermore, those studies lacked the necessary "no probe" controls that would have demonstrated the amoebae's native fluorescence characteristics that would allow false positives to be identified. PCR positive "*Pfiesteria*" amoebae were similarly reported (Burkholder et al. 2001a), but these data are also suspect for the following reasons: 1) Negative controls to detect cross contamination with *Pfiesteria* DNA were not performed, (2) No amoebae-specific primer sets were used to confirm that positives did not contain true amoebae DNA and that amplification in those samples was not due to contamination with co-occurring *Pfiesteria* cells or DNA and (3) Most of the identified "*Pfiesteria*" amoebae that were screened showed no amplification with the *Pfiesteria* species-specific primers.

The finding that *Pfiesteria* lacked "amoeboid" life cycle stages is significant. These amoebae were a key component of the "Ambush Predator Hypothesis" put forward to account for how *Pfiesteria* kills fish in nature (Burkholder 1999; Burkholder and Glasgow 1997b; Burkholder, Glasgow and Steidinger 1995). At temperatures between 12 and 15°C these amoebae were claimed to be the only stage capable of killing fish (Burkholder 1999). These amoebae were also described as abundant in sediments and capable of exploiting a variety of food sources thereby significantly increasing *Pfiesteria*'s effective population size. Upon sensing fish excreta these amoebae were reported to transform into free swimming dinospores, some of which released toxins that killed fish. When food became more limiting, the dinospores were said to be capable of transforming back to amoebae. The absence of any amoebae in the life cycle obviously calls into question much of the Ambush Predator Hypothesis, particularly at 12 to 15°C where the amoebae were the only life cycle stage reported as capable of killing fish.

GEOGRAPHIC DISTRIBUTION OF THE PLDS

For many years the reports of *P. piscicida* and other PLDs were restricted to the US mid-Atlantic coast from Delaware Bay to North Carolina where the initial reports of associations with fish kills originated (Burkholder and Glasgow 2001; Burkholder, Glasgow and Steidinger 1995; Burkholder et al. 1992; Coyne et al. 2001; Lin, Zhang and Dubois 2006; Zhang and Lin 2005 Tables 2 & 3). Development of PCR assays to detect these organisms provided the ability to screen for the DNA of cells in water samples from all over the world. *Pseudopfiesteria shumwayae* DNA was detected in Europe, Asia, South America, Australia and New Zealand (Jakobsen et al. 2002; Rublee et al. 2004; Rublee et al. 2005; Zhang and Lin 2005). Likewise, *Pfiesteria piscicida* was reported in Norway, China, Hawaii, New Zealand, in ballast water from Indonesia, along the Chilean coast and in a saline lake in Antarctica using molecular techniques (Jakobsen et al. 2002; Lin, Zhang and Dubois 2006; Park et al. 2007; Rublee et al. 2004; Rublee et al. 2005). *Luciella masanensis*, one of the PLDs, was primarily reported in the mid-Atlantic, but was also found in Korea (Mason et al. 2007). In addition to PCR detection, cells of *P. shumwayae* were observed and analyzed by SEM in New Zealand (Rhodes et al. 2002), and in Norway cultures of *P. shumwayae* were

established (Jakobsen et al. 2002). *Luciella masanensis* from Korea was cultured to allow a complete characterization of this species in two geographic locations (Mason et al. 2007). *Cryptoperidiniopsis brodyi* was found recently outside of the US in Portugal (Moestrup et al. 2014).

In several locations where *P. piscicida* or *P. shumwayae* first had been detected using PCR, including South America, most locations in Asia and Europe, and in Antarctica, there were no live cells observed nor cultures established for further morphological or genetic characterization. Park et al. (2007) attempted to observe *P. piscicida* cysts and live cells microscopically in water samples from the saline Antarctic lake, Ace Lake, and added several potential prey species to PCR positive samples in an attempt to confirm the presence of viable cells or prompt excystment but were unsuccessful. Therefore, it is not clear whether *P. piscicida* was viable under the current conditions in Ace Lake or the DNA that was detected by the PCR assay was from an exogenous source.

Most studies using quantitative methods determined that, although *Pfiesteria* and PLDs could be detected in many locations, environmental cell concentrations were very low, generally < 10 cells L^{-1} and seldom exceeding 100 cells L^{-1} (Coyne et al. 2001; Lewitus et al. 2002; Lin, Zhang and Dubois 2006). Although there were some fish kills where elevated levels of *Pfiesteria* were reported (Burkholder, Glasgow and Deamer-Melia 2001), there were kill events in the mid-Atlantic region associated with other HAB organisms (see Fish Kill section below and Tables 2 & 3). Furthermore, no fish kills had been reported in association with the presence of *Pfiesteria* or PLDs other than in the mid-Atlantic US estuaries until 2012 when *P. piscicida* was reported to be present during large fish kills in a saline lake in Greece (Oikonomou et al. 2012). Although the authors suggested that *P. piscicida* likely played a role in the two fish kills, there were many other toxic organisms present in water samples that were collected including several cyanobacterial species such as *Planktothrix* and *Anabaena* species, the haptophyte, *Prymnesium parvum*, and fish pathogens, including *Dermocystidium* species. They were unable to provide definitive data on cell concentrations from their molecular analyses and their microscopic cell counts indicated that PLDs were only 0.4% of the plankton biomass by visual counts in the water samples (Oikonomou et al. 2012). Further, a BLAST search of the reported SSU rDNA sequence from the species revealed it to be a PLD more closely related to the genera *Luciella* and *Cryptoperidiniopsis*.

ECOLOGY AND NUTRITION

Pfiesteria piscicida and the PLDs have largely been reported from shallow estuaries, although as mentioned above, there have been rare instances where PLD DNA has been detected in saline lakes in Greece and Antarctica (Oikonomou et al. 2012; Park et al. 2007). *Pfiesteria* and PLDs are typically found in nutrient-rich estuarine waters with salinities ranging between 5-20 ppt and at temperatures ranging between 15-30°C, although from laboratory experiments it is known that they can tolerate an even wider range of temperatures (i.e., 6–31°C) (Burkholder, Glasgow and Steidinger 1995; Lin, Zhang and Dubois 2006; Rublee et al. 2004). As this group of dinoflagellates is heterotrophic, it is not surprising that a light intensity optimum for growth has not been reported for these organisms (Burkholder, Glasgow and Steidinger 1995; Eriksen, Hayes and Lewitus 2002).

Table 2. Fish kills in Maryland waters of Chesapeake Bay during 2007. The date and number of dead fish observed are indicated for each event. The location indicates the site where water samples were collected at or near the site where dead fish were observed. The number of Pfiesteria-like dinoflagellate cells (PLD) counted (cells mL-1) are indicated, as well as results for karlotoxin 1 (KmTx1) levels (ng L-1). PCR assay results for Karlodinium veneficum, Amoebophyra spp., Pfiesteria piscicida and Pseudopfiesteria shumwayae are indicated as positive (Pos) or negative (Neg) for each species

Location	Date	PLD Cells mL^{-1}	Est. Number Fish Dead	KmTx 1 (ng L^{-1})	*K. veneficum*	*Amoebophrya*	*P. piscicida*	*P. shumwayae*
Weems Creek Original Sample	6/29/07	24159	18190	254.4	Pos	Pos	Neg	Neg
Weems Creek Station 1	6/29/07	8680	same as above	34.8	Pos	Pos	Pos	Neg
Weems Creek Station 1 Red Streak	6/29/07	4282	same as above	24.3	Pos	Pos	Pos	Neg
Weems Creek Station 2	6/29/07	28845	same as above	125.2	Pos	Pos	Neg	Neg
Weems Creek Station 3	6/29/07	8484	same as above	4.5	Pos	Pos	Neg	Neg
Weems Creek Station 4	6/29/07	31027	same as above	238.6	Pos	Pos	Neg	Neg
Herring Bay 0601	6/29/07	NC	6	20.8	NR	NR	NR	NR
Brownies Beach 0602	6/29/07	NC	4	68.6	NR	NR	NR	NR
Plum Point Beach 0603	6/29/07	NC	2461	96.7	NR	NR	NR	NR
Breezy Point 0604	6/29/07	NC	same as above	58.9	Pos	Pos	Neg	Pos
Mill Creek	7/2/07	NC	NC	0.00	Pos	Pos	Neg	Neg
Heron Lake	7/2/07	27391	2240	267.6	NR	NR	NR	NR
Stoney Creek County Wharf	7/11/07	23351	9354	125.4	Pos	Pos	Pos	Neg
Stoney Creek Marina	7/11/07	71670	same as above	367.3	Pos	Pos	Pos	Neg
Dividing Creek Station 1	7/13/07	840	2200	5.0	Pos	Pos	Pos	Neg
Dividing Creek Station 2	7/13/07	2160	same as above	9.6	Pos	Pos	Pos	Neg
Main Creek	7/20/07	49450	9810	133.2	Pos	Pos	Neg	Pos
Main Creek	7/27/07	44925	4080	204.8	Pos	Pos	Neg	Neg
Nabbs Creek	7/28/07	<1000	3600	8.4	Pos	Pos	Neg	Neg
Stoney Creek	7/30/07	<1000	7320	5.0	Pos	Pos	Neg	Pos
Weems Creek Original Sample	6/29/07	24159	18190	254.4	Pos	Pos	Neg	Neg
Weems Creek Station 1	6/29/07	8680	same as above	34.8	Pos	Pos	Pos	Neg
Weems Creek Station 1 Red Streak	6/29/07	4282	same as above	24.3	Pos	Pos	Pos	Neg
Weems Creek Station 2	6/29/07	28845	same as above	125.2	Pos	Pos	Neg	Neg
Weems Creek Station 3	6/29/07	8484	same as above	4.5	Pos	Pos	Neg	Neg
Weems Creek Station 4	6/29/07	31027	same as above	238.6	Pos	Pos	Neg	Neg

Table 2. (Continued)

Location	Date	PLD Cells mL^{-1}	Est. Number Fish Dead	KmTx 1 ($ng\ L^{-1}$)	*K. veneficum*	*Amoebophrya*	*P. piscicida*	*P. shumwayae*
Herring Bay 0601	6/29/07	NC	6	20.8	NR	NR	NR	NR
Brownies Beach 0602	6/29/07	NC	4	68.6	NR	NR	NR	NR
Plum Point Beach 0603	6/29/07	NC	2461	96.7	NR	NR	NR	NR
Breezy Point 0604	6/29/07	NC	same as above	58.9	Pos	Pos	Neg	Pos
Mill Creek	7/2/07	NC	NC	0.00	Pos	Pos	Neg	Neg
Heron Lake	7/2/07	27391	2240	267.6	NR	NR	NR	NR
Stoney Creek County Wharf	7/11/07	23351	9354	125.4	Pos	Pos	Pos	Neg
Stoney Creek Marina	7/11/07	71670	same as above	367.3	Pos	Pos	Pos	Neg
Dividing Creek Station 1	7/13/07	840	2200	5.0	Pos	Pos	Pos	Neg
Dividing Creek Station 2	7/13/07	2160	same as above	9.6	Pos	Pos	Pos	Neg
Main Creek	7/20/07	49450	9810	133.2	Pos	Pos	Neg	Pos
Main Creek	7/27/07	44925	4080	204.8	Pos	Pos	Neg	Neg
Nabbs Creek	7/28/07	<1000	3600	8.4	Pos	Pos	Neg	Neg
Stoney Creek	7/30/07	<1000	7320	5.0	Pos	Pos	Neg	Pos

Table 3. Aquatic animal mortalities observed during HAB events in Virginia waters of Chesapeake Bay from 2006-2016. Location and dates of events are indicated. Water samples were collected at or near sites where dead animals were observed and on the same day as the observation unless otherwise indicated. Standard PCR assay results are indicated as positive (Pos) or negative (Neg) and cell numbers are indicated for results from qPCR assays. Assays were done for the following species; Karlodinium veneficum, Chatonella subsalsa, Pfiesteria piscicida, Pseudopfiesteria shumwayae, Alexandrium monilatum and Margalefidinium (formerly Cochlodinium) polykrikoides. For some events brevetoxin and karlotoxin concentrations were determined

Location	Date	*K. veneficum* (Cells mL^{-1})	*C. subsalsa*	*P. piscicida*	*P. shumwayae*	*A. monilatum* (Cells mL^{-1})	*M. polykrikoides*	Notes
Potomac River	6/2/06	Neg	Neg	Neg	Neg	ND	ND	
York River	6/22/06	Neg	Neg	Neg	Neg	ND	ND	
Rappahannock River	8/1/06	1	Neg	Neg	Neg	ND	ND	
York River	9/7/07	<1	Neg	Neg	Neg	40,000	Pos	Rapa whelk mortality @VIMS
York River	9/15/08	Neg	Neg	Neg	Neg	1000 – 3,000 (2-3 days before mortalities observed)	Neg	Cow nose ray mortality @VIMS

Location	Date	*K. veneficum* (Cells mL^{-1})	*C. subsalsa*	*P. piscicida*	*P. shumwayae*	*A. monilatum* (Cells mL^{-1})	*M. polykrikoides*	Notes
Ware River	5/5/10	3,136	Neg	Neg	Neg	Neg	Neg	
Cobb Creek	8/10/10	<1	Pos	Neg	Neg	Neg	Neg	
Cobb Creek	8/16/10	Neg	Pos	Neg	Neg	Neg	Neg	Water extract = 0.1 ng mL^{-1} brevetoxin
Cobb Creek	8/30/10	Neg	Pos	Neg	Neg	Neg	Neg	Water extract = 0.01 ng mL^{-1} brevetoxin; Menhaden tissue <4.5 ng g^{-1} Brevetoxin
Owen's Creek	8/31/10	<1	Pos	Neg	Neg	Neg	Neg	Water extract 0.125 ng mL^{-1} Brevetoxin
Antipoison Creek	9/5/10	Neg	Pos	Neg	Neg	Neg	Neg	
Antipoison Creek	9/8/10	Neg	Pos	Neg	Neg	Neg	Neg	
Antipoison Creek	7/5/12	<1	400	Neg	Neg	Neg	Neg	
Rappahannock River	8/13/12	<1	Neg	Neg	Neg	Neg	28,380	
Chesapeake Bay – Oceanview	9/13/12	<1	Neg	Neg	Neg	193	Neg	
York River	9/14/12	ND	Neg	Neg	Neg	1,200	Neg	
York River	6/12/13	Neg	Neg	Neg	Neg	Neg	Neg	
Piankatank River	7/8/13	101	Neg	Neg	Neg	Neg	Neg	
Lamb's Creek	7/10/14	167	Neg	Neg	Neg	Neg	Neg	*Prymnesium* sp. bloom
Virginia Beach	9/11/14	Neg	Neg	Neg	Neg	Neg	Neg	
Elizabeth River	9/11/14	Neg	Neg	Neg	Neg	Neg	Neg	
Potomac River	5/27/15	752,525	Neg	Neg	Neg	Neg	Neg	KmTx 1 and KmTx3 at 670 ng mL^{-1}
Nandua Creek	6/10/15	32	Neg	ND	ND	Neg	Neg	Oyster death
York River	8/19-23/15	1-10	Neg	ND	ND	Up to 233,032	Neg	Crab, fish, eel and oyster mortalities
Perrin Creek	8/20/15	6	Neg	ND	ND	25,006	Neg	Oyster death (1 yr. old animals)
Mobjack Bay	8/23/15	Neg	Neg	ND	ND	19	Neg	Oyster death (1 yr. old animals)
Big Island, York River	8/25/15	7	Neg	ND	ND	Up to 100,000 (5 days before mortalities observed)	Neg	Oyster death (1 yr. old animals)
Potomac River	6/2/16	700	Neg	ND	ND	Neg	Neg	*Prorcentrum minimum* = 63,400 cells ml^{-1}
York River	8/15/16	<1	Neg	ND	ND	88	2,924	Oyster spat mortality
York River	8/30/16	Neg	Neg	ND	ND	3,400	Neg	Oyster spat mortality

The available data indicate that *Pfiesteria*, as well as PLDs, feed on prey items ranging from bacteria to multi-cellular eukaryotic cells (Burkholder and Glasgow 1995; Burkholder, Glasgow, and Deamer-Melia 2001; Jeong et al. 2006; Parrow et al. 2005). They grow well on certain microalgae, particularly cryptophyte species, their preferred prey (Burkholder and Glasgow 1995, 1997a; Jeong et al. 2006, 2007, 2008; Kim et al. 2019; Lim et al. 2014). In the field, these dinoflagellates have a much higher probability of encountering microalgal prey than invertebrates or fish and, therefore, microalgae probably constitute their primary food source. This dependency on microalagae is indirectly supported by laboratory studies where the growth of *P. piscicida* was stimulated by nutrient addition, including the addition of diluted swine waste (Burkholder and Glasgow 2001; Burkholder et al. 1992). Those nutrient additions stimulated the growth of co-occurring microalgal prey, secondarily fostering *Pfiesteria* growth (Burkholder et al. 2001a; Burkholder, Glasgow, and Lewitus 1998). When presented with cryptophyte prey (*Rhodomonas sp.*) in the laboratory, *P. piscicida* increases its speed, radius and angular velocity but slightly reduces the pitch of its right-handed helix trajectory, suggesting the preferred predation tactics of an "active hunter" (Sheng et al. 2007) and not an ambush predator. The slower *K. veneficum*, in contrast, exhibits "ambush predator behavior", by reducing its velocity, radius and pitch to near zero. It increases its angular velocity, a change that reduces its hydrodynamic signature, while still scanning its environment as "a spinning antenna" (Sheng et al. 2007). Furthermore, field measurements showed a direct correlation between phytoplankton biomass (as measured by chlorophyll *a* concentration), primary production and the density of *Pfiesteria*-like dinospores in the Neuse River estuary, NC, one of the areas identified as the primary *Pfiesteria* fish kill sites (Burkholder, Glasgow, and Lewitus 1998; Pinckney et al. 2000).

The preference of *Pfiesteria* and *Pseudopfiesteria* for protists other than microalgae and their ability to grow on them is little studied. Existing information suggests that their ability to do this may be poor given *Pfiesteria piscicida*'s inability to grow on rotifers (Burkholder and Glasgow 1995) or *Mesodinium rubrum* (Lee et al. 2014). Parrow et al. (2005) also examined the nutritional value of a salmon cell line to axenic (bacteria free) *P. piscicida* and *P. shumwayae* cultures. That study found that although *P. shumwayae* could grow well on fish tissue culture cells, *P. piscicida* (fish killer) could not. This suggested *P. piscicida* required certain phytonutrients or critical microbial associates and could not grow on fish tissue alone.

Pfiesteria itself is readily consumed by numerous predators. These include the heterotrophic dinoflagellates *Oxyrrhis marina* and *Gyrodinium* spp. (Jeong et al. 2017), the ciliate *Strombidinopsis* sp. (Jeong et al. 2017), large tintinnids and oligotrichous ciliates (Stoecker, Shaw and Gustafson 2000), and rotifers and copepods such as *Acartia tonsa* (Mallin et al. 1995). Field measurements have suggested that grazing pressure from these predators is generally high enough to keep PLD densities at relatively low levels (Stoecker and Gustafson 2002; Stoecker, Shaw and Gustafson 2000).

IMPACTS

During the 1990's, the massive fish kills in North Carolina estuaries and Chesapeake Bay, usually of Atlantic menhaden with ulcerative lesions (Figure 7), were attributed to toxic

Pfiesteria (Burkholder 1999; Burkholder and Glasgow 1997a, 1997b; Burkholder, Glasgow and Deamer-Melia 2001; Magnien 2001). The identification of small heterotrophic dinoflagellates, i.e., PLDs, in water samples collected from fish kill events in which many of the fish exhibited deep ulcerative lesions (Figure 8) led to the inference that *Pfiesteria* and its toxin caused both the mortality and the lesions (Burkholder, Glasgow and Deamer-Melia 2001; Glasgow et al. 2001b; Burkholder, Glasgow and Steidinger 1995). However, other researchers disputed the assertion that the fish kills or the lesions were caused by *Pfiesteria* or the action of a *Pfiesteria* toxin. The following sections specially address these various controversies in greater detail.

Figure 7. Acute fish kill event (menhaden, *Brevoortia tyrannus*) in Narragansett Bay (RI) attributed to an anoxia/upwelling event. Photo courtesy of Thomas Ardito, Narragansett Bay Estuary Program, www.nbep.org.

Figure 8. a) Gross appearance of ulcerative mycosis in juvenile Atlantic menhaden, *Brevoortia tyrannus*. These ulcers were commonly attributed to *Pfiesteria* spp. during the 1990s but are now known to be caused by the oomycete, *Aphanomyces invadans*. Ulcers are commonly located peri-anally (arrow) and penetrate deeply into underlying muscle and viscera.

Fish Kills

The causal link between *Pfiesteria* and the fish kills was based on the following logic: High densities of *Pfiesteria*-like cells were present in estuaries, and *Pfiesteria* in tanks have complex life-cycle stages that kill fish. Ergo *Pfiesteria* was responsible for killing fish in estuaries. This conclusion was reached even though the conditions in the tank bioassays and field were vastly different and without confirmation that the complex life cycle of *Pfiesteria* existed in the field. In addition, all *Pfiesteria*-like organisms at fish kill sites were counted using light microscopy and classified as *Pfiesteria*, which inflated estimates of *Pfiesteria* abundance. In actuality, many other small heterotrophic dinoflagellates not easily distinguished from *Pfiesteria* using light microcopy were present (see Taxonomy section).

One of these species was *Karlodinium veneficum*. This mixotrophic species often co-occurs with *Pfiesteria*, forms dense blooms, and produces a well-defined toxin that kills fish in the environments where the original *Pfiesteria* studies were conducted (Adolf et al. 2015; Bachvaroff et al. 2008; Hall et al. 2008). *Karlodinium veneficum* uses its toxin to immobilize cryptophyte prey prior to ingestion (Sheng et al. 2010) and fish kills are collateral damage from a bloom (Place et al. 2012). It should be noted that unlike *Pfiesteria*, whose growth is dependent on consuming prey, *K. veneficum* can acquire C equally well by heterotrophy and photosynthesis. This ability to grow photosynthetically thereby supplying its own C makes achieving higher cell densities much easier for *K. veneficum* relative to *P. piscicida* which rapidly depletes its food supply. *Pfiesteria piscicida* can consume 6 ± 2 prey cells on average in a 10-minute period (Burkholder and Glasgow 1997b), with other experiments showing it removes on average between 2% and 15% of a microalgal prey population in just 1 h (Jeong et al. 2006). It is quite possible that in many instances the high densities of *Pfiesteria* originally reported in the 1990's (e.g., Burkholder et al. 1992; Burkholder and Glasgow 1995) were actually *K. veneficum* (Coyne et al. 2001; Fensin 2004; Handy et al. 2008; Marshall 1999; Marshall, Seaborn and Wolny 1999). The original samples were never checked for chlorophyll fluorescence which would have helped to distinguish the photosynthetic *Karlodinium* from heterotrophic *Pfiesteria*. Support for this potential confusion comes from subsequent surveys that have shown that *Pfiesteria* cells are typically present in natural system at ~10 cells L^{-1} and are not observed to form dense blooms (Coyne et al. 2001; Handy et al. 2008; Lewitus et al. 2002; Lin, Zhang and Dubois 2006; Marshall 1999; Marshall, Seaborn and Wolny 1999; Zhang and Lin 2005).

In addition, Paerl et al. (1998) reported that 80-85% of the fish kills in Pamlico Sound could be attributed to hypoxia/anoxia (oxygen depletion) of the bottom waters caused by eutrophication, which led to dense algal blooms of other species and subsequent die-offs and bacterial degradation that depleted the oxygen. Burkholder, Mallin and Glasgow (1999) challenged the Paerl et al. (1998) reasoning of oxygen depletion as an alternative explanation to *Pfiesteria* toxin as a cause of the fish kills. However, Paerl et al. (1999) provided a plausible mechanism whereby surface-dwelling menhaden might succumb to oxygen-depleted bottom waters in their response. A common problem with ascribing causality to fish kills is that they are frequently observed and reported many hours or even days after the fish have died. The conditions where the dead fish are found may no longer reflect conditions that preceded the mortality event or fish may have been transported long distances away from the initial location of the event. Dissolved oxygen levels measured in the presence of floating dead fish are often not relevant to the conditions that were present when the fish died.

Concentrations of several HAB species in water samples collected at sites during and following reported fish kill events in both Virginia and Maryland waters of Chesapeake Bay have been monitored for the past two decades. As mentioned above, often oxygen depletion may have contributed to a kill event and ascribing cause to a particular HAB species may be erroneous in many instances. Nonetheless, several kill events in Chesapeake Bay have been associated with HAB species, but these are species other than *P. piscicida* and *P. shumwayae*, both of which have been notably absent during most of these fish kills (see Tables 2 & 3). In Maryland, many fish kills have been associated with *K. veneficum* blooms with high cell numbers and karlotoxins being detected in water samples collected from sites where dead fish are reported and at nearby stations (Table 2). Frequently the gill pathology in moribund fish is consistent with that described for laboratory exposure to karlotoxins (Deeds, Reimschuessel, and Place 2006). Likewise, in Virginia, there have also been fish kills associated with *K. veneficum* blooms with karlotoxins detected in a Potomac River sample from 2015 (Table 3). Mortality events of fish, rapa whelks, oysters, rays, eels and crabs have also been observed in Virginia in association with high cell numbers (i.e., > 1,000 cells/ml) of other HAB organisms including *Margalefidinium* (formerly *Cochlodinium*) *polykrikoides*, *Alexandrium monilatum*, *Chattonella subsalsa* and *Prorocentrum minimum* (Table 3). It is interesting to note that, in association with some of the water and fish samples collected from the *C. subsalsa* bloom and kill events, low levels of brevetoxin were detected (Table 3).

Lesions

In the late 1990's, the claim was made that *Pfiesteria* caused the deeply penetrating skin ulcers observed on finfish, predominantly menhaden (*Brevoortia tyrannus*), from massive fish kill events in North Carolina estuaries (Burkholder and Glasgow 1997a; Burkholder, Glasgow, and Deamer-Melia 2001; Burkholder et al. 2001b; Glasgow et al. 2001b). This contention fueled tremendous negative publicity and resulted in significant economic losses by the restaurant, charter boat and seafood industries in North Carolina and Chesapeake Bay (Lipton 1998; Meyer 1997). Whenever people observed or heard of reported lesions, they assumed recreational waters and seafood products were unsafe and avoided them accordingly. This negative perception predominated despite a clear understanding at the time that many factors can cause the appearance of skin lesions on fish. Those factors include a variety of infectious agents, immunological responses, nutritional or metabolic stress and physical damage, as well as toxic compounds such as polycyclic aromatic hydrocarbons (PAHs) or those produced by HABs (Kane, Oldach and Reimschuessel 1998; Law 2001; Noga 2000; Sindermann 1988). This basic knowledge led scientists to question the tacit assumption that all fish kills in North Carolina and Chesapeake Bay estuaries where finfish ulcers were observed were caused by *Pfiesteria* (Blazer et al. 1999; Vogelbein et al. 2001).

There were sound reasons for this skepticism. Historically, studies had shown that the deeply penetrating, and largely single, ulcerous lesions observed on menhaden along the US East coast including Chesapeake Bay and North Carolina were most often associated with oomycete (water molds) infections (Dykstra et al. 1986; Dykstra et al. 1989; Noga 1993; Levine et al. 1990a, 1990b; Noga and Dykstra 1986; Noga et al. 1988). This disease was termed ulcerative mycosis (UM) and characterized by the presence of severe dermatitis, underlying granulomatous myositis and tissue destruction associated with fungus-like hyphae

(Dykstra et al. 1986; Noga and Dykstra 1986). Additionally, lesions typically exhibited a diverse mixture of microbial contaminants colonizing the outermost necrotic tissues (Dykstra et al. 1986, 1989; Noga et al. 1988, 1993; Levine et al. 1990a; Blazer et al. 1999). Initial attempts to fulfill Koch's postulates by re-infecting the fish with oomycetes such as *Saprolegnia* sp. and *Aphanomyces* sp. ATCC 62427 isolated from affected menhaden failed to reproduce the typical lesions seen in wild fish. This left unresolved whether the mixture of microbial agents, including the deeply invasive oomycete found in the ulcers, were (a) opportunists colonizing a superficial lesion caused by unknown environmental stressors or biological insults, or (b) whether one of the microbes was a primary pathogen capable of causing UM directly without other stressors playing a role (Noga et al. 1996; Kane, et al. 1998; Noga 2000). Noga et al. (1996) and Sindermann (1999) proposed that ulcerative lesions in finfishes were multi-factorial in their etiology.

At the time the hypothesis that *Pfiesteria* was the underlying cause of UM in US East Coast menhaden was gaining global attention, a similar ulcerative disease was reported in over 100 species of wild and cultured freshwater and estuarine fishes from the Indo-Pacific region (Callinan 1994a, 1994b). This disease was also characterized by the occurrence of deep tissue necrosis and granulomatous inflammation associated with fungus-like hyphae. The occurrence of water mold infections around the world was variously termed epizootic ulcerative syndrome (EUS) in Asia, red spot disease (RSD) in Australia and mycotic granulomatosis (MG) in Japan (Callinan et al. 1995; Egusa and Masuda 1971; Fraser, Callinan and Calder 1992; Hatai 1980; Hatai et al. 1977; Lilley et al. 1998; Willoughby, Roberts and Chinabut 1995). However, unlike the North American experience with *Aphanomyces* strain ATCC-62427, numerous laboratories independently established that one highly pathogenic oomycete species could by itself produce the typical ulcerative lesions observed in Indo-Pacific fishes through multiple laboratory exposure routes (Catap and Munday 1998; Cruz-Lacierda and Shariff 1995; Hatai et al. 1977; Khan et al. 1998; Lilley and Roberts 1997; Roberts, Willoughby and Chinabut 1993; Wada et al. 1996). This included aqueous challenges of healthy fish to secondary zoospores, the infective stage of this oomycete. These studies clearly established the agent in the Indo-Pacific region, *Aphanomyces invadans*, as a primary pathogen (Callinan, Sammut and Fraser 1996), which is now believed to be the cause of major fish kills affecting both wild and farmed fisheries on a global scale. This water mold is recognized as a single species of cosmopolitan distribution and is listed in the Index of Fungi as *Aphanomyces invadans* (David and Kirk 1997). The disease has since that time been collectively referred to as EUS (Kar 2016; Lilley and Roberts 1997).

Given the experience in the Indo-Pacific region, Blazer et al. (1999) re-investigated menhaden mortality and lesion events attributed to *Pfiesteria* in the Chesapeake Bay. They reconfirmed that the "*Pfiesteria*" lesions in fish were identical to those reported as menhaden UM from eastern US estuaries during the 1980s (e.g., Noga and Dykstra 1986). Additionally, new oomycete isolates obtained from Chesapeake Bay menhaden were definitively identified as *A. invadans* based on growth characteristics, PCR and fluorescent *in situ* hybridization (FISH) assays (Blazer et al. 2002; Lilley et al. 2003). Subsequently, molecular analyses also identified *A. invadans* in all ulcerative lesions found on menhaden collected from North Carolina estuaries where *Pfiesteria* was first reported to have caused fish kills and fish lesion events (Vandersea et al. 2006). Additional controlled laboratory challenges of menhaden with Chesapeake Bay and North Carolina *A. invadans* isolates definitively confirmed this water

mold as a primary pathogen and the causative agent of menhaden EUS (Figure 9) (Kiryu et al. 2002; 2003; Vandersea et al. 2006).

Other observations increasingly led to questions regarding the purported role of *Pfiesteria* in fish kill and lesion events (Dykstra and Kane 2000; Vogelbein et al. 2001). *Pfiesteria* pathology occurring in laboratory fish bioassays and attributed to toxin production was described as widespread surficial erosion of epidermis that formed rapidly in as little as several hours (Noga et al. 1996). In contrast, the *A. invadans* lesions found on wild menhaden exhibited a typical granulomatous inflammatory response and the consistent presence of oomycete hyphae (Figure 9). This inflammatory response takes days to weeks to develop and could not have formed rapidly in response to acute toxin exposure. These data further argued against *Pfiesteria* causing the deep ulcerative lesions observed in wild menhaden. Despite these findings, claims that EUS in Mid-Atlantic USA estuaries was caused by *Pfiesteria* toxin have persisted (Burkholder and Marshall 2012; Burkholder and Marshall 2018).

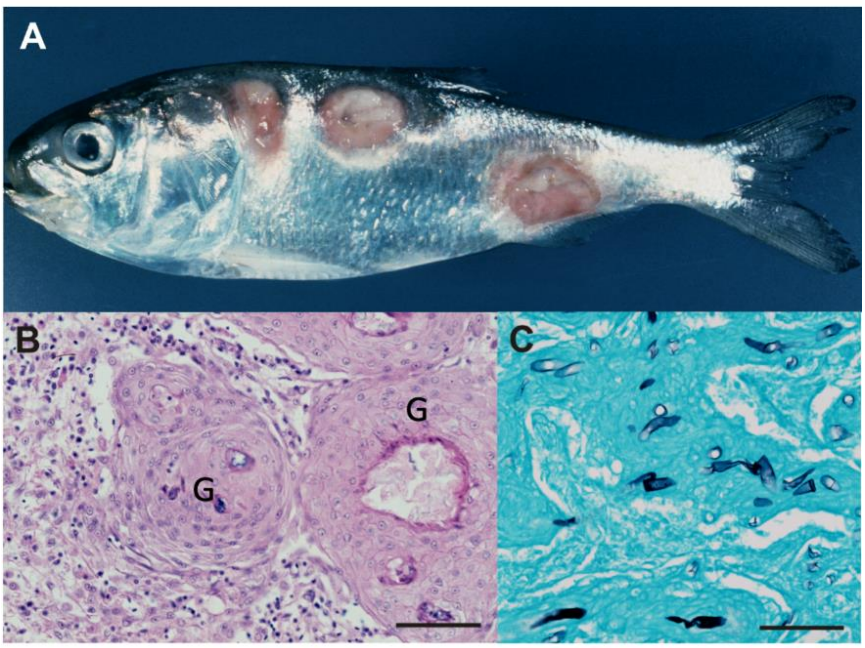

Figure 9. Gross and histologic pathology of experimental ulcerative mycosis of Atlantic menhaden, *Brevoortia tyrannus*. A. Gross pathology of fish 18 days after aqueous challenge with 2° zoospores of *A. invadans*. B. Histopathology showing chronic granulomatous inflammation. G – granulomas consisting of host defensive cells called macrophages encapsulating fungal hyphae. C. Grocott's silver stain for fungi showing black staining hyphae within affected tissues. Scale bars, for figs. B and C, 45 µm. Panel A is reproduced by permission of the journal *Diseases of Aquatic Organisms*, from Kiryu, Yasunari, Jeffery D. Shields, Wolfgang K. Vogelbein, Howard Kator, and Vicki S. Blazer. 2003. "Infectivity and Pathogenicity of the Oomycete *Aphanomyces invadans* in Atlantic Menhaden *Brevoortia tyrannus*." *Diseases of Aquatic Organisms* 54:135–46. https://doi.org/10.3354/dao054135. Panels B and C reproduced from Vogelbein, Wolfgang K., Jeffrey D. Shields, Leonard W. Haas, Kimberly S. Reece, and David E. Zwerner. 2001. "Skin Ulcers in Estuarine Fishes: A Comparative Pathological Evaluation of Wild and Laboratory-Exposed Fish." *Environmental Health Perspectives* 109(Suppl 5):687–93. https://doi.org/10.1289/ehp.01109s5687.

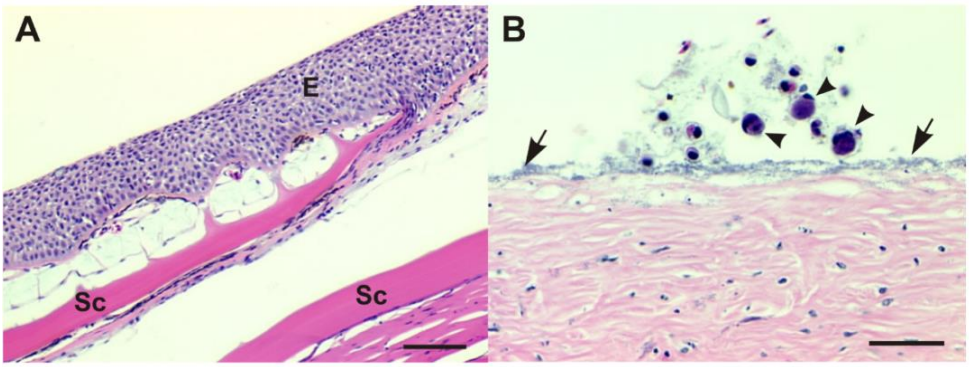

Figure 10. Histology of the skin of tilapia (O*reochromis* sp.) prior to and following challenge with *Pseudopfiesteria shumwayae*. A. Unexposed healthy tilapia (Control) exhibiting intact skin, with multi-layered epidermis (E). Sc: denotes scales. B. Exposed tilapia showing complete erosion of epidermis and bacterial colonization of the exposed dermis (arrows). *Pseudopfiesteria shumwayae* dinospores attached to the eroded skin surface (arrowheads). Scale bars: A, 100 µm; B, 50 µm. Reproduced from Vogelbein, Wolfgang K., Jeffrey D. Shields, Leonard W. Haas, Kimberly S. Reece, and David E. Zwerner. 2001. "Skin Ulcers in Estuarine Fishes: A Comparative Pathological Evaluation of Wild and Laboratory-Exposed Fish." *Environmental Health Perspectives* 109 (Suppl 5):687–93. https://doi.org/10.1289/ehp.01109s5687.

Because menhaden are mobile, the presence of a chronic lesion days to weeks old may have no bearing on current local conditions where fish are collected. Vogelbein et al. (2001) distinguished menhaden UM from laboratory-induced pfiesteriosis in tilapia based on histopathology (Figure 10). Tilapia (*Oreochromis* sp.) experimentally exposed to *P. shumwayae* using the standard fish bioassay approach (Burkholder, Glasgow, and Deamer-Melia 2001; Burkholder et al. 2001a; Burkholder, Glasgow and Steidinger 1995), exhibited direct attachment of *Pseudopfiesteria* dinospores to surficial tissues and widespread loss of the epidermis, mucus and scales (Vogelbein et al. 2001; see section on *Fish Bioassays and Micropredation* below). This physical attachment of *Pfiesteria* dinospores was a new finding and the associated diffuse, widespread superficial pathology was clearly distinct from the deeply penetrating, single chronic ulcers in wild-caught menhaden with EUS. The diffuse epidermal damage reported in laboratory-exposed fish has never been observed in wild menhaden from fish kill or lesion events (Law 2001) and was essentially identical to that previously reported by Noga et al. (1996) as an effect of *P. piscicida* toxin. These data further argue against *Pfiesteria* as causing fish lesions in the wild.

Over the intervening years it has become well established that *A. invadans* is the causative agent of EUS and represents a global threat to wild and cultured finfishes in both fresh and saltwater globally. Since its first report from Japan, the disease was more recently observed in several southern African countries (Iberahim, Trusch and van West 2018). Although pathogenic viruses, bacteria, fungi and protozoan and metazoan parasites are routinely co-isolated from EUS-affected fishes, there is no evidence that these other pathogens causally contribute to the disease (John and George 2012; Kamilya, Dibyendu and Baruah 2014). Nor are we aware of any more recent evidence that *Pfiesteria piscicida* or *Pseudopfiesteria shumwayae* play any role in the pathogenesis of this infectious disease, as claimed in the past. Additionally, an *A. invadans* strain (NJM9701) was recently sequenced

(Makkonen et al. 2016; Russ Diéguez-Uribeondo, and van West 2019), and genomic analysis has identified virulence factors called effector proteins that function to circumvent finfish host defenses (Iberahim, Trusch and van West 2018). These studies provide additional strong support to the idea that some strains of *A. invadans* are primary pathogens. However, under optimal environmental conditions, the skin defenses are usually sufficient to prevent infection. Immune suppression associated with poor environmental quality may, however, augment the potential for this pathogen to infect fishes and cause disease (Iberahim, Trusch and van West 2018; Kamilya, Dibyendu and Baruah 2014; Kiryu et al. 2005). Although superficial skin damage may enhance infectivity of *A. invadans* (Kiryu et al. 2002), it is not a necessary criterion for infection. Given the low *Pfiesteria* cell densities found in nature, and the accompanying low probability of encountering fish, it is highly unlikely they ever inflict sufficient epidermal damage to facilitate an *A. invadans* infection. In conclusion, all available evidence indicates that *Pfiesteria* can be definitively ruled out as a cause of ulcerative lesions in wild fish.

Fish Bioassays and Micropredation

In the early days the reported biological and chemical behavior of *Pfiesteria* was based on observations from a "Gold Standard" fish bioassay developed specifically to test *Pfiesteria* toxicity (Burkholder and Glasgow 2002; Burkholder, Glasgow, and Deamer-Melia 2001; Burkholder, Glasgow and Steidinger 1995; Burkholder et al. 2001b; Glasgow et al. 2001b). In this system, juvenile tilapia were exposed in aquaria (9 - 38 liters) to *Pfiesteria* from sediment samples, water samples or laboratory cultures. Aquaria were maintained for weeks to months until fish mortalities occurred. As fish died, they were removed and replaced with live fish without changing the water. During the incubation, *Pfiesteria* and bacterial populations increased to densities that greatly exceeded those observed in the field. Because of the complex community of microorganisms and degradation of water quality that developed over time, concern was raised regarding "Gold Standard Assay" validity. Because there was no standard timeframe for assays, results were not reproducible and clearly assigning causality to fish mortality was not possible (Drgon et al. 2005; Lovko et al. 2003; Vogelbein et al. 2002). Microbial contaminants in these systems included amoebae, bacteria, chrysophytes, ciliates, diatoms, rotifers and other protists (Burkholder and Glasgow 1997a, 1997b; Burkholder, Glasgow, and Deamer-Melia 2001; Burkholder, Glasgow and Steidinger 1995; Burkholder et al. 2001b; Drgon et al. 2005; Peglar et al. 2004; Vogelbein et al. 2001). Replicating the standard aquarium-format bioassay Drgon et al. (2005) found bacteria, including pathogenic *Vibrio* spp., and *Aeromonas* spp., among the vast assemblage of microorganisms that developed. The ability of bacteria commonly associated with harmful algal species to produce toxins, or contribute to their production, had already been documented (Doucette 1995).

In response, alternative, small-scale temporally standardized bioassays (96 hr) were developed to address concerns associated with the standard bioassay of Burkholder et al. (2001b). These studies exposed larval or juvenile fishes to *Pfiesteria* in tissue culture plates or small culture flasks (Figure 11). This new format with a shorter assay duration, eliminated many of the variables that were present in the Burkholder Gold Standard Bioassay. The new assay format allowed viewing the tissue culture plates under the microscope and led to better elucidation of the mechanisms and biological factors playing a role in fish-killing by

Pfiesteria. Concurrent studies exhibited widespread epidermal erosion in *P. shumwayae*-exposed tilapia and reported a direct physical association between *Pfiesteria* dinospores and the eroded surface epithelia of exposed fish (Vogelbein et al. 2001). Histologically, dinospores were found to be attached to damaged skin, oral mucosa, gills, and deep within the lateral line canal and olfactory organs (Figure 11). An alternative mechanism of fish pathogenicity was hypothesized whereby the dinospores attach to and feed on fish surficial epithelial tissues (Vogelbein et al. 2001). In contrast, earlier studies had held that *Pfiesteria* toxins were responsible for adverse effects in fish and invertebrates, rather than that there was direct physical association with, or ingestion of, *Pfiesteria* (Burkholder, Glasgow, and Deamer-Melia 2001; Burkholder et al. 2001a; Burkholder, Mallin and Glasgow 1999; Burkholder, Glasgow and Steidinger 1997; Kane, Oldach and Reimschuessel 1998; Noga 2000; Noga et al. 1996; Samet et al. 2001; Silbergeld et al. 2000). In fact, the original paper describing the effects of *P. piscicida* specifically stated: "the alga has not been observed to attack fish directly" (Burkholder et al. 1992). However, intense chemoattraction to fish and heterotrophic feeding on single-celled algae, protozoans and isolated vertebrate cells such as human red blood cells had been described previously (Burkholder and Glasgow 1997a; Burkholder, Glasgow and Steidinger 1995).

Definitive studies were conducted to assess the relative importance of toxin production versus direct feeding by *P. piscicida* and *P. shumwayae* in fish mortality. Using the 96 hour larval fish bioassay of Lovko et al. (2003), Vogelbein et al. (2002) demonstrated that physical attachment and myzocytotic feeding on fish skin by *P. shumwayae* (Figure 5) resulted in epidermal damage and fish mortality identical to what had previously been reported as a toxin effect (Burkholder, Glasgow, and Deamer-Melia 2001; Noga et al. 1996). Fish mortality (100%) occurred only in treatments where fish and *P. shumwayae* dinospores were in physical contact (Figure 12). No mortalities occurred in treatments where a semi-permeable membrane prevented physical contact. Mortalities (60-100%) occurred only in fractions that contained live *Pfiesteria* dinospores, while no mortalities occurred in cell-free (soluble) or bacteria-enriched fractions or controls. These findings were further supported by exposure studies using cell-free supernatants of fish killing cultures, lysates of whole *Pfiesteria* cells and organic extracts of lyophilized *Pfiesteria* cells, all of which failed to kill larval fish (Berry et al. 2002). The most parsimonious conclusion of the studies was that finfish pathology and mortality were due to direct epidermal damage derived from a feeding process described as "micropredation" in the absence of toxin production (Vogelbein et al. 2001, 2002). This novel mechanism of pathogenicity without toxin was rapidly confirmed in studies with larval and juvenile finfish (*Cyprinodon variegatus*, *Oreochromis* sp.) (Burkholder, Glasgow, and Deamer-Melia 2001; Burkholder et al. 2001a, 2001b; Gordon et al. 2002), bay scallops (*Argopecten irradians*) and eastern oysters (*Crassostrea virginica*) (Springer et al. 2002). However, these investigators continued to attribute mortalities to an unidentified *Pfiesteria* toxin, insisting that micropredation was a trivial contributor to mortality.

Fish death continued to be used to identify a culture as "toxic" (TOX), whereas strains failing to cause fish distress, disease, or death were consistently referred to as non-toxic, or NON-IND, in many instances without consideration of cell densities in the assays (Burkholder, Glasgow, and Deamer-Melia 2001; Burkholder et al. 2001a; Glasgow et al. 2001a, 2001b). Variations in mortality rate were viewed mainly as differences in toxin production among strains, while the critically important role of *Pfiesteria* cell density and its effect on mortality was ignored. This interpretation became problematic because cell numbers

in assay systems were known to change over time. With an adequate food source (such as fish), dinoflagellate populations in culture can increase over a relatively short time period (24-48 hours) (Gordon and Dyer 2005; Shumway, Burkholder and Springer 2006). Higher cell densities in these assays resulted in increased feeding, greater tissue damage and shorter times to fish death. Lovko et al. (2003) demonstrated a clear dose-response in larval fish exposed to a range of *P. shumwayae* dinospore concentrations (Figure 12). The relationship between fish mortality and cell density was subsequently confirmed for *P. piscicida* using a fish tank bioassay system (Drgon et al. 2005). Ichthyocidal activity of *P. piscicida* was ascribed mostly to micropredation, rather than to a soluble toxin.

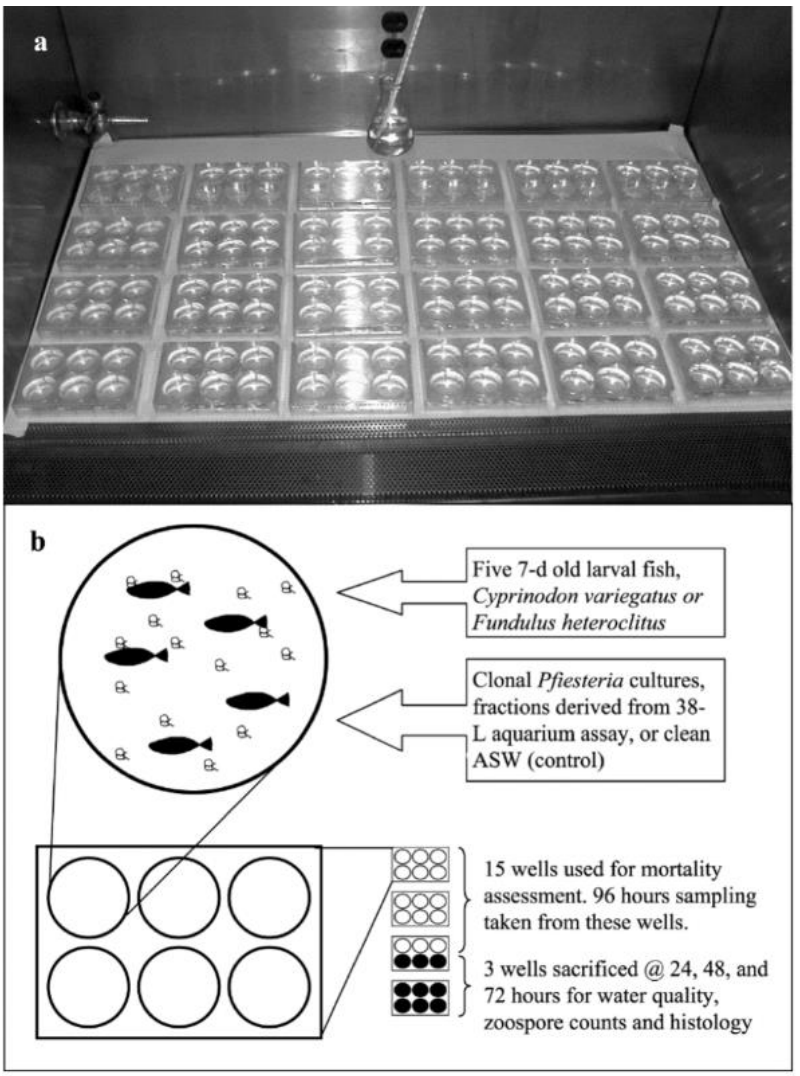

Figure 11. Experimental design and laboratory setup of the 96 hr larval fish bioassays. Figure reproduced by permission of The Phycological Society of America, from Lovko, Vincent J., Wolfgang K. Vogelbein, Jeffrey D. Shields, Leonard W. Haas, and Kimberly S. Reece. 2003. "A New Larval Fish Bioassay for Testing the Pathogenicity of *Pfiesteria* spp. (Dinophyceae)." *Journal of Phycology* 39(3):600–609. https://doi.org/10.1046/j.1529-8817.2003.02106.x.

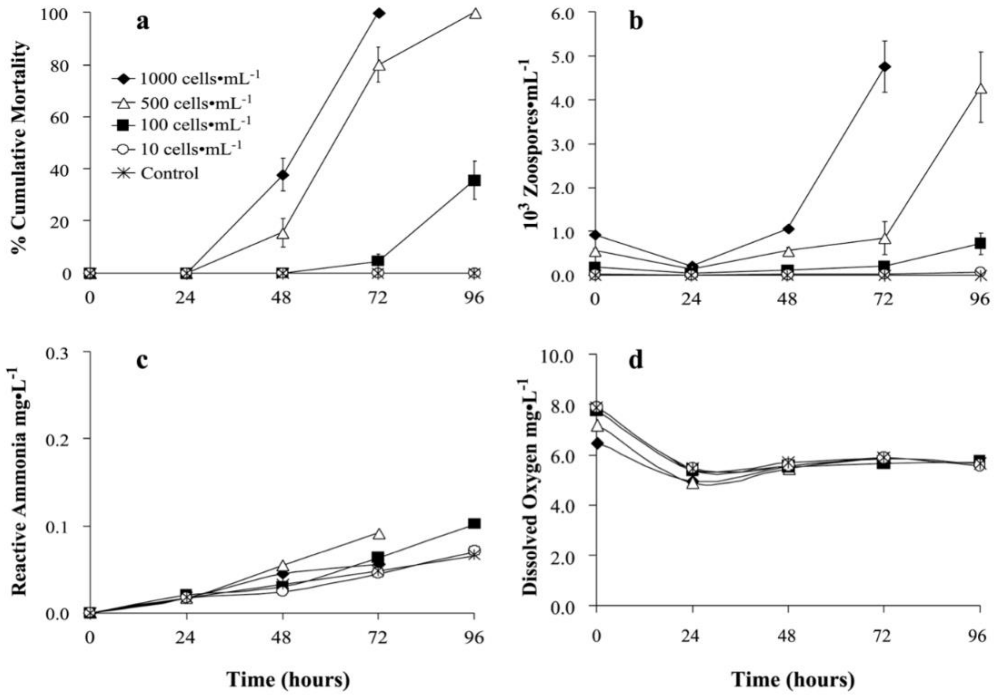

Figure 12. Dose-response curves for larval finfish exposed to *P. shumwayae*: (a) cumulative mortality over 96 hr with initial dinospore densities of 0, 10, 100, 500, and 1000 cells•mL^{-1}. Bars represent standard errors (n = 45), (b) change in dinospore density in each treatment over 96 hr (n = 3), (c) mean reactive ammonia from each treatment (n = 3), and (d) mean dissolved oxygen from each treatment (n = 3). Reproduced by permission of The Phycological Society of America, from Lovko, Vincent J., Wolfgang K. Vogelbein, Jeffrey D. Shields, Leonard W. Haas, and Kimberly S. Reece. 2003. "A New Larval Fish Bioassay for Testing the Pathogenicity of *Pfiesteria* Spp. (Dinophyceae)." *Journal of Phycology* 39(3):600–609. https://doi.org/10.1046/j.1529-8817.2003.02106.x.

Cages with varying mesh sizes also were used to demonstrate that fish mortality was directly proportional to how well the mesh allowed the passage of intact *Pfiesteria* cells into the cage. Only 3%-10% total mortality was observed in tanks with mesh sizes that excluded *Pfiesteria* cells, suggesting the presence of a soluble factor making a minor contribution to overall fish mortality. However, the investigators could not attribute these mortalities to *Pfiesteria* directly, as the tanks contained a rich microbial flora, including bacteria, fungi, and protists, some of which are known fish pathogens and may also produce soluble toxic factors (Drgon et al. 2005). Subsequent studies applying the methods of Lovko et al. (2003) and Vogelbein et al. (2002), have confirmed micropredation as the primary mechanism of killing by *P. shumwayae* in both finfish and shellfish (Gordon and Dyer 2005; Shumway, Burkholder and Springer 2006). We are aware of no more recent investigations that have revisited this aspect of *Pfiesteria* biology, and most of the HAB community stands at consensus that the mechanism of finfish "pathogenicity" in these two organisms is through myzocytotic feeding on the surficial epithelial tissues. To date, a *Pfiesteria* toxin has not been fully characterized and a completed structure is not available. It is well-established that these dinoflagellates are heterotrophs (animal cells) that feed on autotrophic unicellular algae using a specialized feeding structure called a peduncle, rather than obtaining their energy via photosynthesis.

We consider the mortalities that have been described in laboratory exposures to be an artifact of holding the finfish in a captive environment in which dinoflagellate cell densities can rapidly rise to concentrations that are lethal, and from which fish cannot escape. This suggests that these organisms have the ability to become a problem in closed recirculating finfish culture operations. The best documented case comes from two fish farms in Denmark, which use closed recirculating systems, and where both *P. shumwayae* and *L. masanensis* were recently documented to kill fish (Moestrup et al. 2014). Previously *Luciella* spp. had not been linked to any fish kills. In both situations, the fish could not escape and therefore, provided a nutrient rich, high density food source upon which the PLDs could graze via micropredation (see Fish Kills section). *Cryptoperidiniopsis brodyi*, has also been implicated in causing fish kills in closed recirculating systems (Noga, personal communication). Thus, in these limited circumstances, PLDs can represent a threat to certain aquaculture operations. It is unlikely however, that the proper conditions would ever occur in natural systems where finfish can simply swim away and at the low PLD densities typically observed in nature.

Human Health

Filter-feeding shellfish and finfish can accumulate algal toxins in their tissues and thereby are often vectors for transfer of these toxins to humans through consumption (Van Dolah 2000; Friedman and Levin 2005). Human exposure can also occur through direct contact or aerosol inhalation, as in the case in human brevetoxicosis at beaches, or through drinking water, which can become contaminated with cyanotoxins, for example. Many of the algal toxins are neurotoxins that cause a range of primarily gastrointestinal and neurological symptoms including nausea, vomiting, diarrhea, memory and learning deficits, abnormal sensations in the extremities (paresthesia), headaches, dizziness, fatigue, mood disorders, seizures and hallucinations (Baden, Fleming and Bean 1995; Van Dolah 2000). Although *Pfiesteria* has been reported to produce a toxin, the described properties are very different from those of other algal toxins in that it does not bioaccumulate and degrades very rapidly (Jaykus and Green 1998; McClellan-Green, Schmechel and Koltai 2001). The primary route of human exposure is thought to be through aerosols and direct contact with contaminated water.

Human health effects from *Pfiesteria* were reported during the 1990's by laboratory workers and by watermen in North Carolina and Chesapeake Bay. The concept of a human clinical syndrome associated with *Pfiesteria* was first developed based on acute exposure of three laboratory workers to *Pfiesteria* cultures and the bioassay tanks where fish were dying in a single lab that was studying the organism (Glasgow et al. 1995). One of these individuals received additional exposure through the ventilation system that was incorrectly routed from the *Pfiesteria* laboratory into an adjacent office (Schmechel and Koltai 2001). Gastrointestinal difficulties, fatigue, cognitive impairment, as well as mucosal and skin irritation and sores were among the symptoms described by these workers. The individual who had been exposed both in the laboratory and office settings was most severely affected and underwent extensive medical testing two weeks after the onset of symptoms and termination of exposure. Routine blood and urinalysis tests, as well as the neurological tests for gross motor skills were essentially normal. However, a neurophysiological exam revealed memory deficits, and impaired arithmetic, verbal learning and reading skills. Six weeks later, a follow-up screening

revealed that the patient's deficits had been largely resolved (Schmechel and Koltai 2001). One of the other two individuals affected in this laboratory was tested and results were normal; the other individual did not seek medical treatment. To our knowledge, no symptoms have been reported by workers in other laboratories where *Pfiesteria* spp. or other PLDs have been studied.

In addition to the laboratory exposures in the mid-late 1990's, as the press reports surrounding *Pfiesteria* and public awareness of this dinoflagellate increased, there was a surge of reports of symptoms such as confusion, headaches and skin irritations from citizens who had been in or near waters where a fish kill had occurred (CDC 1997; CDC 1999; Moe et al. 2001). Following the Pocomoke River, MD fish kills in 1997 a single-blind clinical study was done on a group of 24 persons who reported direct contact with lesioned fish or waters during an ongoing fish kill (Griffith 1999; Lowitt and Kauffman, 1998; Turf et al. 1999). Nineteen subjects exhibited unexplained significantly decreased performance compared to normal on neuropsychological tests, and five had skin lesions that could not be attributed to a specific cause. The researchers believed that the evidence supported the involvement of *Pfiesteria* in the observed symptoms, although a definitive cause and effect relationship with a *Pfiesteria* toxin specifically could not be found (Grattan et al. 1998). Several years later, Friedman and Levin (2005) criticized the Grattan et al. (1998) study for being limited by small sample sizes and a lack of clear evidence that *Pfiesteria* spp. cells were present in waters to which the subjects were exposed. In addition, Friedman and Levin suggested that the "impairment" observed in the neuropsychological exams could have been due to chance. Moe et al. (2001) pointed out other shortcomings of the Gratten study, including the small number of subjects, the fact that subjects were self-selected, the lack of pre-exposure cognitive testing, and that the medical team was not blinded to the exposure status of the subjects during the evaluation process. As discussed below, several other studies were unable to find an association between human health and occupational exposures to estuaries in which fish kills and lesion events attributed to *Pfiesteria* spp. had occurred (Griffith 1999; Griffith et al. 1998; Smith and Music 1998; Turf et al. 1999; Morris 2001).

In an effort to better understand the association, if any, between *Pfiesteria* and human health, in 1998, the US Congress directed CDC to fund a multi-state surveillance study in coastal North Carolina, Virginia and Maryland to investigate "possible estuary associated syndrome (PEAS)". Citizens frequenting the waterways where fish kills occurred and lesioned fish had been found had been reporting a variety of gastrointestinal, and primarily dermal, respiratory and neurological symptoms, and they thought that their symptoms were due to *Pfiesteria* exposure. It was not clear, however, whether the sensationalized media coverage was leading to the reporting of symptoms from citizens or there really were human health effects associated with exposure to impacted waterways. The reports primarily came from those with occupational exposure to the Chesapeake Bay or North Carolina coastal waters. The symptom and exposure criteria for PEAS were: 1) exposure to estuarine water with a fish kill or fish with lesions consistent with *Pfiesteria* spp., 2) symptoms of memory loss and confusion or three or more of the following symptoms: skin rash/sensation of burning skin at the site of water contact, headache, eye irritation, upper respiratory irritation, muscle cramps and gastrointestinal symptoms, any of which persist for more than 2 weeks, 3) symptoms developing within two weeks of exposure to estuarine water and persisting for two weeks or longer, and 4) inability of a health care provider to identify another cause for the symptoms (CDC 1997; CDC 1999). The results from the Virginia cohort study found no

indication of PEAS (Turf et al. 1999). Similarly, the investigators conducting a four-year CDC-funded study in Maryland found no evidence of a correlation between exposure to waters containing *Pfiesteria* spp. and PEAS symptoms (Morris et al. 2006).

There were other human health studies conducted, but results were equivocal and failed to provide clear evidence of a link between human health effects and exposure to *Pfiesteria*. Visual contrast sensitivity (VCS) testing was done on a group of 82 people reporting symptoms similar to PEAS symptoms after occupational (N=5), recreational or residential (N=77 for both) exposure to waters where fish kill and lesion events attributed to *Pfiesteria* had occurred in Maryland (Shoemaker 2001; Shoemaker and Hudnell 2001). VCS is a measure of visual pattern detection ability and is used as a proxy for neuropsychological testing. The investigators reported significantly reduced VCS in the exposed group compared to a control group of 87 individuals; 34 with exposure to marine or estuarine waters and 53 with no exposure to these waters. Another group of investigators studied 22 individuals exhibiting PEAS symptoms after exposure to waters where *Pfiesteria* had presumably caused fish kills and lesions (Hudnell 2005; Hudnell et al. 2001). Their study included 20 control individuals who had not been exposed to marine or estuarine waters. As with Shoemaker and Hudnell (2001), using the VCS test they also found significant deficits in the exposed group. The results of these VCS tests for *Pfiesteria* exposure, however, came under considerable criticism (Friedman and Levin 2005; Morris 2001; Swinker 2003a, 2003b; Swinker et al. 2001a, 2001b, 2002). The VCS test was viewed as inferior to a comprehensive neuropsychological evaluation and diagnostically non-specific for any particular disease or toxicant exposure. Abnormal VCS test results might be obtained due to chronic sunlight exposure, use of anti-convulsant medications or alcohol and occupational exposure to solvents, petrochemical fuels, heavy metals or combustion products. Critics of the studies noted that those who worked as fishers may have been exposed to these and other toxicants and would have had chronic exposure to sunlight.

Swinker et al. (2001a, 2001b) conducted two studies on individuals who had been exposed to waters where fish kills had presumably been caused by *Pfiesteria* had occurred or where lesioned fish or *Pfiesteria* cells were present. One study included only those with occupational exposure (22 cases and 21 controls) and the other was composed of cases with individuals who called a *Pfiesteria* hotline following occupational and recreational exposure (11 cases and 11 controls). They did not find an increased likelihood of PEAS symptoms in cases in either study, nor did they find evidence of persistent physical health or neuropsychological effects in exposed individuals.

Some patients exposed to *Pfiesteria* also reported development of a variety of skin lesions (Lowitt and Kauffman 1998; Shoemaker 1998). These reports and the conclusions that they were caused by *Pfiesteria* exposure, however, were based on a correlation between people having exposure to the waters where *Pfiesteria* was reportedly present, and the people reporting skin lesions of various types. The studies lacked any means of clearly demonstrating exposure and, therefore, were only correlative. Subsequent studies using *Pfiesteria* extracts from a high concentration of *Pfiesteria* cells induced mild, localized irritation in mice, but not dermal sensitization, and had no effect on humans (Burke and Tester 2002; Patterson, Noga and Germolec 2007). The original human skin irritations attributable to *Pfiesteria* were consequently thought to be due to “chronic irritation and trauma associated with fishing and crabbing work, estuarine microbes, allergens such as ragweed or pollen, intense sun and water

exposures, and preexisting medical and neuropsychologic conditions" (Patterson, Noga and Germolec 2007).

IDENTIFICATION AND CHARACTERIZATION OF *PFIESTERIA* SPP. TOXINS

Perhaps the most troubling aspect of the *Pfiesteria* controversy has been the long-standing inability to obtain the structure of a purported toxin. Given the description of an alternate mechanism of pathogenicity (myzocytosis) in the absence of toxin and the obligate requirement of direct physical contact to cause mortality (Vogelbein et al. 2002), the existence of a *Pfiesteria* toxin has been questioned. It was initially claimed that *Pfiesteria* must feed on fish to produce the toxin (Burkholder and Glasgow 1997a); however, later studies suggested bioactivity in algae-grown strains (Moeller et al. 2007). Secondly, the toxic activity was originally described as hydrophobic (lipophilic) and of high molecular weight (>10,000 daltons) (McClellan-Green, Jaykus and Green 1998). As noted by Drgon et al. (2005), the fish bioassay tanks contain numerous organisms, some of which are identified pathogens, which makes attribution of a toxic compound from this complex matrix impossible. A putative phthalate ester was isolated from these cells (Moeller et al. 2001) but turned out to be a plasticizer contaminant originating from the Instant Ocean salts used to make up the culture media used to grow *Pfiesteria*. The *Pfiesteria* toxin was summarized in an article in *Science* to be "…a glycoside, a molecule that's half sugar, half some other chemical group that has not been identified" (Kaiser 2002), and a partial structure for the molecule was announced at the 10th Annual Harmful Algal Bloom Symposium. The final structure of this toxin has never been reported.

Around the same time other groups began investigating the toxicity of *Pfiesteria* using alternative approaches. One group examined organic extracts of lyophilized *P. shumwayae* cells, all of which failed to kill larval fish (Berry et al. 2002). Since most known algal toxins have a polyketide structure, these researchers used molecular primers to demonstrate that *P. shumwayae* most likely lacked the polyketide synthases needed to synthesize this class of toxins (Berry et al. 2002).

In 2007, a very different structural framework for a putative *Pfiesteria* toxin was described to be a ligated copper compound with numerous congeners (Moeller et al. 2007) (Figure 13). It was hypothesized that the "rapid, free-radical-mediated toxicity of *Pfiesteria* toxins may occur via production of a redox-cycling metal center and free radical(s) that can lead to specific reactions with "pro-toxins" which, in turn, can produce more active toxic species." Under this scenario, sunlight and metal exposure are the two primary environmental factors that would combine to initiate *P. piscicida* toxicity during blooms. "Light exposure could initiate redox cycling of the metal ion(s) resulting in radical formation and release of the toxin species." Unfortunately, direct evidence that free radical formation actually takes place in estuarine waters was not provided and given the high organic load in these waters it seems unlikely that the reaction distance would be very great. More importantly, no dose-response data were presented to confirm the concentration required to kill fish. Without these data it is not possible to rigorously establish the environmental relevance of the described activity. Toxicity was inferred primarily from the work presented by Burkholder et al. (2005),

however, there are several inconsistencies in the authors' interpretation of their data. The first issue is the toxin cell quota. *Pfiesteria* spp. have ~ 100 pg total C per cell (Menden-Deuer and Lessard 2000; Miller and Belas 2004). Yet the reported toxin concentrations for several of the TOX *P. shumwayae* cultures was on the order of 43-251 pg cell^{-1} or 0.5-2.5 X the total C of a *Pfiesteria* cell. These toxin cell quotas are not possible because they equate to more than 100% of all C in the cells. This would provide nearly 200 mg of toxin from a 90-liter culture of 10,000 cells per ml. Next, the toxicity of the extracts was reported as time to death for a fish exposed to 50 μL of *Pfiesteria* toxin (Burkholder et al. 2005). It is not at all clear, however, why they reported a volume of toxin added rather than the actual concentration of toxin, which is what is normally reported for a dose response assay when they had quantified the toxin. Also, the activity of the *Pfiesteria* toxin as measured by cytotoxicity to cultured cells was nonexistent in cells grown on algae, but high in cells grown on fish (Figure 3 of Burkholder et al. (2005), yet the chromatogram shown in Figure 4 of the original online version of Burkholder et al. (2005) showed that the NON IND strain CCMP 1832 grown on algae had the same peak as that of the toxic 2089 strain grown on fish. Other researchers, however, were not able to reproduce results reported from the TOX cultures. As mentioned above these purportedly TOX cultures were neither deposited in a public repository nor were other research groups able to obtain subsamples of these cultures (from the NC State Research group) for conducting experiments. Many of the other research groups, nonetheless, used cultures that had been established from Neuse River samples, the same location from which the purported "TOX' culture samples had been collected. In 2007, Moeller et al. reported that they had purified the toxin from cultures grown on algae.

Standard fish bioassays were used to assess the killing ability of *Pfiesteria* which allowed direct contact between fish and *Pfiesteria* making it impossible to determine what portion of the observed fish mortalities were due to micropredation versus the action of the toxin. As discussed above, over 90% of the observed mortality was likely due to micropredation based on observation of other studies such as Vogelbein et al. (2002). Another aspect of the Burkholder et al. (2005) study was the use of plating and DGGE gel techniques to assess the bacterial similarity or differences between the control and experimental treatments where fish mortality was occurring. The goal was to demonstrate that there were no pathogenic bacteria in the cultures of *P. piscicida* or *P. shumwayae* that were killing fish. Unfortunately, the plating techniques used were only capable of supporting the growth of less than 2% of estuarine bacteria. Since 98% or more of the bacteria would not show up using this assay; any similarities or differences noted between treatments were therefore meaningless. The premise behind the DGGE experiment is that the absence of a unique randomly amplified 16S band in the killing cultures would indicate bacteria are not involved in the production of the toxin. Unfortunately, DGGE tends to pick up only the most abundant bacteria. The lack of a uniform band cannot therefore be used to exclude bacteria as a potential source of the toxin as implied in the paper. This leaves the potential bacterial source of the toxin in limbo. Although, *Pfiesteria* species can be grown axenically on fish cells, it is critical to demonstrate that the toxin can be isolated from these cultures. The study also showed cytotoxicity data from two cultures of *P. shumwayae*. One culture showed no activity at all and the other marginal activity likely within the noise level of the assay (Figure 3 of Burkholder et al. 2005). No 16S ribosomal DGGE data are presented to confirm these cultures are actually axenic despite the fact that the techniques were used to assay bacterial abundance in other

samples. It is on the basis of these data that it was concluded that bacteria-free *Pfiesteria* cultures produced toxin, thereby eliminating bacteria as the source of the toxin.

Another inference in the paper included data showing that *Pfiesteria* cell densities at a fish kill can be high (>1000 cell mL^{-1}). The samples analyzed in Burkholder et al. 2005 were collected in 1998 and 2000 and analyzed years later. These samples stored in Lugols will have gradually deteriorated over time implying that the cell concentrations were even higher than reported. Interestingly these are the only qPCR data published from fish kill sites and are several orders of magnitude higher than previously reported by other investigators. Moreover, the 2000 sample obtained from a fish kill recorded in Arnell Creek, Delaware, had already been shown to contain large numbers of *K. veneficum* by qPCR (Humphries 2002).

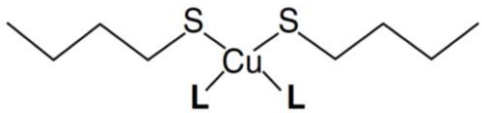

Figure 13. Putative *Pfiesteria* toxin molecular framework. The approximate mass of this framework is 311 amu with a range up to greater than 700 amu. The L stands for ligands of diverse nature.

These data indicate that much research remains to be done in order to confirm the structure and source of "*Pfiesteria*" toxin, despite the fact that identical ^{13}C NMR spectra were shown from standard fish bioassay tanks and in the toxin description paper (Burkholder et al. 2005; Moeller et al. 2007). In 2007, Moeller et al. reported that they had purified the toxin from cultures grown on algae. Clearly a bioactive compound can be extracted from these cultures given that some unknown concentration was able to cause epithelial ulcerations (Figure 5 of Burkholder et al. 2005). The most important remaining question, however, is whether this bioactive compound is environmentally relevant and unique to *Pfiesteria*. The answer is likely to be no for the following reasons: 1) cell densities in nature are generally low (<10 cells mL^{-1}) (Lin, Zhang and Dubois 2006) meaning that there are too few cells generally present to cause a problem even if the toxin proved highly potent, 2) the histology of lesioned fish from fish kill sites does not mimic that seen in the laboratory and is inconsistent with exposure to significant amounts of *Pfiesteria* toxin in the environment, 3) the toxin is very unstable which severely limits the period of effective exposure and 4) extensive epidemiological studies have found no evidence of adverse human health effects associated with this organism. Not until quantitative dose vs. mortality curves are established for the *Pfiesteria* toxin and the toxin is found to be present in the environment at quantities indicated by the dose mortality curve to be of concern, can a *Pfiesteria* toxin be considered of consequence ecologically or toxicologically. *Karlodinium veneficum* fish kills, occurring in the same locations as the "*Pfiesteria* kill sites", incidentally, have met all of the above criteria. Until a fish kill meeting the criteria can be found in which *K. veneficum* is not observed and only *P. piscicida* or *P. shumwayae* are found, can the "cell from hell" be implicated.

CONCLUSION

The emergence of the *Pfiesteria* issue in the late 1990's was highly controversial and had a major impact on research funding for harmful algal bloom issues. It spurred the rapid

development and adoption of molecular techniques for detection, quantitation and monitoring of HAB species. *Pfiesteria* spp., PLDs and many other HABs are now readily identified and quantified in natural waters and sediment samples. This review of the available *Pfiesteria* literature to date forms the basis of the following conclusions regarding this controversial scientific issue.

1. *Pfiesteria piscicida, Pseudopfiesteria shumwayae* and related PLD's are not photosynthetic, but rather heterotrophic dinoflagellates (animal cells) that obtain C by the process of myzocytotic feeding on prey using a unique feeding structure called a peduncle.
2. Field investigations to date are at consensus and reaffirm the typically low abundance (<10 cells mL^{-1}) of *Pfiesteria spp.* and other PLD's in natural estuarine systems.
3. The proximal causes of the mass finfish mortalities in Mid-Atlantic US estuaries during the late 1990's are unknown; however, their attribution to *Pfiesteria* spp. is not supported by the body of literature. More parsimonious hypotheses have been put forward including (a) hypoxia/anoxia events and/or (b) concurrent blooms of *K. veneficum,* a known producer of potent ichthyotoxins having been misidentified as *Pfiesteria*.
4. Attribution of ulcerous lesions in menhaden from Mid-Atlantic US estuaries to *Pfiesteria* toxin(s) is now contradicted by a large body of literature. Ulcers in menhaden are chronic (weeks old) lesions now understood to be caused by a highly pathogenic oomycete called *Aphanomyces invadans*. This pathogen is known to impact over 100 species of finfish and impacts wild and cultured fisheries worldwide.
5. The complex 35-stage life history initially ascribed to *Pfiesteria* is incorrect. More recent studies have shown these organisms to have simple life histories typical of other dinoflagellates. Amoebae and chrysophyte-like cysts identified as stages in the *Pfiesteria* life cycle are now known to be free-living microbial contaminants of the culture systems or the fish used in the original assay protocols.
6. The primary mechanism of *Pfiesteria* pathogenicity in finfishes is not secretion of potent exotoxins, but rather, the micropredatory feeding strategy employed by these organisms. If bioactive molecules are ultimately found to be produced by *Pfiesteria* (as suggested in some studies), the body of literature indicates that it will likely be a minor component of finfish pathogenicity. In a captive environment like a bioassay vessel where the fish cannot escape, these dinoflagellates quickly attach to the epidermis and begin to feed, with resultant mortality being highly dose-dependent. It is unlikely that this occurs in the natural estuarine environment; however, we predict that impacts of these heterotrophic dinoflagellates will become more common under intensive recirculating finfish culture operations in the future.
7. The evidence for existence of a "*Pfiesteria*" toxin is weak. To date, the complete chemical structure of a *Pfiesteria* toxin has not been solved. Even if a toxin is present, it has not been isolated in pure form or demonstrated to produce the reported toxic effects. In addition, the low cell levels observed in nature suggest any negative impact on aquatic animals or humans from bioactive compounds produced by *Pfiesteria* would be negligible.

8. Human health impacts, although reported in the early literature, to date, have not been verified.
9. Given the well-established pathogenicity of micropredatory feeding, it is unlikely that a *Pfiesteria* toxin, should one be characterized, will be of significant consequence in causing fish mortality.

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Chapter 145

KARLODINIUM VENEFICUM: STILL BLOOMING AND TOXIC SIXTY-TWO YEARS LATER

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ABSTRACT

For decades, high densities of the dinoflagellate *Karlodinium veneficum* have been associated with aquatic faunal mortalities worldwide. This small (<8–12 μm) athecate phytoplankton, common in coastal aquatic ecosystems, has a mixed nutritional mode, relying on both photosynthesis and phagotrophy for growth (mixotrophy). It is frequently present in relatively low cell abundance (10^2–10^3 cells mL^{-1}), but is capable of forming intense blooms of 10^4–10^5 cells mL^{-1} that are often associated with fish kills. A suite of toxic compounds (karlotoxins) have been characterized, both in the laboratory and in the field, with hemolytic, ichthyotoxic, and cytotoxic properties. These toxins have been shown to generate pores in membranes with desmethyl sterols and increase the ionic permeability resulting in membrane depolarization, disruption of motor functions, osmotic cell swelling and lysis. The biological *raison d'etre* for karlotoxin production appears to be grazing deterrence and prey capture, both of which stem from the impairing effects of karlotoxin on susceptible organisms. Strain variation in types of karlotoxins and toxin cell quotas is extensive. Since the initial description of *K. veneficum* (FKA *Gymnodinium veneficum)* in 1956 by Dorothy Ballentine, toxic and nontoxic strains have been observed. Despite numerous name changes it is now clearly recognized as a cosmopolitan species with extensive ecosystem impacts. Current knowledge on the physiology, ecology, and life history of *K. veneficum* is summarized.

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Keywords: blooms, fish kills, grazer-deterrent, karlotoxin, life cycle, mixotrophy, parasitism

INTRODUCTION

In the classic "Nature Adrift - The Story of Marine Plankton" by James Fraser [1] it is written,

> *"A poisonous dinoflagellate lives in British waters: It is Gymnodinium veneficum, which has been grown in culture at the Marine Biological Station at Plymouth"* (Figure 1).

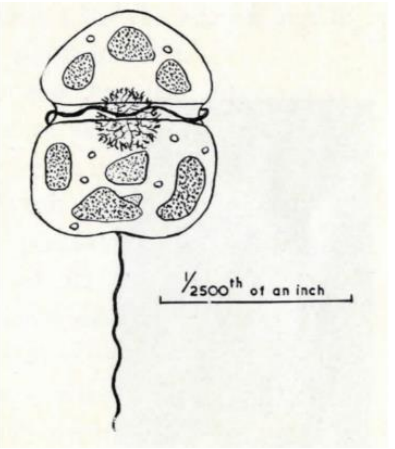


Figure 1. *Gymnodinium veneficium*, a dinoflagellate that produces a poison.

This statement is based on the work of B. C. Abbott and Dorothy Ballantine [2] who characterized the toxin activity of *G. veneficum*, to paraphrase their findings,

> *"…The toxin molecule must be large, as it cannot penetrate a dialysis membrane; it is soluble in water and the lower alcohols, but insoluble in ether and chloroform. It is unstable in acids, … though in neutral solution is more or less thermostable. …With regard to mode of action it depolarizes nerve and muscle membranes. …This depolarization probably occurs by interference with the sodium exchange mechanism, allowing rapid entry of sodium into the cells."*

The discovery of this remarkable toxin began with the isolation of a small dinoflagellate from the region near Plymouth Sound, a bay on the English Channel at Plymouth in England (lat. N. 50° 19'30," long. W 04°10'), on June 8th, 1949 by Dr. Mary Parke (Figure 2, Left) of the Plymouth Laboratory of the Marine Biological Association. The following year on June 28th she isolated a second very similar dinoflagellate from the Hamoaze, over Rubble Bank, off King William Point, South Yard, Devonport (lat. N. 50° 21'50," long. W 04°10'55"). Both were deposited in the Type Culture Collection (Plymouth collection #102 and #103, respectively). Subsequently, Dorothy Ballantine (Figure 2, Right) described and named the two species, *Gymnodinium veneficum* and *Gymnodinium vitiligo.* Ballantine stated that neither species was shown to eat. The greatest difference between the two species was

physiological, as *G. vitiligo* was harmless and a good food for oysters, whereas *G. veneficum* produced a very powerful toxin which was lethal to fish and nearly every other organism tested including mice and a 3ft shark [2]. In 1957, B.C. Abbot and D. Ballantine described the partial purification and characterization of the toxin from *G. veneficum* [2].

Figure 2. Dr. Mary Parke (Left) and Dorothy Ballantine (Right).

Characterization of *Gymnodinium (Karlodinium*) *veneficum* PLY103

G. veneficum remained in culture at the Plymouth Laboratory until 2015 (See Figure 3 for photomicrograph taken in 2006). Fortunately, an opportunity to characterize the strain, examine its mixotrophic capabilities, and purify the toxin from PLY103 arose before it was lost. PLY103 was found to possess the ventral pore and apical groove diagnostic for *K. veneficum* (e.g., Figure 4C for CSC1). The internal transcribed spacer region (ITS) sequence of PLY103 was identical to that of CCMP 415 & 416 (Figure 4B) but differed by a 2-base insertion from all North American isolates (Figure 4A).

Figure 3. PLY103 taken in 2006 by J. Adolf. Scale bar = 10 μm.

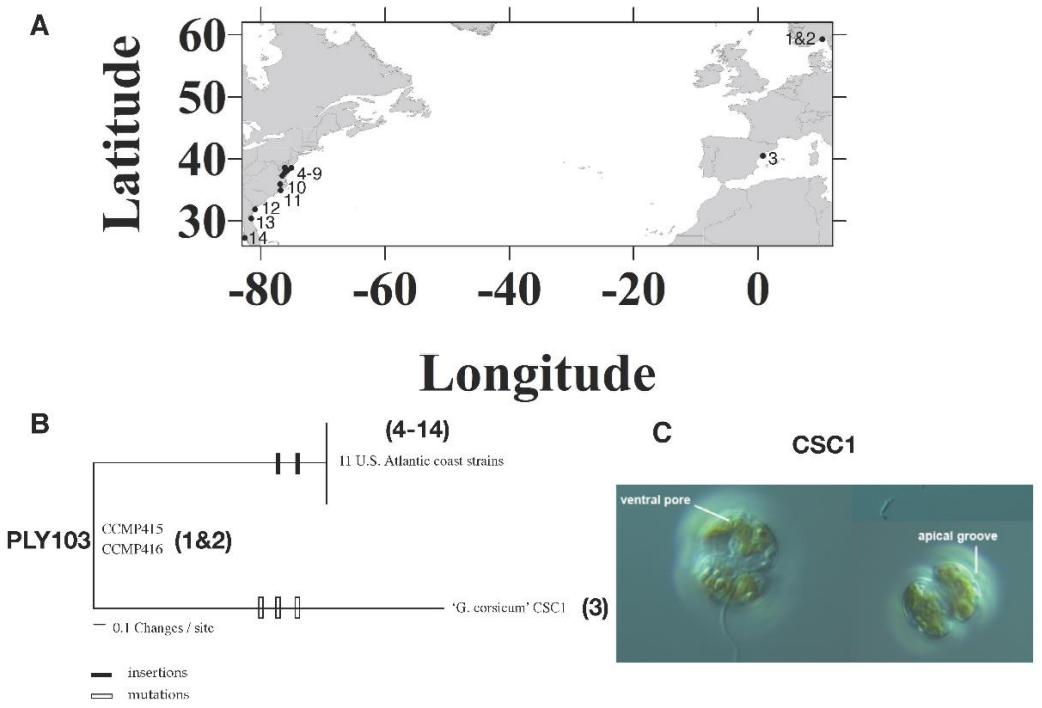


Figure 4. Location of origin for *Karlodinium veneficum* strains analyzed. (A) CCMP 415 [1]; CCMP 416 [2]; CSC1 [3]; CCMP 1975 [4]; MD5 [5]; WC156 [6]; CCMP 1974 [7]; VBL001 [8]; VBL007 [9]; Slocum [10]; MBM1 [11]; PD-6 [12]; F4 [13] and CCMP 2778 [14]. (B) A parsimony tree based on ITS sequences from 14 *K. veneficum* strains examined. Note that there are no sequence differences between the 11 strains from the U.S. Atlantic coast. (C) Photomicrography of CSC1 showing the ventral pore and apical groove found in all strains.

Pigmentation

Ballantine [3] wrote:

> *"The absorption spectra of total pigments, obtained from methanol extracts in a Unicam spectrophotometer are identical. Both show peaks at 444 and 673 mμ, with suggested peaks of pigments other than chlorophyll a at 425-430, 460-470 and 620-639 mμ."*[1]

As seen in Figure 5 and Table 1, the pigments from PLY103 and two additional European strains CCMP 415 & 416 are identical with the previous 14 strains of *K. veneficum* [4]. Each strain exhibited a complete absence of peridinin with the presence of fucoxanthin, 19' hexanoyloxyfucoxanthin, 19'' butanoyloxyfucoxanthin, and gyroxanthin diester pigments [5], similar to its sister genera *Karenia* and *Takayama*. Different from *Karenia* were the nearly equivalent quantities of the two gyroxanthin diesters in all North American strains of *K. veneficum* but not seen in PLY103. The UV spectra were identical for both gyroxanthin diesters peaks, and the LC–MS analysis using APCI ionization found an identical mass

[1] Note. The correct unit used today for *mμ* is *nm*.

(m/z 866) for both peaks. These two peaks were therefore attributed to the 9-*cis* and all *trans* gyroxanthin diesters isolated from *K. veneficum* (*Gymnodinium galatheanum*) and characterized by Bjørnland et al. [6].

When we performed a factor analysis of the pigment profiles, a clear separation between the North American strains and European strains was evident (Figure 5 A&B). Factor 1 accounted for 30% of the variance in the pigment ratios data and was loaded with 19- hexanoyloxyfucoxanthin, 19- butanoyloxyfucoxanthin, gyroxanthin diester, chlorophyll c2, and fucoxanthin (Figure 5A). Factor 2 accounted for an additional 30% of the variance in the pigment ratios data set and was loaded with: chlorophyll c3, carotene, and *cis*-gyroxanthin diester (Figure 4A).

The biplot of factor 1 versus factor 2 scores (Figure 5B) shows a tight cluster along the factor 1 axes and contains all *K. veneficum* strains except 415 and CSC1, indicating that *K. veneficum* strains from the eastern seaboard of the U.S.show similarities among their fucoxanthin:chl a ratios and their *cis*-gyroxanthin diester:chl a values.

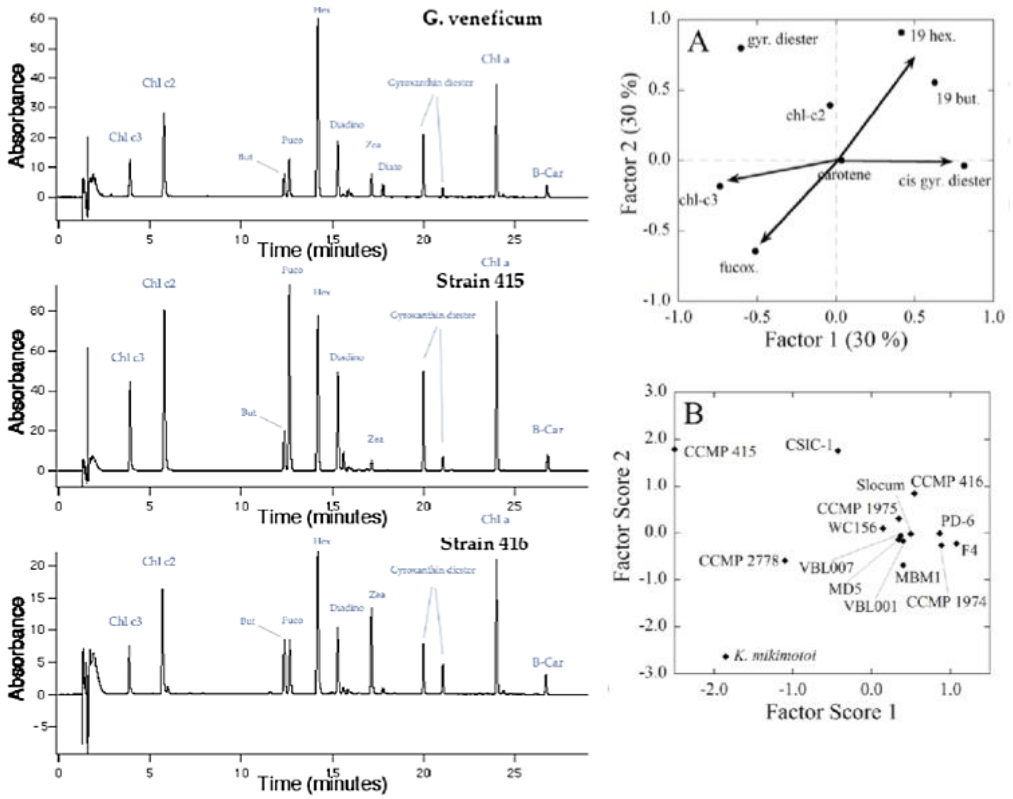


Figure 5. HPLC pigment analysis of PLY103, CCMP 415 and CCMP 426. Pigment designations are: Chl c3 (chlorophyll c3), Chl c2 (chlorophyll c2), Chl c1 (chlorophyll c1), Per (peridinin), But (19'-butanoyloxyfucoxanthin), Fuco (fucoxanthin), Hex (19'-hexanoyloxyfucoxanthin), Diadino (diadinoxanthin), Zea (zeaxanthin), Chl a (chlorophyll a), β-Car (β-carotene). Factor analysis of accessory pigment: Chl a ratios for strains of *K. veneficum* and an outgroup, *K. mikimotoi*. (A) Factor loadings, indicating two axes of variability within the pigment ratios data set. Points grouped along either the x- or y-axis are positively correlated to each other, while points opposite each other along either axis are negatively correlated. (B) Factor scores biplot. Clustered points are similar with regard to the attributes represented by factors 1 and 2, while separated points differ.

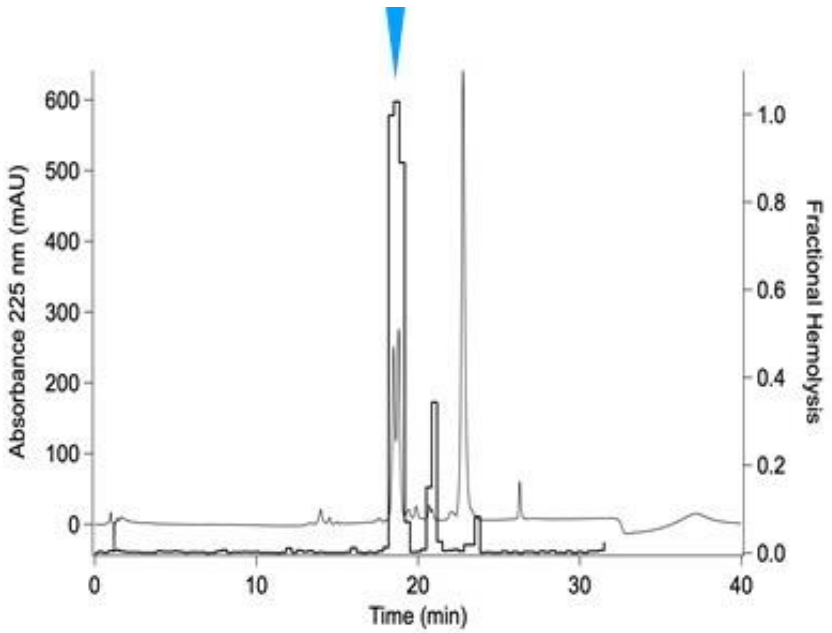
Table 1. *Karlodinium veneficum* pigments

Culture name	Chlorophyll c3 / Chlorophyll *a* (ng/ug)	Chlorophyll c2 / Chlorophyll *a* (ng/ug)	Chlorophyllide *a* / Chlorophyll *a* (ng/ug)	Phaeophorbide *a* / Chlorophyll *a* (ng/ug)	19'Butanoyloxy Fucoxanthin/ Chlorophyll *a* (ng/ug)	Fucoxanthin / Chlorophyll *a* (ng/ug)	Violaxanthin / Chlorophyll a (ng/ug)	19'hexanoyloxy Fucoxanthin / Chlorophyll *a* (ng/ug)
PLY103	63.4	117.2	8.0	3.0	50.9	83.7	0.00	379.6
415	93.2	143.1	2.2	0.0	59.6	278.8	0.00	223.5
416	55.0	104.6	41.5	0.0	94.5	95.3	0.00	246.2
517	95.4	115.9	44.6	1.7	132.7	120.1	0.00	332.1
G. corsicum	44.6	153.2	1.3	0.0	99.3	166.8	0.00	270.4
K. mikimotoi	86.5	118.4	1.1	0.0	10.8	388.7	0.00	12.57
PLY103	77.4	29.3	0.0	79.2	10.8	0.992	7.1	26.0
415	93.7	9.8	0.0	87.7	11.9	0.998	4.5	24.9
416	72.2	86.7	8.6	50.4	28.4	0.959	14.3	33.3
517	89.5	57.2	9.8	90.6	7.4	0.955	6.8	24.2
G. corsicum	113.8	15.7	0.0	69.4	11.0	0.999	6.0	33.8
K. mikimotoi	60.2	5.0	0.0	31.1	2.4	0.999	7.1	35.2

Pigments not detected: chlorophyll c1, perdinin, neoxanthin, prasinoxanthin, anteraxanthin, alloxanthin, myxoxanthophyll, lutein, canthaxanthin, chlorophyll b, divinyl chlorophyll *a*

The Toxin from *Gymnodinium veneficum*: It's a Karlotoxin

Karlotoxins are released by filtration of cells on glass fiber filters, and can be removed from the filtrate by adsorption to C18 resins, and are eluted by 60 to 80% methanol/water solutions [7]. On a C8 reverse phase HPLC separation using a methanol to water gradient, karlotoxins in the methanol fractions are detected through their UV absorption at 225 nm (KmTx 1) and/or 235 nm (KmTx 2) with little UV absorption at 280 nm. Biologically active fractions are detected through hemolysis of red blood cells. In these HPLC runs an additional later-eluting peak is frequently observed when growing cultures in natural seawater. This peak produces multiple MS ions differing by 44 daltons (DA) and is likely to be a plasticizer polymer contaminant which shows hemolytic activity.



	R_1	R_2	R_3	R_4	R_5	R_6	n	m/z
KmTx 2 **(1)**		OH	CH_3	OH	H	Cl	2	1345
44-OH-KmTx 2 **(2)**		OH	CH_3	OH	OH	Cl	2	1361
KmTx 1 **(3)**		OH	CH_3	OH	H	H	4	1338
10- SO_4-KmTx 1 **(4)**		SO_4	CH_3	OH	H	H	4	1419
KmTx 3 **(5)**		OH	CH_3	OH	H	H	3	1324
KvTx 1 **(6)**		OH	H	H	H	H	2	1192
59-*E*-Chloro-KvTx 1 **(7)**		OH	H	H	H	Cl	2	1226

Figure 6. Reverse phase HPLC chromatogram of PLY103 80% methanol fraction assessed by absorbance at 225 nm. The individual fractions collected simultaneously were assayed using a hemolytic assay (bars). The blue arrow to the left indicates the fractions used to solve the structure of the resulting karlotoxins which are shown on the right.

Like Abbot and Ballantine [2], 85 to 90% of toxic activity was found to be released from PLY103 upon filtration and can be purified exactly as previously described for karlotoxins [7]. Hemolytic activity can be used in guided bioassay fractionation and purification (Figure 6). Two structures were elucidated using similar techniques as those previously described [8]. *Karlodinium veneficum* PLY103 produces KmTx 4 and chloro-KmTx 4 karlotoxins which are hemolytic to rainbow trout erythrocytes and found in nearly equivalent cell quotas (0.93 pg/cell vs 1.25 pg/cell). This is a relatively high cellular toxin quota among known *K veneficum* strains [4]. These structures are the smallest congeners elucidated to date and differ significantly from KmTx 2 in the C1-C18 region. KmTx 4 and chloro-KmTx 4 differ from each other only by the absence of a chlorine on the terminal diene of KmTx 4. The structure is five carbons shorter than KmTx 2, all in the polyol region.

Karlotoxin Identification for the Norway Strains

We find the two strains from Norway produce different hemolytic toxins with CCMP 415 toxin having a 225 nm absorption maximum (KmTx 4) and a molecular weight of 1231.7 amu (M+Na) and CCMP 416 toxin having a 235 nm absorption maximum (KmTx 2) and a molecular weight of 1367.8 DA (M+Na) which is identical to the previously described KmTx 2. The toxin from CCMP 415 elutes (Figure 7A) earlier from the C8 column than the karlotoxins detected in the North American strains (i.e., KmTx 2). As with the North American KmTx 2, the toxins from CCMP 416 co-elute as hydroxylated congeners containing mass ions that are 16 DA larger, as well as other less abundant ions (Figure 7B).

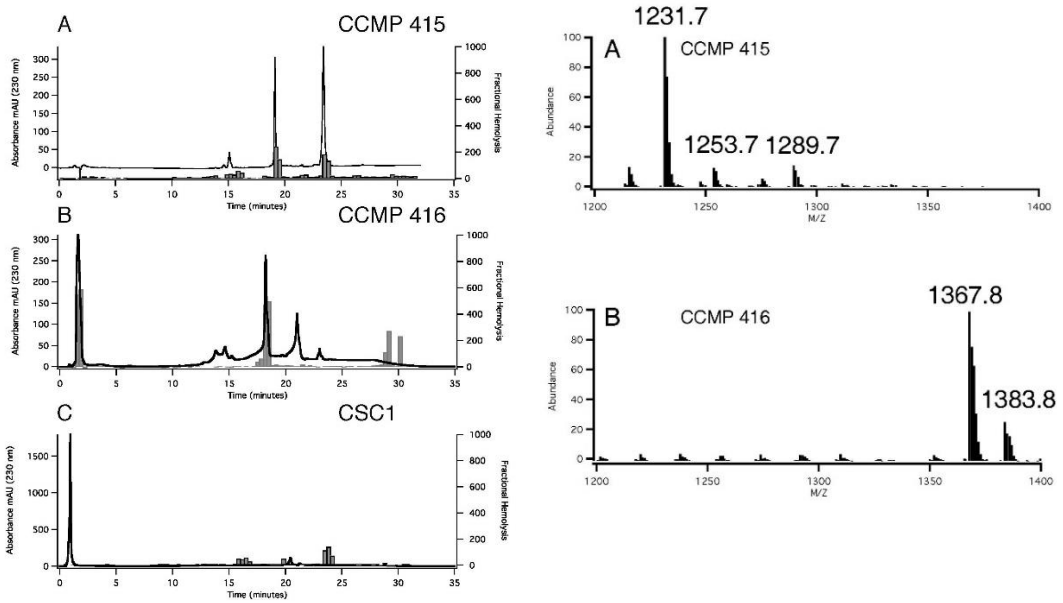


Figure 7. (Left) Reverse Phase HPLC Chromatogram of the hemolytic (bars) fractions from CCMP 415 (A), CCMP 416 (B), and CSC1 (C). CSC1 is nontoxic. (Right) MS spectra of hemolytic fractions from CCMP 415 (A) and CCMP 416 (B). Based on the mass spectra CCMP 415 makes Kmtx 4 while CCMP 416 makes KmTx 2.

Karlotoxin Potency

Using a hemolytic assay of fish red bloods cells the potency of purified toxins from the Norwegian strains were examined using a dose response curve with purified karlotoxins (Figure 8). In all cases the purity of each karlotoxin exceeded 90% based on UV chromatographs. It should be noted that each karlotoxin examined possessed a suite of hydroxylated congeners. Fitting the data from the hemolytic assays to a Hill Equation provides estimates for the karlotoxin concentration giving 50% hemolysis (HD50%). There was nearly a 500-fold difference in hemolytic potency (HD50%) between the most active (KmTx 1) and least active (CCMP 415) karlotoxin. The overall ranking was KmTx 1 > KmTx 3 > KmTx 2, CCMP 416 > CCMP 415, KmTx4 > Saponin (on a per weight basis).

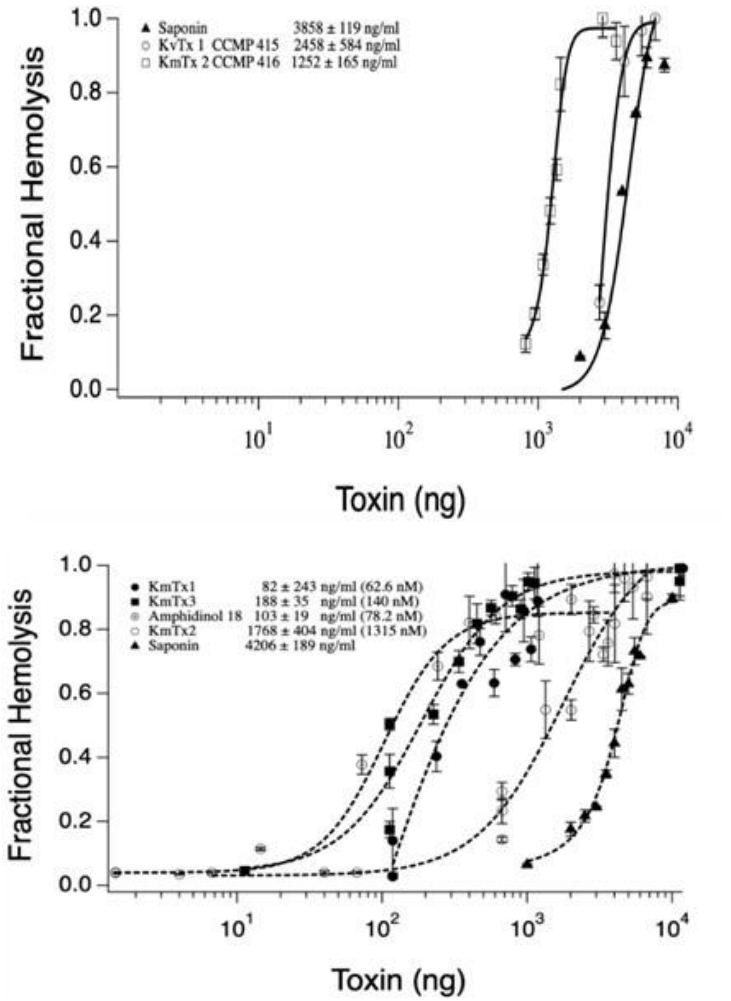


Figure 8. Comparative hemolytic activity for the karlotoxins isolated from the Norwegian strains using rainbow trout erythrocytes compared to saponin (Top). Comparative hemolytic activity for karlotoxins isolated from North American strains compared to saponin and amphidinol (Bottom). Note the logarithmic scale for toxin amount. The fitted line is based on the Hill equation, and the HD50% estimates for the fitted curves are presented in the legend.

Determination of Goby Kill Units

In order to compare different batches of toxin extracts, Abbott and Ballantine [2] defined a toxin unit "*approximate lethal concentration"* necessary to cause death in 10-20 minutes for gobies *(Gobius niger* and *Gobius virescens).* Since these species of goby were not available to us, we used *Gobiosoma bosc* to determine the unit equivalency for KmTx 2. From exposures to *G. bosc* we determined that a kill unit is equivalent to 700 ng and a time to loss of balance is 355 ng for KmTx 2 (Figure 9). Using this conversion, we estimate that Abbott and Ballantine's most toxic extract contained 4.2 µg/ml of KmTx 4 and chloro-KmTx 4.

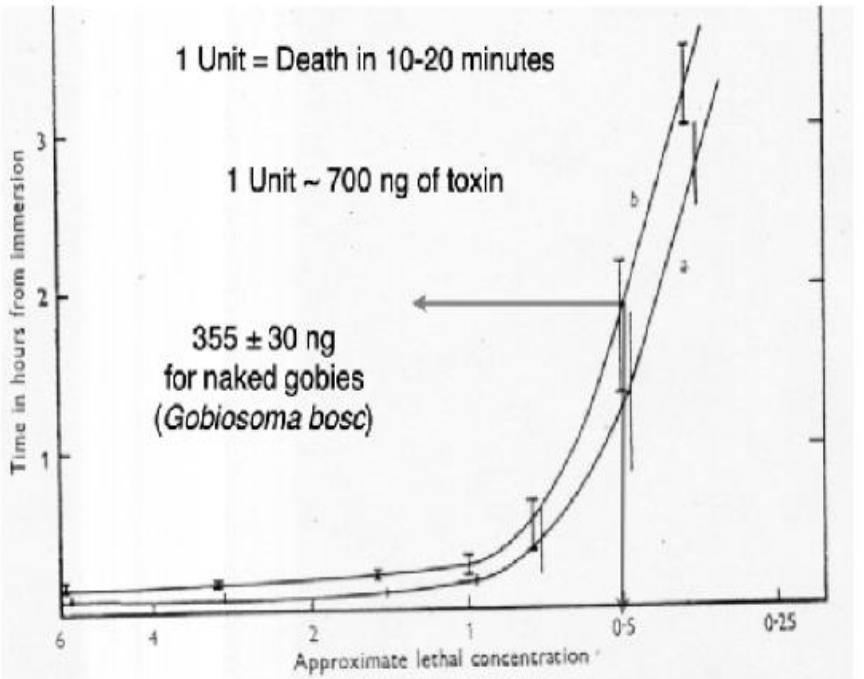
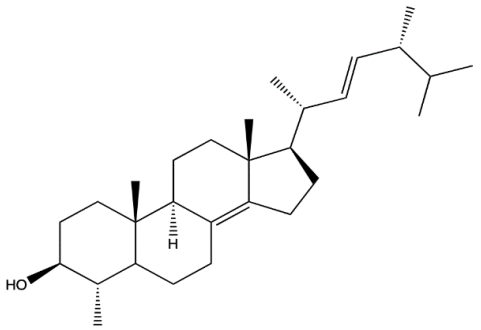


Figure 9. The original Figure 1 from Abbott and Ballantine. [2] Assay curve for toxin strength using gobies. Curve a, time to complete loss of balance in fish. Curve b, time to death. Extremes shown by vertical lines. Concentration in arbitrary units defined in text [2].

Sterol Dependency of Toxicity

In amazing forsight, Abbott and Ballantine [2] found adding an ethanol suspension of cholesterol (0.2 mg/ml) to the goby bioassay completely inhibited death for a 4 unit exposure to gobies. Consistent with this finding we find that cholesterol also inhibits hemolysis karlotoxins in a concentration dependent manner but the endogenous sterol in *K. veneficum* including PLY103, gymnodinosterol (Figure 10), poorly inhibits hemolysis [9]. Hence, toxic *K. veneficum* strains have a protective mechanism against self-intoxication and sterol dependent lysis through their unique 4α-methyl sterol profiles. In contrast, all known prey for *K. veneficum* possess des-methyl sterols [10].



(24S)-4α-methyl-5α-ergosta-8(14), 22-diene-3β-ol

Figure 10. Gymnodinosterol, the dominant sterol of *K. veneficum*.

Can PLY103 Eat?

Although Ballantine [3] reported that in her strains of *Gymnodinium veneficum 'Ingestion has not been observed, though both species have been given a large variety of food, both natural and artificial', K. veneficum* is now recognized as what Mitra et al. [11] refers to as a constitutive mixotroph – a phagotroph that has inherent phototrophic capabilities. In the case of *K. veneficum* this is expressed as the capacity to grow phototrophically in inorganic nutrient media, coupled with the ability to grow as much as 2-3 fold faster when also feeding on particulate prey (often cryptophytes in experimental work), [12–14]. During mixotrophic growth, the relative contribution of phototrophy declines and heterotrophic C acquisition dominates growth [15], with some strains capable of prolonged survival under pure heterotrophic conditions [14]. We found that *Karlodinium veneficum* PLY103 did not feed on any of ten cryptophyte strains tested, which ranged in size from 3.9 μm to 9.3 μm equivalent spherical diameter, although 13 other *K. veneficum* strains, including CCMP 415 & 416, did feed on these cryptophytes under similar conditions [16]. Changing culture medium (from Erdschreibers to ESAW) and salinity did not change these results. This result confirms the original observation of Ballantine [3] that PLY103 is not phagotrophic, and is evidence against prolonged maintenance in culture as a reason for 'losing' the ability to feed.

Prior work had confirmed the production of karlotoxin by PLY103 [17], but the effects of different culture conditions on growth and toxicity had not been previously characterized. PLY103 had been maintained in Erdschreiber's growth medium long term at the PCC, a general purpose seawater medium that contained a soil extract. Transfer of *K. veneficum* PLY103 to a controlled, artificial seawater medium was attempted in order to produce a more stable culture for shipping, which was unsuccessful, and to characterize nutritional effects on growth and toxicity. Modified Enriched Seawater Artificial Water (ESAW) medium [18] with 880 μM NO_3, 34 μM PO_4, 1 mM HEPES buffer, pH 8.0 was prepared (ESAW 'Complete'). Alternative ESAW medium was also prepared containing 1/10th N (-N), 1/10th P (-P), no Se (-Se), or alternative sole nitrogen sources, 54 μM NH_4, or 27 μM urea $(NH_2)_2CO$, or sole P source of 2.1 μM glycerophosphate (GP). PLY103 was transferred in ESAW complete medium by pipeting 6 ml of the PLY stock culture (maintained in Erdschreiber's medium) into 30 ml of ESAW complete that had been pre-cooled to 15°C. These cultures were

monitored for four days and then diluted again with the appropriate ESAW medium 9:1, resulting in a final 98.4% dilution of the initial medium. PLY cryptophytes in wells of a 24-well plate (in Erdschreiber's medium) were diluted 50% (1 ml) with ESAW complete and then this was used to inoculate 15 ml of ESAW complete the next day. After the first dilution of PLY103 from Erdschreiber to ESAW, all cultures grew well, likely due to carry over effects from the inoculation. Effects of the ESAW varieties of medium on growth of PLY103 were recorded after a second 10x dilution that occurred four days following inoculation. Growth rate was highest in ESAW complete and lower in other ESAW varieties (Figure 11). Cell yield was highest in ESAW complete, -N and –P, and lower in NH_4, -Se, Urea, and GP medium. Examination of cellular toxin quotas (KmTx4 + chloro-KmTx 4, pg KmTx cell^{-1}) as a function of growth rate (Figure 11) in the different varieties of ESAW medium produced a negative trend. The ratio of KmTx4 /chloro-KmTx 4, ranged between 0.15 and 0.28 and was not correlated with growth rate. Growth in ESAW medium with NH_4 as the sole N source produced the most toxic cells at both the day four and final timepoints (14 days). These results are consistent with the idea that slower growth rates equate to more toxic cells. Another way to look at this is that toxin synthesis proceeds out of balance with cell division under culture conditions that slow or halt cell division. Further evidence of this is the observation that the total KmTx yield in the different ESAW cultures was relatively constant (301 ± 96.0) ng KmTx ml-1. The data also suggest that NH4 may be an elicitor of KmTx as values were higher than for other nutrient treatments that grew at similar growth rates.

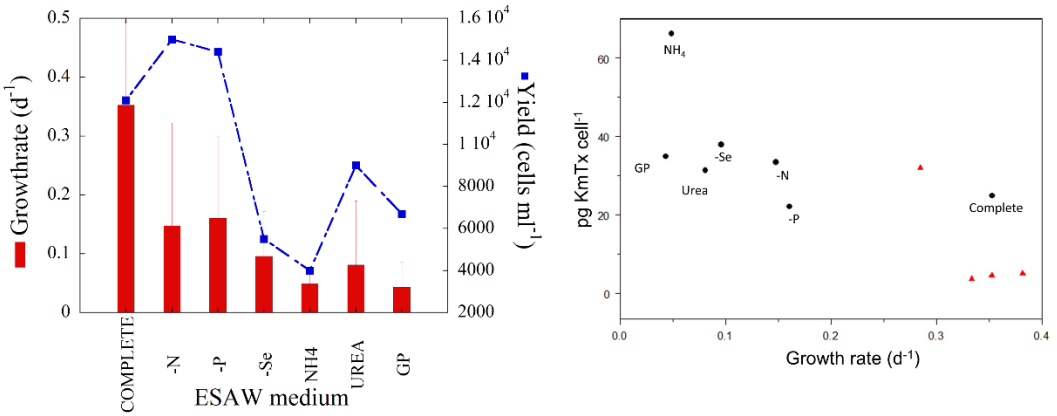


Figure 11. Growth rate and cellular KmTx quotas for PLY103 upon transfer from Erdschreiber's to different varieties of ESAW growth medium. Red triangle symbols are values measured after the first dilution (4d). Some symbols overlap and are not visible. Black circle symbols are final measurements made after an additional dilution in respective ESAW media and an additional 10d of growth.

Toxin Production during Batch Culturing for the Norwegian Strains

We observed a generally stable or increasing toxin cell quota as cells moved from exponential to stationary or senescent growth phase in most strains of *K. veneficum* [4]. For the two Norwegian strains, CCMP 415 and 416, toxin cell quotas remained reasonably constant throughout the growth curve (Figure 12). Both the division rate (μ, 0.35 ± 0.072 vs 0.27 ± 0.029) and maximum densities (321 ± 53.92 vs 78.0 ± 16.9 X 10^3 cell mL^{-1}) were

greater with CCMP 415 than CCMP 416. The Spanish isolate CSC1 produced no toxin and had significantly lower division rates (μ, 0.11 ± 0.053) and maximum densities (68.3 ± 14.8 X 10^3 cell mL^{-1}). Both CCMP 415 and CCMP 416 have been shown to eat cryptophytes [16]. In this study no attempt was made to determine the optimal growth conditions for each strain but rather to compare them under uniform laboratory conditions so these data do not necessarily define the maximum toxin production for each strain.

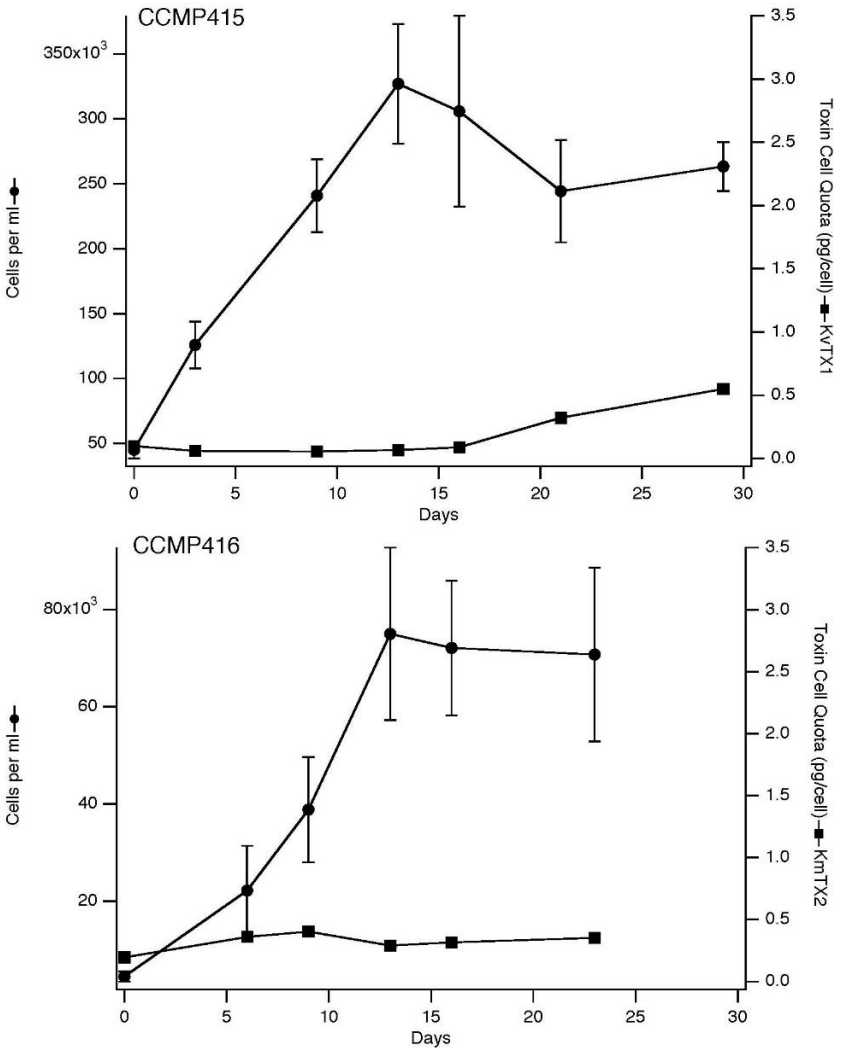


Figure 12. Growth rate (filled circles) and cellular KmTx quotas (filled squares) for *K. veneficum* CCMP 415 and 416.

Genome Size Variation in *K. veneficum* Strains

Consistent with extensive strain variation in growth rates, pigments, phagotrophic ability, types of karlotoxins and toxin cell quotas in *K. veneficum* [4], we find genome size varies

among strains (Figure 13). Dinoflagellates generally have large, unusually-structured genomes compared to most other eukaryotes [19, 20]. But as with most protists, genome size in dinoflagellates seems to correlate well with cell size [21, 22]. Therefore, a relatively small dinoflagellate like *K. veneficum* would be expected to have a relatively small genome, and that is more or less the case. Genome sizes among *Karlodinium* spp. strains were determined by Parrow et al. [23], using flow cytometric measurement of cellular DNA content as compared to a trout erythrocyte nuclei DNA standard (Figure 13). The measured basal (1C, functionally haploid) genome size measured in picograms of *K. veneficum* strains varied significantly from 7.8 ± 0.7 pg DNA cell^{-1} in a strain from Norway (CCMP 415) to 14.7 ± 1.5 pg DNA cell^{-1} in a strain from Spain (CSIC-1). The five tested strains from the U.S. eastern coast (Maryland to South Carolina) also differed significantly from these European strains, but not from one another, with an average 1C genome size of 11.2 ± 0.6 pg DNA cell^{-1}. The estimated 1C genome size of *Karlodinium armiger* (GC-3) was 32.9 ± 2.4 pg DNA cell^{-1}, significantly higher than any *K. veneficum* strain. It was notable that CCMP 415 from Norway, which may be the type specimen for *K. veneficum* (given the loss of PLY103) [24, 25], had a significantly smaller genome size than the other strains, making it a good potential candidate for genome sequencing. This strain has also been in culture the longest (since 1976), but does make low levels of toxin (see Figure 7). An apparent trend towards genome size reduction with extended time in continuous culture has been observed among strains of some dinoflagellates [26, 27], but not in others [21, 28]. Although no cause or mechanism for genome size changes are known to be attributed to clonal cultivation, natural selection likely influences genome size over time [29], and selective forces over long periods of time in serial culture are likely not the same as those acting on wild populations out in nature.

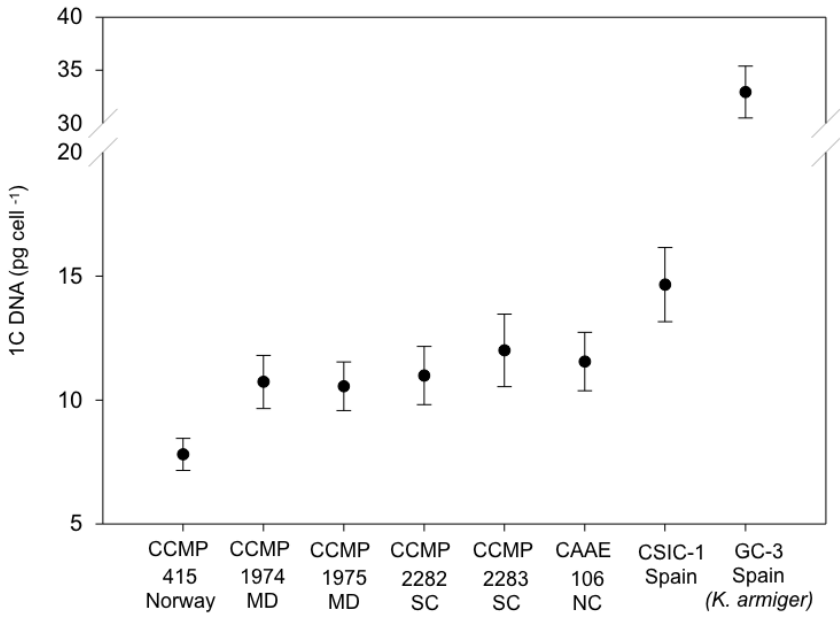


Figure 13. 1C DNA mass/genome size estimates for *Karlodinium spp.* strains. All are currently considered to be *K. veneficum*, with the exception of GC-3, which is *K. armiger* [33]. Locations of strain origins are given. Bars are ±1 SD. Parrow et al. [23]

Lajeunesse et al. [21] also measured genome size by flow cytometry of two of the same *K. veneficum* strains from the eastern US coast (CCMP 1974 and 2282) by flow cytometry and reported higher values of 16.9 and 16.3 pg DNA cell^{-1}, respectively. However, Lajeunesse et al. [21] used a different, smaller DNA mass standard in their studies than Parrow et al. [23] (chicken red blood cells versus trout erythrocyte nuclei), and furthermore, chose a high estimate of DNA mass for chicken red blood cells of 3.0 pg DNA based on [30]. Their values would have been lower and in close agreement with Parrow et al. [23] had they assumed a lower chicken red blood cell DNA standard mass of 2.33 pg (as in [22, 27, 31, 32]). The significantly higher genome size of *Karlodinium armiger* [33] from coastal Spain reported by [23] was confirmed by flow cytometry with a very similar estimate of 34.8 pg DNA cell^{-1}[34]. This same study found two strains of *K. veneficum* from coastal Spain to have genome sizes of 19.9 – 22.4 pg cell^{-1}, higher than any U.S. strain. Figueroa et al. [34] used a previously published genome size for the dinoflagellate *Scrippsiella hangoei* as their DNA mass standard, which would be tied back to an estimate based on DNA fluorescence relative to triploid trout erythrocyte nuclei [35]. The three different studies on genome size in *Karlodinium* spp. dinoflagellates to date each used different DNA mass standards and different DNA fluorophores: SYBR green [23], DAPI [21], and propidium iodide [34]. Such differences in standards, and DNA flurophores (i.e., 'intercalating' dyes with little or no sequence preference such as SYBR green and propidium iodide versus 'non-intercalating' dyes such as DAPI (binds preferentially to AT-rich sequence)), and even factors such as dye concentration and incubation duration can affect relative estimations of target cell DNA fluorescence [22, 27, 30, 35].

These differences in technique may account for some of the variability in reported genome sizes for *K. veneficum*. However, the half-peak coefficients of variation (CV's) and/or standard deviations reported for the target cell DNA measurements in each of these studies were quite acceptable as compared to previous studies on protists (reviewed in [26] and even DNA measurements for clinical pathology [36], a strong indicator of high quality/certainty in the genome size estimates. Intraspecific variability in genome size has been measured for other protists, especially when isolates from different geographic regions were included in the analysis [27, 32, 37–39]. Hayhome et al. [38] suggested that this variation may due to clonal isolation and high degree of genetic redundancy in dinoflagellate genomes, allowing them to tolerate a less than precise division of nuclear DNA during cell division. Alternatively, since genome size is generally thought to be a well-conserved biological parameter in a given species, these data may indicate that not all of the strains presently regarded as *K. veneficum* are the same species. The high degree of variability in physiological parameters found among *K. veneficum* strains certainly suggests that numerous 'ecotype species' may occur [4, 14].

Overall, the *K. veneficum* genome size range (7.8 – 22.4 pg cell^{-1}) as determined so far falls near the range of genome sizes reported for other dinoflagellates of similar size. For example, similarly sized and geographically-overlapping dinoflagellates *Pfiesteria piscicida* and *P. shumwayae* (~7-17 µm in diameter) [27, 35] have 1C genome sizes of 6.7 ± 1.1 and 16.2 ± 1.7 pg DNA cell^{-1}, respectively [27]. Similarly-sized *Prorocentrum* species range from 6.9 – 8.3 pg DNA cell^{-1}, along with *Polarella glacialis* (7.0 pg DNA cell^{-1}), and *Gymnodinium simplex* (11.6 pg DNA cell^{-1}) (reviewed in LaJeunesse et al. [21]. *K. armiger* is larger in size than *K. veneficum*, and has a larger genome size (32.9 – 34.8), closer to the similarly-sized *Alexandrium minutum* (29.9 pg DNA cell^{-1}) [23, 34]. Very small (6-9 µm

diameter) *Symbiodinium* spp. dinoflagellates can have genomes as small as 1.9 pg cell^{-1} [21]. Much larger dinoflagellates can have genomes as large as 250 pg cell^{-1} [21, 22, 40], although such massive genomes may reflect increased redundancy rather than gene coding capacity [41].

DNA Base Composition Variation in *K. veneficum* Strains

When Peter Rae set out to described the ribosomal gene arrangement in the dinoflagellate *Crypthecondium cohnii* he noticed a large discrepancy between the GC content as determined by buoyant density *versus* the T_m of the melting curve for the isolated DNA [42]. He was able to explain this discrepancy when he found the thymidines (T) were extensively replaced by the modified based 5-hydroxylmethyluracil (hmdU; Figure 14). This base has the effect of raising the density and lowering the thermal stability of the DNA. Rae went on to show this replacement was a natural feature of all dinoflagellates he examined and is now considered a diagnostic character for the phylum. Consistent with other dinoflagellates, *K. veneficum* exhibits extensive replacement (51.6 - 66.9%; Table 2) of thymidine by 5-hydroxylmethyluracil in its genomic DNA [43, 44].

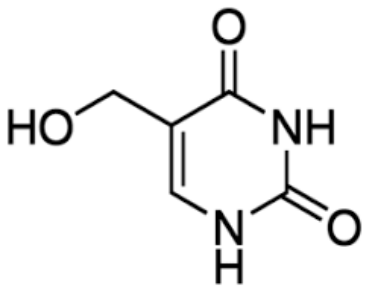


Figure 14. 5-Hydroxylmethyluracil (hmdU).

Table 2. Percent GC and 5-Hydroxylmethyluracil content in genomic DNA

Strain	Percent GC	Percent hmdU of hmdU plus dT
Karlodinium veneficum (Plymouth 706)	51.6	34.9
Karlodinium veneficum (CCMP 2778) (N=4)	52.7	43.2
Karlodinium veneficum (CCMP 1609)	66.9	35.5
Karlodinium veneficum (CCMP 2936) (N=4)	53.5	25.6

Cell Cycle

In situ studies of *K. veneficum* cells progressing through the cell cycle are rare. Garcés et al. [46] studied a field bloom in the Mediterranean sea of what was then called *Gyrodinium corsicum* [47] via microspectrophotometry, and reported that the cell division cycle was well-phased with the ambient light: dark cycle. In the blooming population, cells in S-phase reached a maximum abundance near the middle of the dark period, and the G2+M maximum

fraction occurred at the end of the dark period. Both fractions had a maximum of 15-39% of the total population over the sampling period. The duration of S-phase was computed to be 2.1-8.7 h, and G2+M phase lasted 2.6-7.7 h. These data were later understood to likely be averaged values from a mixed population of *K. veneficum* and *K. armiger* [33], taxa with broadly differing genome sizes and growth characteristics, but nevertheless showed a distinct *Karlodinium* spp. population level cell cycle progression that was strongly linked to the photocycle.

Cell cycle progression was also studied in multiple geographic strains of *K. veneficum* in culture via flow cytometric analysis of cells stained with the DNA fluorophore SYBR Green [23, 45]. Each strain exhibited distinct 1C, intermediate, and 2C DNA subpopulations indicative of respective eukaryotic cell cycle phases G1, S, and G2M that were tightly synchronized to the light:dark cycle (Figures 15 and 16) with very good G1 DNA peak CV's of 6.8 – 9.2%.

In logarithmically growing *K. veneficum* cultures, a cohort of cells in G1 phase enter S-phase every 24 h near the end of the light cycle (Figure 17). S-phase required approximately 4-5 hours for completion, and then the cells entered G2+M phase. G2 +M phase required approximately 5 hours for completion, and the cells completed cell division during the end of the dark period. By the beginning of the next light period, >95% of cells were in G1. The proportion of cells entering S-phase each cycle in exponential phase cultures was approximately 15-45% of the total, and those cells that did not enter S-phase were apparently entrained in G1 until the next photocycle (Figure 15). Stationary phase *K. veneficum* cells arrested in G1, as is typical for eukaryotes. Therefore S and G2+M phases in *K.veneficum* occur during the dark period and have distinct durations, whereas the cellular growth (G1) phase occurs primarily during light but has a variable duration depending on growth (or non-growth) conditions.

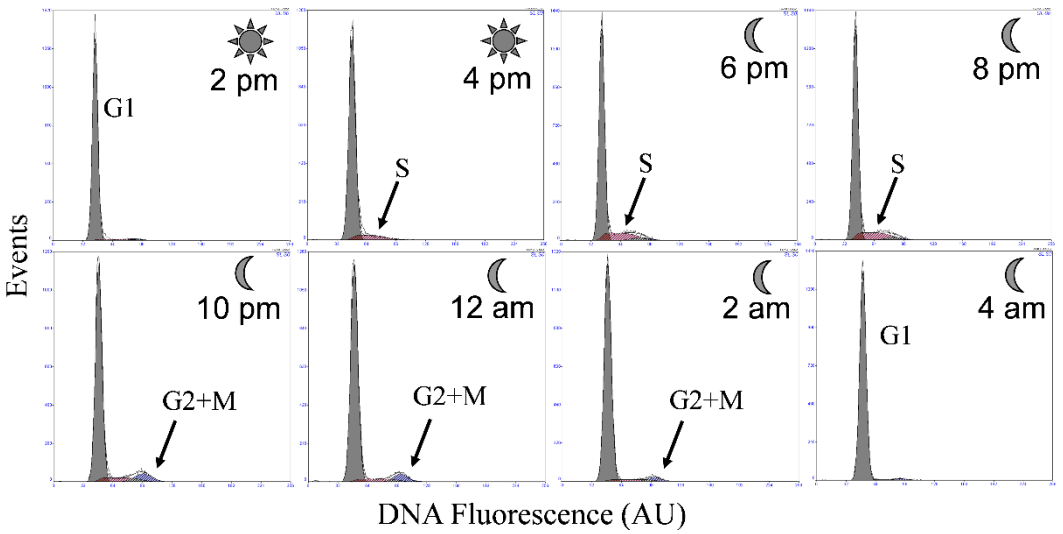


Figure 15. Cell cycle phases and progression of *K. veneficum* (CCMP2282) over time, showing relative proportions of cells in G1, S, and G2M phases. AU = arbitrary units [23].

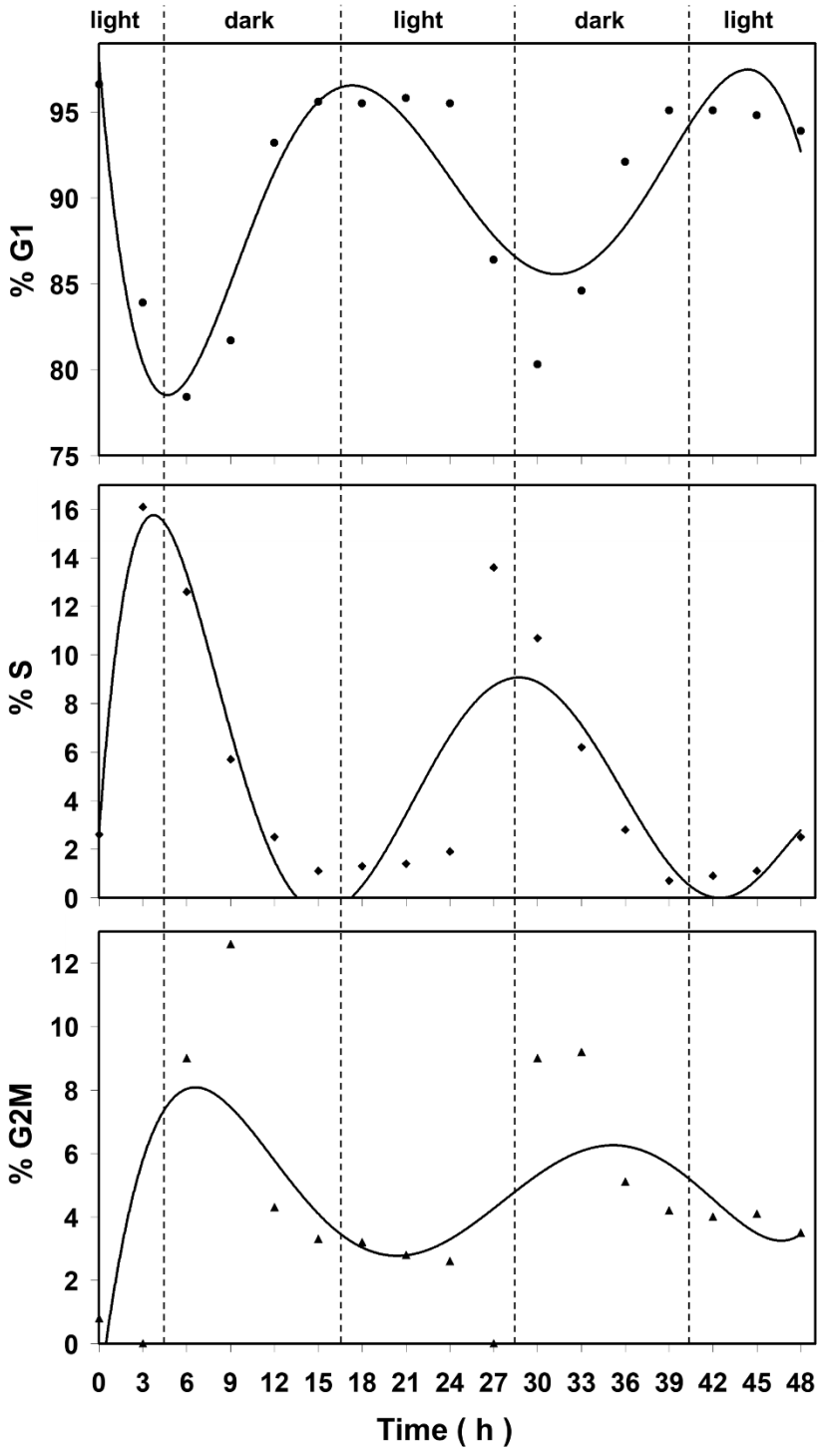


Figure 16. *K. veneficum* (CCMP 415) cell cycle phase fractions G1, S, and G2M over 48 h showing diurnal entrainment with light-dark cycles. Curves are 5th degree polynomials fitted to the phase fraction data. G1 maxima occur in light, G2+M maxima occur in darkness, and S-phase maxima occur at the light-to-dark transition [23].

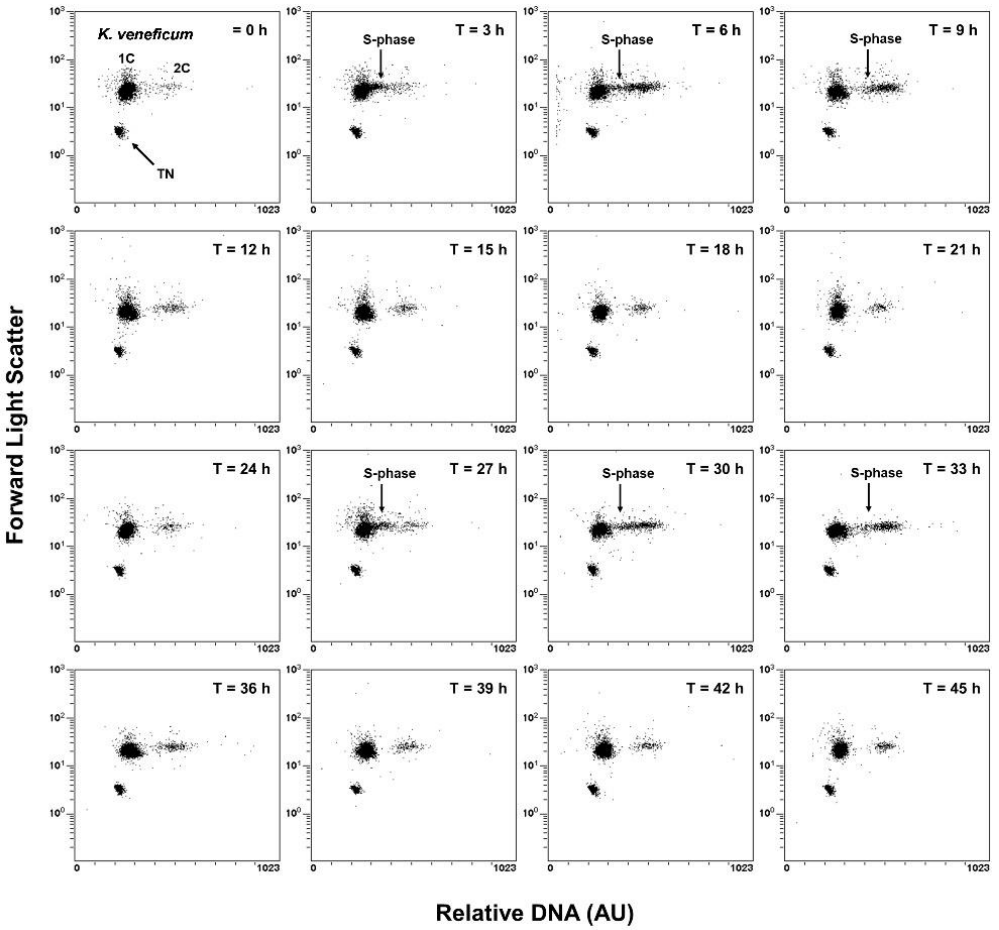


Figure 17. Representative flow cytometric histograms of forward light scatter versus relative DNA fluorescence measured every 3 h over 45 h for a strain of *K. veneficum* (CCMP 415). The pattern of phased cell cycle progression is demonstrated, with S-phase being constrained to a specific portion of the diel cycle. TN = trout nuclei, an internal DNA standard; AU = arbitrary units [23].

Therefore, both a field study [46] and culture studies [23, 45] are in general agreement on both the photoperiodicity and cell cycle phase proportions and durations in growing populations of *K. veneficum*. Other cell cycle studies on *K. veneficum* either did not measure cell cycle progression but rather infer ploidy [34], or examined whether an algicide would disrupt the cell cycle [48]. In regards to natural growth of *K. veneficum*, a major question yet to be addressed is the potential influence of mixotrophic nutrition on cell cycle progression. During mixotrophic growth cryptophytes provide significant contributions to the carbon, nitrogen, and phosphorous needs of *K. veneficum* [49], and significant differences in net growth of *K. veneficum* have been reported when comparing strains growing autotrophically (~ 0.3 divisions day^{-1}) versus when feeding on cryptophyte prey (~0.9 divisions day^{-1}) [12]. The higher growth rates observed under mixotrophic conditions suggests that either a compression of the duration of certain cell cycle phases or a decoupling of the cell cycle and photocycle occurs during mixotrophic growth. If so, this could greatly impact our understanding the role of mixotrophy in K. veneficum bloom development.

Cell Cycle and Toxicity

The biosynthetic pathway for karlotoxin production is currently unknown, but previous research has shown that cell quotas of karlotoxin can vary widely among different bloom and cultured populations, and within populations over time [4, 50]. Deeds et al. [51] observed 10-fold higher cell karlotoxin quotas in natural samples compared to clonal cultures. Significantly, research has demonstrated that *K. veneficum* karlotoxin cell quotas increase in stationary and declining phase cultures (Figure 18), particularly under growth-limiting conditions as caused by low N, P, or the micronutrient selenium [50]. In particular, it was determined that *K. veneficum* cellular quotas of karlotoxin increased 6-fold or more in stationary or declining phase cultures, and that in general, cellular karlotoxin accumulation was inversely related to growth rate. If extrapolated out into the field, these results suggest that environmental conditions that favor slowed or halted growth (e.g., stationary phase bloom populations) also favor cellular accumulation of karlotoxins, and are likely a significant factor underlying *K. veneficum*-related fish kills that require both high cell densities and higher cellular karlotoxin quotas than those generally observed in nutrient replete cultures [50]. Furthermore, it has been found that karlotoxin production is light-dependent (Figure 19), and excess reduced carbon chains produced by photosynthesis are quickly incorporated into karlotoxin molecules (Place, unpub. data). It is important to note that the molecular structure of karlotoxin does not contain N, P, or other nutrient known to typically limit phytoplankton growth but does start with glycolate, a photorespiration product. So enzymatic biosynthesis of karlotoxin could conceivably continue using only photosynthetically-reduced CO_2 and water even under conditions where low N or P limits production of nucleic acids and proteins needed for cell proliferation.

Although the influence of cell cycle events on toxin production in harmful algae has been studied in relatively few species, in each case it was found that toxin synthesis was phased to specific portions of the cell cycle. In *Alexandrium fundyense*, saxitoxin synthesis was restricted to 8-10 h in the G1 phase of the cell cycle [52]. In *Prorocentrum lima*, DTX4 production began in G1 and persisted into S phase, whereas DTX1 production occurred later during S and G2 phases [53]. In *Chrysochromulina polylepis*, toxicity was highest in G1 phase [54]. In most species, the photocycle had a clear role in synchronizing the cell cycle and thus toxin synthesis. These and other results suggest a simple but strongly predictive inverse relationship between the rate of cell proliferation and cellular toxin accumulation in toxin-capable *K. veneficum* cells and populations, wherein rapidly proliferating *K. veneficum* cells exhibit relatively low karlotoxin quotas while growth-limited (slowly or non-proliferating) *K. veneficum* cells arrest in G1 for multiple light/dark cycles, undergo several cycles of light-induced karlotoxin synthesis, and become significantly more toxic. This model has yet to be tested for *K. veneficum*, or for any other HAB species: we know of no previous studies that have quantifiably correlated HAB toxin production with *in situ* growth rates in natural bloom populations. Doing so for *K. veneficum* could be a big step forward in explaining why some bloom populations are highly toxic while others seem less so: the most toxic cells might be the ones that are not growing, but just persisting.

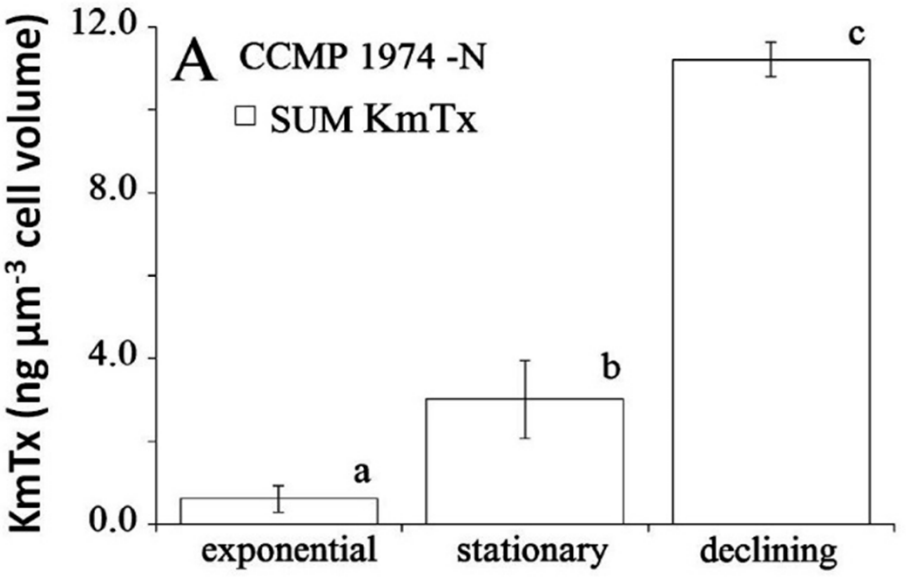


Figure 18. Example of KmTx cell quotas in different stages of *K. veneficum* culture growth. Adolf et al. [50].

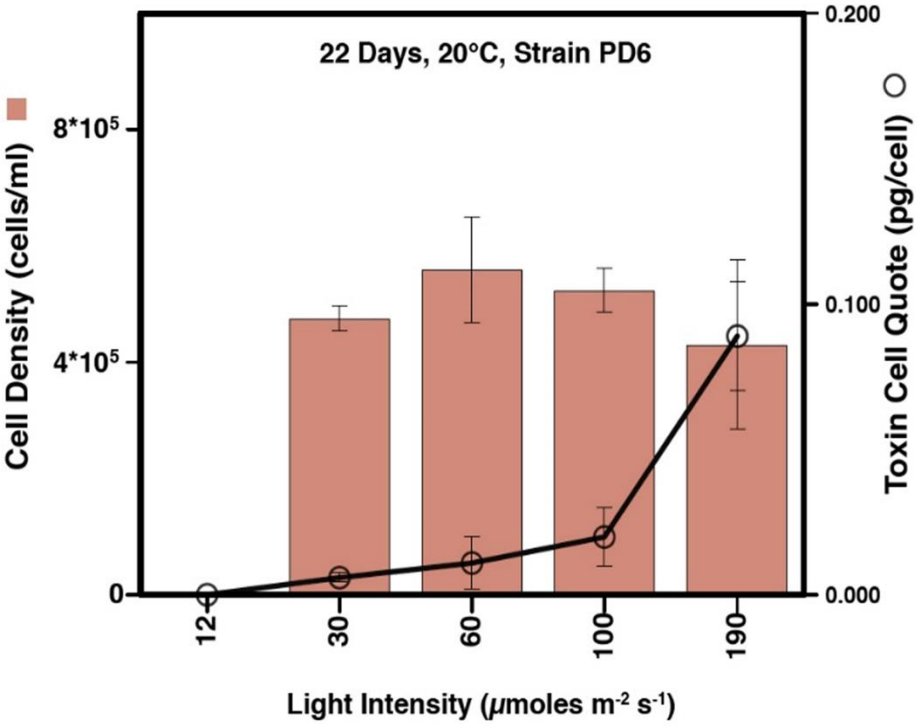


Figure 19. Karlotoxin production (right axis) in *K. veneficum* as a function of increasing light intensity (x-axis). A. Place, unpublished data.

Life Cycle

Asexual Reproduction

Life cycle observations for *K. veneficum* began with the first paper describing the species in 1956 [3]. In this seminal paper, under the section "*Notes on Cell Division*," Ballantine described and drew the gross morphology of cell division, and also (unwittingly) drew a stage of gamete fusion (Figure 20). As depicted, cell division in *K. veneficum* begins with a cleavage indentation at the antapex of the hyposome that proceeds through the cell towards the cell apex. Cells in the process of division thus appear to have a broad, split hyposome. In culture, dividing cells typically swim less vigorously than non-dividing cells [3]. Once the division plane reaches a point above the cingulum, division proceeds from both ends of the cell and the two offspring cells are left connected by a cytoplasmic channel at their episomes. The cells swim connected this way for a short duration (minutes), often orienting themselves at right angles to one another, until the connection breaks and the offspring cells are freed. The entire cell division process takes only 2-3 h to complete [3], or as little as 1.5h (Parrow pers. obs.). Since it also typically occurs late at night in cultures grown on a light: dark cycle (and in field blooms) [45, 46] cell division in *K. veneficum* can be easily missed, although early-morning observation of cultures often allows some stragglers or failures in the process to be viewed.

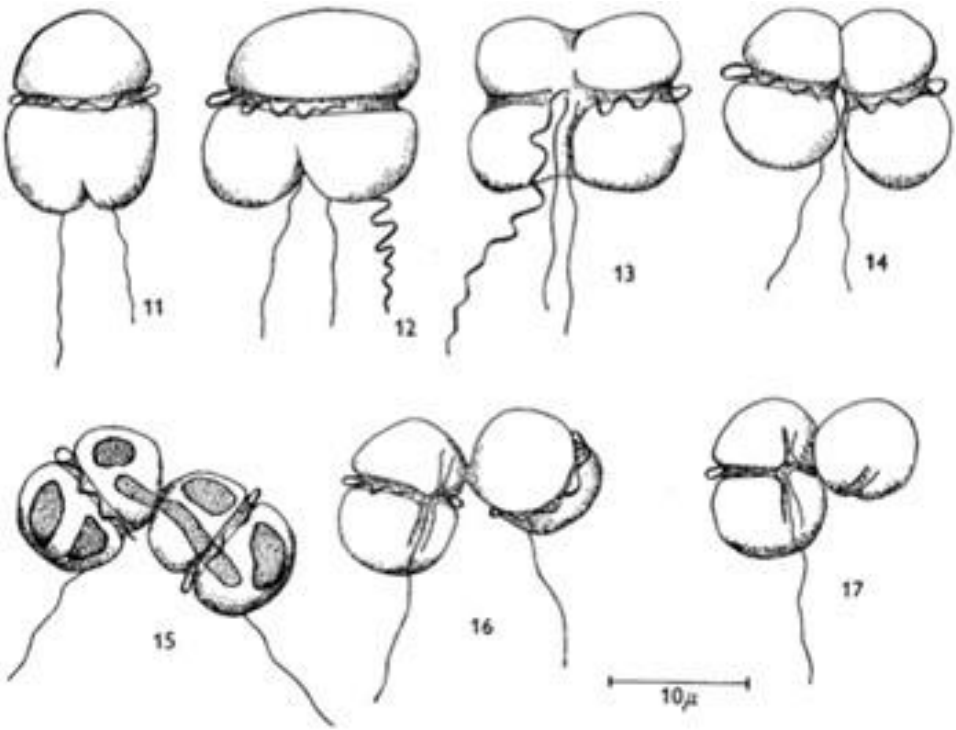


Figure 20. *K. veneficum* cell division stages as reported by Ballantine [3], then as *Gymnodinium veneficum.* This sequence is correct except illustration 14, which depicts gamete fusion, not cell division as interpreted.

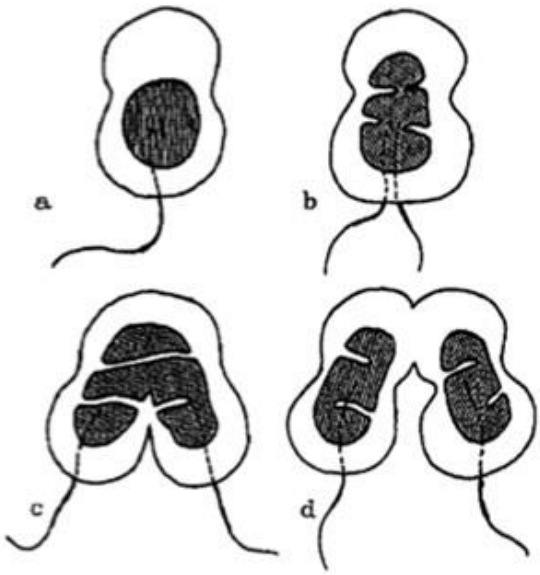


Figure 21. Flagellar, nuclear, and cell division in *Karlodinium veneficum* as depicted by Leadbeater and Dodge [55], then as *Woloszynskia micra*. Nuclear and cell division occur simultaneously, and offspring pairs remain briefly attached at their episomes.

Ten years after Ballantine described *K. veneficum*, Leadbeater and Dodge [55] used this dinoflagellate (provided to them by Dr. M. Parke at the Plymouth Laboratory) to study mitosis by transmission electron microscopy in one of the first studies of its kind. Aside from providing details on how the nuclear envelope remains intact during mitosis and is penetrated by microtubule-containing cytoplasmic channels, they also confirmed the observations of Ballantine [3] on the morphology of cell division, and added that nuclear and cell division occur simultaneously in *K. veneficum*. (Figure 21). Ribosomes and mitochondria were observed inside the trans-nuclear mitotic channels (!), [55] and this was at a time when it was still openly wondered if dinoflagellates might occupy an intermediate position between prokaryotes and eukaryotes [56].

Sexual Reproduction

The first depiction of a sexual stage in *K. veneficum* was drawn by Ballantine [3], who interpreted it as cell division (Figure 20, inset 13). It is clear from her writing that Ballantine was uneasy with her placement of this observation, as it does not fit with the rest of her described sequence of cell division. By her description, indentation of the cell apex does not occur until cleavage from the antapex has proceeded "*to a point above the girdle*." The cell(s) depicted in her Figure 14 does not fit that description, and, furthermore, is the only proposed "ventral" view in the sequence. In fact, what she depicted was not cell division but rather the typical morphology of gamete fusion in *K. veneficum*. It is also not a ventral view as described, but a lateral view, from the side of the two fusing gametes, with a clear illustration of the 'copulation globule" between them, predating von Stosch's description [57]. In Ballantine's [3] defense, sexuality in dinoflagellates was essentially unknown when she made her observations [57, 58], so it may be understood why she considered all paired cells as a stage of cell division.

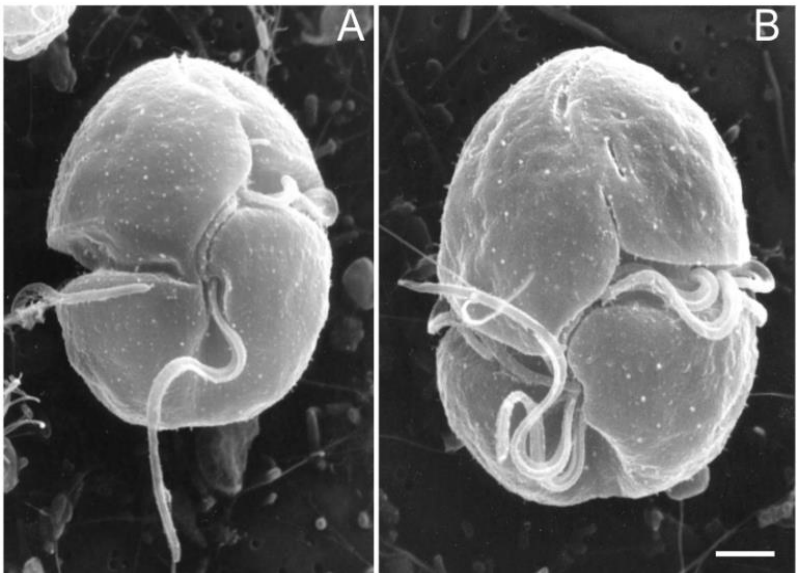


Figure 22. *K. veneficum* cells, from field samples of the Neuse River Estuary, NC. Scanning electron micrographs of (A) a biflagellate dinospore and (B) a quadriflagellate planozygote showing two transverse and two longitudinal flagella. Scale bar = 1 μm [Photos by M. Parrow].

Sex and meiosis in *K. veneficum* was reported by Parrow et al. [45], who first became aware of sex in this dinoflagellate (and, of its very existence) due to a graded project in a hands-on electron microscopy course in graduate school in the late 1990's. The specimens of choice for his project were mixed dinoflagellates from a field sample in a North Carolina estuary, and the best pictures he developed (on film, in the darkroom) were of dinoflagellates he later realized were most likely *K. veneficum* (then as *Gyrodinium galatheanum*) (Figure 22). The most significant of the specimens photographed was a relatively large cell with two transverse and two longitudinal flagella (Figure 22B), which was, of course, a planozygote. Beginning with von Stosch [57], a feature consistently known to be displayed by motile dinoflagellate zygotes is retention of the both longitudinal flagella from the two component gametes that formed them, allowing planozygotes to be presumptively identified and equated with sexuality in both cultures and field populations (reviewed in Parrow and Burkholder [59]. What is less common, or at least less commonly observed, is retention of both transverse flagella as well, making the planozygote quadriflagellate. *K. veneficum* is special in this regard, but not unique [60].

Since then, all known sexual stages in the life cycle of *K. veneficum* have been documented [45] (Figure 23). Gamete fusion can be found in most, but not all, *K. veneficum* cultures, particularly in dense, healthy populations. The gametes are pigmented, in the smaller end of the size range for *K. veneficum* cells, and essentially isogamous. Fusion takes place by mid-ventral union of gametes, a morphology that is quite different from cell division and thus easy to distinguish (Figure 23). This mid-ventral fusion of gametes is very similar to that of *Gymondinium pseudopalustre* (= *Biecheleria pseudopalustris*) [57, 61], *Gyrodinium uncatenum* (= *Levanderina fissa*) [60, 62], and *Polykrikos kofoidii* [63]. Like them, *K. veneficum* gamete fusion begins with formation of an attachment structure in the ventral/sulcal area that may be homologous to the "copulation globule" described by von

Stosch [57] (Figure 23 B and F). This structure was also clearly illustrated by Ballantine [3] (Figure 20, inset 13). *Karlodinium veneficum* gametes orient ventrally for fusion, but the two gametes do not need be oriented in the same direction to fuse. Fusions often occur with gametes oriented ventrally but pointed in opposite directions (Figure 23 B and F), or at right angles to one another. Fusion is completed in about 1 h, with karyogamy immediately following plasmogamy, resulting in a quadriflagellate planozygote. Altogether, the morphology of life cycle events in *K. veneficum* match very closely with Coats et al.'s [60] descriptions for *Gyrodinium uncatenum* (= *Levanderina fissa*) (Figure 24). Similarities between *K. veneficum* and *Levanderina fissa* include morphology of cell division and fusion, retention of four flagella by the planozygote, a similar feeding process involving sulcal phagotrophy, and even the appearance of delicate surface striations on some cells (e.g., upper gamete hypocone in Figure 24B). These features should be investigated for homology.

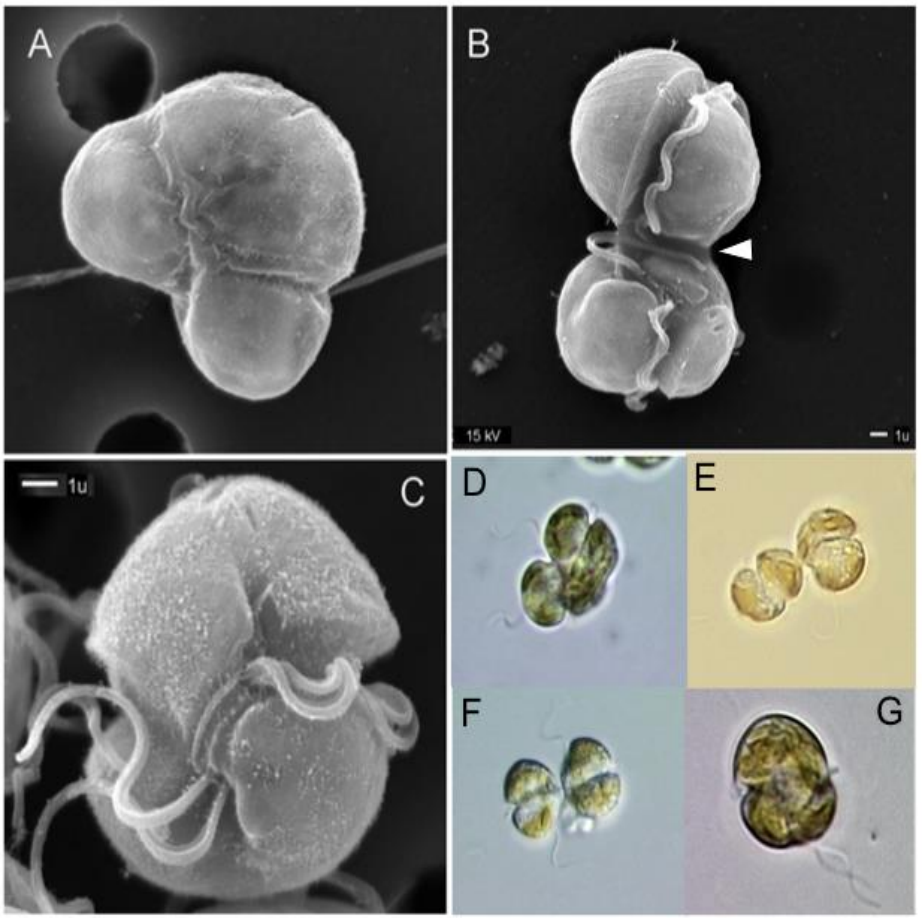


Figure 23. Scanning electron (A-C) and light (D-G) micrographs of *K. veneficum* life cycle stages. (A) Early asexual cell division. (B) Gametes in early fusion. Note mid-ventral union and formation of a "copulation globule" sensu von Stosch (1973) between the gametes (arrow). (C) Planozygote with two transverse and two longitudinal flagella. (D-G) Living cells. (D) Early to mid asexual cell division. (E) Late cell division, offspring cells almost separated. (F) Early gamete attachment/fusion, with early formation of the "copulation globule." (F) Planozygote with two longitudinal flagella apparent [23]. Photos A-C by E. Allen (NCSU) and M. Parrow, D-G by M. Parrow.

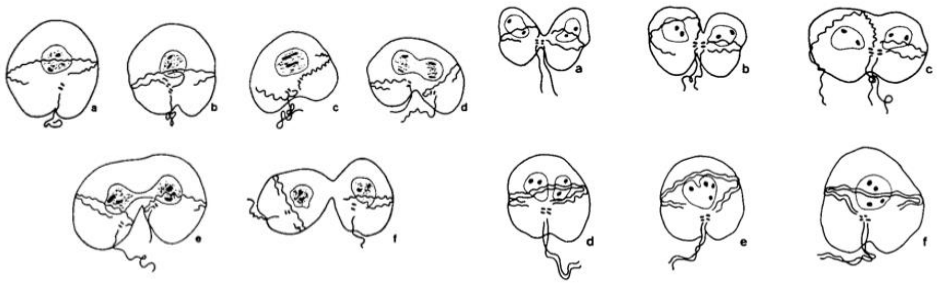


Figure 24. Depiction of cell division (Left, a-f) versus gamete fusion (Right, a-f) in *Levanderina fissa* by Coats et al. [60]. Both processes are essentially the same in morphology as cell division and gamete fusion in *Karlodinium veneficum*. Note the two transverse and two longitudinal flagella in the planozygote (Right, e-f), as also occurs in *K. veneficum*.

Another feature common to dinoflagellate zygotes is nuclear cyclosis [57, 58], a dramatic swirling of the chromosomes that occurs within the nuclear envelope prior to zygote nuclear division. This process is associated with the pairing of homologous chromosomes in early-to-mid-meiotic prophase, has been observed to occur specifically in dinoflagellate zygotes in a number of species (reviewed in [59, 63]). It is therefore considered to be a clear marker for meiosis in dinoflagellates. In *K. veneficum*, nuclear cyclosis can be observed in mature planozygotes with large nuclei. These cells appear to often become sluggish during nuclear cyclosis, and even settle to the bottom of the culture vessel (Parrow, pers. obs.). High resolution microcopy is required to see nuclear cyclosis in a small dinoflagellate like *K. veneficum*, but in many cases both the dual longitudinal flagella and nuclear cyclosis can be observed simultaneously in sluggish specimens. In *K. veneficum*, nuclear cyclosis occurs as a smooth unidirectional swirling of the chromosomes, and lasts for at least 2h [https://www.youtube.com/watch?v=xyWy3muIu1g].

All dinoflagellates for which sufficient information exists appear to exhibit a haplontic life cycle with zygotic meiosis, with one notable exception: the coenocytic/pseudocolonial dinoflagellate *Polykrikos kofoidii* [63], which is diplohaplontic due to a single apparent mitotic nuclear division in the diploid state. This "exception that proves the rule" may be related to the coenocytic condition of *P. kofoidii*, which is relatively unusual among dinoflagellates. In unicellular dinoflagellates exhibiting a haplontic life cycle, mitosis (asexual division) occurs only in functionally haploid 1C DNA cells. Haploid cells produced by mitosis can also act as gametes and fuse to produce a diploid zygote. The zygote divides by meiosis, restoring the haploid, 1C DNA state. All the evidence collected so far supports *K. veneficum* as having a haplontic life cycle, with zygotic meiosis, like the vast majority of dinoflagellates studied thus far appear to have. In particular, the regular, phased occurrence of 4C DNA planozygotes has been documented in *K. veneficum* cultures, and a pattern of conventional meiotic division demonstrated by DNA cell cycle analysis and flow cytometric sorting of phases for imaging of nuclear states [45] (Figure 25). In principle, it is not possible to distinguish early planozygotes with 2C relative DNA from G2 cell cycle phase asexual cells, unless they occur distinctly out of phase with the rest of the uniformly cycling population. This is difficult to prove, and requires the assumption that measured 2C DNA cells are proof of diploidy rather than potentially being G2 phase mitotic cells but has been presented as proof of sex in *K. veneficum* [34]. However, demonstration of populations of 4C DNA cells, each with single nucleus or a pair of 2C nuclei in division, is clear evidence of

diploidy and canonical meiosis. In any eukaryotic life cycle (haplontic, diplontic, or diplohaplontic), the 4C DNA nucleus exists in only 1 position: as a diploid nucleus, immediately after pre-meiotic S-phase, and before meiosis I nuclear division.

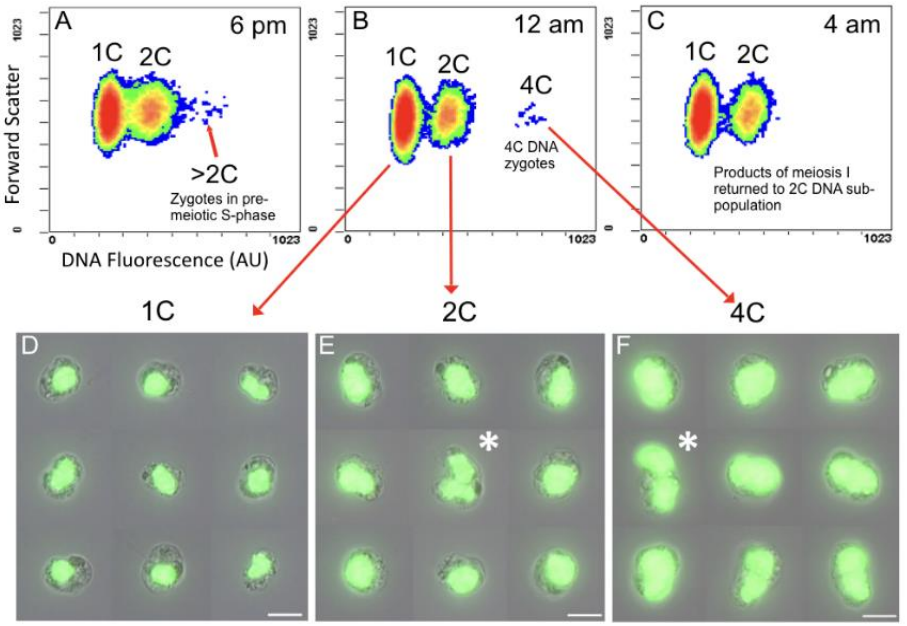


Figure 25. (A-C) Time series of DNA measurements on *Karlodinium veneficum* (CCMP2283). Meiotic DNA synthesis began at 6 pm, and produced a 4C DNA subpopulation (zygotes in meiotic prophase) by 12 am. By 4 am, zygotes had divided and offspring apparently re-entered the 2C DNA subpopulation. (D-F) Individual flow-sorted cells of *K. veneficum* from sample (B) with fluorescently stained DNA (green nuclei). 1C, 2C, and 4C DNA cells, only cells with 2C or 4C DNA were found in the processes of nuclear division (asterisks), scale bar = 10 µm [45].

In *K. veneficum*, planozygotes appear to attain a 4C DNA content following premeiotic DNA replication prior to division, as the conventional pattern of meiosis would predict. This 4C DNA nucleus would be where nuclear cyclosis occurs (i.e., meiotic prophase I). Early planozygotes, before meiotic S-phase, would be 2C in DNA and indistinguishable from mitotic (asexual) cells in G2 of the cell cycle following the typical eukaryotic pattern of asexual cell division. However, in *K. veneficum*, cells advancing through pre-meiotic S-phase (Figure 25A), and the resulting mature planozygotes with 4C DNA content (Figure 25B) have been detected in several (but not all) strains examined by flow cytometry [45]. In general, *K. veneficum* planozygotes also appear to undergo meiosis in phase with the light-dark cycle (Figure 25, A-C), just as mitosis is phased with photocycle (above). Pre-meiotic S-phase (replication of homologous chromosomes into sister chromatids) occurs late in the light cycle, and zygotes with resulting 4C DNA divide (meiosis I) during the dark period, presumably to form two offspring cells each with 2C DNA. These cells presumably then go through meiosis II to restore the 1C nuclear state, following the pattern of conventional meiosis. The occurrence of cells with individual nuclei containing 1C, 2C, and 4C DNA has been confirmed by sorting individual cells with each DNA content onto microscope slides and photographing them (Figure 25, D-F). Importantly, and as expected, only cells with 2C or 4C DNA were found in the process of nuclear division (Figure 25, E and F). 4C nuclei in division

represent meiosis I, whereas 2C nuclei in division could represent meiosis II or mitosis (they are a similar process). These findings for *K. veneficum* are significant because, to our knowledge, actual quantification of a meiotic process in a dinoflagellate has only been reported once before. In the dinoflagellate *Prorocentrum micans*, DNA measurements by microfluorometry were used to demonstrate that vegetative cells and gametes had 1C DNA and early planozygotes had 2C DNA, which was then doubled to 4C DNA before nuclear cyclosis followed by zygote division in a 4C→2C→1C DNA reduction sequence that was quantitatively determined to prove meiosis [65, 66]. Based on these data on observations and cell features, Bhaud et al. [65] generated a life cycle diagram for *P. micans*. With similar data and following their example, we diagram the life cycle of *Karlodinium veneficum* as it is presently understood (Figure 26).

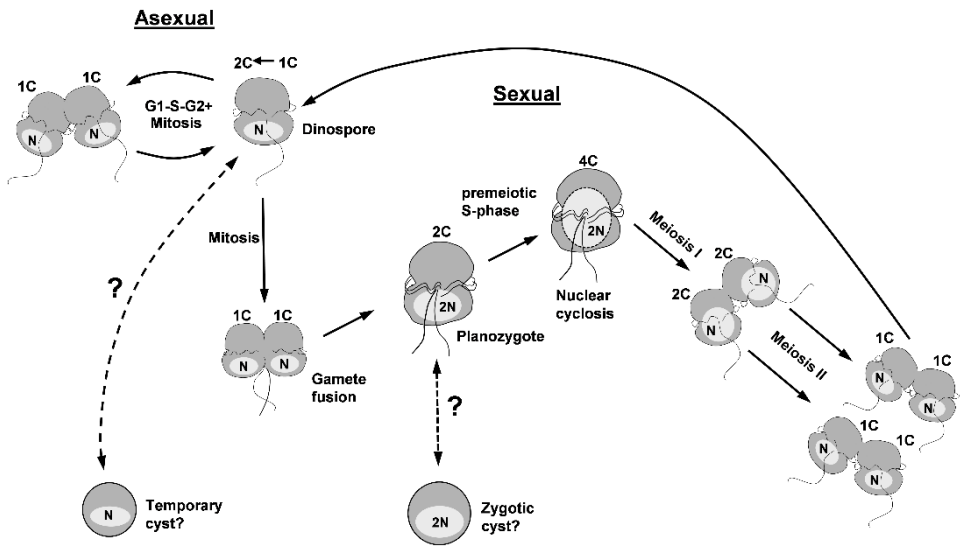


Figure 26. Life cycle of *Karlodinium veneficum*, based on observations and DNA content measurements to date. Dotted arrows/question marks are stages that are more speculative. N indicates ploidy (inferred), whereas C indicates DNA content (determined).

Resistant, nonmotile stages commonly known as cysts have not been observed as a consistent phenomenon in any *K. veneficum* strain under any conditions. In many, but not all dinoflagellates [35], resting cysts are a product of the sexual cycle. *K. veneficum* may be heterothallic, like closely-related *Karenia brevis* [67], which might explain why sexuality is apparent in some cultured strains but not others (i.e., some cultures may have been initiated with a planozygote). To investigate whether heterothallic sexuality might lead to resting cyst formation, we have pair-wise crossed multiple *K. veneficum* strains, but no resting cysts have yet been observed in any treatment of crossed strains. Treatment of clonal and crossed strains with reduced temperatures (10°C and 4°C) has occasionally resulted in non-motile cells that might be considered temporary cysts, but as with sister taxon *Karenia,* [67] nothing resembling these have ever been found in nature. As such, they cannot be ruled out as culture artifacts rather that *bona fide,* demonstrable and consistently repeatable life history stages. At present, it seems that *K. veneficum* is holoplanktonic, and the possibility of yet-undiscovered resting cysts, along with where *K. veneficum* cells overwinter, remains a mystery.

Swimming Behavior

On swimming behavior, Ballantine [3] writes,

> "These organism move in a characteristic fashion. They swim forward in a jerky irregular spiral, usually with the ventral side downwards. Movement is quite rapid and the cell rotates from time to time. The transverse flagellum undulates rapidly in the girdle during movement and the longitudinal flagellum wither vibrates rapidly (during active swimming) or remains almost still, trailing behind (sluggish movement).

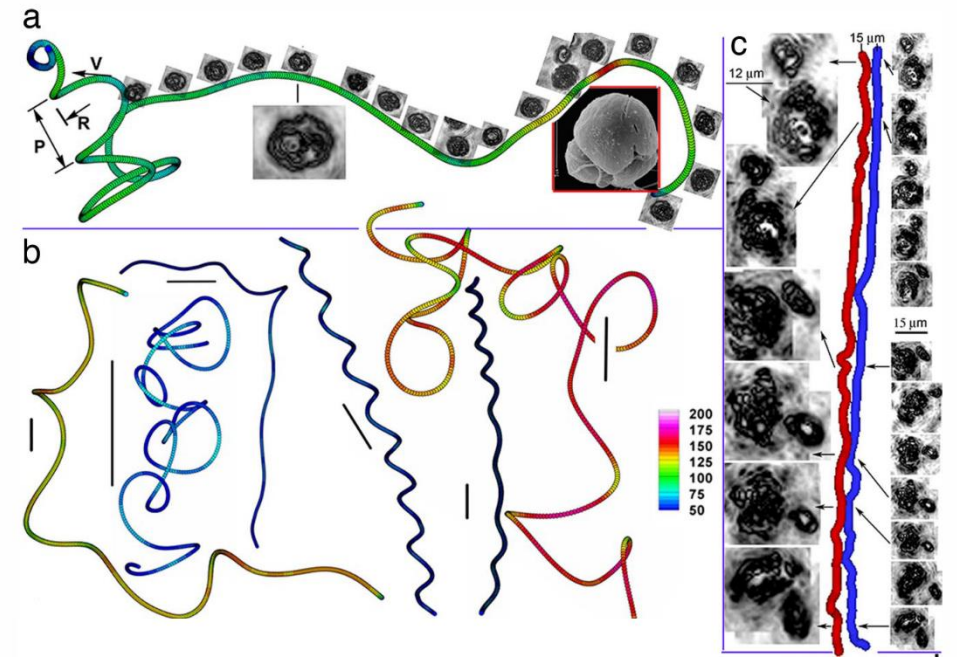


Figure 27. Gallery of swimming patterns observed with *K. veneficum* [69]. (a) A 3D trajectory of *K. veneficum*, color-coded with cell swimming velocity, superimposed with in-focus images sampled at every 40 time steps (0.33 s). (Insets) SEM and reconstructed image of *K. veneficum*. (b) Sample characteristic trajectories of *K. veneficum* cells. (Scale bars: 50 microns) (c) Sample trajectories of *K. veneficum* and S. major moving in unison. To the right of trajectories are shown selected in-focus images of predator and prey cells, with arrows indicating timing of image. To the left of the trajectories are shown some of the images magnified x4 with arrows indicating corresponding timing. [Reproduced with permission (Copyright (2007) National Academy of Sciences, U.S.A.].

Using digital holographic microscopy Sheng et al. [68] showed *K. veneficum* exhibited complex highly variable swimming behavior (Figure 27) that could be characterized by radius and pitch of helical swimming trajectories and by translational and angular velocity. *K. veneficum* moved in both left- and right-hand helices. When presented with its prey (*Storeatula major*), *K. veneficum* reduced its velocity, radius, and pitch but increased its angular velocity, changes that reduce its hydrodynamic signature while still scanning its environment as "a spinning antenna." In the nontoxic strain MD5 which we view as model for *Gymnodinium vitiligo (*PLY102*)* there was no change in swimming behavior in the presence of prey [69]. MD5 did not eat, nor did it care whether prey were present or not! [68]

Ecology and Toxicity in *K. veneficum*

Among HAB species, the ability to link toxin production to growth strategies and life histories has proven generally difficult, but examples are accumulating that support the idea that toxic substances produced by phytoplankton do serve a role in the ecology of the organism. Sometimes these toxins are different from the toxins associated with public health impacts of HAB species [70], such as the allelochemicals exuded by *Karenia brevis* that are distinct from brevetoxins [70]. Among mixotrophic species, Skovgaard and Hansen [71] and Tillman [72] described prey immobilization that led to enhanced feeding in *Prymnesium parvum*. Blossom et al. [73] showed prey immobilization through secretion of a toxic mucous trap in *Alexandrium pseudogonyaulax*, which constituted a novel mode of prey capture and uptake. A follow up study [74] confirmed phagotrophy by use of this method in four of five strains of *A. pseudogonyaulax* tested, but not in any of the other eight species of *Alexandrium* tested. Xu and Kiorbe [75] demonstrated that 10 of 12 toxic dinoflagellates tested showed true grazer defense properties – that is they prevented grazing rather than toxifying predators after ingestion, an evolutionarily stable strategy.

In *K. veneficum*, evidence exists that the same toxin (karlotoxin) that is responsible for fish kills also shows grazing deterrent [76–78, 89], allelopathic [10], and prey-immobilization effects [69, 78]. Conceptually, this suggests a critical role of karlotoxin in the ecology of *K. veneficum* as a strategy for maintaining positive net population growth rate by increasing cell division rate and/or decreasing grazing losses. However, understanding how karlotoxin affects potential prey / competitors and how different growth modes (e.g., nutrient replete / deplete, phototrophic / mixotrophic) affects the cellular toxin quota of *K. veneficum* remain important questions to address in order to understand the role of karlotoxin in *K. veneficum* ecology.

Although several studies have documented the effects of KmTx on prey motility and/or survival [68, 69], none have examined the sub-lethal effects on prey photosynthesis. Exposure of a common prey item (*S. major*) to purified KmTx 1 affected cell morphology and PS II efficiency (Figure 28). Exposure to KmTx 1caused *S. major* to swell, a result consistent with the mechanism of KmTx as a non-specific pore former in membranes containing des-methyl sterols [9, 79]. This coincided in a dose-dependent manner with inhibition of PS II (e.g., Fv/Fm), as measured with a Tox-Y-Pam fluorometer. Based on the mechanism of KmTx, pore-formation in chloroplast membranes is unlikely given the lipid profile, but depolarization of the cell membrane (as evidenced by cellular swelling) may have an effect on cellular ion balances that translates to impaired photosynthesis. During a *K. veneficum* bloom KmTx levels of 100 to >1000 ng mL^{-1} have been measured [80, 81], well above the range at which effects on cell morphology and PSII were observed. Since most toxin in living cells is cell-associated, cell-cell contact or cell lysis is likely an important factor in exposing potential prey/competitor cells to toxin *in situ*. The inset picture shows *K. veneficum* with a captured *S. major* that appears to be swollen, perhaps due to the effects of karlotoxin delivered via cell-cell contact.

Changes in cellular toxin quota associated with a shift from phototrophic to mixotrophic growth were examined in *K. veneficum* strain 2778 (Figure 29). A phototrophically-maintained culture (f/2 –Si P/10) was fed cryptophyte (*Storeatula major*) prey and tracked in

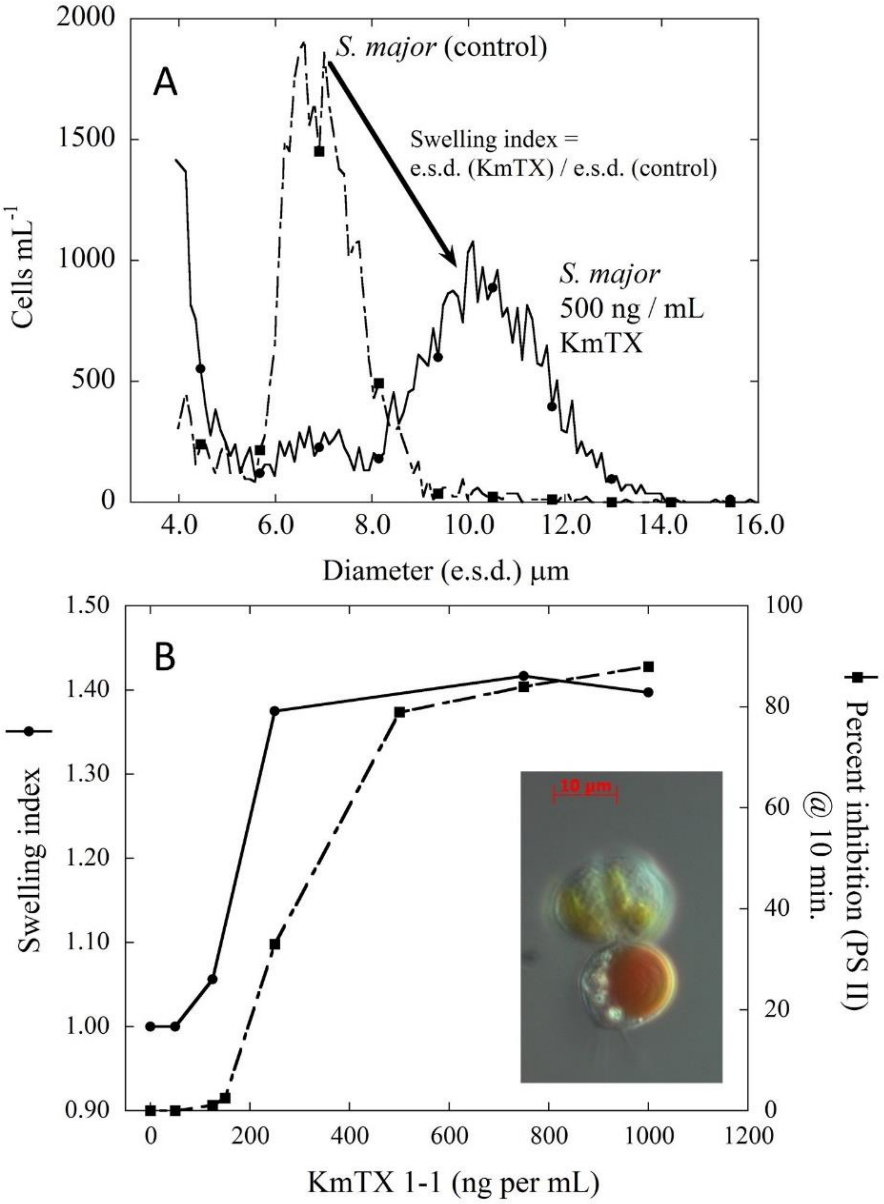


Figure 28. Dose-dependent effect of purified KmTx 1 on *S. major* (A) cell morphology and (B) PS II.

semi-continuous culture. Prey addition was controlled to allow depletion between dilution cycles. Feeding rate during mixotrophic growth averaged 2.7 ± 1.4 prey predator^{-1} d^{-1}. Lower cellular toxin quotas and higher growth rates were observed during mixotrophic growth periods compared to phototrophic periods. Further experiments investigated whether or not this reduction in cellular toxicity was associated with cell division or simply with prey ingestion, taking advantage of the fact that for the first day after feeding a phototrophically-maintained culture, prey ingestion typically occurs without cell division (Figure 30A). Over the first 10h of the experiment, during which *K. veneficum* strain 2278 cultures consumed cryptophytes (Figure 30B), cellular toxin levels did not differ from the phototrophic control

cultures. However, between the 24h time point and the end of the second day of the experiment, when mixotrophic cultures had grown to a higher density than the phototrophic controls, the cellular toxin level of mixotrophic cultures was lower (Figure 30C). A conclusion for this strain is that mixotrophic cells are less toxic than phototrophic cells, and this appears to be related to growth rate since toxin content did not change during the period when cryptophytes were initially consumed but *K. veneficum* cells did not divide. This conclusion contrasts with what Lin et al. [82] observed. They found significant toxicity (oyster larval bioassay) of both nutrient-stressed and mixotrophic cultures of *K. veneficum* (CCMP 1975). Since two different measures of toxicity were used (KmTx detection vs. bioassay) it is difficult to draw conclusions, but further investigation of the impact of mixotrophic feeding on *K. veneficum* toxicity is warranted. Lowering glycolate pools in mixotrophic growth could also explain the differences. It should be noted that *K. veneficum* has another toxic component that is not karlotoxin. The abundant fatty acid all-cis-3,6,9,12,15-octadecapentaenoic acid (18:5n-3) is highly toxic [83].

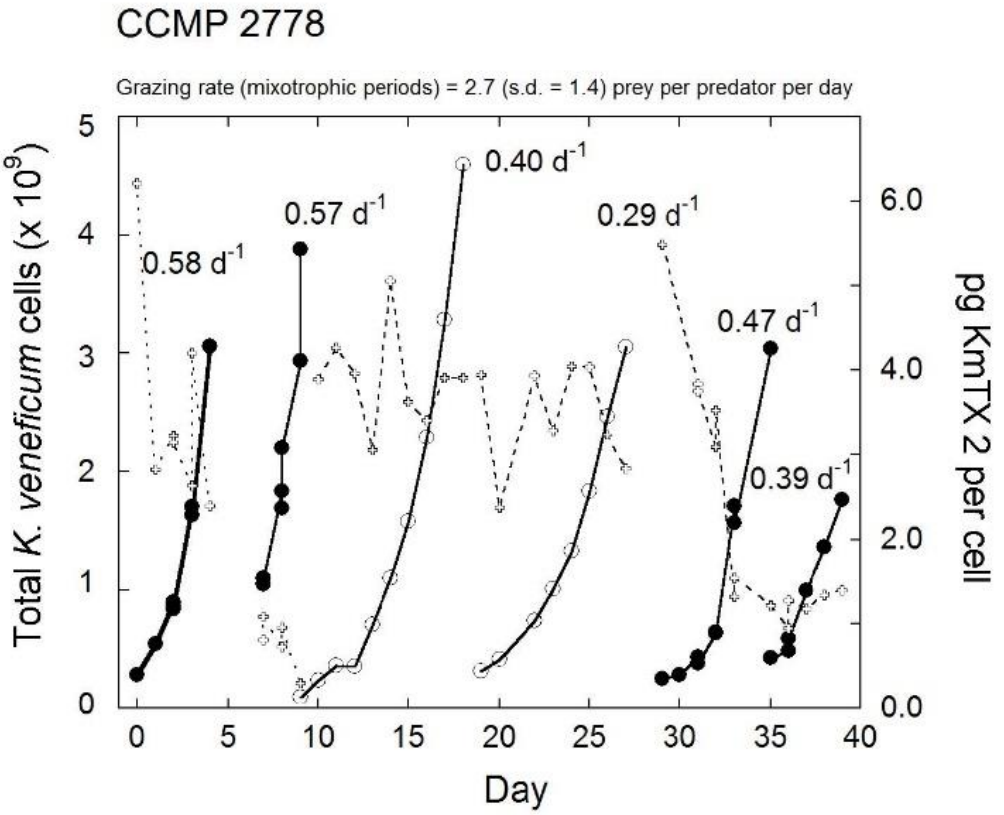


Figure 29. Growth experiment where *K. veneficum* strain CCMP 2278 that had been maintained as phototrophic was pulse fed prey cryptophytes (*Storeatula major*). Alternating phototrophic and mixotrophic growth periods were achieved by allowing prey to be grazed down to depletion before dilution of cultures with inorganic growth medium (f/2 with 1/10 P). Daily measurement of cell density (●&○) and KmTx (+) were made to track changes in cellular toxin quote with changes in growth mode.

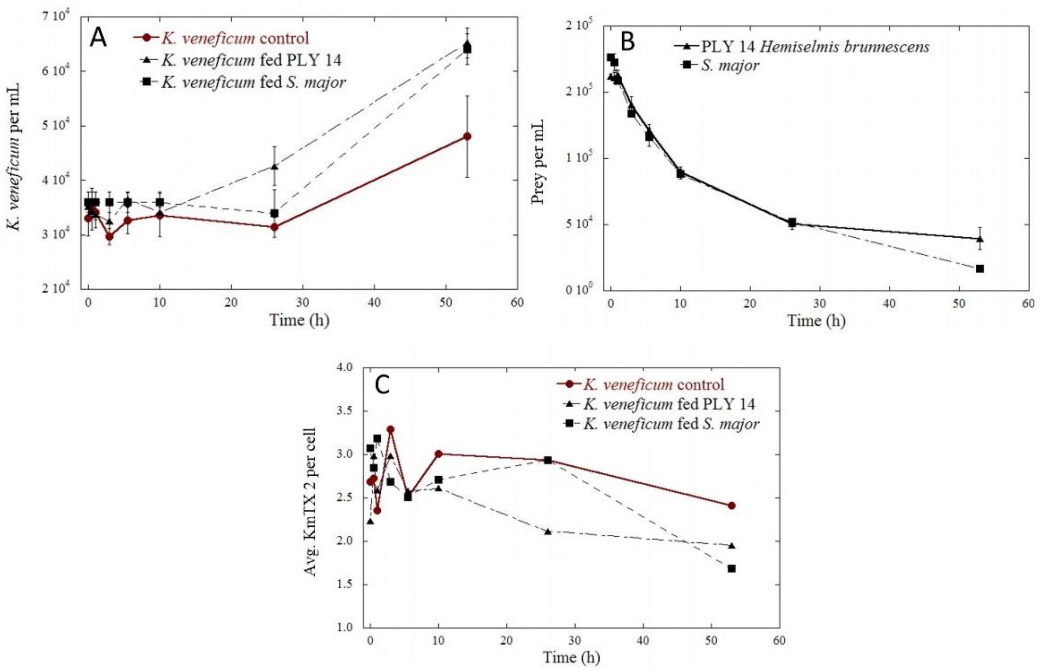


Figure 30. Changes in cellular toxin quota associated with short term phototrophic vs mixotrophic growth in *K. veneficum* strain 2778.

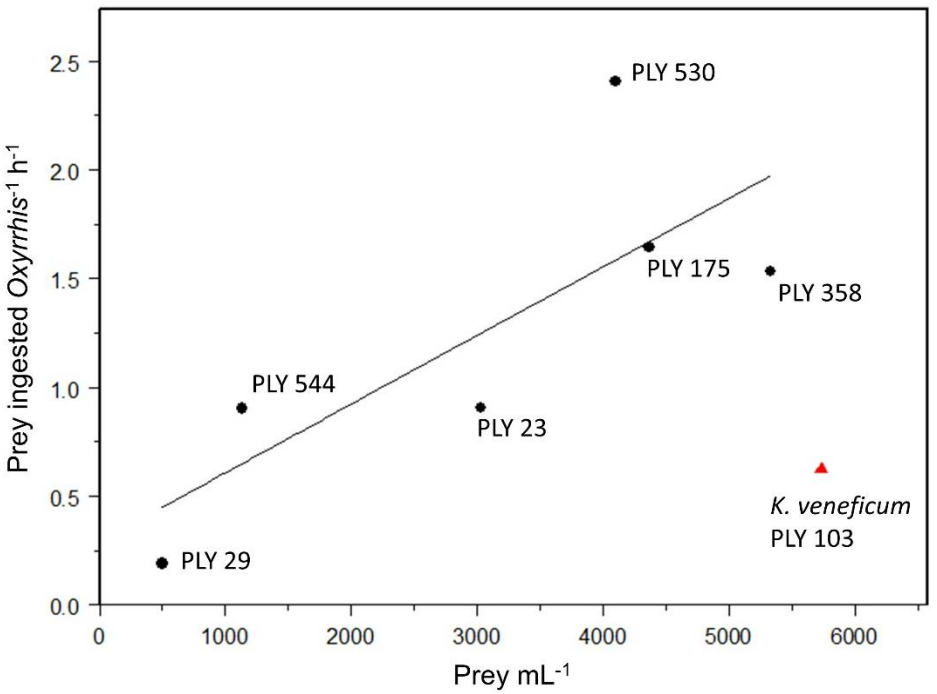


Figure 31. *Oxyrrhis* grazing rates vs. prey density. PLY103 is *Karlodinium veneficum*, all others are cryptophytes of similar size as PLY103.

Various studies have described anti-grazing and allelopathic effects of karlotoxins on other protists with which *K. veneficum* interacts. Experiments were conducted at the MBA Citadel Hill laboratory to examine the grazing of the heterotrophic dinoflagellate, *Oxyrrhis marina*, on *K. veneficum* PLY103 as compared to other prey. *Oxyrrhis* strains PLY 697, 997A, and 2098 were tested in a preliminary experiments using cryptophyte PLY 175 as prey. PLY 697 and 997A were the same isolate maintained on bacteria (697) or *Rhodomonas* algae (997A). *Oxyrrhis* strain 2098 was used for all further experiments. Unfortunately no other strains of *K. veneficum* were available so grazing of *O. marina* on cryptophytes prey of similar size to *K. veneficum* was used as a comparison. *Karlodinium veneficum* PLY103 and cryptophyte strains PLY 544, 530, 358, 175, 23, and 29 (in ESAW complete) were used as prey at densities below 7,000 ml^{-1}. *Oxyrrhis* density was approximately 680 ml^{-1} in each grazing assay. Assays were conducted in triplicate in 24-well plates. All prey were examined in triplicate with and without predator. Grazing rates were calculated by dividing the difference in prey abundance between prey with predators and prey without predators after a 2 h incubation period at 15°C.

Oxyrrhis marina grazing rates varied for prey tested and that among the cryptophytes tested *O.* marina grazing rate trended up with prey density (Figure 31). Viewed in the context of the prey density – grazing relationship, *O. marina* grazing on PLY103 was relatively low (Figure 31), consistent with other studies finding reduced grazing of *O. marina* on toxin-producing strains of *K. veneficum* [76].

Global Distribution and Potential Roles of Karlotoxin in *Karlodinium veneficum*

Karlodinium veneficum was originally described as toxic and is now recognized as a harmful algal bloom (HAB) species - an ichthyotoxic dinoflagellate with a history of fishkills and other marine life moralities worldwide [49]. Karlotoxin is the causative agent of *K. veneficum*-associated fish and marine life kills [51, 84] but also has demonstrated allelopathic [76, 85], anti-grazing [78, 86, 87], and prey-immobilization effects [49, 69, 87] although recent evidence suggests allelochemicals besides karlotoxins may also be present in some strains [88]. Thus, a role for karlotoxins in the ecology of *K. veneficum* consistent with maximizing net population growth rate through increased cell division rate or decreasing losses due to grazing is suggested. However, cellular toxicity varies widely among strains, including non-toxic strains [4] that also sometimes form blooms in nature.

K. veneficum Distribution and Reported Blooms

This section will review the distribution of *K. veneficum* globally (Figure 32), focusing on reports published since the last review [49]. It is worth noting that current global distributions of *K. veneficum*, as for other protists, likely reflect natural patterns as well as anthropogenic movements of species due to processes such as ballast water uptake and discharge (e.g., [89]- Steichen et al. [90] employed genetic techniques to detect *Karlodinium* and other dinoflagellates in ballast water discharged by ships in Port of Houston, Texas, USA. Additionally, contemporary models of *K. veneficum* abundance have incorporated potential

prey availability in addition to the more commonly used physical / chemical parameters that typify phytoplankton models.

The Chesapeake Bay region has a history of *K. veneficum* blooms and fish kills [51] and recent analyses using long term data sets have shed light on the ecological controls of *K. veneficum*. Li et al. [91] analyzed the relationship between three HABs (*Prorocentrum minimum*, *K. veneficum*, and an index of cyanobacterial HABs) and Chesapeake Bay water quality between 1991 – 2008. In comparing water quality conditions associated with *K. veneficum* blooms relative to *P. minimum* blooms, *K. veneficum* blooms tended to occur at higher SST, lower DIN, and higher DIP (e.g., lower DIN:DIP) [91]. Subsequent time series analyses [92] of water quality and phytoplankton community composition in Chesapeake Bay resulted in optimal models in which prey availability (identified as *Cryptomonas* spp. and unidentified microflagelates < 10 μm) was an important predictor of *K. veneficum* abundance in addition to the more commonly examined phyico-chemical parameters. An important outcome of both of these studies was that *K. veneficum* abundance shows a significant increase over time in the mesohaline part of Chesapeake Bay [91, 92] related to nutrients delivered in the dissolved and /or particulate form as prey.

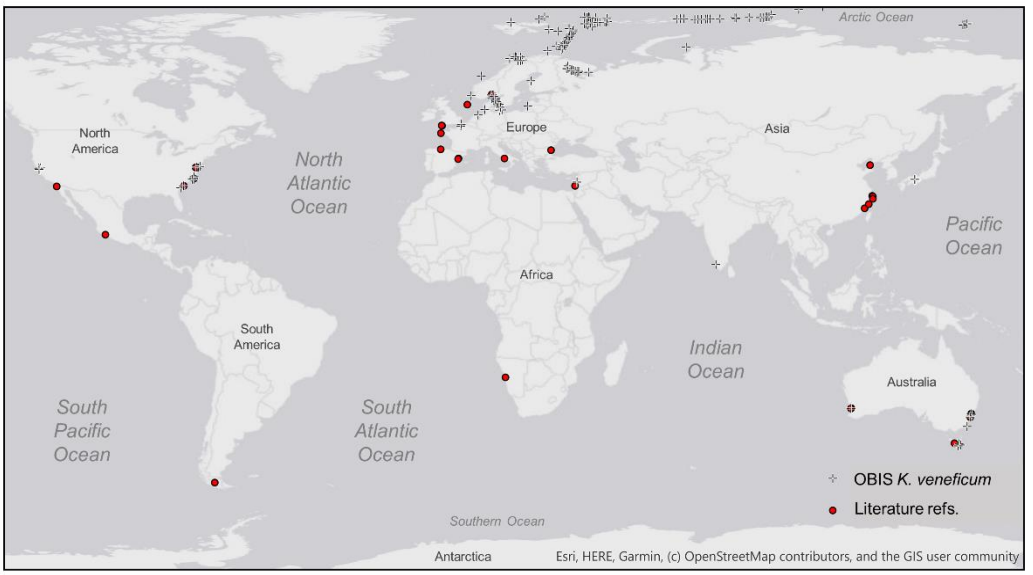


Figure 32. Distribution of *Karlodinium veneficum* as recorded in primary literature (red) and the Ocean Biogeographic Information System (https://obis.org/taxon/233037) (black x's). Sources include Adolf et al. 2015 [80], Ajani et al. 2001 [121], A. Tatters (USC, pers. Comm.), Balantine 1956 [3], Bjornland and Tangen 1979 [130], Braarud 1959 [131], Busch 2016 [132], Deeds et al. 2002 [51], deSalas et al. 2005 [51], Dzhembekova et al. 2018 [134], Escobar-Morales and Hernandez-Becerril 2015 [135], Garcés et al. 2006 [33], Guallar et al. 2016 [93], Hall and Paerl 2011 [136], Hall et al. 2008 [33], Huang et al. 2019 [33], Kempton et al. 2002 [84], Li et al. 2000 [13], Li et al. 2015 [91], Lin et al. 2018 [92], Luo et al. 2018 [137], Moreno-Pino et al. 2018 [99], Siano et al. 2009 [138], Raveh et al. 2019 [100], Toldrà et al. 2018 [139], Wang et al. 2011 [94].

Other forecast models designed to predict *K. veneficum* do not consider potential prey items as predictors. Guallar et al. [93] used an artificial neural network to model *Karlodinium* spp. abundance in Alfacs Bay (NW Mediterranean), and area of known and recurrent *K. veneficum* blooms and ichthyotoxicity. A combination of lagged *Karlodinium* spp. abundance and environmental variables produced useful week-ahead forecast models. Although potential prey items were not tested as an input variable in these models and factors relating to freshwater inputs to the Alfac Bay stood out as strong predictors, the authors did speculate that some of the environmental drivers associated with *K. veneficum* forecasts (e.g., low light) might reflect a role of mixotrophic nutrition [93].

Wang et al. [94] first recorded *K. veneficum* in the East China Sea (ECS) but Dai et al. [95] presented the first record of *K. veneficum* blooms in the ECS, co-occurring with *Prorocentrum donghaiense* in seasonally temperature-stratified waters. Subsequent culture studies [96] showed that *K. veneficum* toxin inhibited (in co-culture or through addition of isolated toxin) the growth of *P. donghaiense* and that *K. veneficum* fed upon *P. donghaiense*, suggesting that feeding upon *P. donghaiense* may be an important factor supporting *K. veneficum* blooms in the ECS. Huang et al. [97] found *K. veneficum* in 12 of 38 samples analyzed by qPCR assay in Xiangshan Bay of the ECS, with positive hits occurring in the bottom waters (between 9-100m depth) and interpreted as possibly the result of *K. veneficum* using heterotrophic nutrition to sustain itself under the deep, low-light conditions, but this speculation requires further investigation.

The Swan River (SRE) in Western Australia is an anthropogenically impacted subtropical estuary that has experienced *K. veneficum* blooms and fish kills as far back as 1999. The co-occurrence of fish kills and *K. veneficum* blooms beginning in July 2003 prompted further study of the relationship between these conditions. Adolf et al. [80] reported a sequence of events including a large *K. veneficum* bloom with high toxicity levels, a stratified water column in a narrow section of the SRE with hypoxic bottom waters, and a fish kill of occurring toward the end of the *K. veneficum* bloom. The blooming population of *K. veneficum* was observed to ingest added cryptophyte prey, indicating the potential for mixotrophic nutrition, although this was not quantified nor widely observed in the bloom population. Analyses of fish gills collected at the site showed symptom of karlotoxin exposure (e.g., [98]), but the co-occurrence of *K. veneficum* with hypoxic conditions prompted questions of possible synergistic effects between these two well-known ichthyotoxic agents.

The more widespread use of molecular genetic techniques to detect *Karlodinium* spp. has resulted in additional records of its occurrence. Moreno-Pino et al. [99] noted presence of *Karlodinium* by NGS in Strait of Magellan near the southern tip of South America, where SST was 2 – 5ºC and salinity was 27.5 – 32.5. Raveh et al. [100] found *K. veneficum* in 18S rRNA sequences and confirmed its presence by microscopy following bioassay additions of high nitrate well amelioration brines to oligotrophic southeast Mediterranean Sea water in winter (18ºC, 38.5 salinity) and winter (30.5ºC, 38.5 salinity) experiments. The small size and 'naked' nature of *K. veneficum* makes the availability of molecular probes for detection an important tool for those interested in understanding the distribution of this organism.

Top Down Forces Regulating *K. veneficum* Abundance

Blooms of *K. veneficum*, as with most harmful algal bloom species (HABs), likely depend on a complex suite of physical and biological conditions favoring their growth over that of competitors. Hall et al. [81] and Garcés et al., [47] both stress the importance of physical water conditions in explaining observed natural accumulations of *K. veneficum* (referred to as *G. corsicum* in Garcés et al., [47]. Bloom initiation and growth were favored by high nutrient availability and reduced dispersal during the period of declining riverine discharge after storm events.

One possible bloom control factor for *K. veneficum* is the co-occurrence of a parasitic dinoflagellate belonging to the genus *Amoebophrya*. Members of this group were first described by the French protistologist Cachon and have been reported to infect dinoflagellates, ciliates, radiolarians, chaetognaths and siphonophores [101, 102]. Forty different free-living dinoflagellate species representing over 20 genera are estimated to be infected by *Amoebophrya spp*. [103]. *Amoebophrya ceratii* [101] was originally described as a species having a broad dinoflagellate host range, however molecular data and controlled laboratory infection experiments have shown that this is a species complex comprised of *Amoebophrya ex. K. veneficum, A. ex. Akashiwo sanguinea* and *A. ex. Gymnodinium instriatum* [104, 105].

Amoebophrya likely plays an important role in the ecology of estuaries and open ocean environments by consuming its host and contributing to the microbial loop [106]. While the ecology of *Amoebophrya* in specific estuarine and coastal systems has been investigated [107], the global importance of *Amoebophrya* and related organisms was not clear until the development of environmental clone libraries. These libraries revealed surprising diversity and abundance of sequences related to *Amoebophrya* in the world's oceans, particularly at depth [108–110]. This emerging lineage, dubbed the marine alveolates in environmental molecular phylogenies, roughly corresponds to the Syndiniales, as described by Cachon and Cachon [111].

Infection by *Amoebophrya* species can occur in the cytoplasm, nucleus, or both as in the case of *A. ex. K. veneficum* [112]. *Amoebophrya* species enter their host as a dinospore, grow inside the cell in a vegetative form known as a trophont, and begin to multiply within the cell to form the vermiform (or beehive) stage which ultimately causes the host to burst and release many new *Amoebophrya* dinospores [101, 102]. In order to survive and propagate, these new dinospores must find new host cells within a couple of days as they are good food for ciliates and other heterotrophic protists [113]. In contrast, dinospores of *Amoebophrya ex. K. veneficum* can remain infective for up to eleven days [112]. The infectivity rate is generally lower (90%) in laboratory experiments with *A. ex. K. veneficum* compared to experiments with *A. ex. A. sanguinea* and *A. ex. G. instriatum* (100%; [112]). It was proposed that this difference was due to toxin production by the host, however Bai et al. [114] showed that infection of *K. veneficum* by *Amoebophrya ex. K. veneficum* was positively correlated with host toxicity, i.e., nontoxic strains are poorly infected. Moreover, karlotoxin cell quotas remained similar between infected and uninfected hosts until parasite emergence [114]. Place et al. [115] found that *Amoebophrya ex. K. veneficum* contained the same dominant sterol (Gymnodinosterol, Figure 10) as *K. veneficum*. This sterol profile grants *K. veneficum* immunity from the effects of its own toxin, which lead to the hypothesis that host/parasite pairs that had a similar sterol profile would possess the same protection against karlotoxin.

Toxicity of the host species also was shown to not be a factor in the infection of natural populations of *Dinophysis* by *Amoebophrya sp.* [116, 117].

Time series sampling of *K. veneficum* and *Amoebophrya* (Figure 33) showed a tight coupling ($R = 0.69$ $p < 0.001$) on approximately daily time scale. In time series such as this, it is difficult to say whether changes in cell abundance are due to growth, death or advection. For instance, the increase in cell abundance from day 189 – 190 would equate to a growth rate of >9 d^{-1}, which has not been observed in *K. veneficum* previously and is likely due to advection of cells to the sampling area. On the other hand, declines in cell abundance (e.g., day 190-191) are equally difficult to ascribe to advection vs. biological processes (including *Amoebophrya* infection). *Karlodinium veneficum* cultures exposed to *Amoebophrya* showed ~ 90% infection rates by 10h, and suffered drastic declines in cell abundance within 60h, [118] so a role of *Amoebophrya* in day to day population dynamics is possible. The tight coupling between *K. veneficum* and *Amoebophrya* sp. in natural populations shown here underscores the potential top-down regulation of *K. veneficum* populations in nature and needs to be considered when trying to understand population controls.

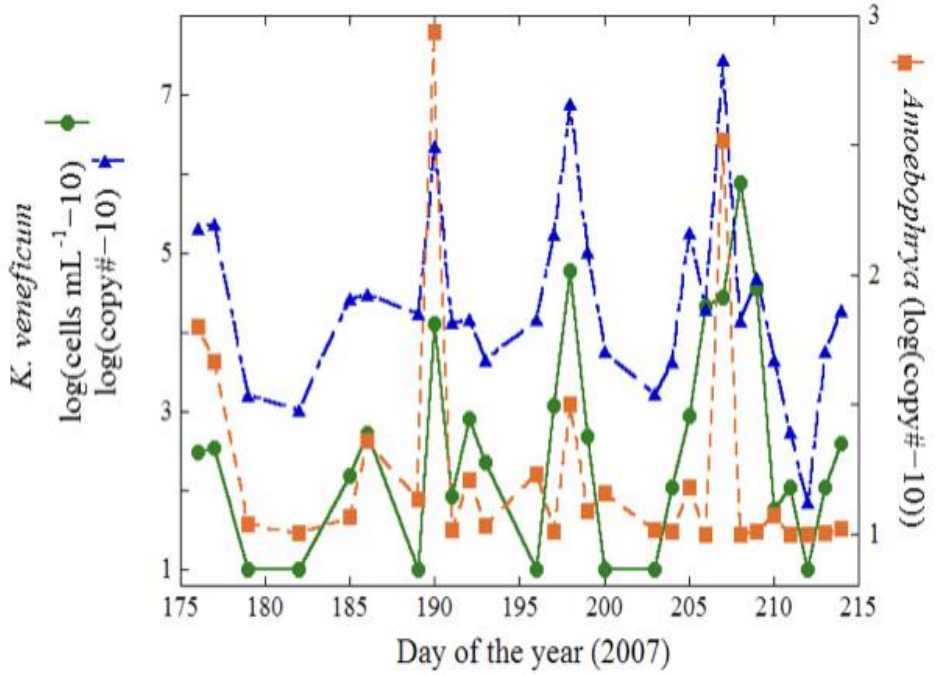
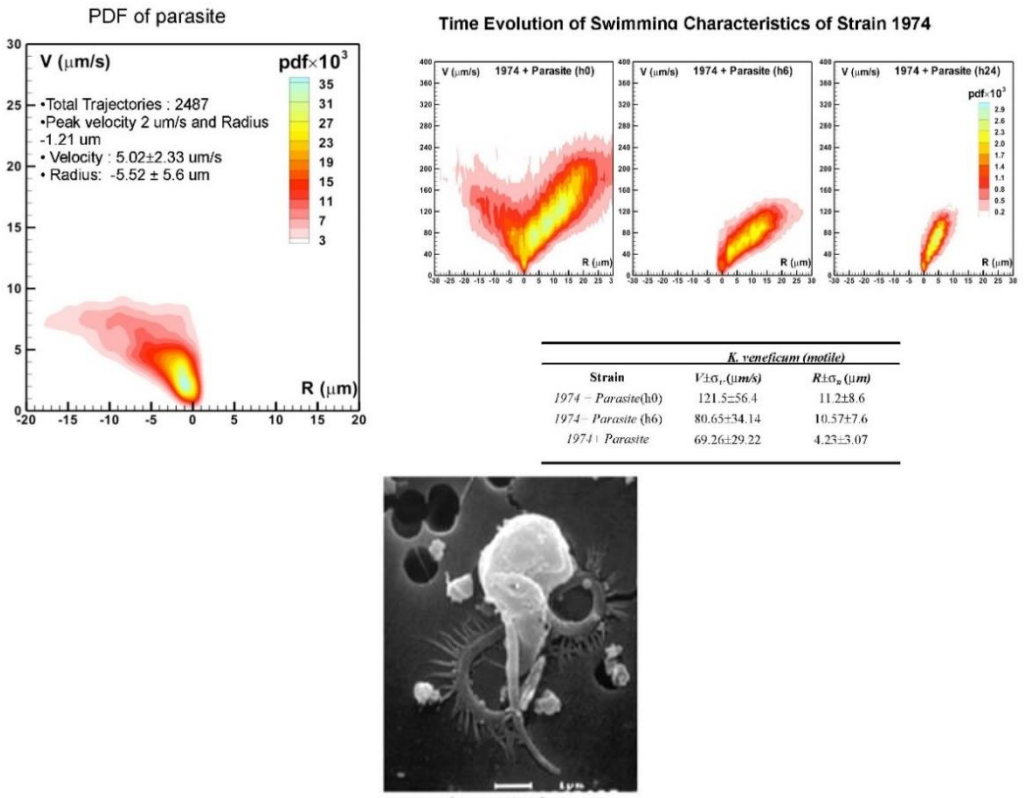


Figure 33. Time series of *K. veneficum* and *Amoebophyra ex. K. veneficum* in the Inner Harbor, Baltimore, MD.

Infection in the laboratory by *Amoebophrya ex. K. veneficum* of *K. veneficum* CCMP 1974 results in a reduction of swimming speed and turning but did not stop mobility of the host (Figure 34). This is consistent with field observations [112] which showed infected host was found deeper in the water column. The parasite swims in a left handed helix at the amazing slow speed of 2 um/sec, nearly 60 fold slower than the host, which indicates infection must occur when the host is still.



	K. veneficum (motile)	
Strain	$V \pm \sigma_V$ (*μm/s*)	$R \pm \sigma_R$ (*μm*)
1974 – Parasite(h0)	121.5±56.4	11.2±8.6
1974 – Parasite (h6)	80.65±34.14	10.57±7.6
1974 Parasite	69.26±29.22	4.23±3.07

Figure 34. Joint probability density functions (PDFs) of velocity magnitude (V) and radius of helical trajectories (R) from digital holography of *Amoebophrya* ex. *K. veneficum* exposed to *K. veneficum* CCMP 1974 in laboratory. Right - Parasite alone; Left - Host *K. veneficum* CCMP 1974 exposed to parasite (Sheng and Place, unpublished). Inset, SEM of *Amoebophrya* ex. *K. veneficum*. (Courtesy of W. Coats).

Synthesis: Why Does *K. veneficum* (Sometimes) Form Ichthyotoxic HABs?

"Success" among the phytoplankton requires maintenance of net population growth above losses, and the formation of dense 'blooms' by HAB species is often considered to occur as a result of an uncoupling between population growth and loss terms facilitated by interactions between the physiological capabilities of the HAB organism and the physical/chemical environment [119]. There are several described life-history strategies that are thought to contribute to uncoupling of growth and losses among the HAB-forming algae, including toxin-mediated grazer avoidance, cyst formation, diel vertical migrations, and mixotrophic nutrition. *K. veneficum* is not known to produce a resting cyst (this chapter), so the strategy involving this life history stage may not be significant for this species. Diel vertical migrations (DVM) have been observed in *K. veneficum* [81, 120] and its blooms are often associated with stratified water column conditions wherein light and nutrient sources are physically separated, suggesting an important role of DVM in *K. veneficum*'s life history. However, DVM is unlikely to distinguish *K. veneficum* from other flagellated phytoplankton, as this appears to be a common trait among HAB and non-HAB motile forms alike [81].

Karlotoxin affects susceptible cells through non-specific pore formation resulting in cytotoxicity through osmotic shock and lysis [79]. Sub-acute exposure of susceptible cells results in a variety of effects described as either allelopathic, grazing deterrent, or prey-immobilizing depending on the experimental context in which interactions between *K. veneficum*, potential prey and/or grazers are being viewed. An idea put forward by Xu and Kiørboe [75], that toxins evolved as an adaptation for grazer deterrence may have other side effects that are non-adaptive and only emerge at high cell densities, seems to fit *K. veneficum* well. Karlotoxin does fit the definition of a grazer deterrent given by Xu and Kiørboe [75] in that it deters (some) grazers before they ingest cells, and it also confers grazing protection on co-occurring non-toxic strains [76]. This idea that phytoplankton 'allelochemicals' actually evolved primarily as grazer deterrents is also consistent with findings of Jonsson et al. [121] who concluded that allelopathy cannot explain HAB formation.

In nature, *K. veneficum* likely experiences heavy grazing pressure from micro-zooplankton. Johnson et al. [122] studied *K. veneficum* in Chesapeake Bay and showed that grazing losses are significant for *K. veneficum* and highly correlated to grazer biomass, particularly *O. marina*. Thus, in order to bloom, growth needs to be uncoupled from grazing at least short-term (e.g., [87]). Physical factors such as freshwater flow can uncouple grazers from phytoplankton prey (e.g., [123]), while the production of grazing-deterrent toxins is likely a more reliable means. The increase in cellular toxicity associated with lower growth rates due to nutrient limitation [50, 82] is consistent with an increased role for grazing deterrence to preserve net population growth when cell division rates decline. The high cellular toxin quota of *K. veneficum* PLY103 coupled to the apparent absence of phagotrophic abilities also argues that karlotoxins evolved primarily as a grazer deterrent and perhaps play an emergent role as a means to increase prey capture rates. In practice, however, karlotoxin serves both purposes simultaneously: in three-way culture experiments with *K. veneficum*, a prey cryptophyte (*S. major*) and potential general predator (*Oxyrrhis marina*), toxic *K. veneficum* ended up dominant by avoiding *O. marina* grazing and consuming cryptophytes, whereas non-toxic *K. veneficum* was consumed by *O. marina* along with the cryptrophytes [76]. Cryptophytes are not the only prey consumed by *K. veneficum*, but they are likely an important prey item in the field for *K. veneficum* as they are too many other grazers [124], making the idea that KmTx inhibits the grazing of co-occurring heterotrophs on a common pool of prey an important advantage conferred by KmTx production.

Mixotrophic nutrition is clearly a critical component of the *K. veneficum* ecology. Several studies note the co-occurrence and feeding activity of *K. veneficum* on prey items in the field [13, 96, 125], although there are several studies in which mixotrophy is not observed or obvious during the high biomass bloom period [80, 81]. This might indicate that mixotrophy is less important to maintaining established blooms and more important to bloom initiation [76]. Along these lines, Egerton et al. [126] observed a rainfall event, followed by a *Cryptomonas* sp. bloom, followed by a bloom of mixotrophic *Gyrodinium instriatum* in the Chesapeake Bay region. New, dynamic models of mixotrophic *K. veneficum* growth underscore the importance of considering environmental influences that are mediated through initial effects on potential prey populations [92].

It is probably overly simplistic to look at these results and hang all of the successes of *K. veneficum* blooms on production of KmTx without considering the cost of toxin production and the known existence of non-toxic strains. If toxin production, in *K. veneficum* or other species, was costless and highly beneficial then why is toxin production not a universal trait

among phytoplankton (e.g., [75])? Chakraborty et al. [127] modeled PST production by *Alexandrium* spp. to demonstrate that under resource-limiting conditions the physiological cost of toxin production is non-trivial, but the benefits (e.g., grazer deterrence) outweigh the costs. Non-toxic *K. veneficum* strains are more susceptible to grazing losses and perhaps less voracious grazers [76, 76], but they are also less susceptible to infection by the potentially bloom-ending parasite, *Amoebophrya* ex. *K. veneficum* [114]. It is likely that *in situ K. veneficum* populations consist of multiple strains having distinct phenotypic and genotypic characteristics including variable toxicity as was found for *A. tamarense* [128]. Documenting this and understanding where being a non-toxic *K. veneficum* is advantageous are two important question for future research. Understanding the strategies employed by a given HAB species and the conditions that characterize blooms will aid management, preparedness and our ability to forecast HABs.

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Chapter 146

CIGUATERA-CAUSING DINOFLAGELLATES IN THE GENERA *GAMBIERDISCUS* AND *FUKUYOA*: DISTRIBUTION, ECOPHYSIOLOGY AND TOXICOLOGY

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ABSTRACT

Ciguatera poisoning results from the consumption of fish and marine invertebrates contaminated with lipid soluble toxins known as ciguatoxins (CTXs) that are produced by benthic dinoflagellates in the genera *Gambierdiscus* and *Fukuyoa*. Overall, 16 species of *Gambierdiscus* and three closely related *Fukuyoa* species are now recognized worldwide.

Occurrence data clearly highlight the current geographical expansion of these organisms from tropical and sub-tropical waters to temperate-like areas, a likely consequence of climate change. Numerous studies have examined *Gambierdiscus/ Fukuyoa* spp. *in vitro* growth responses under varying environmental factors. Results confirm that differences in both tolerance and optimum growth ranges exist not only across species, but across strains as well.

Gambierdiscus/Fukuyoa spp. are the potential source of at least six families of cyclic polyether compounds whose contribution to ciguatera syndrome (except for CTXs) as well as ecological relevance remain to be ascertained. Factors governing toxinogenesis in these organisms are not well understood, but several studies have provided evidence that this functional trait may depend on a combination of abiotic and biotic (including genetic) factors.

Despite the significant advances achieved in the understanding of this phenomenon, ciguatera incidents remain difficult to predict, and their recent expansion to novel areas

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continues to pose a serious threat to the public health, lifestyle and economy of world populations. Suggested areas for future research efforts will be discussed.

Keywords: ciguatera, algal turfs, ecophysiology, biotic factors, toxinogenesis, impacts

1. INTRODUCTION

Ciguatera poisoning represents the most common non-bacterial seafood intoxication globally, and results from the consumption of fish that have accumulated lipid soluble toxins known as ciguatoxins (CTXs) [1 and references therein]. Yet, other marine organisms that are also highly prized by island communities such as bivalves (e.g., giant clams), echinoderms (*e.g.*, sea urchins) and gastropods (e.g., trochus) have been found to be potential vectors for human poisonings by CTXs [2-5]. CTXs are produced by benthic dinoflagellates in the genus *Gambierdiscus* and *Fukuyoa* (Figure 1) that grow preferentially within mixed algal 'turfs' covering degraded coral substrates [6], although these organisms may also be found in sand, coral, detritus and other surfaces (Figure 2). CTXs enter the coral reef food web through grazing by herbivores and detritivores and are further accumulated and bio-transformed in carnivores through predation [7].

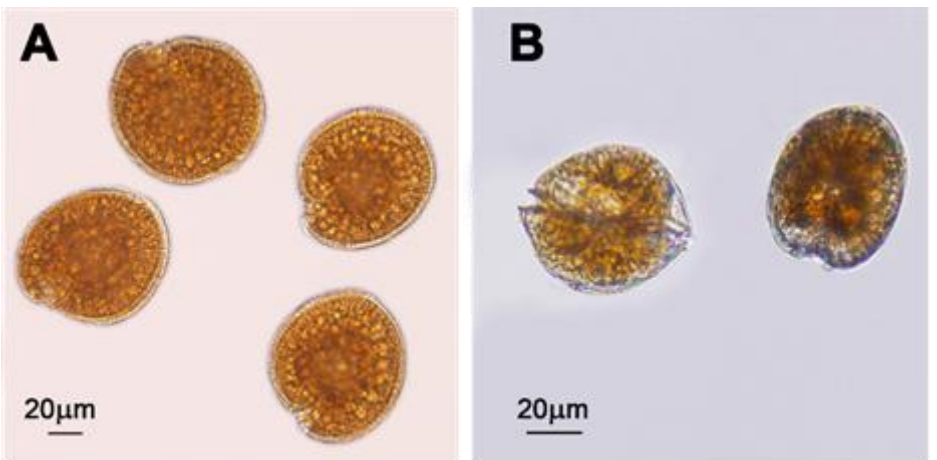


Figure 1. *Gambierdisxus* and *Fukuyoa* cells under light microscopy. (A) *Gambierdiscus toxicus* (© ILM); (B) *Fukuyoa paulensis* (© Lesley Rhodes, Cawthron Institute).

Ciguatera syndrome is characterized by a complex symptomatology, including gastrointestinal, neurological, cardiovascular and general disorders [8, 9, and references therein], that vary between individuals [10] and is often complicated by chronic manifestations lasting months to years [11, 12]. Fortunately, mortality is rare (<0.1%), but the high morbidity of this debilitating and sometimes long-lasting illness makes it a prominent problem for recreational and subsistence fisheries worldwide. Over the past decades, the frequency and distribution of ciguatera have allegedly expanded, a likely consequence of increased human activities and climate change [13-18].

A wealth of data on the main causative agent of ciguatera, *Gambierdiscus* spp., can be found in the literature. However the resurgence of scientific interest and increasing research

efforts noticeable worldwide in the past decade have led to recent advances in the understanding of this organism, particularly with respect to the discovery of novel species and novel compounds. The purpose of this book chapter is to provide an update of the current state of knowledge on the taxonomy, distribution, ecophysiology and toxicology of the ciguatera-causing dinoflagellates *Gambierdiscus* and *Fukuyoa*. A further section will deal with the global socio-economic impacts of ciguatera poisoning, in light of the recent expansion of ciguatera to novel (temperate) areas and the significant burden it represents for world populations. The concluding section will focus on some of the remaining knowledge gaps in this field of research in order to develop reliable control strategies of ciguatera poisoning.

2. BIODIVERSITY OF *GAMBIERDISCUS* AND *FUKUYOA* SPP.

Species composition of *Gambierdiscus* assemblages, and particularly the presence of highly toxic species or strains in a given area, is arguably one of the main driving factors for elevated ciguatera incidence rates and severity of outbreaks [4, 19, 20]. The accurate identification of *Gambierdiscus* species and their associated toxicity is therefore crucial to help predict the potential emergence of ciguatera risk.

Until 1995, *Gambierdiscus* was regarded as a monotypic taxon with all *Gambierdiscus* cells recorded as *G. toxicus* [21]. However, the significant progress achieved over the past two decades in taxonomic studies of this genus, most notably the identification of reliable molecular markers, has allowed for the description of new species of *Gambierdiscus*, suggesting that many of the reports of *G. toxicus* in the early literature may actually concern other species. As a matter of fact, it was further concluded that the original description of *G. toxicus* by Adachi and Fukuyo (1979) [21] likely included multiple species [22], and eventually led to the description of a new epitype of *G. toxicus* in 2009 [23, 24].

Modern taxonomy of *Gambierdiscus* species currently uses both morphological characteristics and phylogenetic analyses. Morphotaxonomy traditionally considers distinguishing characteristics such as cell shape (globular vs. lenticular), cell surface (smooth vs. areolated), cell size, or size and shape of specific thecal plates (e.g., 1p, 2', or 4" using the Kofoid tabulation system) to differentiate between isolates. These key characteristics can be found in detail in the original descriptions of *Gambierdiscus*/*Fukuyoa* species. Molecular analyses rather focus on molecular markers such as nuclear-encoded ribosomal RNA genes (5.8S, SSU and LSU rDNA), since noted plasticity in the morphology of cells can lead to inaccurate identification, particularly when comparing cultured clones to cells collected from the field [25]. Different molecular techniques are currently available, including Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism (RFLP), Fluorescent *In Situ* Hybridization (FISH) probes or complete sequencing of the targeted genes [20, 26].

Overall, 16 species of *Gambierdiscus* are now recognized worldwide: *G. toxicus*, *G. belizeanus*, *G. australes*, *G. pacificus*, *G. polynesiensis*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *G. excentricus*, *G. scabrosus*, *G. silvae*, *G. balechii*, *G. cheloniae*, *G. lapillus*, *G. honu* and *G. jejuensis* [21, 23, 25, 27-35]. In addition, two globular *Gambierdiscus* species have recently been reclassified as *Fukuyoa yasumotoi* and *F. ruetzleri*, with a new species described as *F. paulensis* [23, 36, 37]. Formal classification is also pending for several

unnamed genetic clades (i.e., *Gambierdiscus* sp. ribotypes and *Gambierdiscus* sp. types) which could represent new undescribed species, as it was the case for *G. silvae*, *G. scabrosus*, *G. jejuensis* and *G. balechii* previously known as *Gambierdiscus* sp. ribotype 1, *Gambierdiscus* sp. type 1, 2 and 6, respectively [29, 30, 35, 38]. It is likely that more species will be characterized in the coming years as more extensive samplings are conducted in areas that are still understudied such as the Indian Ocean or temperate locales.

Of note, the generalization of the molecular approach to ciguatera species identification has also fostered the development of novel detection tools operational straightforward in ciguatera risk assessment programs. Indeed, semi-quantitative PCR assays are now available for the detection and enumeration of the following species/types in environmental samples: *G. belizeanus, G. caribaeus, G. carpenteri, G. carolinianus, G. ruetzleri* and *Gambierdiscus* sp. ribotype 2 [26], *G. scabrosus*, and *Gambierdiscus* sp. type 2 and 3 [39], *G. polynesiensis, G. toxicus, G. pacificus* and *G. australes* [4], *G. lapillus* [40], and *G. excentricus* and *G. silvae* [41]. Recently, Smith et al. (2017) [42] have proposed the use of metabarcoding techniques as a useful approach for the high-throughput screening of ciguatera-related harmful algal blooms (HAB) species.

3. GLOBAL DISTRIBUTION OF *GAMBIERDISCUS* AND *FUKUYOA* SPP.

Numerous reports of the presence of *Gambierdiscus* in samples collected from the eastern Atlantic region, the Caribbean and Western Atlantic region, Central and Western Pacific, Southeast Asia and Indian Ocean can be found in the literature [24 and references therein]. For several of these locales, the species diversity still remains unknown. As mentioned above, earlier reports of *G. toxicus* as a single cosmopolitan species with a seemingly worldwide circumtropical distribution needs to be reconsidered [23, 24 and references therein, 43, 44].

Gambierdiscus spp. are preferentially found in tropical and sub-tropical waters of the globe between 35°N and 35°S [45], but recent studies show that *Gambierdiscus* and/or *Fukuyoa* spp. are presently established in temperate-like areas as well, including Korea [35, 46] Japan [39], the Kermadec Islands (New-Zealand) [47, 48], Southern Australia [49], the Northern Gulf of Mexico [50], and the Mediterranean Sea [51, 52] (Table 1). There is concern that the geographic range of these two genera, and subsequently of ciguatera outbreaks, will continue to expand as sea surface temperatures rise [53, 54]. Indeed, cyclical weather patterns such as El Niño - which is associated with unusual warming waters in the Pacific - have resulted in spike in ciguatera cases in Kiribati, Western Samoa, Tuvalu and Cook Islands [55]. Similarly, Gingold et al. (2014) [56] found an association between ciguatera poisoning (CP) incidence and warmer sea surface temperatures (SST) in the Caribbean basin. Based on water temperature projections over the coming century, a substantial shift in both the distribution and abundance of ciguatera dinoflagellates is to be expected [57, 58], with some species becoming dominant whereas others will become less prevalent. In the long-term, however, temperatures may get too warm according to Llewellyn (2010) [59], thereby hindering *Gambierdiscus*/*Fukyoa* growth and resulting in a lower risk of ciguatera.

Table 1. Global distribution of *Gambierdiscus* and *Fukuyoa* species and phylotypes. For some of the locations, (*) indicate species which were identified using solely morphological characteristics. Positive confirmation of their identity will require further phylogenetic analyses

Locations	Species or phylotypes	References
Pacific Ocean		
Australia	*G. belizeanus, G. carpenteri, G. honu, G. lapillus, G. toxicus, F. paulensis*	[33, 34, 47, 49, 161, 195, 235, 236]
Cook Islands	*G. australes, G. pacificus, G. polynesiensis, G. cheloniae, G. honu, G. lapillus*	[4, 25, 32, 34, 44, 47, 115, 162, 237]
Fiji	*G. carpenteri, G. toxicus*	[44, 47]
French Polynesia	*G. australes, G. caribaeus, G. carpenteri, G. pacificus, G. polynesiensis, G. toxicus*	[4, 21, 25, 44, 85, 238]
New Zealand	*G. australes, G. carpenteri, G. pacificus, G. polynesiensis, G. honu, F. paulensis*	[42, 47, 143, 239]
Guam	*G. carpenteri, G. toxicus*	[23, 47, 238]
Hawaii	*G. australes, G. caribaeus, G. carpenteri, G. toxicus*	[23, 44, 47, 85, 238]
Kiribati	*G. balechii, G. belizeanus, G. carpenteri, G. pacificus, Gambierdiscus* sp. type 4, 5	[38, 240]
Marshall Islands	*G. pacificus, G. toxicus*	[44, 47]
New Caledonia	*G. toxicus*	[25]
Palau	*G. caribaeus, G. toxicus*	[23, 44, 47]
Tonga	*G. australes, G. honu, G. pacificus*	[47]
Caribbean Sea		
Aruba	*G. carolinianus, G. carpenteri*	[19, 237]
Belize	*G. belizeanus, G. caribaeus, G. carolinianus, G. carpenteri, G. silvae, F. rueztleri, Gambierdiscus* ribotype 2	[23, 27, 44, 85, 237, 238]
Cayman Islands	*G. caribaeus*	[23, 44, 85, 238]
Curaçao	*G. silvae*	[237]
Jamaica	*G. caribaeus, G. carolinianus, G. carpenteri,*	[23, 44, 85, 238]
Martinique	*Gambierdiscus* ribotype 2	[44, 85, 238]
Mexico	*G. belizeanus, G. caribaeus, G. carolinianus, G. carpenteri, G. toxicus, F. yasumotoi*	[41, 85, 237, 238, 241]
Puerto Rico	*Gambierdiscus* ribotype 2	[44, 85, 238]
St Barthélemy	*G. belizeanus*	[20, 23, 85, 238]
St Maarten	*G. belizeanus, G. carolinianus, Gambierdiscus* ribotype 2	[85, 237]
Turks & Caicos	*G. belizeanus*	[237]
US Virgin Islands	*G. belizeanus, G. caribaeus, G. carolinianus, G. carpenteri, G. silvae, Gambierdiscus* ribotype 2	[20, 85, 237, 238]
Gulf of Mexico		
Dry Tortugas	*G. caribaeus, G. carpenteri*	[85, 238]
Florida Keys	*G. belizeanus, G. caribaeus, G. carolinianus, G. carpenteri, G. excentricus, G. silvae, F. yasumotoi, Gambierdiscus* ribotype 2	[20, 23, 44, 105, 237, 238]
Flower Garden Banks	*G. belizeanus, G. caribaeus, G. carolinianus, G. carpenteri, F. rueztleri, Gambierdiscus* ribotype 2	[19, 85, 237, 238]
Indian Ocean		
La Réunion	*G. belizeanus*, G. toxicus**	[25, 242]
Mauritius	*G. toxicus**	[243]
Tanzania	*G. toxicus**	[244]
Atlantic Ocean		
Bahamas	*G. caribaeus, G. carolinianus*	[20, 44]

Table 1. (Continued)

Locations	Species or phylotypes	References
Bermuda	*G. carolinianus*	[44]
Brazil	*G. excentricus, G. silvae, F. paulensis*	[37, 85, 245, 246]
Canary Islands	*G. australes, G. caribaeus, G. carolinianus, G. excentricus, G. silvae, Gambierdiscus* ribotype 3	[28, 30, 85, 163, 247]
Florida	*G. belizeanus, G. caribaeus, G. carolinianus, G. carpenteri, G. excentricus, F. rueztleri*	[19, 237, 238]
Madeira	*G. australes*	[163, 238]
Morocco	*G. excentricus*	[30]
North Carolina	*G. carolinianus, F. rueztleri*	[19, 23, 238]
Mediterranean Sea		
Balearic Islands	*G. australes, F. paulensis*	[52, 125]
Crete	*G. carolinianus*	[85, 238]
Red Sea		
Jordan	*G.* cf. *belizeanus, F. yasumotoi*	[248]
Saoudi Arabia	*G. belizeanus*	[249]
Kuwait	*F. yasumotoi*	[248]
Persian Gulf		
Kuwait	*F. yasumotoi*	[248]
Arabian Gulf		
Pakistan	*G. australes, G. belizeanus, G. polynesiensis, G. toxicus, F. yasumotoi*	[250]
Asia		
Borneo	*G. belizeanus*, G. pacificus**	[251, 252]
China	*G. australes, G. caribaeus, G. pacificus, F. rueztleri*	[237, 253]
Indonesia	*G. balechii*	[31]
Japan	*G. australes, G. scabrosus, F. yasumotoi, Gambierdiscus* sp. type 2, 3	[20, 29, 84, 85, 99, 164]
Korea	*G. caribaeus, G. jejuensis, F. yasumotoi*	[35, 46, 254]
Malaysia	*G. balechii, G. caribaeus, G. carpenteri, G. honu, G. pacificus, F. yasumotoi*	[38, 47, 85, 190, 237]
Phillipines	*G. carpenteri*	[69]
Singapore	*F. yasumotoi*	[36]
Thailand	*G. caribaeus*	[255]
Vietnam	*G. pacificus*, G. toxicus*, G. polynesiensis*, F. yasumotoi**	[126, 256]
China	*G. australes, G. caribaeus, G. pacificus, F. rueztleri*	[237, 253]

Table 1 provides an updated summary of the current distribution of all known *Gambierdiscus* and *Fukuyoa* species. These global occurrence data were collated from studies which used either morphological and/or molecular techniques to identify species. Interestingly, in some of these locations, the presence of ciguatera-related dinoflagellates is consistent with the report of ciguatera cases decades earlier [60-63]. In addition, *Gambierdiscu*s and/or *Fukuyoa* have also been reported from other regions of the world, such as Tonga [42], several locations in Latin America such as Mexico, Cuba, Guatemala, El Salvador, Costa Rica, Colombia, etc. [64 and references therein, 65, 66], Morocco and Crete [51], Hong Kong waters [67], and the Lesser Antilles [68], nevertheless, the exact species are yet to be determined.

Occurence data suggest that some of these species seem to be limited to specific areas of the globe (i.e., *G. excentricus* and *G. scabrosus)*, whereas others are characterized by a more cosmopolitan distribution (i.e., *G. belizeanus, G. caribaeus, G. carpenteri* and *F. yasumotoi*) (Table 1), consistent with the wider range of tolerance to environmental conditions exhibited

by these species *in vitro* [69, 70] (see section 4). Nevertheless, it is likely that, with additional samplings, species now designated as specific to a region will eventually be reported from other locales.

Assessing *Gambierdicus*/*Fukuyoa* spp. abundance in the environment proves very challenging due to the high variations observed both at a temporal and spatial scale. Too, the notorious patchy distribution of these dinoflagellate even over small distances, and the differences in the algal hosts morphology which can greatly influence abundance estimates are other major impediments to an accurate assessment of population densities [71]. Despite these limitations, the literature survey conducted by Litaker et al. (2010) [44] on *Gambierdiscus* abundance data for the Atlantic and Pacific Oceans showed that 85% of the average values were < 1,000 cells.g^{-1} wet weight (ww) algae, while 10% of the abundance estimates fell between 1,000 and 10,000 cells.g^{-1} ww algae. Such findings have led the authors to speculate that densities > 1,000 cells.g^{-1} ww algae likely correspond to localized blooms conditions that have the higher potential of causing ciguatera events. On the other hand, some studies have stressed the importance to also consider algal host-dinoflagellate associations, which may act as toxin source or sinks, depending on macroalgal palatability [72]. In other words, algae that are actively consumed by herbivores could be responsible for a high toxin flux even at low dinoflagellate densities, whereas unpalatable algae with higher dinoflagellate densities might contribute little to toxin transfer in marine food webs.

4. ECOPHYSIOLOGY

Gambierdiscus/*Fukuyoa* spp. are regarded as benthic epiphytic organisms, but can occasionally exhibit free-swimming behavior [73-76], suggesting that they may behave as mixotrophs [77]. Although members of this genus survive as photoautotrophs, this hypothesis is consistent with recent findings that complex pathways of N and C utilization are indeed present in this taxon [77]. According to these authors, cells of *G. caribaeus* were able to sustain significant growth in the absence of light when cultured in medium supplemented with an exogenous carbon source. Nonetheless, such findings still await further confirmation since these authors used non-axenic cultures, and previous research showed that this dinoflagellate may require the presence of specific bacteria to maintain growth [78].

The life cycle of *Gambierdiscus* is still poorly documented. Early studies by Hokama et al. (1996) [79] described the succession of six distinct stages in culture: i) a motile free-swimming cell phase at the beginning of the culture, ii) a pre-cyst phase after 2 to 3 weeks, iii) a cyst phase followed by iv) a secondary cyst phase, v) a mitotic phase in which cysts divide, and vi) a terminal phase that may last up to 4-6 months preceding a new cycle. Next, Van Dolah et al. (1995) [80] demonstrated that cell division in *Gambierdiscus* was phased to the diurnal cycle, with cells dividing only during a 3h-window late in the dark phase, when grown in a 16:8 hour light:dark cycle. These authors also documented the role played by a CDC2-like kinase which, like in higher eukaryotes, was expressed constitutively in *Gambierdiscus* throughout the cell cycle but activated only during mitotis. Results of a recent study conducted on a *G. balechii* strain allowed to confirm previous findings on a circadian regulation of cell division [81] and that the life cycle of *Gambierdiscus* likely involves both asexual and sexual processes, as speculated by Taylor (1979) [82]. All these observations

(cyst-like structures and sexual reproduction) may help explain the exceptional ability of these species to adapt, survive, and even thrive in harsh environmental conditions (see following sections 4.1 and 4.2).

Compared to most planktonic dinoflagellates, *Gambierdiscus*/*Fukuyoa* spp. are considered slow-growing organisms, with growth rate never exceeding 0.5 div.d^{-1} [19, 35, 58, 69, 83-86]. The highest growth rate ever reported for *Gambierdiscus* isolates is 0.55 div.d^{-1} and concerns a Hawaiian strain [87]. Whether there is a relationship between growth rate and cell potency remains unclear, although highly toxic clones in the genus *G. polynesiensis* were found to exhibit relatively low reproductive rates ($\simeq$ 0.13 div.d^{-1}) as compared to non-ciguatoxic strains [83].

4.1. *Gambierdiscus/Fukuyoa* spp. Preferred Habitats

High densities of *Gambierdiscus*/*Fukuyoa* spp. are often observed in disturbed/degraded coral reef habitats [15-17, 44]. Indeed, Lewis (1986) [15] reported that *Gambierdiscus* grows prodigiously following both natural and man-made disturbances of coral reefs. Similarly, while studying the fluctuations of *G. toxicus* populations in a ciguateric site of French Polynesia, Chinain et al. (1999) [17] found that there was an increase in both the density and frequency of *Gambierdiscus* blooms following a severe coral bleaching episode affecting large areas of the study site. A general consensus is that the massive colonization of dead corals by macroalgae provides more substrate for the settlement of epiphytic *Gambierdiscus*/*Fukuyoa* spp. populations, as first hypothesized by Randall (1958) [88]. This may explain why reef disturbances due to extreme climatic events (*e.g.*, hurricanes, heavy rains, coral bleaching) or human activities (*e.g.*, dredging and filling, constructions, military activities) frequently precede ciguatoxic events [1, 13, 14, 89].

It is not clear whether *Gambierdiscus* abundances are subject to seasonality or not, as field studies sometimes showed contradictory conclusions. Chinain et al. (1999) [17] reported that *Gambierdiscus* cell densities were the highest at the beginning and end of the hot season in French Polynesia, consistent with observations from a field survey conducted in Hawaii [57]. Conversely, other studies did not observe seasonal patterns or concluded that highest densities were rather reported in the fall season [79, 90, 91]. In any case, it is likely that seasonal patterns, if any, will be primarily determined by the different temperature tolerances characterizing the various *Gambierdiscus*/*Fukuyoa* species (see the following section 4.2).

Gambierdiscus populations are generally more abundant in habitats where temperatures are comprised between 25 and 30°C [17, 54]. However, early observations from the Florida Keys, U.S. Virgin Islands, Hawaii, and Queensland, that *Gambierdiscus* abundance peak at cooler temperatures seem to contradict this generalization [24 and references therein].

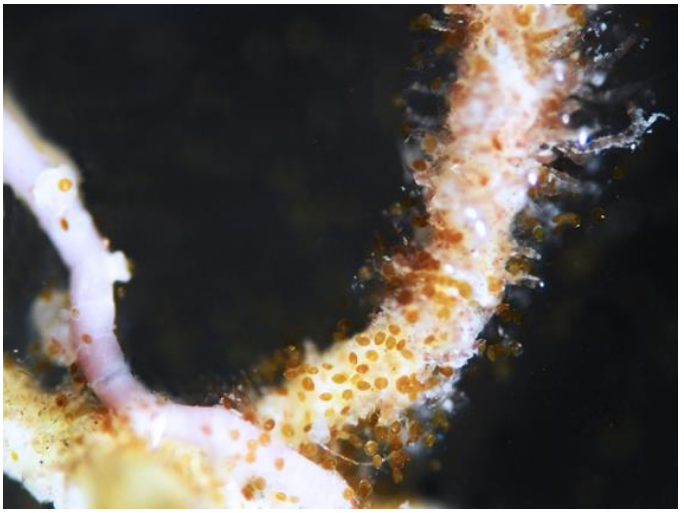
Like other dinoflagellates, *Gambierdiscus spp*. are sensitive to excessive agitation and show a clear preference for calm and stable environments [44, 45, 92]. There are several field observations, however, that tend to contradict this general idea: for instance, in areas such as Mayotte Island, Hawaii or Canary Islands, higher densities of *Gambierdiscus* were actually found in turbulent winward locations [28, 93, 94]. Small-scale turbulence has been shown to directly influence the physiology of some dinoflagellates by causing cell disruption, alteration of cell division and cell cycle phase duration, and even by affecting toxin production [95, 96]. Nakahara et al. (1996) [75] have observed that turbulence may cause *Gambierdiscus* cells to

quickly attach to the surface of the macrophyte. Cells are also often found attached to their algal host by a mucus thread, so as to limit cells dispersal.

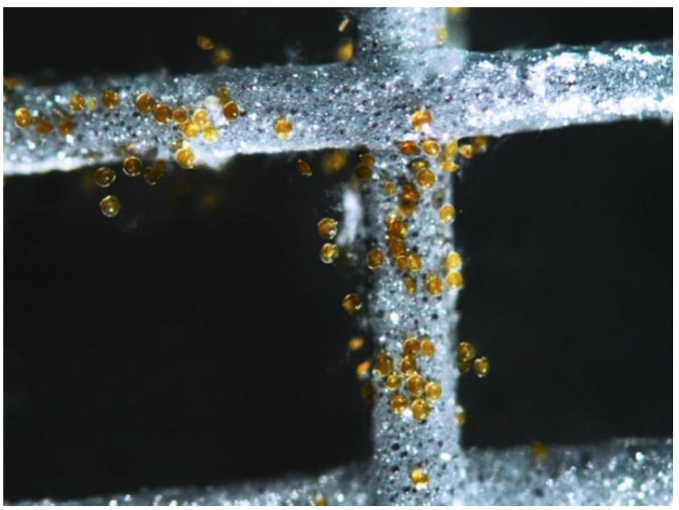
Gambierdiscus appears to prefer environments with high, stable salinities, between 28-35 [24 and references therein, 44] but has been reported from areas with extreme lower or upper salinity values such as river outlets [65] or mangrove environments where salinity can exceed 40 [97]. There is evidence, however, that salinity preferences may significantly differ among species [24, 69] (see following section 4.2.).

Many studies have documented the apparent preference of *Gambierdiscus* for specific macroalgal hosts [24 and references therein, 68], but the exact nature of this preference remains elusive. There is some evidence that various macroalgae may supply important growth factors [75, 93, 98-100], but other factors such as algal structure and surface area, or algal class may also play a critical role [6, 72, 76, 98]. According to Bienfang et al. (2008) [45], the occurrence of *these epiphytic organisms* on many macroalgae species suggests opportunism in regard to macroalgal substrate, rather than a regulation of their abundance by specific macroalgal metabolites. Results of a study which examined the epiphytic relationship between *Gambierdiscu*s and 24 different macroalgal species seem to contradict this statement since it was shown that i) cells attached to specific algal species while avoiding others, and ii) certain algal host inhibited cells proliferation while others allowed for their growth [76]. In addition, in the environment, cell densities of epiphytic pennate diatoms on macroalgae have been shown to affect *Gambierdiscus* spp. abundance and therefore, may represent another growth determinant in the same way as sea water temperature, salinity and nutrients [101]. In any case, multiple benefits may result from the association of *Gambierdiscus* spp. with macrophytes: e.g., fixation on a substrate, protection from turbulence, shading from direct sunlight, and access to organic compounds within the thallisphere [102].

Gambierdiscus spp. are able to grow in shaded as well as high-lighted water habitats [50]. In shallow tropical waters, for instance, *Gambierdiscus* populations are often found attached to benthic macrophytes or to drifting algae or detritus [103] where surface irradiances can sometimes exceed 2,000 µmol photons.m^{-2}.s^{-1} [102]. In cultures, however, these organisms seem to be best adapted to relatively low light conditions (50-230 µmol photons.m^{-2}.s^{-1}) and can even sustain growth at irradiances as low as 10 µmol photons.m^{-2}.s^{-1}, which would allow cells to survive at depths > 80-100m in tropical environments [84, 104]. There are actually limited data studies on the vertical distribution of *Gambierdiscus*/*Fukuyoa* spp. [50, 105, 106] but early observations are consistent with *in vitro* observations since the presence of *Gambierdiscus* cells was reported at depths between 30-50m [23, 50, 93, 97]. Furthermore, recent findings clearly suggest the existence of species-specific responses to changing light regimes, with species capable of maintaining growth even when exposed to very high irradiances (> 500 µmol photons.m^{-2}.s^{-1}) [69, 107]. Several behavioral strategies have been suggested to help this organism cope with high irradiances, including cell aggregation, mucus production, use of the three-dimensional structure of the algal host to minimize light exposure and prevent photodamage, as well as physiological responses such as the plasticity of cell size and chlorophyll content [102, 107, 108].



(A)



(B)

Figure 2. Cells of *Gambierdiscus* sp. attached to natural and artificial substrates. (A) on the thallus of the red algae *Jania* sp. (Rhodophyte); (B) on the grilling of a window-screen device (© ILM).

4.2. *In Vitro* Growth

Data regarding *Gambierdiscus/Fukuyoa* spp. growth responses under varying environmental factors are useful to inform predictive models of their abundance, seasonality and global distribution. Early laboratory studies have primarily investigated *Gambierdiscus* sp. optima and limits for temperature, salinity and light factors. Overall, these studies generally agree with field surveys although conflicting results were sometimes reported [see 24 for a review and references therein].

The influence of ambient nutrient concentrations on *Gambierdiscus* populations remains ambiguous, since this topic had been the subject of a limited number of field studies, with

sometimes contradictory conclusions [94, 105, 109]. Similarly, only two laboratory studies have sought to determine the effect of nutrients on *Gambierdiscus* growth: in the first study by Sperr and Doucette (1996) [110], the authors found that growth rates within five isolates remained constant under different N:P ratios, indicating an ability of all clones to maintain high reproductive rates under different nutritional regimes. The second study by Lartigue et al. (2009) [111] examined the effects of different nitrogen sources on the growth of two clones and concluded that nutrient physiologies likely differed between strains. Interestingly, these clones were subsequently classified as two distinct species, namely *G. caribaeus* and *Gambierdiscus* sp. ribotype 2.

As mentioned previously, most of these studies were conducted at a time when *Gambierdiscus* taxonomy was unresolved, so the extent to which growth responses may vary across the multiple species now known in *Gambierdiscus* and *Fukuyoa* genera remains to be clarified. Recent studies have started to provide growth data for the following species/phylotypes: *G. polynesiensis*, *G. australes*, *G. pacificus*, *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *G. silvae*, *G. scabrosus*, *F. ruetzleri*, and *Gambierdiscus* spp. types 2, 3, 4 and 5 [69, 70, 83-85, 104, 112]. Results confirm that differences in both tolerance and optimum growth ranges exist not only across species, but across strains as well [70].

5. TOXICOLOGY

5.1. Chemodiversity of Secondary Metabolites

Increasing attention is paid to secondary metabolites produced by microalgae, particularly dinoflagellates due to their potential uses in the biology, biomedical and toxicological fields [113]. These compounds are also ecologically relevant since they are suspected to act as allelochemicals influencing biotic interactions (see section 5.4), and may therefore be involved in structuring microbial communities [114]. The recent development of metabolomics is furthermore a unique opportunity to investigate the chemodiversity of such dinoflagellates, in order to e.g., describe new compounds (including toxins), assess the variations of their productions in response to different stressors, or identify chemical markers at different taxonomic levels (chemotaxonomy) [115].

Gambierdiscus/Fukuyoa species produce several non-structurally related groups of secondary metabolites with a ladder-shaped cyclic polyether backbone: ciguatoxins (CTXs), maitotoxins (MTXs), gambieric acids (GAs), gambierol, gambieroxide and gambierones (Figures 3 & 4). The Table 2 presents the compounds that have been detected in the different *Gambierdiscus*/*Fukuyoa* species described to date (except for the newly described *G. jejuensis* and *F. yasumotoi* species for which no information are available), through isolation or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analyses. As outlined above, before 1999 and the study by Chinain et al. [25] that has introduced molecular analyses, the identification of *Gambierdiscus* species was made solely on the basis of morphological characteristics, leading to potential misidentifications. Thereby, strain identifications prior to 1999 should probably be reassessed, particularly concerning *G. toxicus*.

Table 2. List of polyether compounds that have been detected (through isolation or LC-MS/MS analyses) in *Gambierdiscus*/*Fukuyoa* genera

Species[a]	P-CTXs	MTXs	Other compounds	References
F. paulensis	P-CTX3 (= 54-deoxy-P-CTX1B)[b] *No P-CTX3B, P-CTX3C, P-CTX4A, P-CTX4B*	*No MTX1*	Gambieric acid A[b] 44-methylgambierone[c]	[49, 125, 143, 257]
F. ruetzleri	2,3-dihydroxy-P-CTX3C[d,e] *No P-CTX3C and P-CTX4A/B*	*No MTX1, MTX2, MTX4*	44-methylgambierone[c]	[66, 126, 129]
G. australes	2,3-dihydroxy-P-CTX3C[d,e] *No P-CTX3B, P-CTX3C, P-CTX4A, P-CTX4B*	MTX1 *No MTX2, MTX4*	44-methylgambierone[c]	[32, 47, 48, 115, 119, 126, 129, 134]
G. balechii	*No information available*	*No MTX1, MTX2, MTX4*	44-methylgambierone[c]	[129]
G. belizeanus	*No information available*	*No MTX1, MTX2, MTX4*	Gambierone 44-methylgambierone[c]	[129, 135, 140]
G. caribaeus	2,3-dihydroxy-P-CTX3C[d,e] *No P-CTX3C and P-CTX4A/B*	MTX2[d] *No MTX1, MTX4*	44-methylgambierone[c]	[126, 129]
G. carolinianus	*No information available*	*No MTX1, MTX2, MTX4*	44-methylgambierone[c]	[129]
G. carpenteri	2,3-dihydroxy-P-CTX3C[d,e] *No P-CTX3B, P-CTX3C, P-CTX4A, P-CTX4B*	*No MTX1, MTX2, MTX4*	44-methylgambierone[c,f]	[119, 126, 129, 142]
G. cheloniae	*No P-CTX3B, P-CTX3C, P-CTX4A, P-CTX4B*	*No MTX1*	44-methylgambierone[c]	[32, 119]
G. excentricus	M/L-*seco*-P-CTX4A/B[b,d] M/L-*seco*-P-CTX3C/B[b,d] Tetraseco-P-CTX3C/B[b,d]	MTX2[d] MTX4[d] *No MTX1*	44-methylgambierone[c]	[128, 129]
G. honu	*No P-CTX3B, P-CTX3C, P-CTX4A, P-CTX4B*	*No MTX1*	44-methylgambierone[c]	[34, 119]
G. lapillus	*No P-CTX3B, P-CTX3C, P-CTX4A, P-CTX4B*	*No MTX1*	44-methylgambierone[c]	[33, 119, 142]
G. pacificus	2,3-dihydroxy-P-CTX3C[d,e] P-CTX3C/B[d] 51-hydroxy-P-CTX3C[d] M-*seco*-P-CTX3C[d] P-CTX4A/B[d] M-*seco*-P-CTX4A[d] C/I-CTX1/2[d]	MTX2[d] *No MTX1, MTX4*	44-methylgambierone[c]	[32, 115, 119, 126, 127, 129]
G. polynesiensis	P-CTX3B P-CTX3C M-*seco*-P-CTX3C 2-hydroxy-P-CTX3C P-CTX4A P-CTX4B M-*seco*-P-CTX4A/B[d]	*No MTX1*	44-methylgambierone[c]	[32, 48, 82, 115, 116, 118, 119, 258]
G. scabrosus	*No information available*	*No MTX1, MTX2, MTX4*	44-methylgambierone[c]	[129]
G. silvae	*No information available*	*No MTX1, MTX2, MTX4*	44-methylgambierone[c]	[129]

Species[a]	P-CTXs	MTXs	Other compounds	References
G. toxicus[g]	P-CTX4A P-CTX4B M-*seco*-P-CTX4A/B P-CTX3B P-CTX3C P-CTX2 (= 52-*epi*-54-deoxy-P-CTX1B) P-CTX3 (= 54-deoxy-P-CTX1B) M-*seco*-P-CTX3C 51-hydroxy-P-CTX3C M-*seco*-P-CTX3C methyl acetal 2,3-dihydroxy-P-CTX3C[d,e]	MTX1 MTX2[d] *No MTX4*	Gambieric acids A-D Gambierol Gambieroxide 44-methylgambierone[c]	[119-124, 126, 130-133, 137-139]
Gambierdiscus sp. ribotype 2	*No information available*	*No MTX1, MTX2, MTX4*	44-methylgambierone[c]	[129]

[a]No information available for *F. yasumotoi* and *G. jejuensis.* [b]Trace amounts. [c]Compound previously identified as MTX3 and renamed 44-methylgambierone by Murray et al. (2019) [134] and Boente-Juncal et al. (2019) [135] who characterized its structure by NMR and MS experiments. [d]Tentative identification on the basis of low resolution LC-MS/MS analyses without reference materials. [e]In Pisapia et al. (2017) [129], authors presume that the study by may have misidentified 2,3-dihydroxy-P-CTX3C, as it is likely that the actual compound present was the ubiquitous 44-methylgambierone (former MTX3). [f]Five temperate *G. carpenteri* strains were found free of 44-methylgambierone (former MTX3) [142], the only ones among 89 *Gambierdiscus* strains tested by Murray et al. (2019) [134]. [g]Potential misidentifications, most likely *G. polynesiensis* for some Polynesian strains.

Many congeners of CTXs, comprising 13 or 14 rings transfused by ether bonds, have been isolated from dinoflagellates and/or fish [for a review see 116]. They are grouped into three sub-families according to their geographical origin, i.e., Pacific, Caribbean, and Indian CTXs (P-CTXs, C-CTXs and I-CTXs, respectively). Several P-CTXs have been detected in *Gambierdiscus*/*Fukuyoa* species, unlike C-CTXs or I-CTXs which have been found only in fish samples. To date, 11 P-CTXs (Figure 3) were definitely detected in *G. polynesiensis* strains (TB92 and RG92 from French Polynesia, CAWD212 from Cook Islands) [32, 83, 117-121], and/or in some *G. toxicus* strains (GII-1 and RGI-1 from French Polynesia, potential misidentifications of the species, most likely *G. polynesiensis* currently known to be the major toxic species in the South Pacific [116]) [122-126]: P-CTX4A, P-CTX4B, M-*seco*-P-CTX4A/B, P-CTX2 (52-*epi*-54-deoxy-P-CTX1B), P-CTX3 (54-deoxy-P-CTX1B), P-CTX3B, P-CTX3C, 51-hydroxy-P-CTX3C, 2-hydroxy-P-CTX3C, M-*seco*-P-CTX3C, M-*seco*-P-CTX3C methyl acetal. Moreover, trace amounts of P-CTX3 (54-deoxy-CTX1B) was detected in one strain of *F. paulensis* (Dn135EHU from Mediterranean Sea) [127]. In other species of *Gambierdiscus*/*Fukuyoa*, P-CTXs were only tentatively identified on the basis of low resolution LC-MS/MS analyses without reference materials [128-130].

MTX1 (Figure 4a) is the largest non-polymeric molecule described to date in nature, with 32 ether rings and two sodiated sulfate ester groups, having a molecular weight around 3400 Da. It was detected in some *G. australes* [32, 47, 48, 117, 121, 131-133] and *G. toxicus* strains [134-136]. Two other analogues have been reported in the literature but their structures haven't been elucidated yet: MTX2, tentatively detected in some strains of *G. caribaeus*, *G. excentricus*, *G. pacificus* and *G. toxicus* [131, 136], and MTX4, recently detected exclusively in *G. excentricus* strains [131]. Another putative analogue was initially identified as MTX3 [137] but had a molecular weight much lower than other MTXs and was recently renamed 44-methylgambierone when its structure was finally fully characterized by RMN analyses (see

below) [138, 139]. It should be noted that a recent study conducted by Lewis et al. (2016) [140] indicated that several strains of *Gambierdiscus*/*Fukuyoa* produce multiple MTX congeners, actually more than four for one particular strain of *G. belizeanus*, suggesting a likely broader chemical diversity than what is known so far within MTX group.

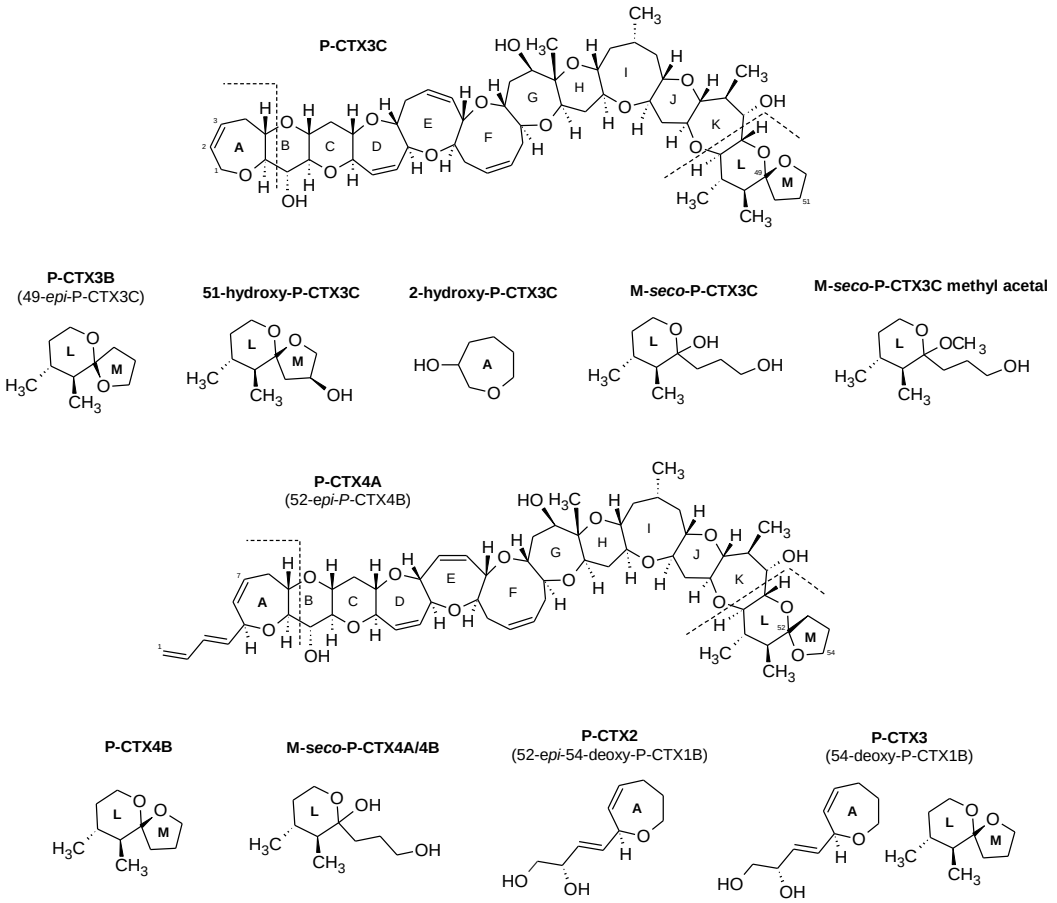


Figure 3. Structures of the P-CTXs definitely detected in *Gambierdiscus*/*Fukuyoa* genera.

Gambieric acids (GA)-A, -B, -C and -D (Figure 4b) were isolated from a strain identified as *G. toxicus* (GII-1 from French Polynesia, potential misidentification of the species) [141] and are composed of nine contiguous ether rings and one isolated tetrahydrofuran. GA-A was also detected in trace amounts in *F. paulensis* (Dn135EHU from Mediterranean Sea) [127].

Gambierol (Figure 4c) was isolated from a strain identified as *G. toxicus* (RGI-1 from French Polynesia, potential misidentification of the species) [142]. It contains a transfused octacyclic polyether core and a skipped triene.

Gambieroxide (Figure 4d) was more recently isolated from a strain identified as *G. toxicus* (GTP2 from French Polynesia) [143]. This polyether compound comprises 12 transfused ether rings and two side chains at the extremities. Its structure is very similar to that of yessotoxin.

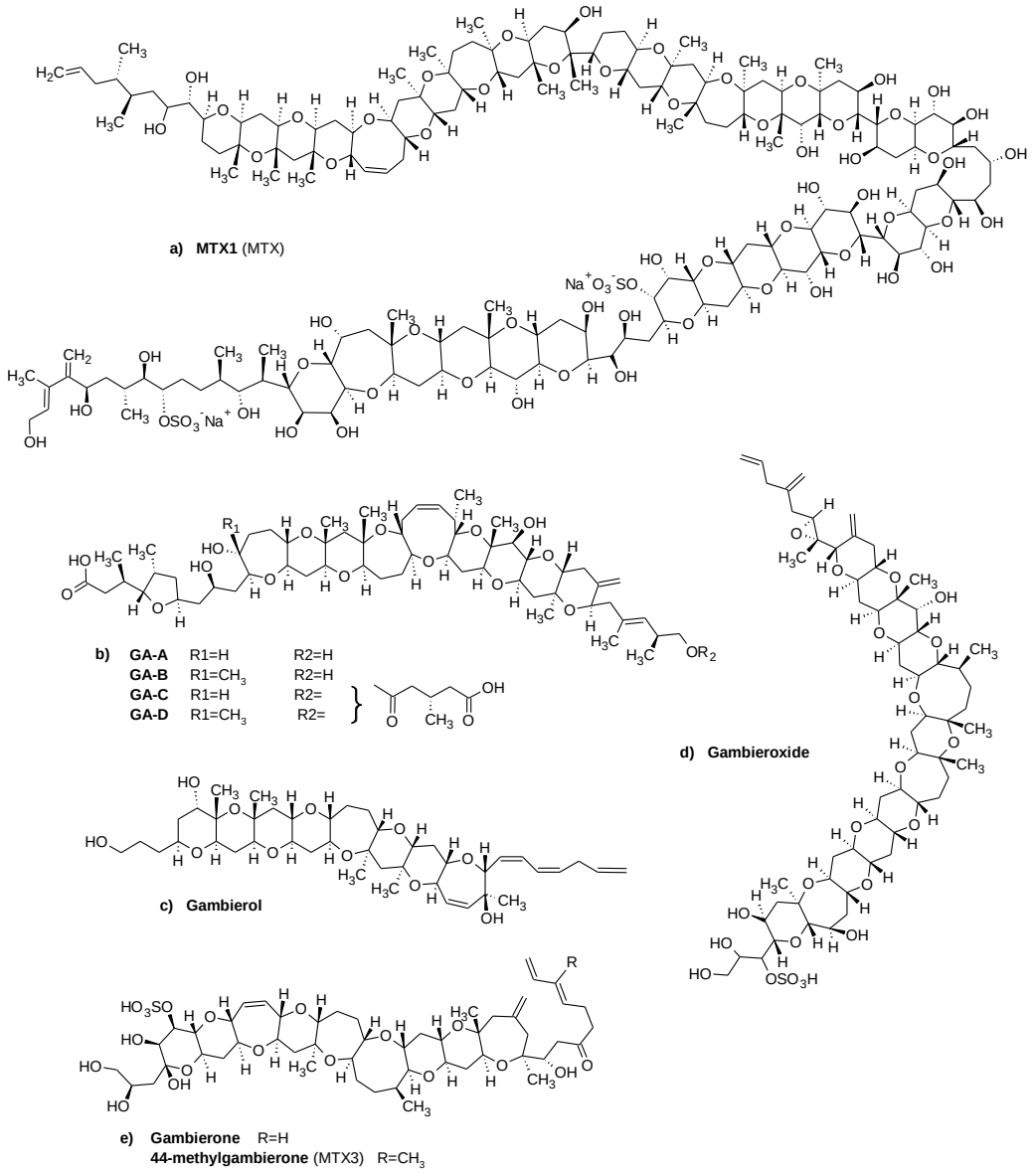


Figure 4. Structures of other polyether compounds isolated from *Gambierdiscus*/*Fukuyoa* genera.

Finally, gambierone (Figure 4e) was recently isolated from a *G. belizeanus* strain (CCMP401 from Caribbean Sea) [144], as well as its analogue, the 44-methylgambierone (Figure 4e) [138, 139]. The structure of this last molecule was elucidated by another team at the same time after isolation from four *G. australes* strains (Kermadec Islands) [138, 139]. Gambierones are composed of nine transfused rings, a sulfate ester group and a conjugated vinyl end. Before its structure was definitely elucidated, the 44-methylgambierone was reported as MTX3 in numerous studies and has been detected in all *Gambierdiscus*/*Fukuyoa* species analyzed to date, except for *G. jejuensis* and *F. yasumotoi* for which data are currently unavailable [32-34, 47-49, 67, 117, 121, 131, 132, 145]. An exception concerns temperate clones of *G. carpenteri* which have been found free of 44-methylgambierone [121, 145].

Further characterization of toxin profiles of all known species of *Gambierdiscus/Fukuyoa* is crucial since this information is useful to inform ciguatera risk models. Some authors even speculated that the toxin profiles in the causative dinoflagellates may contribute to shape the toxin profiles in fish [146] – which have been shown to vary across regions and fish species [123, 147].

5.2. Modes of Action of Bioactive Compounds

CTXs are by far the most studied group due to their direct involvement in ciguatera poisoning. Their major mechanism of toxicity expresses through the binding to site 5 of the α-subunit of the voltage-gate sodium channels (VGSCs) on the neuronal membrane, leading to the persistent activation (opening) of VGSCs which results in cell depolarization at rest and disruption of peripheral and central nerve transmission (e.g., increase in Na^+ influx, enhancement of neuronal excitability, spontaneous and repetitive actions potentials) [for reviews see 148, 149]. The activation of VGSCs by CTXs has been shown to also indirectly disturb the Ca^{2+} homeostasis. Furthermore, some studies indicate that CTXs interact with other targets, such as the voltage-gated potassium channels (VGPCs), resulting in their inhibition and accentuation of the neurocellular perturbations [for reviews see 148, 149]. The multiple effects of CTXs are believed to underly the complex symptomatology observed in CP syndrome. On the contrary, the contribution of the other polyether compounds produced by *Gambierdiscus*/*Fukuyoa* genera to the clinical picture of ciguatera is not yet fully elucidated.

MTX1 is among the most potent marine toxins known to date with an LD_{50} in mice of 50 $ng.kg^{-1}$ [134-136]. Its activity results in a massive calcium influx and a rapid cell death, likely due to a combination of different ion channels' modulation (e.g., nonselective cation channels, voltage-gated calcium channels, transient receptor potential channels) and altering many cellular functions, nevertheless, its primary target and mechanism of action are still unknown [for a review see 139]. MTX2 was shown to produce similar symptoms in mice to those elicited by MTX1, although with lower potency [136], whereas MTX4 was reported to exhibit a toxic effect similar to the one of MTX1 in neuroblastoma cells [131], their mechanisms of action has however not been described yet.

GA-A and GA-B are potent antifungal agents, displaying a remarkable activity against filamentous fungi, while being ineffective against yeasts [150]. GA-A is able to displace the binding of tritiated brevetoxin-3 ($[^3H]$-PbTx-3) to the site 5 of VGSCs in excitable membranes, which is also the binding site of CTXs as describe above [151]. As PbTxs, GA-A could thus be a useful pharmacological tool to study the binding of CTXs to VGSCs.

As GA-A, gambierol acts as a functional antagonist of neurotoxin site 5 on VGSCs [151-153]. In addition, gambierol inhibits VGPCs in neurons, and two of its synthetic analogues have shown great potential for the development of therapeutic tools in autoimmune diseases [154]. Finally, gambierol and some synthetic analogues appear as promising molecules for the treatment of Alzheimer's disease (effects on *N*-methyl-d-aspartate, tau, and amyloid β expressions) [155].

Gambierones seem to share similar biological activities with CTXs. Indeed, gambierone causes VGSCs activation in a similar pattern as CTXs (appearance of sodium currents at hyperpolarized potentials), although with much less potency [144]. Likewise, both

gambierone and 44-methylgambierone also induce a small rise in the cytosolic calcium concentration in human cortical neurons, as CTXs [139]. However, a decrease in cell viability in undifferentiated neuroblastoma cells is observed at higher concentrations of 44-methylgambierone, opening new research avenues on the potential therapeutic effects of this newly characterized molecule [139]. Finally, the expression of ionotropic glutamate receptor has been shown to be modified by chronic exposure of human neurons to gambierones, an alteration that could be involved in the neurological manifestations observed in human CP [139].

5.3. Toxin Production in *Gambierdiscus* and *Fukuyoa* spp.

As mentioned previously, until the late 90s, *Gambierdiscus toxicus* was regarded as the sole origin of CTXs, but recent studies clearly show that this species is only slightly to non-toxic: actually, cultures of *G. toxicus* seem to produce considerable quantities of MTXs but only meager amounts, if any, of CTXs [25, 111]. Too, the lack of assay methods that clearly differentiate CTXs from MTXs in *G. toxicus* cultures may have puzzled early attempts to separate these two toxins [87, 136, 156].

Following the description of novel species in *Gambierdiscus* and *Fukuyoa* genera, many studies aimed at documenting CTXs and MTXs production in these two genera, since these two toxin groups are regarded as the primarily causes of CP (see section 5.2). To this aim, various analytical methods were used, e.g., the mouse biological assay (MBA), mode of action-based methods such as the receptor binding assay (RBA) and the cell based assay using neuroblastoma (CBA-N2a), as well as chemical methods such as LC-MS/MS [157-159].

Tables 3 and 4 provide a tentative overview of all the toxicity data reported across species/strains and geographic locations available in the literature. However, several issues clearly limit data interpretation and comparison between studies: i) the general lack of consensus in extraction protocols and analytical methods used, ii) the fact that these results were obtained under different culture conditions and/or harvesting growth phases, iii) the use of different units to express toxin contents, and iv) several of these studies do not provide quantitative values but, instead, simply inform on the presence/absence of specific toxic compounds.

5.3.1. CTXs Production

Table 3 is a summary of the CTX-related toxicity detected in most of the *Gambierdiscus/Fukuyoa* species known to date (data not available for *F. yasumotoi* and the newly described *G. jejuensis* species).

This table highlights the significant variations observed in CTXs production across species, but also between strains from different geographic origins (intra-specific variance): this is the case for instance for a *F. ruetzleri* strain originating from the Caribbean Sea which was found to be 27-fold and 3.8-fold more toxic than strains from the Gulf of Mexico and the Atlantic Ocean, respectively (Table 3) [19]. Similarly, the CTX-related toxicity in strains of *G. balechii* from the Pacific Ocean was at least 6-fold higher than in strains from Asia. In some instances, toxic and non-toxic strains can even be observed within a given species, as evidenced in a *F. paulensis* strain isolated from the Mediterranean Sea which was found

MBA^+, whereas strains originating from the Pacific and Atlantic Oceans showed no toxicity in MBA [37, 117, 127].

Table 3. CTX production in *Gambierdiscus* and *Fukuyoa* species/phylotypes. The units commonly used for CTX quantification are indicated in the first line of the table for each detection method, but different units can also be found in the literature: ([a]) mg.Kg^{-1}, ([b]) cell.MU^{-1}, ([c]) pg P-CTX1B/ P-CTX1 eqv.cell^{-1}, ([d]) pg C-CTX1B eqv.cell^{-1}, ([e]) pg PbTX-3 eqv.cell^{-1}

Species[(*)] and sea	Geographical area	MBA (MU.1,000 cell^{-1})	LC-MS/MS (pg CTX.cell^{-1})	rRBA (pg PCTX3C eqv.cell^{-1})	CBA-N2a (pg PCTX3C eqv.cell^{-1})	References
F. paulensis						
Pacific Ocean	New-Zealand	MBA^-	$LC\text{-}MS/MS^-$			[115]
Atlantic Ocean	Brazil				CBA-N2a^{--}	[37]
Mediterranean Sea	Balearic Islands	MBA^+	$LC\text{-}MS/MS^+$			[125]
F. ruetzleri						
Caribbean Sea	Belize		$LC\text{-}MS/MS^+$		(24.5 ± 0.12) x10^{-3}	[19, 126]
Gulf of Mexico	Northwestern				(6.5 ± 1.47) x10^{-3}	[19]
Atlantic Ocean	North Carolina USA				(0.88 ± 0.40) x10^{-3}	[19]
G. australes						
Pacific Ocean	French Polynesia	4x10^{-4}		[0.017-0.03]		[25, 82]
	Cook Islands	12x10^{-5} [0.8 - 6.3][a]			0.04[c]	[115, 162]
	Hawaii		$LC\text{-}MS/MS^-$		(2.7 ± 0.9)x10^{-3}	[85, 126]
	Kermadec Islands		$LC\text{-}MS/MS^-$			[47, 48]
Atlantic Ocean	Canary Islands				[0.6 - 1.4]x10^{-3} [280 - 697]x10^{-3c}	[85, 163]
	Portugal				[200 - 515]x10^{-3c}	[163]
Asia	Japan	670x10^{-4}				[164]
G. balechii						
Pacific Ocean	Kiribati Islands				[0.14 - 19.9]x10^{-3}	[38]
Asia	Malaysia				(1.11 ± 0.19) x10^{-3}	[38]
	Indonesia	MBA^+			(3.4 ± 1.5) x10^{-3}	[31, 85]
G. belizeanus						
Caribbean Sea	St Barthélémy St Maarten Virgin Island Belize Turks &Caicos			0.123	(0.65 ± 0.10) x10^{-3} (0.49 ± 0.26) x10^{-3} (0.43 ± 0.38) x10^{-3} (2.49 ± 1.28) x10^{-3} (0.51 ± 0.16) x10^{-3}	[19, 25]
Gulf of Mexico	Florida Keys				(0.54 ± 0.03) x10^{-3}	[19]
Red Sea	Saoudi Arabia				[1.02 - 6.5] x10^{-5c}	[249]
G. caribaeus						
Pacific Ocean	Hawaii				(1.6 ± 1.0) x10^{-3}	[85]
Caribbean Sea	Belize Cayman Island Mexico				[0.52 - 0.80] x10^{-3} (0.48 ± 0.04) x10^{-3} (1.29 ± 0.4) x10^{-3}	[19]
Gulf of Mexico	Florida Keys				(0.19 ± 0.03) x10^{-3}	[19]
Asia	Thailand	≥ 100x10^{-4}				[110]
G. carolinianus						
Caribbean Sea	Aruba Jamaica Puerto Rico St Maarten				(1.03 ± 0.94) x10^{-3} (0.10 ± 0.03) x10^{-3} (0.02 ± 0.01) x10^{-3} (0.18 ± 0.06) x10^{-3}	[19]

Species(*) and sea	Geographical area	MBA (MU.1,000 cell^{-1})	LC-MS/MS (pg CTX.cell^{-1})	rRBA (pg PCTX3C eqv.cell^{-1})	CBA-N2a (pg PCTX3C eqv.cell^{-1})	References
Mediterenean Sea	Crete				$(3.3 \pm 0.6) \times 10^{-3}$	[85]
G. carpenteri						
Pacific Ocean	Hawaii				$(1.4 \pm 0.6) \times 10^{-3}$	[85]
Caribbean Sea	Aruba Belize Jamaica Mexico				$(0.71 \pm 0.21) \times 10^{-3}$ $(0.29 \pm 0.16) \times 10^{-3}$ $(0.93 \pm 0.17) \times 10^{-3}$ $(1.14 \pm 0.18) \times 10^{-3}$	[19]
Gulf of Mexico	Flower Garden Banks				$(1.37 \pm 0.30) \times 10^{-3}$	[19]
Asia	Philipines			[1 – 7.48][e]		[69]
G. cheloniae						
Pacific Ocean	Cook Islands	[0.32 - 1.58][a]	LC-MS/MS^{-}			[32, 34]
G. excentricus						
Gulf of Mexico	Florida				0.469 ± 0.01	[19]
Atlantic Ocean	Canary Islands				1.43 ± 0.05 [0.37 - 1.10][c]	[28, 85]
G. honu						
Pacific Ocean	Cook Islands	0.2[a]				[32, 34]
G. lapillus						
Pacific Ocean	Australia	[0.78 - 150][a]	LC-MS/MS^{-}			[33]
G. pacificus						
Pacific Ocean	French Polynesia	9×10^{-4}		r-RBA^{-}	[5.44×10^{-4} - 13.5×10^{-3}]	[5, 25, 82, 85]
	Cooks Islands	0.8[a]	LC-MS/MS^{-}			[115]
	Republic of Kiribati				$(0.011) \times 10^{-3}$[c]	[240]
Asia	Malaysia				[31.7 - 75.8]$\times 10^{-3}$	[127]
G. polynesiensis						
Pacific Ocean	French Polynesia	[13×10^{-5}- 1500×10^{-4}]		[2.8 - 4.5]	[1.2 - 20.9]	[4, 5, 25, 82, 116, 158, 159, 258]
	Cook Islands	1.0[a]	18.2			[32, 115]
G. scabrosus						
Asia	Japan	20×10^{-4}			$(27.9 \pm 3.8) \times 10^{-3}$	[85, 164]
G. silvae						
Caribbean Sea	Curacao				$(19.6 \pm 4.21) \times 10^{-3}$	[19]
Atlantic Ocean	Canary Islands				[10.3 - 12.4] $\times 10^{-3}$	[85]
G. toxicus						
Pacific Ocean	French Polynesia	1 100 ± 164[b] MBA-				[25, 82, 154]
	Australia	MBA-		[0.022-0.028]		[132]
Caribbean Sea	Belize Martinique				[1.3-8.7]$\times 10^{-3}$[d] [30.7-54.3]$\times 10^{-3}$[d]	[109]
Gulf of Mexico	Florida, USA	[250 - 16 392][b]				[89]
Gambierdiscus sp. ribotype 2						
Caribbean Sea	Martinique Puerto Rico Virgin Islands St Maarten Belize				$(10.9 \pm 0.36) \times 10^{-3}$ $(6.63 \pm 0.54) \times 10^{-3}$ $(4.99 \pm 0.38) \times 10^{-3}$ $(4.66 \pm 1.01) \times 10^{-3}$ $(5.9 \pm 0.8) \times 10^{-3}$	[19]

(*) No information available for *F. yasumotoi* and *G. jejuensis.*

Based on Table 3, toxic species can be classified into three distinct groups:

I. a first group of species characterized by a CTXs production of the order of femtograms (< $4.0x10^{-3}$ pg P-CTX3C eqv.cell^{-1}, as assessed by CBA-N2a). This group comprises *G. belizeanus*, *G. caribaeus*, *G. carolinianus* and *G. carpenteri*, with the lowest toxicity recorded in a *G. belizeanus* strain collected from the Red Sea (Table 3).

II. a second group of species which display a toxicity within the range of femtograms (> $4.0x10^{-3}$ pg P-CTX3C eqv.cell^{-1}) up to < 1 pg P-CTX3C eqv.cell^{-1}, which includes *G. australes*, *G. pacificus*, *G. scabrosus*, *G. silvae*, *G. toxicus*, and *Gambierdiscus* ribotype 2. An exception concerns *G. excentricus* whose toxicity ranged from 0.47 up to 1.43 pg P-CTX3C eqv.cell^{-1} in the Atlantic Ocean, although the exact nature of the CTX analogues involved remains to be determined (Tables 2 and 3) [19, 28, 86]. Further investigations are also required to characterize the CTX profiles in *G. cheloniae*, *G. honu* and *G. lapillus* whose extracts showed a significant CTX-like activity on mice that could not be linked however to the presence of either P-CTX3B, -3C, -4A or -4B (Table 2) [32-34].

III. a third group composed exclusively of strains of *G. polynesiensis*, a species allegedly endemic to the Pacific Ocean which is recognized as the highest CTXs producer known to date. Published toxicity data for this species range from 1.2 up to to 20.9 pg P-CTX3C eqv.cell^{-1}, as assessed by either CBA-N2a, r-RBA or LC-MS/MS [4, 5, 25, 83, 117, 118, 160-162].

Although *G. excentricus* strains have been found to be twice to 14-fold less toxic than *G. polynesiensis* strains, these data suggest that the presence of these two species in a given area could be used as a CP high-risk biomarker, since they are the source of a CTXs reservoir that can be rapidly transferred through the trophic chain via the herbivores [160, 163].

5.3.2. MTXs Production

The contribution of MTXs in CP symptomatology is still the matter of current debate: indeed, because of their low intestinal absorption due to their high molecular weight and hydrophilicity, MTXs are believed to poorly contribute to ciguatera poisoning unless gut and liver tissues of fish are consumed [164].

Table 4 summarizes the MTXs production reported in *Gambierdiscus* and *Fukuyoa* species/phylotypes. It should be noted that the putative MTX3 (p-MTX3) reported in all the studies cited in this section, corresponds in fact to 44-methylgambierone which has been recently characterized [138, 139].

Whereas CTXs production is seemingly restricted to a small number of strains/species, MTX compounds appear to be produced by a large number of *Gambierdiscus/Fukuyoa* species, sometimes in copious amounts [86, 131, 132, 165, 166]. Conversely, based on these data, three species/phylotypes seem to be unable to produce MTX compounds: *F. ruetzleri*, *G. belizeanus*, and *Gambierdiscus* ribotype 2, as well as *F. yasumotoi*.

So far, the highest MTX-related toxicity to mice was evidenced in a *G. toxicus* strain originating from La Réunion Island. *G. caribaeus*, *G. carpenteri*, *G. scabrosus*, and *G. silvae* were found to produce less than 10 pg MTX eqv.cell^{-1} as assessed by CBA-N2a, while MTX production in *G. balechii*, *G. carolinianus* and *G. pacificus* produce up to 20 pg MTX

eqv.cell^{-1}. As for *G. australes* and *G. excentricus*, both species show a wider range of MTXs production, ranging from 4.3 to 275 and 0.48 to 85.7 pg MTX eqv.cell^{-1}, respectively (Table 4).

Table 4. MTX production in *Gambierdiscus* and *Fukuyoa* species/phylotypes. The units commonly used for MTX quantification are indicated in the first line of the table for each detection method, but different units can also be found in the literature: ([a]) mg.Kg^{-1}, ([b]) pg pMTX-1.cell^{-1}, ([c]) pg MTX4.cell^{-1}

Species[(*)]	Geographical area	MBA (MU.1,000 cell^{-1})	LC-MS/MS (pg MTX.cell^{-1})	CBA-N2a (pg MTX eqv.cell^{-1})	References
F. paulensis					
Mediterranean Sea	Balearic Islands	MBA^{+}			[125]
Fukuyoa ruetzleri					
Caribbean Sea	Belize		LC-MS/MS^{-}		[126, 129]
Gulf of Mexico	Flower Garden Banks		LC-MS/MS^{-}		[129]
Fukuyoa yasumotoi					
Asia	Singapore	MBA^{+}	-		[36]
G. australes					
Pacific Ocean	French Polynesia	0.2 MBA^{-}			[25, 82]
	Cook Islands		[2.0 - 8.9][b]		[115, 143]
	Hawaii		LC-MS/MS^{-}	(5.5 ± 0.5)	[85]
	Kermadec Islands		[0.3-36.6][b]		[47, 48]
Atlantic Ocean	Canary Islands			[4.3 – 4.7] [227-275]	[85, 163]
Asia	Japan	67x10^{-4}			[164]
G. balechii					
Asia	Indonesia	MBA+		(19.9 ± 2.9)	[31, 85]
G. belizeanus					
Caribbean Sea	St Barthélémy Virgin Island		LC-MS/MS^{-}		[129]
Gulf of Mexico	Florida Keys		LC-MS/MS^{-}		[129]
G. caribaeus					
Pacific Ocean	Hawaii		LC-MS/MS^{+}	(5.3 ± 1.0)	[85, 129]
Caribbean Sea	Belize Cayman Island Mexico		LC-MS/MS^{+}		[129]
Gulf of Mexico	Florida		LC-MS/MS^{+}		[129]
G. carolinianus					
Caribbean sea	Puerto Rico		LC-MS/MS^{-}		[129]
Gulf of Mexico	Dry tortugas		LC-MS/MS^{-}		[129]
Mediterenean Sea	Crete		LC-MS/MS^{-}	(10.6 ± 0.4)	[85, 129]
G. carpenteri					
Pacific Ocean	Hawaii		LC-MS/MS^{-}	(6.3 ± 1.9)	[85]
Caribbean sea	Belize		LC-MS/MS^{-}		[129]
	Jamaica		LC-MS/MS^{-}		[129]
Gulf of Mexico	Flower Garden Banks		LC-MS/MS^{-}		[129]
G. cheloniae					
Pacific Ocean	Cook Islands	[0.32-1.58][a]			[32, 34]
G. excentricus					

Table 4. (Continued)

Species(*)	Geographical area	MBA (MU.1,000 cell^{-1})	LC-MS/MS (pg MTX.cell^{-1})	CBA-N2a (pg MTX eqv.cell^{-1})	References
Atlantic Ocean	Canary Islands		[13.0 – 72.8][c]	[0.48 – 1.38] (85.7 ± 41.5)	[28, 85, 129]
	Brazil		[16.0 -19.8][c]		[129]
Gulf of Mexico	Florida		22.9[c]		[129]
G. honu					
Pacific Ocean	Cook Islands	0.2[a]			[34]
	Kermadec Islands	0.2[a]			[34]
G. lapillus					
Pacific Ocean	Australia	MBA^{+}			[33]
G. pacificus					
Pacific Ocean	French Polynesia	0.7	LC-MS/MS^{+}	20.1 ± 5.9	[25, 85, 129]
Asia	Malaysia		LC-MS/MS^{+}		[127, 129]
G. polynesiensis					
Pacific Ocean	French Polynesia	[0.06-0.1]			[25]
	Cook Islands	7.9[a]	LC-MS/MS^{-}		[115]
G. scabrosus					
Asia	Japan	67x10^{-4}		(1.5 ± 0.2)	[85, 164]
G. silvae					
Atlantic Ocean	Brazil		LC-MS/MS^{-}		[129]
	Canary Islands		LC-MS/MS^{-}	[2.2 – 3.3]	[85, 129]
G. toxicus					
Pacific Ocean	French Polynesia	0.7	LC-MS/MS^{-}		[25, 85]
	New Caledonia	0.6			[25]
Indian Ocean	La Réunion	1.7	LC-MS/MS^{-}		[25]
Gambierdiscus ribotype 2					
Caribbean sea	Belize		LC-MS/MS^{-}		[129]
	Puerto rico		LC-MS/MS^{-}		[129]
	St Maarten		LC-MS/MS^{-}		[129]

(*) No information available for *F. yasumotoi* and *G. jejuensis.*

It has been suggested that the ability to produce CTXs and MTXs is a stable characteristic in *Gambierdiscus* strains, which is maintained even years after their isolation and acclimatation to laboratory conditions [25, 32, 47, 48, 83, 117, 131, 166, 167]. This observation has led to the speculation that toxin production is genetically determined in this dinoflagellate. However, the significant variation observed in toxicity within and among species [e.g., 19, 83, 86, 110, 131, 168, 169] suggests that this functional trait may also depend on a combination of environmental factors, both biotic and abiotic drivers, as in many other phytoplankton taxa.

5.3.3. Biotic Factors Influencing Toxin Production in Gambierdiscus and Fukuyoa Spp.

5.3.3.1. Genetics of Toxin Production

In the Eukaryote kingdom, *Gambierdiscus* species are among the organisms with the largest genomes, e.g., 32.5 and 35 Gbp for *G. australes* and *G. belizeanus*, respectively [170]. However, very little is known on the biogenesis of CTXs and MTXs and even less on the genes responsible for the production of these toxins in *Gambierdiscus/Fukuyoa* genera.

CTXs and MTXs are polyether ladder compounds that have a polyketide origin. Polyketides are synthesized by specific enzymes called polyketide synthases (PKSs) through a series of condensation and reduction steps of acyl monomers. PKS enzymes are multi-functional complexes consisting of a minimal set of catalytic domains, namely ketoacylsynthase (KS), acyl transferase (AT) and acyl carrier protein (ACP), which are required for function. Three further domains ketoacylreductases (KR), dehydrases (DH) and enolreductases (ER) can be optionally present, and when present, are responsible for the broad variety of polyketide structures found in dinoflagellates [171]. Traditionally, these enzymes are classified into 3 types, according to their domain organization, in particular Type I PKSs which correspond to large multifunctional enzymes with modular or iterative activity [172, 173].

To date, the transcriptomes of several CTX-producing species of *Gambierdiscus* have been investigated: *G. polynesiensis*, *G. belizeanus*, *G. australes* and *G. excentricus* [170, 174, 175] and contigs with sequence similarity to Type I PKSs were found, as reported in other dinoflagellates [171, 176, 177]. Coincidentally, all four species also produce 44-methylgamberione [138, 139]. Of note, no differences have been highlighted in the expression of the monofunctional KS domains between these four species [174], although substantial differences exist in these strains' known toxin profiles for CTXs and MTXs: e.g., at least six P-CTX analogs have been detected in *G. polynesiensis* cultures while three unknown CTXs are suspected in *G. excentricus*; moreover, MTX1 is produced by *G. australes* vs. MTX2 and MTX4 in *G. excentricus* (Table 2), whereas low amount and no MTXs has been found in *G. polynesiensis* and *G. belizeanus*, respectively (Table 4).

Additionally, contigs with sequence similarity to type II Fatty Acid Synthase (FAS) genes were also found [174]. This differentiation of PKS and FAS pathways in *Gambierdiscus* is important, as it will likely facilitate approaches to investigate toxin biosynthesis pathways in this dinoflagellate.

In summary, the genetic basis of CTXs and MTXs synthesis is still unresolved and additionnal fundamental studies are required to further understand these perplexing pathways, in order to promote the development of novel methods for the monitoring of these harmful dinoflagellates.

5.3.3.2. Growth Stage

An earlier study showed an increase in total toxicity using MBA from the exponential to the stationnay phase for five *G. toxicus* strains originating from the Pacific and the Atlantic Oceans, regardless of N:P ratio [110]. These results are consistent with the common belief that ciguatoxins are secondary metabolites as their production is enhanced during the stationary growth phase. The variability of toxin production in *G. polynesiensis* was also evaluated at different growth stages via the MBA and r-RBA [83]. Regardless of the test used, CTXs production decline from the beginning to the end of the exponential growth phase to 3.4 ± 0.1 eqv P-CTX3C pg.cell^{-1} followed by an increase during the stationary phase up to 11.9 ± 0.4 eqv P-CTX3C pg.cell^{-1} [83]. It is noteworthy that freshly inoculated cells produce also high amounts of CTXs similarly to the ones monitored at the end of the stationary phase. For MTXs production measured by MBA, the same production pattern was observed in *G. polynesiensis* showing a toxicity of 18 ± 4 x10^{-5} MU.1000 cell^{-1} and almost twice during the stationary phase at 31 ± 9 x10^{-5} MU.1000 cell^{-1} reaching a maximum in freshly inoculated cells 50 ± 10 x10^{-5} MU.1000 cell^{-1} [83]. In *G. australes* isolated from the Macaronesian

Islands, MTXs were not produced during the exponential phase, but only during the stationary phase [166].

When comparing CTXs and MTXs production in *G. polynesiensis*, the same pattern was observed for both except that CTXs were dominant in aged cells at the end of the stationary phase [83]. This CTXs production pattern was also observed in *in vitro* cultures of *G. toxicus* presenting an increase in toxicity in aged cells [111, 156, 178]. So, if one wants to compare levels of toxin production between several strains of *Gambierdiscus* spp., cells should be cultivated under the same laboratory conditions along with a harvest time at the same growth age. For several *Gambierdiscus* species, cell division seems to be slow down during the stationary phase, but the positive correlation of toxin biosynthesis with aged cells is still unclear [19, 83]. It has been hypothetized that the energy required by *Gambierdiscus,* or other toxic dinoflagellates, cannot be allocated at the same time to cell division and toxin production [70, 83, 111, 179]. Additionnally, the high toxin content measured in *G. polynesiensis* was linked to a growth rate slower than for other *Gambierdiscus* species [83] as also observed for *G. excentricus* and *G. carpenteri* [19, 70, 86]. A low growth rate with high production of intracellular toxin could also be considered as a particularly advantageous evolutionary strategy in the natural environment, to improve self-protection in reducing competition with other microalgae or to limit grazing by herbivores [19, 83, 180]. On the contrary, a positive relationship was observed between a high growth rate and "relative" high levels of CTXs production [0.2 - 0.697] pg P-CTX1B eqv.cell^{-1} during the exponential growth phase in *G. australes* strains originating from Macaronesian Islands [166].

5.3.3.3. Contribution of Bacterial Flora

Previous studies have not found an influence of associated bacteria on CTXs production in *Gambierdiscus* since the toxicity of *Gambierdiscus* strains cultured in axenic vs. xenic conditions were found to be similar, and endosymbiotic bacteria could not be observed in transmission electron microscopy (TEM) sections toxigenic cells [78, 156, 168, 181, 182]. However, a recent study by Wang et al. (2018) [180] has provided evidence that the growth and toxin production of *Gambierdiscus* spp. can be regulated by quorum sensing bacteria. Using nine different co-cultured bacteria, these authors showed that eight of them were able to enhance the growth of *Gambierdiscus* spp., suggesting nutrient supply and/or release of growth-promoting bioactive substances to this microalgae [180]. Negative and positive interactions on toxin production were also observed, depending on the bacteria co-cultured with *Gambierdiscus* [180]. Different interactions have been suspected between associated bacteria and *Gambierdiscus*: nutrient transferring, sources competition, toxic substance releasing and photosynthesis inhibition that can play a role on algal survival, acquisition of energy, cell division and the ability of *Gambierdiscus* to produce toxins [180]. Then, this algal-bacterial relationship is complex and more efforts should be made in understanding its ecological roles.

5.3.4. Abiotic Factors Influencing Toxin Production in Gambierdiscus and Fukuyoa spp.

From the first studies on *Gambierdiscus* spp., the influence of environmental factors on toxin production was questioned. Because light, temperature, salinity, pH, and nutrients foster the growth and ecological distribution of *Gambierdiscus* dinoflagellates, these factors might also impact toxin production. However, the first attempts to examine CTXs production

towards environmental factors were hurdled by unresolved taxonomy in *Gambierdiscus* species [70, 79, 110, 128, 169, 183-186].

Since the 2000s, there has been a globalization of the ciguatera phenomenon due to the geographic extension of *Gambierdiscus* spp. ranges to areas previously spared from this disease (e.g., Europe), probably in response to the effects of climate change [58]. Furthermore, in a global context of climate change, a better knowledge of the environmental factors likely to influence the toxinogenesis of the microalga appears essential if one wants to be able to anticipate the severity of outbreaks varying from one region to another. In this context, the effects of environmental parameters on the growth of several *Gambierdiscus* species have been the subject of recent studies [58, 69, 84, 85, 104, 111]. These studies have shown the ability of some species to adapt to wide ranges of temperature, salinity, light or nutrients and to better understand the geographical distributions of endemic or ubiquitous *Gambierdiscus* species. However, how changes in environmental conditions can affect *Gambierdiscus* toxin production is still explored.

Regarding nutrient parameters, the total toxicity of five *G. toxicus* strains measured by MBA were not significantly influenced by increasing N:P ratio whether during the exponential phase or the stationary phase [110]. Similarly, N:P ratio did not seem to influence Na^+ channel activity detected at all N:P ratios for the two toxiest strains MQ2 (Atlantic ocean) and TO4 (Pacific ocean), except at ratio N:P of 5:1 where Na^+ channel activity recorded for MQ2 strain was the highest. Conversely, Na^+ channel activity was detected for the three other strains only at the highest N:P ratios [110]. These authors suggested genetic differences between these strains of *G. toxicus* whereas an unresolved taxonomy was probably one of the explanations. CTXs productions of two strains of *G. toxicus* from the Caribean were not affected by organic (nitrate or ammonium) or inorganic (urea, free amino acids or putrescine) nitrogen sources [111]. The only significant effect was observed for one strain whose cells were found to contain higher CTXs levels in the stationary phase vs. the exponential phase when grown on nitrate [111].

When cells were grown at different salinities of 30, 32 and 35, the toxin profile of *G. toxicus* was modified in terms of the ratio of the most abundant CTX putative precursor [128]. In the same way, *G. carpenteri* originating from the Philippines sampled at a salinity of 30, showed a great adaptability in laboratory conditions towards a large range of various salinity levels from 26 to 41 [70]. Within this tolerable range of growth, CTXs production was optimal under the lowest salinity, i.e., 26, showing the highest cellular content of 7.48 ± 0.49 pg PbTx3 eqv.cell^{-1} followed by a decline in CTXs production starting at 29 until the highest levels of salinity tested [70]. How an increase in salinity can actually interfere with toxin biosynthesis is still unexplained. However, a possible explanation could be that the toxins acting on voltage gated sodium channels (activators or inhibitors) produced by these toxic dinoflagellates may affect osmoregulation of ion concentrations inside the cells [187].

The effect of light on *Gambierdiscus* toxins production has also been investigated. As mentioned previously, this dinoflagellate is able to grow in shaded as well as highlighted water habitats [50]. Toxin production in *G. carpenteri* was high at low light conditions and decrease slowly by 2-fold under highest light intensities [70]. Photo inhibition did not seem to occur as toxin production was still maintained under high light intensities [70]. Little is known about the link between photosynthesis and biochemical pathways needed for toxin production in dinoflagellates, although a study has shown that an increase of chlorophyll *a*

content following an increase in light intensity was observed in parallel with a decrease of toxin production in *Pyrodinium bahamense* var. *compressum* [188].

When *G. carpenteri* was grown under a wide temperature range of 19-33°C, the highest toxin production was recorded at 19°C followed by a 2-fold decrease and stabilization at 27-33°C [70]. Moreover, when its toxin production declined, an increase in growth rate was observed. It has been speculated that this negative relationship, also observed in other *Gambierdiscus* species and toxic microalgae, may be due to the adverse effects of temperature changes that could influence up and down regulation of mRNA during toxin biosynthesis [70 and reference therein].

5.4. Ecological Relevance of Ciguatera-Related Compounds

The ability of microorganisms to produce and release chemicals/toxins that affect potential competitors for resources is well known among dinoflagellates [189-191]. The diversity and structural complexity of toxins synthesized by epiphytic *Gambierdiscus*/*Fukuyoa* spp. have led to speculation that they may be allelochemic agents directed against co-occurring taxa of the thallisphere [45]. In particular, *Gambierdiscus*/*Fukuyoa* spp. are frequently found associated with other dinoflagellates in the genera *Ostreopsis, Prorocentrum, Coolia, and Amphidinium* in benthic assemblages of ciguateric biotopes [42, 192, 193]. The allelopathic effects of ciguatera-related toxins towards these taxa are still poorly documented. Earlier studies showed that *Prorocentrum concavum* and *G. toxicus* were reciprocally inhibited both in exudate-supplemented cultures and in cross-cultures, and that both exudates and CTX extracts of *G. toxicus* were able to inhibit the growth of several diatoms and chlorophytes species [for a review see 194]. A more recent study also showed that, in crossed culturing experiments, culture media preconditioned with filtered-exudates of *G. toxicus* were able to inhibit the growth of *Coolia monotis* and *Ostreopsis lenticularis* [195]. Of note, only one study has addressed the possible role of gambieric acid-A as an endogenous growth enhancer towards *G. toxicus* cultures [196].

Conversely, extracts of several benthic taxa known to co-occur with *Gambierdiscus* spp. in ciguateric biotopes, e.g., *Prorocentrum lima*, *O. lenticularis*, *C. monotis*, are able to suppress *Gambierdiscus* spp. growth or adherence capacity in the laboratory [195, 197, 198]. However, evidence shows that these allelopathic effects may be under the control of various environmental factors such as temperature or salinity (suppression or reversal of the inhibitory effects of *Ostreopsis* sp. on *G. carpenteri* growth) [198].

Since antagonist relationships of *Gambierdiscus* with co-occurring benthic dinoflagellates [17] or diatoms [101] are sometimes observed in the field, it appears interesting to pursue and develop laboratory studies in order to better characterize interspecific interactions among ciguateric phytobenthic communities and to better understand the ecological relevance of the secondary metabolites produced by *Gambierdiscus*/*Fukuyoa* species. Apart from being used to compete for space, these molecules could also eventually be used to repel grazers or repress the growth of pathogenic organisms. Moreover, to this date, lab-experiments haven't really addressed the characterizations of which secondary metabolites are responsible for these allelopathic effects.

6. SOCIO-ECONOMIC IMPACTS OF CIGUATERA POISONING

Ciguatera Poisoning (CP), associated to CTXs exposure, results in a large declination of gastro-intestinal, cardio-vascular, neurological, neuropsychiatric and other systemic manifestations, which may last weeks, months or even years [9], and is considered the most commonly reported marine biotoxins related illness worldwide, with an annual incidence estimated between 50,000 and 500,000 [199]. CP is also known to be responsible for substantial human health and socio-economic impacts for populations living in endemic regions, especially from the South Pacific, and a threat for millions of costal living inhabitants. Nevertheless, the ability to assess CP socio-economic impacts prone to be challenging due to the lack of i) biological diagnosis tools, that contribute to the difficulty diagnosing CP due to the non-specific nature of symptoms, ii) global awareness, especially among the medical community, iii) specific reporting systems and, iv) systematic ciguatoxic fish screening due to expensive CTXs detection tests.

6.1. Ciguatera Poisoning Impact on Health-Related Expenses

CP incidence is not easy to ascertain because of an important under-reporting in part due to the fact that persons affected often do not seek medical attention, especially in endemic regions where populations have become accustomed to heal themselves relying on traditional medicines [200, 201], and that health care workers, may have difficulties to correctly diagnose the disease, and if so, do not know or are not obliged to declare cases to authorities. Indeed, CP specific reporting systems are scarce and CP report is mandatory in only few countries (Cayman Islands, Cuba, Miami, Canary Islands, Hong Kong) [202, 203]. This has for consequence that CP under-reporting can rich 90% in some part of the world [204] and contributes to the rarefaction of studies aiming at assessing health-related costs.

Despite these difficulties, some authors estimated individual CP health expenses at USD $1,513/reported case in Cook Islands [205]; USD $1,613/reported case and USD $749/unreported case in Moorea Island (French Polynesia) [206]; USD $1,000/reported case and USD $700/unreported case in developed countries such as Canada [207], USD $545 in US Virgin Islands and USD $200 in Aruba [203]. Global Public health-related costs were grossly estimated at USD $ 19 million/year in USA tropical jurisdictions (Florida, Hawaii, Puerto Rico, Virgin Islands, Guam, American Samoa, Northern Mariana Islands) for the period 1987-1992 [208] and USD $241,847 for the period of 2007-2013 in Moorea Island (French Polynesia) [206]. Todd (1985) [207] estimated the CP economic impact associated with medical costs and lost-labor productivity for Canada at CAD $2.7 million/year, between 1960's and 1980's. CP economic impacts (including estimated health-related costs, and monitoring and management costs) where estimated around NZD $396,000 in 2011 in Rarotonga (NZD $750,000 during epidemic pick in 2006) [205].

Of note, these estimation were obtained under different contextual circumstances, such as the study period, the level of populations' dependence to marine products, social costume, access to imported food, demographic trends, hospitalization duration or number of days off work, that can interfere in calculations' extrapolation and renders comparisons difficult [54, 92, 209, 210]. It does not take in account either insurance costs purchased by certain seafood

companies to cover potential ciguatera-caused damages [211], or direct arrangements for medical expenses reimbursement between seafood sellers and ciguatera affected consumers, as is customary in some Pacific Islands, such as French Polynesia. Moreover, these cost estimations are limited to the acute maganement of CP and exclude the burden of medical expenses related to chronic sequels, that can last for months or even years, and concern over 20% of the population affected [11, 12]. Finally, if CP is retalivelly easy to diagnose in endemic regions, it can prove to be complicated in temperated areas where the diagnosis can take several months, multiple specialists consultations, and expensive medical examinations (blood and urine analysis, electromyogram, scanner, Magnetic Resonance Imaging, biopsies, etc.), whose expenses can significantly weigh the costs associated to patients' management.

6.2. Ciguatera Poisoning Impact on Local Fisheries

Fish contribute substantially to the livelihoods of Pacific Islands Countries and Territories (PICTs) nations whose annual fish consumption rate are among the highest in the world (between 50 to 110 kg/capita) [212]. Besides providing Pacific islanders with essential dietary proteins and animal fat, marine products also represent an important mainstay for local economies in that they contribute significantly to households incomes [213, 214]. CP therefore represents a major concern and burden for these communities and fisheries industry [215]. Of note, fish species commonly regarded as ciguatoxic fish, such as groupers, snappers, mackerels, jacks, barracudas, wrasses, parrot fish and surgeon fish, are often listed among the main species sold in many market places [9, 216].

In the Cook Islands, the increase of CP occurrence over the past two decades has progressively discouraged local fish consumption [2]. Indeed, results of a survey realized in 2006 indicated a constant decline in average daily *per capita* fish consumption rates since 1989, from 318 g in 1989 to 176 g in 2006. These results are in accordance with observations made in Tuvalu, especially in Funafuti, where the number of fish species affected by ciguatera has increased concurrently with the risk of discouraging consumers to eat marine products [217].

In the absence of global consensus and systematic CTXs detection in marine products, health autorities from endemic regions usually put bans on ciguatera emblematic species, or specimens over a certain size, based on the general assumption that a positive correlation exists between the amount of toxins and fish size. Such regulation exists since 1939 in French Polynesia and has progressively be implemented in American Samoa, Fiji, Hawaii, New Zealand, Australia, Florida, Mexico, Cuba, Puerto Rico, Gualeloupe, Reunion Islands, etc., with, nevertheless, some variations in species listed from a country to another [203, 218-222]. Some countries, such as the Dominican Republic, have also opted for temporal restrictions since they recommend avoiding certain at-risk species between the months of May and August [203].

The economic losses caused by the harvest loss associated with these restrictions have been tentatively estimated around USD $1.1 million/year in French Polynesia [223], USD $10 million in Caribbean [199] and over NZD $700,000 during CP epidemic in Cook Islands according to Rongo and van Woesik (2012) [205].

6.3. Ciguatera Poisoning Impact on International Trade

A typical example of how a single-CP event can lead to the permanent closure of an export industry is the one of the Republic of Kiribati, where fish trade used to be an important source of income for local fishermen (estimated over AUD $8,000/fisherman). In 1999, a major CP outbreak occurred in Hong Kong with fish supposedly imported from Kiribati. This event resulted in the ban of all fish from Kiribati in the Hong Kong market, and in the total closure of the trade in Kiribati, which represented an annual revenue loss of AUD $250,000 [224].

CP cases reported these past nine years in Europe by the European Rapid Alert System for Food and Feed (RASFF) where all due to fish imported mainly from Indian Ocean and South-East Asia [225].

Given that many countries do not have laboratory CTXs detection capabilities, and lack accurate species labelling and traceability procedures, and that no regulatory limits nor reference methods exist, regulation policies regarding the trade of potential ciguatoxic fish relies entirely on the appreciation of local authorities. Based on the understanding that fish are not screened on a routine basis, recommendations, mostly concern the avoidance or prohibition of specimens, particularly large predatory reef-fish that are regarded as "suspect" simply because they originate from affected areas [209].

The Council Directive 91/493/EEC of the European Community states that placing on the market "*fishery products containing biotoxins such as ciguatera toxins or muscle-paralysing toxins*" is forbidden [226], while in its Guidance for industry, the US-Food and Drug Administration (FDA) recommands that "*because ciguatoxic endemic areas are localized, primary seafood processors should recognize and avoid purchasing fish from established and emerging areas of concern*" [227].

Alternatively, other countries such as Australia (Sydney Fish Market) may choose to circumscribe the field of restrictions, by publishing several lists of bans based on different criteria: i) a list of prohibited specific species whatever their origin (chinaman or chinaman snapper, tripletail maori wrasse, humpback maori wrasse, red bass, paddle-tail or humped-back red snapper and moray eel); ii) a list of prohibited specific species from precise locations (coral trout form Fijian waters); iii) a list of prohibited locations whatever the species (Kiribati or Marshall Islands); and iv) a list of species maximum size limits [228].

6.4. Ciguatera Poisoning Impact on Tourism

Globally, travelers are poorly informed about ciguatoxic risk by the tourism industry. According to the United Nations World Tourism Organization, there were over 1.3 billion "international tourist arrivals" in 2017. This data is in constant augmentation since 2010 [229], like the risk of contracting uncommon pathologies, such as ciguatera, for a temperate region traveler due to tourist attractiveness for tropical and subtropical countries [230, 231].

Some countries show a certain reluctance to communicate on their ciguatera incidence for fear of a dramatic decrease in tourist frequentation. In at-risk areas, CP has been associated to a decrease in tourism and recreational fishing [209, 211] and represents a constent source of apprehension to hotels and restaurants industry. Indeed, if a severe poisoning event should

occur, this could derive to a loss of business and a long-term stigmatization of the establishment.

In 2019, 55 countries and island territories were listed as "at-risk destinations" by the International Association for Medical Assistance to Travellers (IAMAT) [232]. For countries regarded as at high risk of ciguatera, the extent to which this ciguatoxic status may actually influence the choice of it as a travel destination in tourists' mind is not documented, as yet.

6.5. Ciguatera Poisoning as a Cause of Lifestyle and Tradition Upheaval

It has been suggested that CP have impacted human migration, and fishing and dietary patterns among Pacific populations [2, 209, 233, 234].

Beyond its direct impact on fisheries, ciguatera can also lead to drastic changes in lifestyle linked to a progressive loss of food capital [204] and a forced dietary shift among local populations, resulting in a strong reliance on imported and/or canned products, with the risk of increasing sugar and fat intake. As an example, a shift in dietary habits was reported among Rarotonga residents (Cook Islands) who modified their fish consumption in favor of an increased intake of chicken and canned fish, particularly during the peak of a ciguatera outbreak [2]. Moreover, some PICTs not only observed changes in their population's diet, but also reported secondary medical consequences, such as diabetes, as an indirect aftermath of CP [214].

Finally, a survey conducted among Rarotonga communities showed that CP may also interfere and disrupt the intergenerational transmission of traditional knowledge in affected communities, particularly with regard to fishing practices [205].

6.6. Ciguatera Poisoning before the Law

With increasing CP incidence, not only appeared a loss in confidence of consumers regarding fishery industry, but also legal actions for non-compliance with regulations or requests for compensation. An example of litigation concerned a CP event involving 46 individuals attending a banquet at a restaurant, which served maori wrasse fish imported from Trunk Reef in Queensland (Australia), in 1997 [235]. One of the affected consumers made a legal claim against the restaurant, which turned against the fish supplier. As a result, both the restaurant and the supplier were declared negligent by the judge in charge [236]. In the coming years, it is expected to assist to an increase of lawsuits of this type.

According to the Food and Agriculture Organization (FAO) of the United Nations "c*iguatera dinoflagellates are predicted to become one of the increasing food safety threats due to climate change*" [237], so that a significant increase in ciguatera burden is to be expected, not only in endemic regions, but also at a global scale**.** The multiple ramifications of ciguatera thus emphasize the need for more thorough studies using a more integrated approach of CP socio-economic impacts, for improved ciguatoxic risk prevention and management.

CONCLUSION

Significant advances have been achieved in the past two decades in the understanding of the ciguatera dinoflagellates *Gambierdiscus* and *Fukuyoa*. Still, ciguatera events occurrence and variability in their severity across geography remain difficult to predict.

Identifying the key-factors likely to contribute to ciguatera flare-ups is very challenging due to the multitude of potential influential factors and their interactions. Although much of the data available in the early work on *Gambierdiscus* spp. is valid for the genus, many studies will have to be redone for the various species now known to exist in *Gambierdiscus* and *Fukuyoa* genera. Too, in laboratory-based studies, experiments designed to explore the concomitant effects of two or more factors on *Gambierdiscus*/*Fukuyoa* spp. growth should be encouraged, since responses may significantly differ from those obtained in single-factor studies. Moreover, the current knowledge on these organisms needs to be reassessed through more observational field studies since natural conditions often produce responses that are different from laboratory-controlled conditions.

Furthermore, most of the studies currently available in the literature were designed to assess inter-specific variability based on the growth response of a single strain for each species, but little is known about the intra-specific variance within each species. Some authors have speculated that isolates within a given species can easily adapt to the changes of environmental conditions, and that the differences observed in growth responses and toxin production between isolates at varying environmental conditions may be habitat dependent rather than species dependent [70, 198]. This has prompted recent studies aiming at investigating the effect of fine-scale habitat heterogeneity (e.g., macroalgal cover, composition of epiphytic communities, etc.) on the dynamics of benthic harmful dinoflagellate assemblages, including *Gambierdiscus* spp. [193].

Gambierdiscus/*Fukuyoa* communities in a given area can comprise multiple co-occurring species that vary with respect to their toxicity. Therefore, species composition of blooms and most importantly the presence of certain highly toxic species and/or strains are likely to play a prominent role in both CFP flare-ups and severity of outbreaks [4, 17, 19]. It is thus of utmost importance to devote future research efforts to: i) fully characterize the toxin profile associated with each of the species and genotypes now known, focusing primarily on species known to produce substantial amounts of toxins, particularly *G. polynesiensis*, and *G. excentricus*; ii) clarify how these algal toxin profiles may contribute to shape the toxin profiles in ciguatoxic fish, and iii) clarify the biological activities and contribution of recently characterized compounds in ciguatera symptomatology. All these data will be useful to inform ciguatera risk models.

Future field ecology studies should also examine the role of the macroalgal community in the transfer of ciguatera toxins to higher trophic levels, particularly with regard to how algal palatability and grazing pressure from herbivores can significantly influence toxin flux injected in marine food webs [105].

Finally, field observations are increasingly showing that other epiphytic dinoflagellates often co-habit with *Gambierdiscus*/*Fukuyoa* communities in benthic assemblages of ciguatera-prone biotopes [42, 92, 192]. These benthic HAB species are known to be the potential source of other potent toxins likely to bio-accumulate in marine food webs, and whose potential contribution to ciguatera symptomatology is still poorly documented. In this

regard, the introduction of high throughput screening techniques such as metabarcoding in current monitoring programs would greatly benefit this area of research.

Undoubtedly, the greatest challenge for the ciguatera scientific community in the coming years will be to translate the recent advances in the knowledge-base of the ecology and toxicology of *Gambierdiscus*/*Fukuyoa* spp. into practical solutions for a more reliable assessment of ciguatera risk and effective mitigation programs.

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Chapter 147

DIARRHETIC SHELLFISH POISONING

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ABSTRACT

Okadaic acid (OA) and dinophysistoxins (DTXs) are the causative toxins of diarrhetic shellfish poisoning (DSP). OA/DTX analogues are produced by dinoflagellates that belong to the genera *Dinophysis* and *Prorocentrum*. In the present chapter, chemical nature, bioactivities, accumulation and metabolisms of toxins in bivalves, analytical methods of OA/DTX analogues are described.

Keywords: okadaic acid (OA), *Dinophysis*, dinophysistoxins (DTXs), diarrhetic shellfish poisoning (DSP), Liquid chromatography-mass spectrometry (LC/MS), metabolism

INTRODUCTION

Diarrhetic shellfish poisoning (DSP) in humans caused by consumptions of bivalves was first reported in Japan in 1978 [1]. DSP human symptoms are diarrhoea, nausea, vomiting and abdominal pain starting 30 min to a few hours after ingestion and complete recovery occurs within 3 days [1]. Okadaic acid (OA) [2] and its analogues dinophysistoxins (DTXs) are the causative toxins of diarrhetic shellfish poisoning (DSP) [3, 4]. OA/DTX analogues are produced by dinoflagellates that belong to the genera *Dinophysis* [5, 6]. Bivalves become contaminated with OA/DTX analogues by feeding on toxic *Dinophysis* species. Although the dinoflagellate *Prorocentrum* is found to be a producer of OA/DTX analogues, involvement of bivalve contamination is unknown. Some predators of bivalves such as crabs and lobsters are also contaminated with OA/DTX analogues by feeding toxic bivalves.

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Table 1. Some DSP cases reported with epidemiological investigations

Countries Years	Toxins	Shellfish species	(a) Numbers of people consumed shellfish (b) Numbers of patients (c) Numbers of people investigated for epidemiological study	Detection methods	Toxicities	Estimated amounts of toxins consumed by people	Ref.
Japan (Miyagi Pref. etc.) 1976, 1977 (June-July)	DTX-1	Mussels (*Mytilus edulis*) Scallops (*Patinopecten yessoensis*, *Chlamys nipponensis akazara*)	(a) - (b) 164 (c) 8 (10-68 ages)	MBA[*1]	5.0-8.5 MU[*2]/g midgut glands	12 MU for mild symptoms 19-70 MU for severe symptoms	1
Japan (Hokkaido) 1982 (June)	-	Mussels (*Mytilus coruscus*) Venus clams (*Gomphina (Maeridiscus/melanaegis*) Scallops (*Patinopecten yessoensis*)	(a) 35 (b) 21 (c) 6 (2-38 ages)	MBA	-	8.6 MU for mild symptoms 5.4-6.5 MU for no symptoms	7
Japan (Gifu Pref.) 1982 (July)	DTX-3	Scallops (*Patinopecten yessoensis*)	(a) 48 (b) 44 (c) 20	MBA	7.0-13.5 MU/g midgut glands	>18 MU	8
Norway 2001 (July)	OA and OA esters?	Mussels	(a) 77 (b) 39 (c) 72	HPLC-FLD[*3]	55.56 ug/100g edible tissues (Hydrolysis of samples was not done)	1-1.5 ug OA equivalent/kg body weight	9
Portugal 2001 (July)	DTX3	Razor clams (*Solen marginatus*)	(a) 6 (b) 5 (c) 6 (9-61 ages)	LC/MS[*4]	50 ug OA equivalent/100g edible tissues (Hydrolysis of samples was done)	25-175 ug OA equivalent	10
UK 2006 (June)	OA and OA esters	Mussels	(a) - (b) 159 (c) -	LC/MS	25.8-30.2 ug OA equivalent/100g edible tissues (Hydrolysis of samples was done)	> 40ug OA equivalent	11
France 2009 (June)	OA and OA esters	Mussels	(a) 49 (b) 18 (c) 11 (13-65 ages)	MBA and LC/MS/MS	1.261 ug OA equivalent/kg edible tissues (Hydrolysis of samples was done)	45 ug OA equivalent for LOAEL 0.8 ug/kg body weight	12

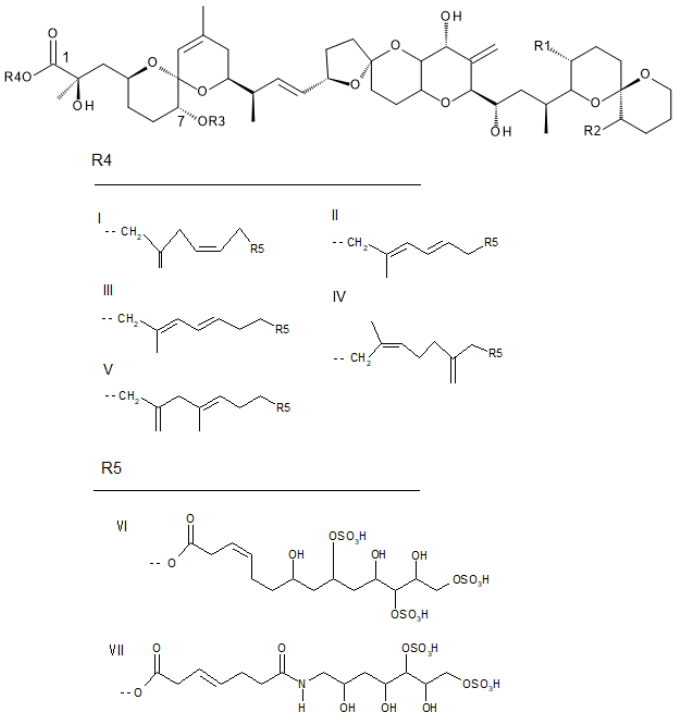
[*1] Mouse bioassay.
[*2] Mouse unit.
[*3] High performance liquid chromatography fluorometric detection.
[*4] Liqiud chromatography mass spectrometry.

DSP has been reported from almost all over the world including North and South America, Europe, East Asian countries, and New Zealand etc. Table 1 shows some DSP cases reported with epidemiological investigations which succeeded in estimating the amount of bivlave molluscs and the toxins consumed by affected people [1, 7-12]. It is estimated that consumption of 48 µg of OA or 38 µg of DTX-1 per person results in diarrhea [13]. The regulation of OA/DTX analogues recommended by Codex Alimentarius (CODEX STAN 292-2008) is 0.16 mg OA equivalent/kg in the edible tissues of bivalves. This regulatory level is widely accepted in the world.

CHEMISTRY AND BIOACTIVITIES

OA was originally isolated from the two sponges, *Halichondria okadai* Kodata in Japan and *H. melanodocia* in the Florida Keys and the structure was elucidated by single crystal X-ray diffraction techniques for *o*-bromobenzyl ester of OA, nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS) [2]. The chemical structures of OA/DTX analogues are shown in Figure 1. In 1982, DTX-1 was isolated from the mussel *Mytilus edulis* involved in the DSP in Japan and the structure elucidated by NMR and MS spectrometry was reported as 35(*S*)-methyl OA [3]. This was the first report identifying that OA analogues are the causative agent in DSP. The stereochemistry at the C-35 was later corrected as 35(*R*)-methyl [14, 15]. DTX-3 was first isolated from Japanese scallops, *Patinopecten yessoensis*, and the structure was elucidated as 7-*O*-acyl-DTX-1 in 1985 [4]. Detailed fatty acid profiles of the DTX-3 in bivalves had later been elucidated by LC/MS and LC/MS/MS techniques [16-21]. OA diol esters were found from the benthic dinoflagellate *Prorocentrum lima* in 1987 [22] and several diol esters had later been found from *Prorocentrum lima*, *P. concavum*, *P. maculosum*, *P. belizeanum* [23-31] and *Dinophysis acuta* [32-35]. In 1992, DTX-2 was isolated from Irish mussel and the structure was elucidated as 35(*S*)-methyl 31-demethyl OA by NMR [36]. A new OA analogue, 19-*epi*-OA, was isolated from *P. belizenaum* and the structure was elucidated as the 19-epimer of OA by NMR [37]. In 2008, two forms of hybrid diol esters esterified with fatty acids were found from blue mussels, *Mytilus edulis*: one with the fatty acid conjugated to the 7-OH of the OA moiety and the other with the fatty acid conjugated to the OH group in the diol moiety [38]. These new esters were tentatively identified by iontrap LC/MS^n technique.

OA is a lipophilic polyether compound of a C_{38} fatty acid. OA/DTX analogues are soluble in organic solvents such as acetone, methanol and chloroform (Figure 1). It shows no absorption maximum in UV region. The IR bands at 3450, 1740, 1080, 880 cm $^{-1}$ are observed due to absorption by the hydroxyl and a carboxyl moiety [2]. There are 17 chiral centers and 3 spiroketal moieties within the molecule of OA. The absolute stereochemistry of OA was assigned as 2*R*, 4*S*, 7*R*, 8*R*, 12*S*, 13*R*, 16*R*, 19*S*, 22*R*, 23*S*, 24*R*, 26*S*, 27*S*, 29*S*, 30*S*, 31*R*, 34*S* [2]. X-ray crystallographic studies of OA have shown that OA has a pseudo-macrocyclic structure due to hydrogen bond between the C-1 carboxyl terminus and the C-24 hydroxyl group [2]. The pseudo-macrocyclic structure of OA in not necessarily the case in 19-epimer of OA [37], therefore 19-*epi*-OA is more polar than OA, as demonstrated by the fact that it is soluble in methanol but not in chloroform.



	R1	R2	R3	R4	R5	EMW
OA	CH_3	H	H	H	-	804.5
DTX1	CH_3	CH_3	H	H	-	818.5
DTX2	H	CH_3	H	H	-	804.5
DTX3	(H or CH_3)		Acyl	H	-	
OA-D7a	CH_3	H	H	I	OH	914.6
OA-D7b	CH_3	H	H	II	OH	914.6
OA-D8	CH_3	H	H	III	OH	928.6
OA-D9a	CH_3	H	H	IV	OH	942.6
OA-D9b	CH_3	H	H	V	OH	942.6
DTX4	CH_3	H	H	III	VI	1472.6
DTX5a	CH_3	H	H	II	VII	1392.6
DTX5b	CH_3	H	H	III	VII	1406.6

Figure 1. Structures of okadaic acid and dinophysistoxin analogues.

OA/DTX analogues are known as an inhibitor of serine/threonine protein phosphatases (PPs) [39]. OA methyl ester does not inhibit PPs due to the loss of the acidic nature of the carboxyl group. It is reported that the carboxyl group as well as the four hydroxyl groups at C-2, C-7, C-24 and C-27 of OA are important for PPs inhibition activity [40]. The reduced activity of PPs has been implicated in the brain of carboxyl group (AD) patients and it is suggested that OA induced neurotoxicity may be a novel tool to study Alzheimer's disease pathology for development of new therapeutic approach [41].

19-*epi*-OA potently inhibited protein phosphatase 2A(PP2A) equally to OA, whereas the inhibitory activity of 19-*epi*-OA for protein phosphatase 1 (PP1) was far lower than OA. 19-

epi-OA selectivity for PP2A versus PP1 surpasses that shown by okadaic acid 10-fold, making it one of the most selective inhibitors of this class [37]. DTX-1 and DTX-2 have a stereochemistry opposite at the C-35. DTX-1 and DTX-2 are 35(*R*)-methyl OA [14, 15] and 35(*S*)-methyl 31-demethyl OA [36, 42], respectively. DTX-2 is about half as toxic in the mouse bioassay and has about half the affinity for PP2A as OA [43]. Molecular modeling studies indicated that 35(*S*)-methyl of DTX-2 had an unfavorable interaction in the PP2A binding site and this has been proposed as the reason for the reduced toxicity of DTX-2 [42, 44].

The C-1 carboxyl terminus and the C-7 hydroxyl group are both commonly modified by esterification. One group is the so-called "OA diol-esters" in which the carboxyl group of OA is conjugated to several different unsaturated diols to form allylic diol-esters. Several OA diol-esters have been found in toxic algae [22-35]. Esterification at the C-1 positions of OA, DTX-1 and DTX-2 markedly decrease the binding of the toxins to PP2A [23, 24]. The other group is a complex mixture of 7-*O*-acyl ester derivatives of OA, DTX-1 and DTX-2. This group is also known as the "DTX-3" complex although DTX-3 was originally named for 7-*O*-acyl-DTX-1 [4]. They can be formed as metabolites of free OA, DTX-1 and DTX-2 in bivalves that have consumed toxic dinoflagellates. The activities of OA were generally decreased by acylation of the C-7 hydroxyl group [45]. The decrease was most significant in the mouse lethality, moderate in cytotoxicity, and only slight in the fluid accumulating potency in mouse intestinal loops. Diarrheagenicity measured by suckling mouse assays was affected little by the acylation. Potency of 7-*O*-acyl ester derivatives generally increased in parallel with the unsaturation in the acyls [45]. Esterified OA, DTX-1 and DTX-2 can be converted to their free parent toxin at strong basic conditions [4]. This also indicates that OA analogues are chemically stable at the strong basic conditions. This alkaline hydrolysis is widely applied to quantify both free toxins and esterified toxins by chemical and biochemical methods [46].

The ratios between the toxicity of the analogues and that of a reference compound within the same toxin group are termed "Toxicity Equivalency Factors" (TEFs). TEFs of marine toxins associated with shellfish were recently summarized [47]. The TEFs recommended by the FAO/WHO expert group for OA/DTX analogues are: OA=1, DTX-1=1, DTX-2=0.5. Recently the oral TEF values on mice was reported [48]. Results confirmed that DTX-1 is more toxic than OA by oral route while DTX-2 is less toxic. The oral TEF values are: OA=1, DTX-1=1.5 and DTX-2=0.3.

TOXIN CONTENTS AND PROFILES IN *DINOPHYSIS* SPP.

OA, DTX-1 and DTX-2, as well as the other lipophilic toxin group pectenotoxins (PTXs) are produced by the genus of *Dinophysis*. Bivalves become contaminated with these toxins by feeding on toxic *Dinophysis* species. Analysis of individually picked cells was historically the only unambiguous way to ascribe a toxin profile and content information to a *Dinophysis* species, until 2006, when cultures of *D. acuminata* became available [49]. The cellular toxin content and profiles of *Dinophysis* species of pooled picked cells reported in previous studies are shown in Table 2 [50–67]. These data would be still useful to predict bivalve contamination with OA/DTX analogues in each monitoring area.

Table 2. Reported toxin content and profiles in *Dinophysis* field specimens

Species	pg/cell				Location	Analysis method	Ref.
	OA	DTX1	DTX2	PTX2			
Dinophysis acuminata	1.6	-	-	-	Le Havre, France	HPLC-FLD	50
	Trace	-	-	-	Tokyo Bay, Japan	HPLC-FLD	50
	9.1	-	-	-	Gullmar, Sweden	HPLC-FLD	51
	9.9-21.7	-	-	-	Galicia, Spain	HPLC-FLD	52
	-	-	-	180	Bahia Inglesa, Chile	LC/MS/MS	53
	-	0.3-0.7	-	10.7-22.4	Abashiri, Japan	LC/MS/MS	54
	-	ND-0.7	-	25.9-50.2	Yakumo, Japan	LC/MS/MS	54
	-	ND-4.7	-	ND-55.6	Yakumo, Japan	LC/MS/MS	67
	ND-1.1	-	-	1.1-10.2	Flødevigen Bay, Norway	LC/MS/MS	55
	3.7	-	-	-	Bueu, Spain	LC/MS/MS	56
	-	ND-2.4	-	ND-63.1	Saroma, Japan	LC/MS/MS	67
	-	-	-	22.0	Kesennuma, Japan	LC/MS/MS	67
	-	-	-	-	Uramura, Japan	LC/MS/MS	67
	1.0-13.4	-	-	-	Takada, Japan	LC/MS/MS	67
	1.2	0.9	-	24.7	Hiroshima, Japan	LC/MS/MS	67
	-	-	-	7.14-7.18	Shimonoseki, Japan	LC/MS/MS	67
Dinophysis fortii	-	13.0-191.5	-	42.5	Mutsu Bay, Japan	HPLC-FLD	50
	ND-8.1	ND-62.2	-	ND-83.6	Mutsu Bay (Noheji, Aomori), Japan	LC/MS/MS	67
	23.0	-	-	-	Inland Sea, Japan	HPLC-FLD	50
	ND-57.7	ND-16.0	-	-	Ofunato, Japan	HPLC-FLD	57
	-	8.4-10.9	-	51.4-63.8	Yakumo, Japan	LC/MS/MS	54
	ND-3.3	ND-97.6	-	ND-74.0	Yakumo, Japan	LC/MS/MS	67
	-	ND-47.2	-	ND-82.8	Saroma, Japan	LC/MS/MS	67
	-	ND-59.6	-	124.6-236.0	Akita, Japan	LC/MS/MS	67
	-	ND-13.9	-	7.6-36.2	Yamada, Japan	LC/MS/MS	67
	-	-	-	10.9-20.4	Kamaishi, Japan	LC/MS/MS	67
	-	9.8-14.2	-	25.0-29.8	Sendai, Japan	LC/MS/MS	67
	-	16.2-17.1	-	77.6-105.8	Onagawa, Japan	LC/MS/MS	67
	-	15.3-30.3	-	52.4-60.4	Ishinomaki, Japan	LC/MS/MS	67
	3.0	-	-	21.4	Hakata, Japan	LC/MS/MS	67
Dinophysis acuta	9.4	-	-	-	Vigo, Spain	HPLC-FLD	50
	4.0	4.2	-	-	Sogndal, Norway	HPLC-FLD	50
	-	6.6	-	-	Gullmar, Sweden	HPLC-FLD	51
	58	-	78	-	Ireland	HPLC-FLD	58
	6.3-33.1	-	1.0-22	-	Galicia, Spain	HPLC-FLD	52
	85	-	77	14	Glandore, Ireland	LC/MS/MS	59
	-	-	-	29.1-32.3	Galicia, Spain	LC/MS/MS	60
	0.7-9.4	-	0.9-6.6	0.3-3.3	Pontevedra, Spain	LC/MS	61
	1.3-10.6	-	-	0.2-3.8	Flødevigen Bay, Norway	LC/MS/MS	55
	2.9	-	1.9	1.5	Bueu, Spain	LC/MS/MS	56
Dinophysis caudata	0.73	-	-	-	Galicia, Spain	HPLC-FLD	52
	7.9-56.5	ND-53.9	-	-	Saipan, Philippines	HPLC-FLD	62
	-	-	-	100.0-127.4	Galicia, Spain	LC/MS/MS	60
	0.6	-	2.8	5.0	Moana, Spain	LC/MS/MS	56
	-	-	-	2.0-14.5	Day Bay, China	LC/MS/MS	63
	-	-	-	ND-55.3	Yakumo, Japan	LC/MS/MS	67
	-	-	-	-	Uramura, Japan	LC/MS/MS	67
	-	-	-	-	Shimotsuura, Japan	LC/MS/MS	67
	-	-	-	29.4-41.8	Kagoshima, Japan	LC/MS/MS	67

Species	pg/cell				Location	Analysis method	Ref.
	OA	DTX1	DTX2	PTX2			
Dinophysis infundibulus	-	-	-	14.8	Yakumo, Japan	LC/MS/MS	54
	-	-	-	-	Yakumo, Japan	LC/MS/MS	67
	-	-	-	-	Yamada, Japan	LC/MS/MS	67
Dinophysis miles	5.7-20.9	ND-10.7	-	-	Saipan, Philippines	HPLC-FLD	62
Dinophysis mitra	-	10.0	-	-	Mutsu Bay, Japan	HPLC-FLD	50
	-	-	-	-	Yakumo, Japan	LC/MS/MS	54
	-	ND-2.6	-	ND-16.4	Yakumo, Japan	LC/MS/MS	67
	-	ND-3.9	-	ND	Saroma, Japan	LC/MS/MS	67
Dinophysis norvegica	-	14.0	-	-	Sogndal, Norway	HPLC-FLD	50
	-	-	-	50.8-67.4	Yakumo, Japan	LC/MS/MS	54
	ND-2.6	ND-1.4	-	ND-89.0	Yakumo, Japan	LC/MS/MS	67
	ND-0.2	-	-	0.3-2.0	Flødevigen Bay, Norway	LC/MS/MS	55
	-	-	-	ND-20.1	Saroma, Japan	LC/MS/MS	67
Dinophysis ovum	7.1	-	-	-	Vigo, Spain	LC/MS/MS	64
Dinophysis rotundata	ND-0.4	-	ND-0.5	ND-0.3	Bueu, Spain	LC/MS/MS	65
	-	101.0	-	-	Mutsu Bay, Japan	HPLC-FLD	50
	-	-	-	-	Yakumo, Japan	LC/MS/MS	54
	-	-	-	-	Yakumo, Japan	LC/MS/MS	67
	-	-	-	-	Saroma, Japan	LC/MS/MS	67
	-	ND-0.9	-	-	Yamada, Japan	LC/MS/MS	67
	-	-	-	-	Ishinomaki, Japan	LC/MS/MS	67
	-	-	-	-	Urakami, Japan	LC/MS/MS	67
	2.3	-	-	-	Hakata, Japan	LC/MS/MS	67
	-	-	-	0.9	Flødevigen Bay, Norway	LC/MS/MS	55
Dinophysis sacculus	16.5	-	-	-	Le Croisic, France	HPLC-FLD	66
	14	-	-	-	Morgat, France	HPLC-FLD	66
	29.6	-	-	-	Kervel, France	HPLC-FLD	66
	12.9	-	-	-	Pont-Aven, France	HPLC-FLD	66
Dinophysis skagii	-	-	-	-	Bueu, Spain	LC/MS/MS	56
Dinophysis tripos	-	36.0	-	-	Kesennuma, Japan	HPLC-FLD	50
	-	-	-	-	Yakumo, Japan	LC/MS/MS	54
	-	ND-1.2	-	ND-467.4	Yakumo, Japan	LC/MS/MS	67
	-	-	-	22.4	Yamada, Japan	LC/MS/MS	67
	-	-	-	80.7-110.2	Onagawa, Japan	LC/MS/MS	67
	-	-	-	181.8	Ishinomaki, Japan	LC/MS/MS	67
Dinophysis rudgei	-	-	-	-	Yakumo, Japan	LC/MS/MS	67

ACCUMULATION AND METABOLISM OF TOXINS IN SHELLFISH

Free OA, DTX-1, DTX-2 and these esters at the C-1 carboxyl terminus in bivalves is absorbed from algae and metabolized. Esters at the C-7 position of OA, DTX-1 and DTX-2 had widely been observed in several bivalve species. Rapid conversion of DTX-1 to 7-*O*-acyl-DTX-1 was observed in scallops, *P. yessoensis*, fed DTX-1-producing *D. fortii* for 5 days [46]. This conversion from DTX-1 to 7-*O*-acyl-DTX-1 in mussels, *Mytilus galloprovincialis* and *M. coruscus*, was relatively slow in comparison with scallops, *P. yessoensis* [21, 68, 69]. In general, *Mytilus* spp. (blue mussels) tended to have the lowest

proportion of esterified toxin of any shellfish species and it is rare to find more than half the OA group toxin content of blue mussels esterified [70]. The conversion of free toxins to their 7-*O*-acyl derivatives markedly reduces the intraperitoneal toxicity in mice [45]. Therefore, the bioconversion of toxins may be a kind of a defense mechanism of bivalves. The most dominant fatty acids esterified to the C-7 position of OA, DTX-1 and DTX-2 are 16:0, 14:0, and 16:1 in several reports [16-21]. It has been suggested that bacteria present in the bivalve gut could contribute substantially to the acylation of the toxins, however treating mussels with antibiotics did not have any significant effect on the acylation of the supplied OA, suggesting that bacteria do not play any significant role in this process [71]. When a purified OA diol ester was incubated with a crude extract from green-lipped mussels, rapid hydrolysis to free OA was observed [72]. In contrast to the rapid hydrolysis of OA diol esters in the green-lipped mussels, two forms of hybrid diol esters esterified with fatty acids were the most abundant toxins in blue mussels, *Mytilus edulis* [38], suggesting slow hydrolysis of OA diol esters in the blue mussels.

The absorption efficiency for DTX-1 and DTX-1 esters combined was estimated to be less than 3% in scallop, *P. yessoensis*, fed DTX-1-producing *D. fortii* for 5 days [46]. Similar absorption efficiency of OA analogues was obtained in Canadian Bay scallop, *Argopecten irradians* (<5%) [73], and mussel, *Mytilus galloprovincialis* (9%) [74]. When Japanese scallops, *Patinopecten yessoensis*, were fed with sufficient cells of cultured *D. fortii* producing DTX-1, the absorption efficiency for DTX-1 and DTX-1 esters combined was 7-23% [75]. Toxins were almost exclusively accumulated in the digestive gland with only low levels being detected in the gills, mantles, gonads, and adductor muscles (Figure 2). When a DTX-1-producing microalgae, *Prorocentrum foraminosum* was fed to Gray's mussels, *Crenomytilus grayanus*, for 12 days, the digestive gland accumulated 91–100% of DTX-1 and DTX-1 esters; and kidneys and gills accumulated up to 8.5% and 4.3%, respectively [76]. Contents of OA/DTX in mussels, *Mytilus edulis*, increased linearly with incubation time exposed to cultured *D. acuta* producing OA and DTX-1b, which is a putative DTX-1 isomer, for a week, and the net toxin accumulation was 66% and 71% for OA and DTX-1b, respectively [77].

Several field studies have suggested marked species-specific differences in diarrhetic shellfish toxin (DST) accumulation among commercial bivalves. Oysters hardly accumulate OA/DTX analogues even when co-occurring mussels accumulate toxins. This trend was demonstrated by a laboratory experiment using oysters (*Crassostrea gigas* and *C. brasiliana*) and mussels (*Perna perna*) exposed to a plankton suspension containing *D. acuminata* [78].

When mussels (*Mytilus galloprovincialis*) were exposed to different concentrations of purified OA and DTX-1 in filtered (0.45 um) seawater for 96 h, mussels accumulated toxins if suspended particulate matter is added in filtered seawater, suggesting a new understanding of the mechanisms of DST accumulation by bivalves in marine aquaculture environments [79].

The residual ratios for OA and DTX-1 analogues in scallops, *P. yessoensis*, administered these toxins via syringe were 2-7% and 5-12%, respectively [80].

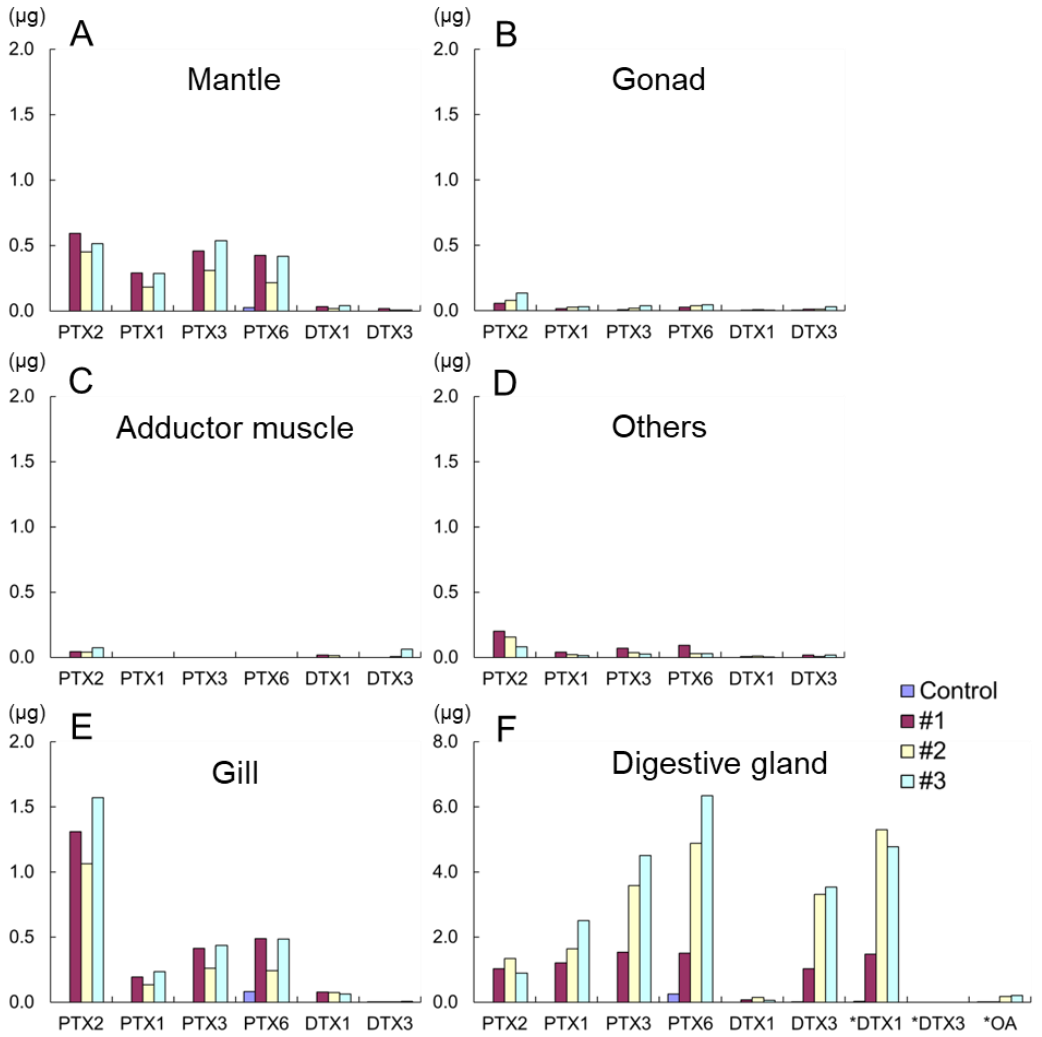


Figure 2. PTXs and DTXs contents in scallops #1-3 and control tissues (A) mantle; (B) gonad; (C) adductor muscle; (D) others; (E) gill; (F) digestive gland. Toxins indicated with and asterisk were obtained for post-hydrolysis extracts. DTX3; 16:0-O-DTX1. (Figure 4 from Toxins 2015, 7, 5141-5154).

INSTRUMENTAL ANALYTICAL METHODS

Liquid Chromatography (LC)/ Mass Spectrometric (MS) Detection

Since the very early ESI LC/MS investigation for OA and DTX-1 was reported in 1990 [81], several studies on the development and application of LC/MS for OA/DTX-1 analogues and other lipophilic toxins had been reported as summarized in previous reviews [82, 83]. Determination of these toxins by LC/MS is usually carried out using a reversed phase chromatography on a C8- or C18-silica column and isocratic or gradient elution with acetonitrile/water or methanol/water mobile phases containing volatile modifiers such as

acetic acid, formic acid, ammonium formate or ammonium acetate. The mobile phase that is most useful for LC/MS of the OA/DTX analogues and other lipophilic toxins is one composed of aqueous acetonitrile with 50 mM formic acid and 2 mM ammonium formate. The acidic conditions (pH 2.3) facilitate good chromatography of acidic toxins including OA/DTX analogues by suppressing ionization of the carboxyl groups and preventing deleterious ion exchange interactions with residual silanol groups in the stationary phase. It is reported that a change of a mobile phase pH to alkaline conditions results in better sensitivity of acidic toxins OA/DTX analogues in negative ionization mode by using the new type of cross-linked silica based C18 column materials which are stable up to pH 12 [84]. Approximately two times higher sensitivity for OA was obtained with alkaline mobile phase when compared with acidic mobile phase. Multiple reaction monitoring (MRM) LC/MS/MS chromatogram of OA/DTX-1 analogues and other lipophilic toxins analyzed with an acidic mobile phase obtained from scallop extract is shown in Figure 3. The most common MRM ion channel to detect acidic free OA/DTX-1is recorded for the $[M-H]^-$ precursor ion to the characteristic ion at *m/z* 255 in the negative mode (Figure 4).

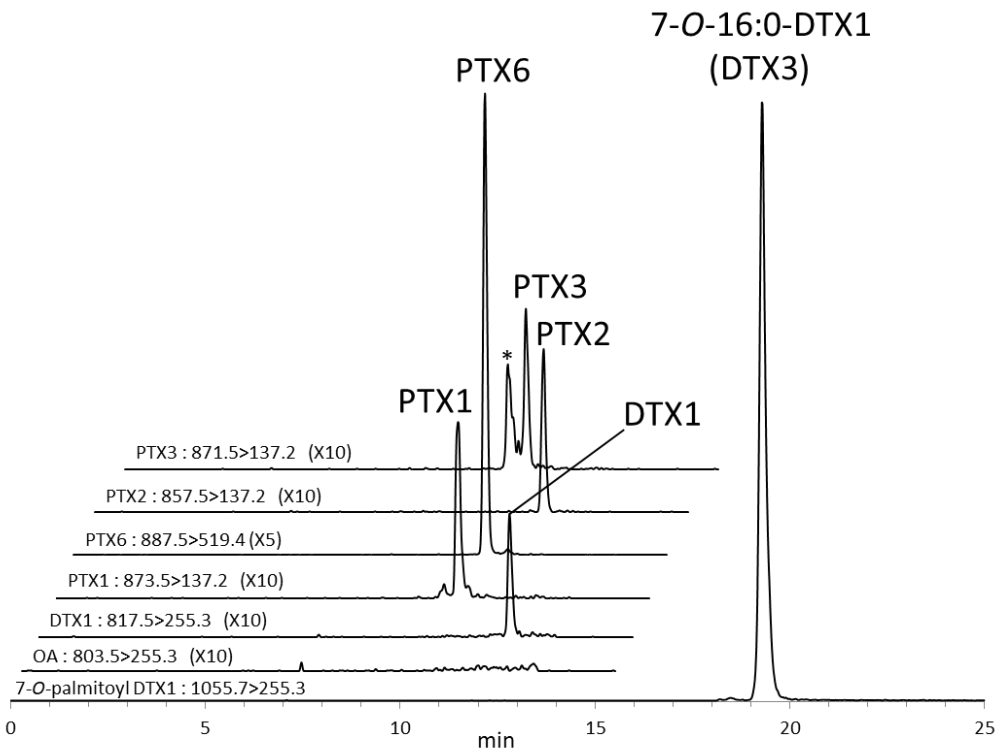


Figure 3. MRM LC/MS/MS chromatogram of OA/DTX analogues and other lipophilic toxins obtained from scallops (Figure 3 from *Toxins* 7 (2015) 5141-5154).

The qTOF MS/MS fragmentation spectra of OA and DTX-1 obtained on the negative and positive modes are shown in Figures 4 and 5, respectively. The qTOF MS/MS spectra obtained on the positive mode give more structural information than those obtained on the negative mode. The spectrum showed a series of ions resulting from the loss of first ammonium and then water molecules from $[M+NH_4]^+$, as well as several characteristic

fragment ions of OA, such as *m/z* 429, 305, 223 and 169 [32, 85]. The corresponding ions were also obtained from DTX1. The MS/MS fragmentation diagram supported by accurate mass measurements on the fragment ions using a qTOF MS/MS and previous study [32, 85, 86, 87] is also shown in Figures 4 and 5. Assignment of *m/z* 111(OA), 125 (DTX1) on the positive mode was carried out by our recent qTOF MS/MS experiment. Elucidation of the mass fragmentation pathways of $[M+Na]^+$ of OA, DTX1 and DTX2 by a hybrid linear ion trap (LTQ) Orbitrap MS was reported [87].

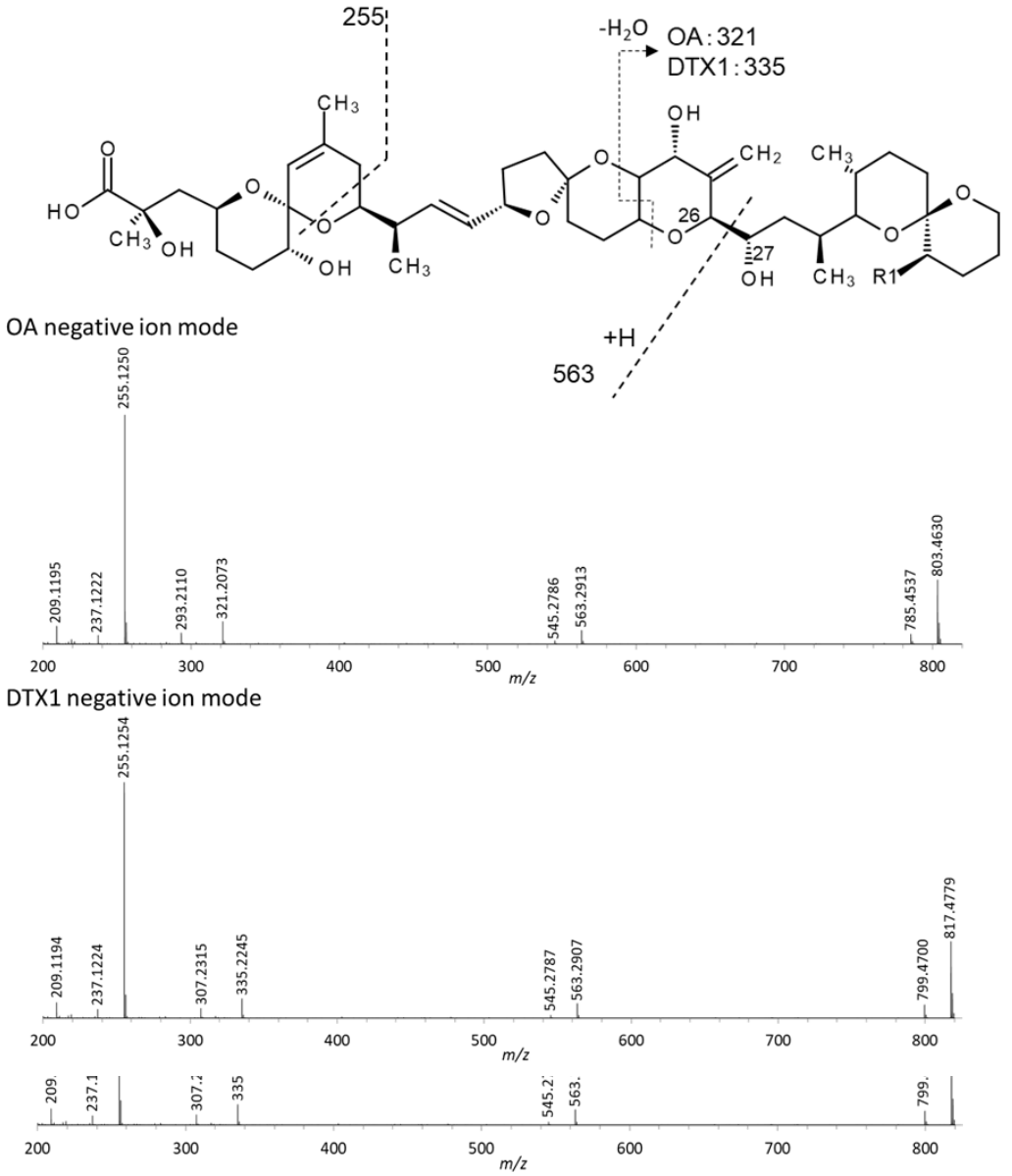


Figure 4. qTOF MS/MS product ion spectra obtained for $[M-H]^-$ of OA and DTX1.

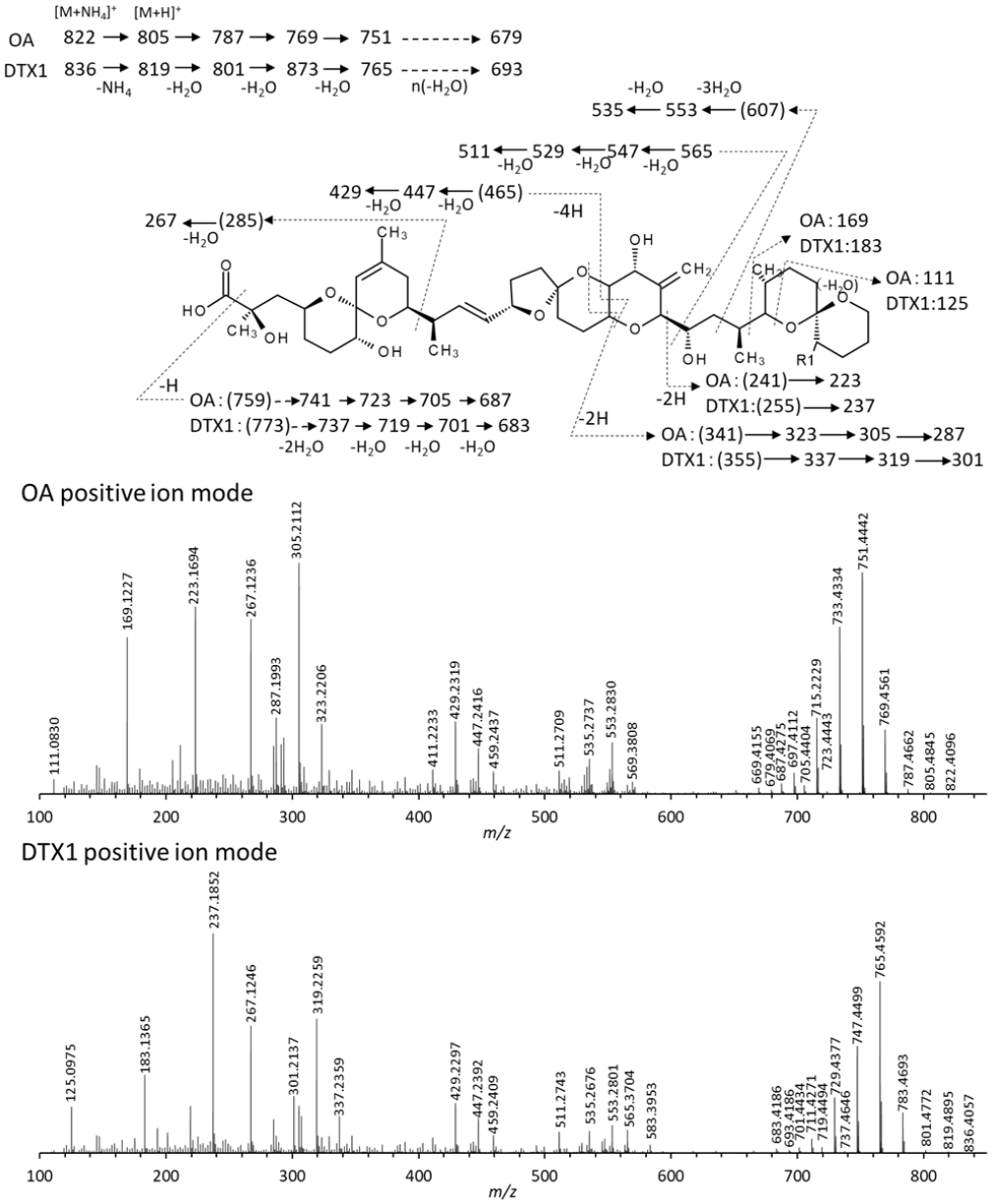


Figure 5. qTOF MS/MS product ion spectra obtained for $[M+HH_4]^+$ of OA and DTX1.

The 7-*O*-acyl derivatives of OA and DTXs are well-suited for negative ion MS/MS as they form intense $[M-H]^-$ ions and the collision-induced fragmentation of ions provides good structural information. Figure 6 shows an example of the negative ion MRM LC/MS/MS chromatogram of several 7-*O*-acyl DTX1 obtained from Japanese scallops [21]. The MRM ion channel was recorded for the $[M-H]^-$ precursor ion to $[M-H]^-$ of the fatty acids.

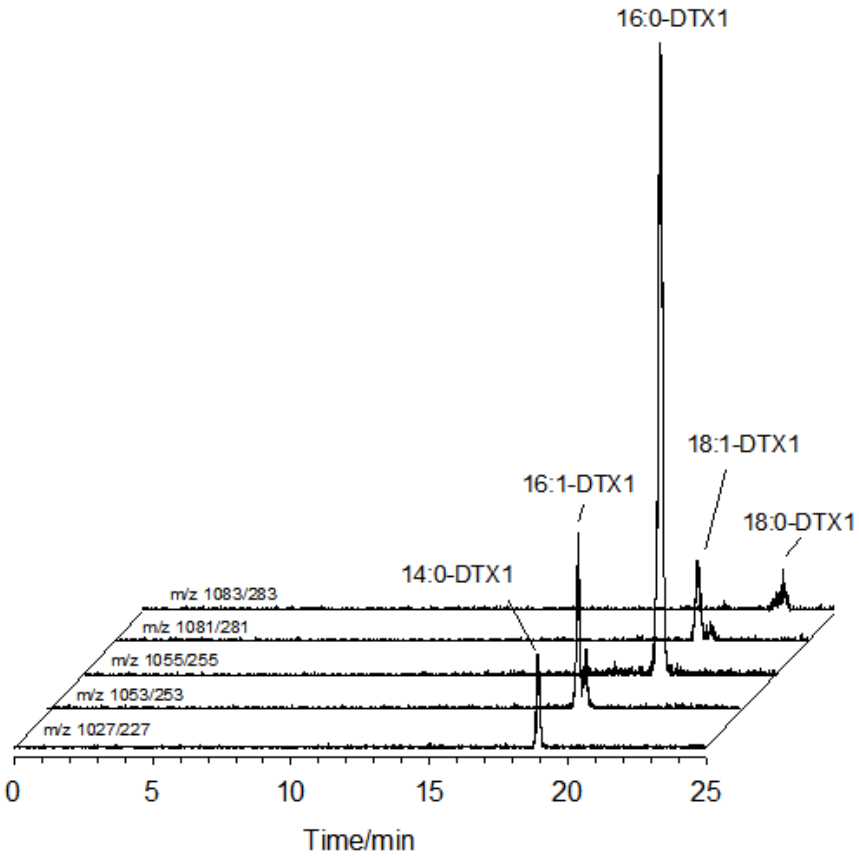


Figure 6. Multiple reaction monitoring (MRM) LC-MS/MS chromatogram of several 7-*O*-acyl-DTX1 analogues detected in scallops. (Figure 6 from *Fish. Sci.* 75 (2009) 1039-1048).

Liquid Chromatography (LC)/ Fluorometric (FL) Detection

Pre-column fluorescence analysis of free OA/DTX by isocratic LC/FL after derivatization with 9-anthryldiazomethane (ADAM) was reported in 1987 [88]. This method had widely been used for plankton and shellfish analyses. The derivatisation of toxins with ADAM has also been used for confirmation of the presence of a carboxyl group in structural studies [89]. Other fluorescence reagents, 1-bromoacetylpyrene (BAP) [90], 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone (BrDMEQ) [91], and 9-chloro-methylanthracene (CA) [91] can be applicable for analyses of toxins with carboxyl groups. Separation of fluorescent toxin derivatives by LC is usually carried out using C18 reversed phase chromatography with acetonitrile/water mobile phases.

Although clean-up of ADAM derivative of OA/DTX analogues by Sep Pak silica cartridge [88] is useful, this cleanup method of 9-anthrylmethyl esters using a cartridge column is unsuitable for the automated handling of large numbers of samples in routine monitoring. Recently a convenient LC/FL for determining OA/DTX analogues as 9-anthrylmethyl esters was developed with the addition of column switching to simplify and automate cleanup [92]. Figure 7 shows the schematic diagram of the LC/FL with column

switching valve. The peak of OA or DTX-1 ADAM derivative eluted from a cleanup column was introduced to an analytical column by switching the valve from A to B. The LC chromatograms obtained from scallops fortified with OA are shown in Figure 8. A clear peak of OA ADAM derivative was obtained from the scallop extracts in the range between 0.08 and 0.8 µg/g. The recoveries of OA and DTX-1 at all fortification levels obtained for bivalve extracts ranged from 90 and 113%, with relative standard deviation (RSD) values of 0.9-9.9% [92].

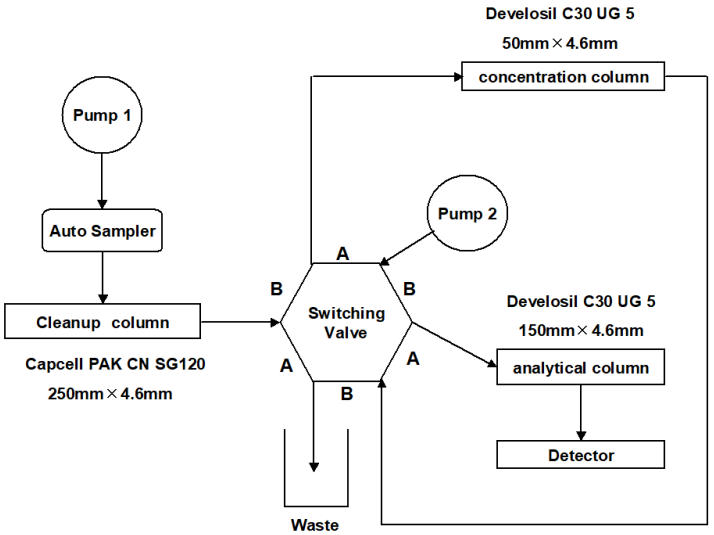


Figure 7. Schematic diagram of HPLC-FLD with an automatic column switching cleanup valve. Pump 1; gradient pump, Pump 2; isocratic pump.

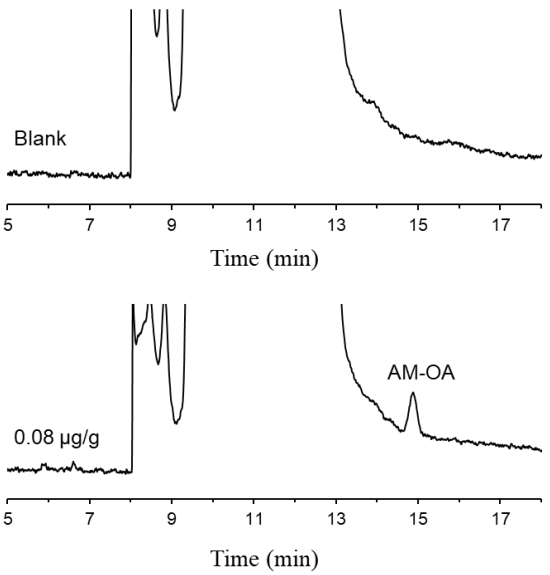


Figure 8. Chromatograms of ADAM derivatives of toxins obtained from scallops fortified with OA on the automatic column switching cleanup LC/FD.

Quantitative Nuclear Magnetic Resonance Spectroscopy (qNMR)

NMR spectroscopy is a powerful analytical technique for structure elucidation of natural and synthetic organic compounds. Recently ^{1}H-NMR has also been applied as a quantitative analytical technique as the NMR signal intensity is proportional to the number of nuclei under specific controlled conditions. Quantitative NMR (qNMR) gives accurate and precise quantitative results for analytes when internal or external standards are used since the concentration of analytes is directly determined via the integral value ratio. Figure 9 shows ^{1}H-NMR spectrum of OA dissolved in CD_3OD. OA and DTX1 were accurately quantified by qNMR with the well-separated proton signals at 5.78, 5.52, 5.36, 5.30 and 5.06 ppm corresponding to H-14, H-15, H-41, H-9 and H-41 of OA [93]. Similar result using other well-separated signals was reported from another laboratory [94].

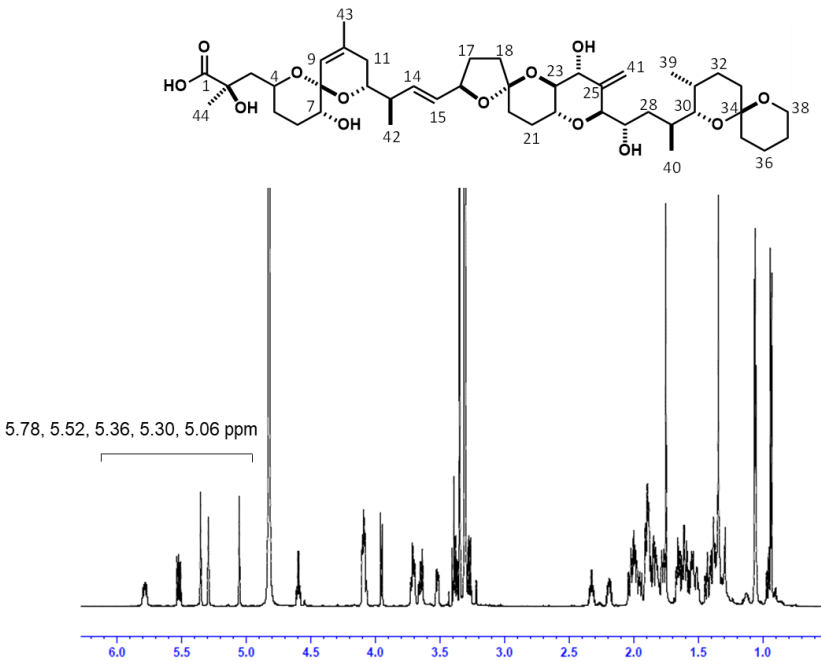


Figure 9. Chemical structure and ^{1}H-NMR spectrum of okadaic acid dissolved in CD 3OD (800 MHz).

Signal choice could depend on very slight amounts of contaminants in analytes (OA/DTX) purified from algae or bivalves. This technique is intrinsically useful to prepare certified reference materials (CRMs) of OA/DTX analogues for instrumental analysis such as LC/MS.

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Chapter 148

THE TOXIC MARINE THECATE DINOFLAGELLATE *PYRODINIUM BAHAMENSE*

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ABSTRACT

The marine thecate dinoflagellate *Pyrodinium bahamense* inhabits the tropical to subtropical waters of the Indo-Pacific, Arabian coasts, eastern Pacific and Atlantic-Caribbean. The species produces the neurotoxins saxitoxins (STXs), a collective compounds that caused paralytic shellfish poisoning (PSP) in human since the 1970s. This review highlights recent findings and information after the previous reviews, focusing on the species classification, and the biological and ecological significances. The varietal identities of *P. bahamense* (var. *compressum* and var. *bahamense*) have been reviewed. Blooms of *P. bahamense* is believed to exhibit a seasonal signature, however, factors that drive the variability in bloom magnitude, frequency and toxin production remain elusive. Some knowledge gaps are identified in this review to advance research of this important toxigenic dinoflagellate species.

Keywords: bioluminescence, human illness, paralytic shellfish poisoning, saxitoxins

INTRODUCTION

Pyrodinium bahamense Plate (1906) is a marine thecate dinoflagellate. This neritic species is widely distributed in the tropical waters of the Indo-Pacific, Arabian coasts, eastern Pacific and Atlantic-Caribbean (Usup et al. 2012). The species, together with *Gymnodinium*

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catenatum and some species in the genus *Alexandrium*, have gained considerable attention when they were found to cause numerous human illnesses and even fatalities in the Southeast Asian countries after consuming contaminated shellfish (e.g., Maclean 1989; Holmes & Teo 2002; Lim et al. 2007, 2012; Yñiguez et al. 2020), a phenomenon termed paralytic shellfish poisoning (PSP). While *Pyrodinium bahamense* has been categorized as harmful in the Pacific region, its blooms are considered positive in the Atlantic, mainly the Caribbean, as the bioluminescence produced during the blooms in the bays and lagoons is an important tourist attraction (Sastre et al. 2013).

While previous publications have provided comprehensive reviews on the taxonomy, biology, and ecology of this harmful dinoflagellate (Hallegraeff and Maclean 1989; Azanza and Taylor 2001; Usup et al. 2012; Morquecho 2019), this chapter highlights recent findings after the review of Usup et al. (2012), focusing on the classification, biological and ecological significances of the species. Significant scientific gaps are identified to advance research of this important toxigenic species.

TAXONOMY AND BIOLOGY

The species *Pyrodinium bahamense* was first described by Plate (1906) from New Providence Island, Bahama, Atlantic Ocean, and later Böhm (1931) described an anterioposteriorly compressed form of the species from Persian Gulf, Indian Ocean. The genus *Pyrodinium* is monotypic, with a single species *P. bahamense* described. Steidinger et al. (1980) recognized morphological variations between the Atlantic and Pacific populations, gave these the infraspecific status of variety. They related *P. bahamense* var. *bahamense* to the Atlantic population, while *P. bahamense* var. *compressum* to the Pacific.

The history of its taxonomic classification was detailed in Taylor and Fukuyo (1989) and Usup et al. (2012). Likewise, phylogenetic placement of the species has been implied in several studies (Fensome et al. 1993; Leaw et al. 2005; Mertens et al. 2015). The genus is classified in the family Ostreopsidaceae, subfamily Pyrodinioideae by Fensome et al. (1993) based on the fossilizable dinosporin cysts and the motile stage of cells. The molecular phylogenetic position of *P. bahamense* inferred from the nuclear encoded ribosomal RNA genes (Usup et al. 2002; Ellegaard et al. 2003; Leaw et al. 2005; Zhang et al. 2005) and the mitochondrial gene of cytochrome b (Zhang et al. 2005) both placed this species in the order Gonyaulacales.

Thecal Morphology

Morphological characteristics of the thecal plates, like many thecate dinoflagellates, are commonly used for species delineation and classification (Figure 1). This morphological feature has been well studied and documented (e.g., Matenauer 1933, Schiller 1937, Osorio Tafall 1942, Taylor 1976, Wall and Dale, 1969, Steidinger et al. 1980, Balech 1985; Morquecho 2008; Usup et al. 2012; Mertens et al. 2015).

Motile cells of *Pyrodinium bahamense* are readily distinguished from other thecate dinoflagellate species by its heavily armoured thecal surface and the ornamented apical

projections (Figure 1). The thecal plate tabulation, after the taxonomic revision of Mertens et al. (2015), is shown as [P_o, P_i, 4′, 0a, 6′′, 6c, 9s, 5′′′, 1p, 1′′′′] (Figure 2). Detailed descriptions of the thecal morphology have been provided in Mertens et al. (2015).

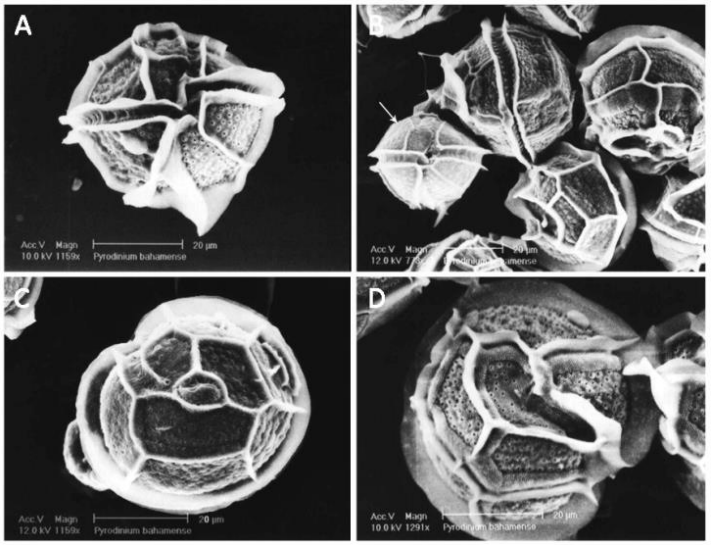

Figure 1. Scanning electron micrographs of *Pyrodinium bahamense*. Culture specimens from Sabah, Malaysia. (A) Ventral view of vegetative cell. (B) Gamete-like cell (arrow). (C) Apical view. (D) Antapical view.

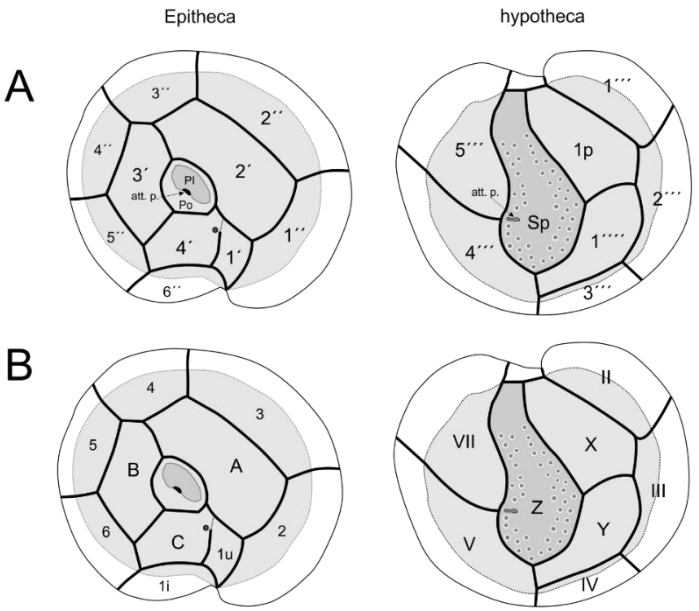

Figure 2. Schematic drawing of *Pyrodinium bahamense* thecal plate tabulation according to the Kofoidian model (after Mertens et al. 2015) (A) as compared to the Taylor-Evitt scheme (B). P_o, apical pore plate; P_i, closing or cover plate; att. p., attachement pore.

Pyrodinium bahamense has always been considered as a sister taxon of *Alexandrium*, on the basis of similar plate tabulation and structural homology (Steidinger et al. 1980; Balech 1985). They were distinguished at the generic level based on the strongly developed theca with apical and antapical spines in *P. bahamense* as compared to the thinly thecate *Alexandrium* species (Balech 1985), variation in the sulcal tabulation (Balech 1985) and distinct cyst morphology (Balech 1985; Mertens et al. 2015); this was later supported by the molecular phylogenetic analysis inferred from the ribosomal RNA cistron (Leaw et al. 2005; Mertens et al. 2015; Cusick et al. 2016). Morphologically *P. bahamense* shares the same features of long and twisted posterior sulcal plate (S.p. in Kofoidian or Z in Taylor-Evitt homology, see Figure 2) and the trapezoid, metasert first apical plate with some *Alexandrium* species (e.g., *A. pseudogonyaulax* and *A. taylori*).

Varietal Status of *Pyrodinium bahamense*

The species was widely accepted to consist of two morphological varieties: *P. bahamense* var. *bahamense* and var. *compressum* (Steidinger et al. 1980) that corresponded to the broadly recognised non-toxic Atlantic and toxic Pacific populations. The former is reported with more developed apical spines and can occur in pairs but never in long chains; the latter is anterioposteriorly compressed and forms long chains of up to tens of cells. Morphological comparative studies between the two varieties depicted contrasting opinions on the varietal separation of *P. bahamense* (Steidinger et al. 1980; Balech 1985; Badylak et al. 2004; Mertens et al. 2015). While Baldylak et al. (2004), who observed Atlantic populations from Indian River Lagoon, Florida, supported separate varieties, Mertens et al. (2015), agreeing with Balech (1985) and others (reveiwed in Mertens et al. 2015), showed no consistent morphological criterion to unequivocally separate both varieties. Nonetheless, the authors suggested that the field-observed variations represent different life-stage forms. The presence of both varieties in the Pacific Ocean off Central America and Mexico further supported the conclusion of no varietal separation (Martínez-López et al. 2007; Morquecho 2008; Gárate-Lizárraga and González-Armas 2011). The phylogenetic analysis by Mertens et al. (2015) based on LSU rDNA sequences, however, revealed two distinct LSU ribotypes within the *Pyrodinium* clade, depicting Indo-Pacific and Atlantic-Caribbean populations, respectively.

Reproduction

The species *Pyrodinium bahamense*, like most dinoflagellate species, produces resting cysts as part of the sexual life cycle in their life history. The fossilized cysts have the paleontological name *Polysphaeridium zoharyi* (Rossignol) Bujak, Downie, Eaton and Williams. It was first described as *Hystrichosphaeridium zoharyi* by Rossignol (1962) from the Israelian Wuaternary coastal sediment dated in late Paleocene to early Eocene (McLean 1976; Williams et al. 1993), and from Pleistocene sediments in the Western Pacific (Matsuoka 1976; Mao and Harland 1993).Wall and Dale (1969) related the motile stage of *P. bahamense* to *Hystrichosphaeridium zoharyi*, and they transferred the species to a new genus *Hemistocystodinium*. It was later transferred to *Polysphaeridum* by Bujak et al. (1980). Matsuoka (1989) related *P. bahamense* var. *compressum* to *P. zoharyi*. Detailed cyst

morphology of *P. bahamense* are documented in numerous studies (Matsuoka et al. 1989; Mertens et al. 2015). Mertens et al. (2015) noted significant intergradation in the cyst morphologies of both varieties.

The life cycle of *P. bahamense* plays an important role in the ecology and bloom dynamics. The species undergoes a haplontic life cycle with sexual and asexual reproduction. Detailed description of the life cycle has been documented (reviewed by Usup and Azanza 1998). The asexual reproduction involved binary fission of the haploidic cells (Usup 1995) or ecdysis from the pellicle cyst stage (Onda et al. 2014). Pellicle cyst formation has been observed *in vitro* by Onda et al. (2014) and found that the pellicle cysts were developed at sub-optimum culture conditions.

The sexual life cycle of *P. bahamense* is similar to most dinoflagellates that form resting cysts (hypnozygotes). The cycle begins with fusion of gametes of the same size (isogamous); this was observed in laboratory cultures (Corrales et al. 1995) but not from natural populations. Gametes are small (Azanza and Larsen 1997; see also Figure 1B) while planozygotes are larger than the vegetative cells; chromatophores were observed in the hypnozygotes (Corrales et al. 1995). The resting cysts produced from laboratory cultures undergo a mandatory dormancy period of 2.5-3 months prior to excystment (Corrales et al. 1995; Usup, per. comm.). While life-cycle transition of dinoflagellate cells in natural bloom condition have been considered to play an important role in bloom dynamics, in particular the toxic *Alexandrium* species (Figueroa et al. 2011; Lau et al. 2017), the environmental drivers for the sexual processes of natural *Pyrodinium* populations are not known.

Bioluminescence

The species is capable of producing and emitting light, also termed bioluminescence, by a chemical reaction involving a substrate luciferin and the enzyme luciferase (Biggley et al. 1969). Cusick et al. (2016) revealed the diversity of the luciferase gene (*lcf*) in *P. bahamense*, and demonstrated that *lcf* sequences formed distinct geographical-defined clusters of the Western Atlantic (Indian River Lagoon and Mosquito Bay, Puerto Rico) and the Indo-Pacific. Evolutionally, *P. bahamense lcf* is closer to *Pyrocystis lcf* than its sister taxon *Alexandrium* (Cusick et al. 2016). While it has been suggested that bioluminescence system in dinoflagellates may act as defense again predation pressure (Esaias and Curl 1972; White 1979; Marcinko et al. 2013; Valiadi and Iglesias-Rodriquez 2013) and oxidative stress (Wilson and Hastings 2013), the ecological function as well as the physiological role of bioluminescence in this species has yet to be determined.

Coastal embayments with natural bioluminescence are unique and are considered popular tourist attractions. In the Caribbean, bioluminescent bays and lagoons are attributed to the persistent blooms of *P. bahamense*, however, the blooms have been reported to be declining over the years (Sastre et al. 2013). Efforts have been made to conserve the continuous *P. bahamense* populations and its bioluminescence in Laguna Grande, Puerto Rico, by maintaining the water quality and to preserve mangrove communities within the basin and adjacent areas (Sastre et al. 2013).

Saxitoxin Production

The species *Pyrodinium bahamense* produces a group of neurotoxins, saxitoxins (STXs), and is often associated with PSP in the Indo-Pacific and Pacific coasts of Central America (Usup et al. 2012). PSP was recorded for the first time in Papua New Guinea in 1972 (Maclean 1973). Reports of PSP associated with *P. bahamense* have been reviewed by Azanza and Taylor (2001), Usup et al. (2012), and Morquecho (2019). Only *P. bahamense* in the Pacific (broadly recognized as var. *compressum*) was assumed to be toxic until a toxic Atlantic population was reported, for the first time, by Landsberg et al. (2002), but no shellfish poisoning incident was implicated, but puffer fish poisoning was believed to be due to the occurrence of *P. bahamense* (Landsberg et al. 2006).

Table 1. Levels and compositions of saxitoxins of *Pyrodinium bahamense* in cultures and/or bloom populations. n, number of samples analyzed

Geographical location		Toxin content	Toxin composition (%)	Detection method	Reference
Pacific					
Koro and Babelthuap Islands, Palau	Bloom (n = 2)	28–30 pg cell^{-1}	STX (46%), neoSTX (31%), dcSTX (5%), B1 (18%)	TLC	Harada et al. 1982
Palau	-	-	STX (28%), neoSTX (15%), dcSTX (4%), B1 (47%)	HPLC	Oshima 1989
Sabah, Malaysia	Culture (n = 1) at various culture conditions	100–1200 fmol cell^{-1}	neoSTX, B1 (>80%), STX, dcSTX, B2	HPLC	Usup et al. 1994, 1995
Sabah, Malaysia	Culture (n = 1)	130 fmol cell^{-1}	neoSTX (28%), B1 (>50%), STX (14%), dcSTX, B2	HPLC	Usup et al. 2006
Masinloc Bay, Zambales, Philippines	Culture (n = 5)	165–400 fmol cell^{-1}	B1 (>40%), neoSTX (>40%)	HPLC	Montojo et al. 2006
Masinloc Bay, Zambales, Philippines	Bloom (n = 2)	1200–1600 fmol cell^{-1}	STX (>20%), neoSTX (>20%), dcSTX (>40%), B1 (10%)	HPLC	Montojo et al. 2006
Masinloc Bay, Zambales, Philippines	Bloom (n = 2)	70 fmol cell^{-1}	neoSTX (<20%), B1 (60-80%)	HPLC	Montojo et al. 2006
Bamban Bay, Zambales, Philippines	Cultures at various culture conditions	80–400 fmol cell^{-1}	STX (90%), dcSTX, B1, (10%)	HPLC	Gedaria et al. 2007
Isla San José, Gulf of California, Mexico	Culture grown at salinity of 15 and 25℃	90 pg STX eq. cell^{-1}	STX only	HPLC	Morquecho et al. 2014
King Abdullah Economic City, Red Sea	Culture	2 pg STX eq. cell^{-1}	ND	ELISA	Banguera-Hinestroza et al. 2016
King Abdullah Economic City, Red Sea	Bloom	68 ng mL^{-1}	ND	ELISA	Banguera-Hinestroza et al. 2016
Atlantic					
Indian River Lagoon, Florida	Culture (n = 11)	1.68–25.57 pg STX eq. cell^{-1}	B1 (91%), STX (8.9%)	ELISA, HPLC	Landsberg et al. 2006
Indian River Lagoon, Florida	Bloom (n = 2)	3.28 pg STX eq. cell^{-1}	B1 (73%), STX (26%), dcSTX (1%)	ELISA, HPLC	Landsberg et al. 2006

With regards to the cellular toxin quota (toxin content in a cell), *P. bahamense* is one of the most toxic paralytic shellfish toxins (PSTs)-producing species, with the toxicity up to 1600 fmol $cell^{-1}$ reported thus far (Montojo et al. 2006; Table 2). Toxin profile of *P. bahamense* was stable in both culture strains and natural bloom populations examined (Table 2; references herein), composed mainly of STX, dcSTX and B1 (GTX5), with traces of neoSTX and B2 (GTX6) (Table 2). The toxin compositions, however, varied among culture strains and natural populations. A summary of the levels and composition of saxitoxins from culture and natural populations of *P. bahamense* is given in Table 2. There is a significant intraspecific variation in *P. bahamense* toxin productions in both culture and natural populations (Table 2). Likewise, very limited field studies have indicated the dynamics of toxin production in the natural populations, making it difficult to draw conclusion on the mechanisms that affect toxin production in this species.

The saxitoxins biosynthesis pathway in the toxigenic marine dinoflagellates has been partly discovered and the related genes have been elucidated (Stuken et al. 2011; Murray et al. 2015; Tsuchiya et al. 2016, 2017; Cho et al. 2019), but the complete route, including of those in *P. bahamense*, remain to be resolved.

Sterol Production

Production of sterols, an array of membrance-reinforming lipids in *P. bahamense* has been elucidated by Usup et al. (2008) and Houle et al. (2018). These macromolecules were used to study chemotaxonomic relationship among eukaryotes, including dinoflagellates (Leblond et al. 2010). The isolates of *P. bahamense* from Florida produced three sterols (cholesterol, dinosterol, and 4α-methylgorgostanol, similar to other cholesterol- and dinosterol-producing thecate dinoflagellate species (Houle et al. 2018).

ECOLOGICAL SIGNIFICANCE

Ecological Niches

While the modern cysts have been found in tropical to subtropical regions with wider distribution, *Pyrodinium bahamense* motile cells in general are confined to tropical coastal waters (reviewed in Usup et al. 2012). Both the motile cells and cysts were recorded from the biogeographical regions of Indo-Pacific, Arabian coasts (Persian Gulf and Red Sea), eastern Pacific coasts of central America, and the Atlantic-Carribean, which spans a narrow latitudinal extent of 30°N and 10°S with seawater temperatures above 20°C. *Pyrodinium* cysts in the Gulf of Tehuantepec, the Pacific coast of Mexico revealed that *Pyrodinium* cysts were present since the 1860s to present (Sanchez-Cabeza et al. 2012). In the eastern Atlantic Ocean, *P. bahamense* cyst type was first recorded by Amorim and Dale (1998).

New reports of *P. bahamense* motile cell and cyst occurrences since the review of Usup et al. (2012) are: San José, Yavaros, El Colorado Lagoon, Gulf of California (Morquecho et al. 2012); Laguna Grande, Puerto Rico (Sastre et al. 2013); Yemeni coastal waters, Red Sea (Alkawri et al. 2016); King Abdullah Economic City, Red Sea (Banguera-Hinestroza et al.

2016); Gulf of Gemlik, Marmara Sea, Turkey (cyst; Balkis et al. 2016); St. Croix, US Virgin Islands (cyst; Reidhaar et al. 2016).

Saxitoxins in the Food Web

Human intoxication is primarily caused by the ingestion of saxitoxin-contaminated shellfish (Table 2). Minimal variation in the toxin profiles was observed between the contaminated shellfishes from Malaysia and the Philippines, and the dinoflagellate (Montojo et al. 2006; Usup et al. 2006). However, different types of shellfish contaminated by *P. bahamense* toxins exhibit a degree of variability in the toxin accumulation (Montojo et al. 2010; 2012). On the other hand, Rañada et al. (2016) demonstrated that smaller *Perna viridis* mussels, an indicator species used in the Philippines saxitoxin monitoring program, are more toxic than larger mussels when exposed to low density of *P. bahamense*. Studies on toxin bioaccumulation in shellfish and other types of vectors by *P. bahamense* are scarce and require further investigation.

In 2013, the species has been implicated, for the first time, as the cause of massive sea turtle mortality in El Salvador, with *G. catenatum* and *P. bahamense* recorded in the water (Amaya et al. 2018).

Paralytic Shellfish Poisoning (PSP) is of great concern due to the potency of PSTs that can cause severe human illness and death. PSP has also expanded globally (Hallegraeff 1993) although of course part of this is due to increased awareness. Most of these PSP cases due to *Pyrodinium* comes from the Southeast Asian region with the Philippines and Malaysia as the two most affected countries (Azanza and Taylor, 2001; Usup et al. 2012; Yñiguez et al. 2020). For example, in the Philippines, there have been more than 2200 PSP cases from 1983 to 2013 with more than 123 fatalities (Bajarias et al. 2006; Arcamo et al. 2014). Fortunately, the illnesses have been declining through time (Arcamo et al. 2014) even though many areas are still affected by *Pyrodinium* blooms. In Malaysia, there have been at least 200 PSP cases and 20 deaths reported (Jipanin et al. 2019; Yñiguez et al. 2020). This decline in PSP cases, especially in the Philippines, is attributed to the active monitoring and warning program spearheaded by the relevant government agencies.

Table 2. Organisms at various levels in the food web affected by saxitoxins derived from *P. bahamense*

Organisms	Location	Reference
Shellfish (*Perna*, *Phirella*, *Spondylus*, *Crassostrea*, *Meritrix*, *Pinna*)	Indo Pacific: Indonesia, Malaysia, Philippines, Palau, Papua New Guinea	Leaw 2005; Usup et al. 2006; Montojo et al. 2010, 2012; Azanza and Taylor 2001
	Eastern Pacific: El Salvador, Mexico, Costa Rica, Guatemala	Mata et al. 1990; Morquecho 2019
Fish (*Sardinella*)	Indo Pacific: Borneo	Oshima 1989
Puffer fish (*Sphoeroides spengleri*, *S. testudineus*, *S. spengleri*)	Atlantic: Indian River Lagoon, Florida	Quilliam et al. 2004; Landsberg et al. 2006

Bloom Dynamics

Blooms of *Pyrodinium bahamense* across geographic areas from the Atlantic (Phlips et al. 2006, 2015; Soler-Figueroa and Otero 2015; Morquecho 2019), eastern Pacific (Moquecho et al. 2012; Morquecho 2019), to the Red Sea (Alkawri et al. 2016; Banguera-Hinestroza et al. 2016) and the Indo-Pacific (Maclean 1989; Usup et al. 1989; Azanza et al. 1998; Montojo et al. 2006; Villanoy et al. 2006; Adam et al. 2011; Mohammad-Noor et al. 2014; Yñiguez et al. 2018), distinctly occurred at sites characterized by warm (tropical to subtropical), relatively shallow (from one to a few tens of meters), semi-enclosed or protected areas with long water residence times, and usually with relatively variable salinity.

A consistent pattern in the initiation of *P. bahamense* blooms is the important role of the germination of cysts from cyst beds (Anderson 1989; Usup et al. 2012; Azanza et al. 2018). These cyst beds are also key in the recurrence of blooms within the same site. Cysts most likely need to be resuspended before they can germinate (Azanza et al. 2018), after which appropriate conditions for higher population growth rates need to be present for a bloom to develop (Yñiguez et al. 2018). Conditions for the increase in cell numbers appear to be pulses of sufficiently high nutrients (though what this threshold is, if there is one, is not clear) and low flushing rates. These conditions, together with the capacity of *P. bahamense* to withstand a large range of salinities, and possibly decrease grazing through its toxin, can be the key factors leading to its blooms (Phlips et al. 2006; Usup et al. 2012). These bloom patches can then be transported to other areas beyond the sites where blooms are initiated.

One primary factor associated with these blooms is either a dry period followed by rainfall (Usup et al. 1989; Azanza 2013; Yñiguez et al. 2018) or rainfall per se (Morquecho et al. 2012; Phlips et al. 2006, 2015). This rainfall is believed to lead to increased run-off that introduces nutrients into coastal waters and satisfies the nutrient requirements of *P. bahamense*. Bloom conditions are also observed to be related to periods of calmer and stratified conditions that potentially comes after wind-induced mixing (Usup et al. 1989; Villanoy et al. 2006; Usup et al. 2012; Yñiguez et al. 2018). There is evidence that blooms can have a seasonal signature, particularly occurring during seasons when rainfall increases and/or the transition periods from drier to rainy conditions. For example, in subtropical Florida, *P. bahamense* blooms during the rainy summer period (Phlips et al. 2006, 2015). In Southeast Asia, which experiences a monsoon system, blooms are typically during the rainy monsoon period: e.g., southwest monsoon in the Philippines (Azanza et al. 2004; Montojo et al. 2006; Adam et al. 2011; Azanza 2013; Yñiguez et al. 2018) and northwest monsoon in Papua New Guinea (Maclean 1989). These blooms have been linked to El Niño Southern Oscillation (ENSO) patterns. Azanza and Taylor (2001) related the occurrence of red tide events of *P. bahamense* with El Niño in Southeast Asia. This relationship is still not fully understood due to the rarity of long-term data on *Pyrodinium* blooms. Phlips et al. (2015) found that in the 16-years of data from Florida lagoons, El Niño conditions (rainy in this area) was correlated to *Pyrodinium* blooms. Sanchez-Cabeza et al. (2012), on the other hand, observed increased flux of *P. baha*mense cysts into the sediments presumably from blooms in the water column during transitions from dry La Niña conditions in Mexico to higher rainfall.

Pyrodinium bahamense blooms can last from weeks to months. What leads to their decline is less understood compared to how they are initiated. One hypothesis is that when *P. bahamense* encounters certain, possibly stressful conditions (e.g., nutrient limitation or high cell densities), they encyst into hypnozygotes, which serve to decrease cells in the water

column (Azanza et al. 2018). Other potential factors contributing to the decline is the shift in hydrographic conditions, i.e., more mixing or turbulent conditions, and top-down control through grazing (Usup et al. 2012; Azanza et al. 2018; Yñiguez et al. 2018).

Bacteria Interaction

Knowledge on the interaction between *P. bahamense* and bacteria is still lacking. Bacterial community compositions associated with *P. bahamense* have been investigated in several studies based on the *P. bahamense* cultures established from the Philippines (Azanza et al. 2006; Onda et al. 2015). Azanza et al. (2006) found STX and neoSTX at levels <73 ng STXs/10^7 cells in the bacterial strains of *Moraxella*, *Erythrobacter* and *Bacillus*, suggesting possible contribution of bacterial endosymbionts in the toxin production of *P. bahamense*.

CONCLUSION: KNOWLEDGE GAPS AND SUGGESTIONS FOR FUTURE RESEARCH

- Is there a sign of geographical expansion in *P. bahamense*? The information on distinct ribotypes of *P. bahamense* has provided a framework for further investigation of the genetic diversity of *P. bahamense* across biogeographical regions.
- What causes the diminishing of *Pyrodinium* blooms in areas that used to have persistent blooms? Does eutrophication at the coastal embayment drive the shift of plankton assemblages?
- Sexual transition and reproduction of paralytic shellfish toxins-producing dinoflagellates have been demonstrated to play an important role in bloom development (Anderson, 1998) but field evidence for *P. bahamense* is still limited. Investigating the life-cycle transitions of *P. bahamense* during bloom periods would provide more insights into the bloom dynamics, and possibly enable prediction of the duration and timing of bloom.
- Environmental drivers such as salinity, temperature and nutrient availability are among the triggering factors in the sexuality of *Alexandrium* species (e.g., Garcés et al. 2004; Figueroa et al. 2011). How do these factors affect the *Pyrodinium* life cycle?
- Considering the distinct toxin profile of *P. bahamense* as compared to the sister group *Alexandrium*, and the total absence of hydroxysulfate toxins (GTX1-4, C1-4), it would be crucial to understand how the evolution of the STX biosynthetic genes and its pathway occurred in this dinoflagellate.
- The genetic basis of saxitoxin production in this species and how the environmental stressors affect the toxigenesis of this species are worth further investigation.

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Chapter 149

ECOLOGY AND RANGE EXPANSION OF *NOCTILUCA SCINTILLANS* IN THE GLOBAL OCEANS

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ABSTRACT

The cosmopolitan bloom forming dinoflagellate *Noctiluca scintillans* exists in two forms, the heterotrophic red *Noctiluca* and the mixotrophic green form which harbors a photosynthetic symbiont *Protoeuglena noctilucae* while also having a phagotrophic mode of nutrition. The combining facets of being a primary producer and a consumer appears to underpin green *Noctiluca*'s recent expansion in the Arabian Sea, where it now appears annually as large-scale blooms outcompeting the well-known winter diatom blooms and posing a threat to regional fisheries and the long-term health of an ecosystem that supports a coastal population of nearly 120 million people. Although more ubiquitous than green *Noctiluca* which until now has been confined to the tropics, blooms of the more cosmopolitan, heterotrophic red *Noctiluca* are also occurring more often, lasting longer and have expanded even into the Southern Ocean. In this review, we discuss the ecology of these two disparate varieties of *Noctiluca*, and synthesize a growing body of evidence that ascribes their recent expansion to anthropogenic activities. We also address some of the ecological and socio-economic impacts of these blooms especially those related to the food web, and summarize recent efforts to provide their spatial and temporal estimates using satellite ocean color remote sensing.

Keywords: green *Noctiluca*, red *Noctiluca*, mixotrophy, hypoxia, climate change

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1. INTRODUCTION

The heterotrophic dinoflagellate *Noctiluca scintillans* Macartney 1810 (Kofoid and Swezy 1921) is one of the most prominent 'red tide' organisms as well as one of the most extensively studied (Elbrachter and Qi 1998, Hallegraeff et al. 2008, Harrison et al. 2011; and references within). It is commonly known as 'Sea Sparkle' because it appears as brightly bioluminescent blooms globally. There exists only one species in the genus *Noctiluca* and the synonym *Noctiluca miliaris* Lamarck (Suriray 1836) which was used in the past has been discontinued. Thus, only the genus name *Noctiluca* will be used throughout this paper. *Noctiluca* is large (200 – 1000 μm in diameter), bulbous and occurs in two forms: red *Noctiluca* which is heterotrophic, and when present, constitutes one of the major microzooplankton grazers in the food web (Elbrachter and Qi 1998, Fonda-Umani et al. 2004, Kiorboe and Titelman 1998, Kirchner et al. 1996, Nakamura 1998) exerting significant feeding pressure on phytoplankton especially diatoms but also feeding on an extensive variety of food items including bacteria, microzooplankton, copepod and fish eggs, protozoans and even detritus (Zhang et al. 2015). Red *Noctiluca* (Figure 1) appears as large reddish streaks and patches in surface waters of coastal and shoreline areas worldwide, because of the presence of small droplets of carotenoids that are thought to be derived from the plankton that it feeds on (Balch and Haxo 1984), although the coloration still remains poorly understood. Green *Noctiluca* (Figure 2a), on the other hand, is a mixotroph that harbors thousands of free-swimming photosynthetic symbionts (Figure 2b), but it can also be phagotrophic when food is available (Hansen et al. 2004, Sweeney 1978, Harrison et al. 2011, Gomes et al. 2008). Green *Noctiluca* blooms form large green slicks that turn yellowish brown as the bloom decays (Furuya et al. 2006a, Gomes et al. 2008, 2009) (Figure 3a-b). Unlike some other 'red tide' organisms (Anderson et al. 2002) both red and green *Noctiluca* are essentially non-toxic but repercussions for the food web from their indiscriminate feeding and from gelatinous zooplankton being their top predators, as well as their accumulation of ammonia, have raised concerns for fisheries, tourism, offshore oil refineries and desalination plants (Suvapepun 1989, Goes and Gomes 2016, Goes et al. 2018).

This review attempts to feature some of the more recent findings related to red *Noctiluca* and the lesser studied green *Noctiluca*, especially its ecophysiology and complex mixotrophic behavior and its appearance and expansion as anthropogenic activities continue to change the planet. Although this review is more focused on the mixotrophic green *Noctiluca*, comparisons are drawn to red *Noctiluca* to elucidate their similarities and dissimilarities. As noted by Harrison et al. (2011), previous reports did not clearly differentiate between the two, creating confusion that still persists even in more recent reports.

We start (Section 2) by comparing the distribution of green *Noctiluca* which is restricted to tropical waters *vis a vis* the more ubiquitous red *Noctiluca*. Following this, Section 3 describes the characteristics of the heterotrophic red and the mixotrophic green *Noctiluca* and describes the endosymbionts of the latter. Section 4 addresses the consequences of *Noctiluca*'s proliferation for the food web, and how phototrophy combined with phagotrophy provide a more flexible strategy in nutrient acquisition, growth, and production for the mixotrophic green *Noctiluca*. Section 5 describes the recent efforts by the oceanographic community to develop methods to identify and discriminate green *Noctiluca* from other phytoplankton based on the optical signature of its photosynthetic endosymbionts, and studies

that use daily, synoptic images from various remote ocean color instruments to provide insights into the dynamics of *Noctiluca* blooms that cannot be achieved by conventional *in situ* sampling alone. Section 6 traces the expansion of *Noctiluca* blooms, especially the mixotrophic green variety from localized and sporadic blooms to widespread annually recurring events like those seen in the Arabian Sea. Finally, Section 7 summarizes various reports on the adverse effects of *Noctiluca*. Albeit nontoxic, outbreaks of thick and widespread blooms of *Noctiluca* seen in the northern Indian Ocean, the Gulf of Thailand and the Sea of Oman can cause severe damage to fisheries, tourism and other marine related industries. It also addresses the potential repercussions of these blooms for the food web.

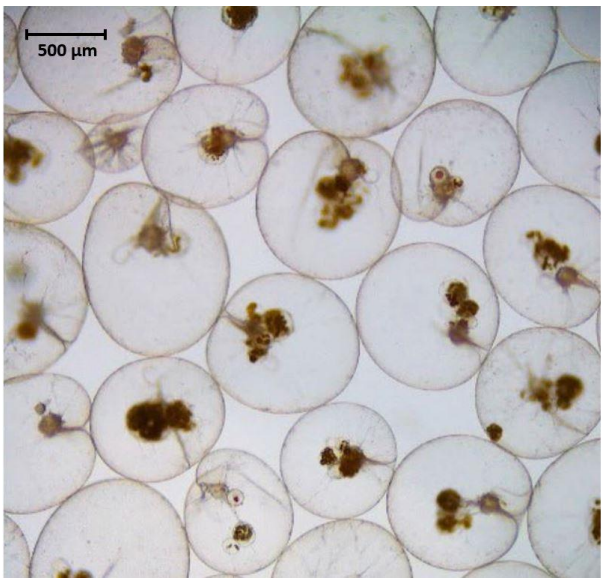

Figure 1. Cells of the red *Noctiluca scintillans* from a surface slick from Sullivans Cove, Tasmania, Australia. Photo kindly provided by Dr. Gustaaf Hallegraeff, Institute for Marine and Antarctic Studies, University of Tasmania, Australia.

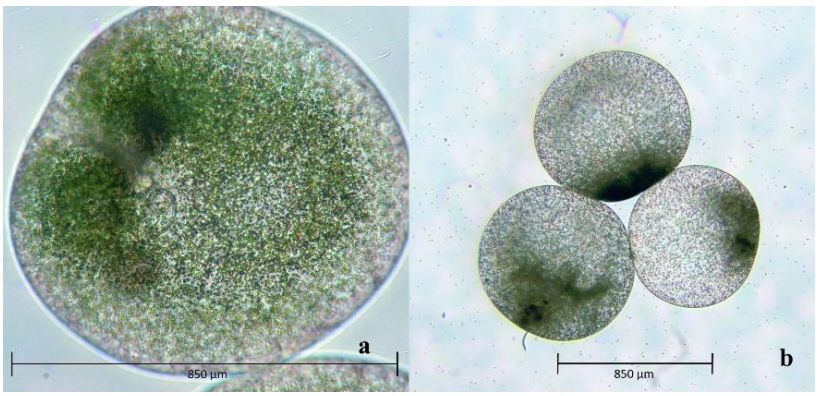

Figure 2. a) A single cell of the green *Noctiluca scintillans* with free-swimming endosymbionts *Protoeuglena noctilucae*. Tentacle used to capture food is seen in the center b) A cluster of cells of the green *Noctiluca scintillans* with healthy endosymbionts floating outside the cells.
Both images were produced with an AmScope 40X-900X phase tissue culture inverted microscope with a 10× and 4× objective.

2. DISTRIBUTION

Noctiluca's ubiquitous distribution world-wide has been very well documented in an excellent review by Harrison et al. (2011) which provides the first map of the global distribution of both red and green *Noctiluca* as well as lists of all reported occurrences with locations, coordinates and dates. Figure 4 is a redrawn and updated map of Harrison et al. (2011) with two new reports of green *Noctiluca* from the coasts of Tanzania and Seychelles.

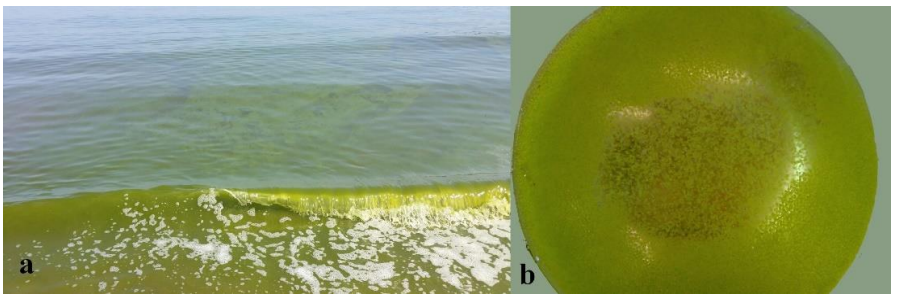

Figure 3. a) Thick surface bloom of *Noctiluca* in the Sea of Oman observed on 7th February 2018 b) *Noctiluca scintillans* cells from the bloom in a beaker showing the density of the bloom.

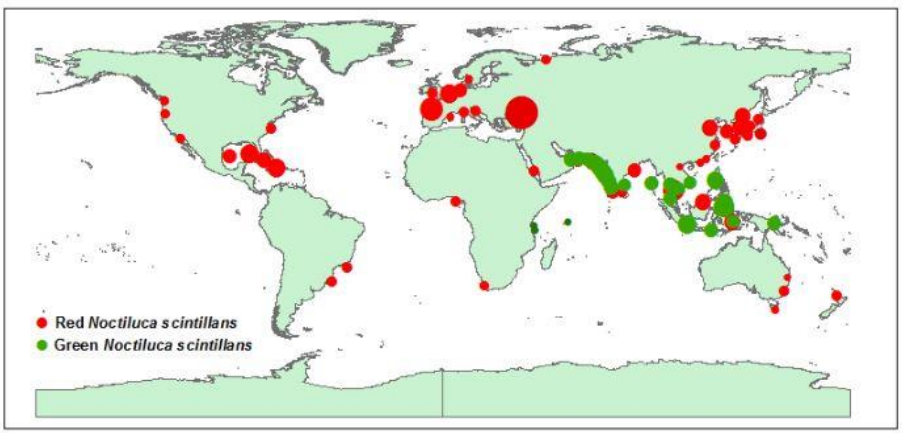

Figure 4. Updated map of the global distribution of red (red dots) and green (green dots) *Noctiluca scintillans* redrawn from Harrison et al. (2011) to include recent reports of green *Noctiluca* off the Tanzanian coast (Lugomela, 2007) and off Seychelles (David Rowat, Marine Conservation Society, Seychelles, pers. comm.). Dots are proportional to the concentration of cells recorded in Tables 1 and 2 of Harrison et al. (2011).

Red *Noctiluca* occurs widely in temperate to sub-tropical coastal regions of the world in a temperature range of about 10-25°C (Harrison et al. 2011) although reports by Qi et al. (2019) for the East China Sea and by Baliarsingh et al. (2016) and Mohanty et al. (2007) for the Bay of Bengal, indicate that it can grow beyond this temperature range (Figure 4). It generally, seems to prefer higher salinities because it has not been observed in estuaries (Harrison et al. 2011). It is particularly abundant in high productivity areas such as upwelling or eutrophic areas where diatoms dominate since they are its preferred food source (Figure 4; Harrison et al. 2011).

Some of the more well studied regions where red *Noctiluca* occurs recurrently, include the German Bight (Uhlig and Sahling 1990, Fock and Greve 2002); the North Sea (Hauke et al. 1998); Northern and Southern Black Sea (Kideys and Romanova 2001, Kideys et al. 2000) and the northern Adriatic Sea (Degobbis et al. 1995, Fonda-Umani et al. 2004). In Asia, red *Noctiluca* appears repeatedly in the Seto Inland Sea, Japan (Pithakpol et al. 2000, Tada et al. 2004); Sagami Bay, Japan (Miyaguchi et al. 2006); Southern Korea in the Jinhae Bay (Baek and Kim 2011), Bohai Sea, China (Zou et al. 1985); East China Sea (Yan et al. 2002); and Tolo Harbor, Hong Kong (Tang et al. 2006, Zhang et al. 2017b). Further south, red *Noctiluca* has been observed in the Gulf of Thailand (Suvapepun 1989, Cheevaporn and Menasveta 2003); Jakarta Bay, Indonesia (Adnan 1989) and off the west coast of India (Sahayak et al. 2005), in the Gulf of Mannar (Edward et al. 2009) and the Bay of Bengal (Mohanty et al. 2007). Red *Noctiluca* has also been reported from the Gulf of Mexico (Gomez-Aguirre 1998), the Chesapeake Bay, USA (Marshall et al. 2006); NSW, Australia (Tong et al. 1998, Dela-Cruz et al. 2002; 2008, Oke and Middleton 2001) and SE, Australia (Dela-Cruz et al. 2003); the Australian coastal waters (Hallegraeff et al. 2008) and off Tasmanian waters (Thompson et al. 2008). For a more detailed survey of the various locations globally where red *Noctiluca* has been found, the reader is directed to Table 1 in Harrison et al. (2011).

In contrast, green *Noctiluca*, because of its pre-disposition to warmer waters (24-30°C) is restricted to coastal and open tropical waters (Figure 4) with the first report of green *Noctiluca* in association with endosymbionts originating from the Dutch Indies (Weber and Bosse 1890). Since then, large green blooms have been observed in the Arabian Sea annually during the winter monsoon (Gomes et al. 2008, Parab et al. 2006, Prasad 1953, Prasad 1958, Subrahmanyan 1954), but, until now, not in the Bay Bengal probably because of the low salinities caused by excessive precipitation and river runoff (Gomes et al. 2016).

Sporadic blooms of the green *Noctiluca* have also been observed in the tropical waters of Southeast Asia including the Gulf of Manila (Furuya et al. 2006a), Gulf of Thailand (Lirdwitayaprasit et al. 2006, Sriwoon et al. 2008) and Jakarta Bay (Praseno and Wiadnyana 1996) and the coastal waters of Vietnam (Lam and Hai 1996). In the Sea of Oman (Gulf of Oman) red *Noctiluca* was documented since 1988, but green *Noctiluca* was seen for the first time in 1999 (Thangaraja et al. 2007), and since then appears annually almost throughout the year (Figure 3a-b) (Gomes et al. 2008, 2009; Al-Hashmi et al. 2015, Al-Azri et al. 2007). There are also recent reports of green *Noctiluca* from the coastal waters of Tanzania (Lugomela 2007).

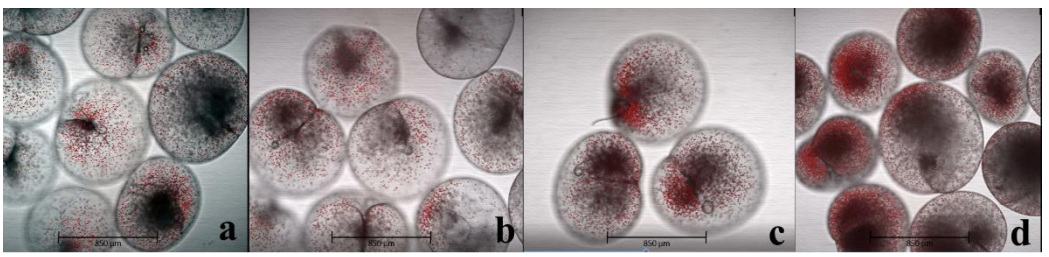

Figure 5. *Noctiluca scintillans* cells grown in media of salinity a) 32 b) 33 c) 34 d) 36. Endosymbionts *P. noctilucae* were much denser at the highest salinity of 36. Images were collected using a confocal microscope.

There are no reports of the co-existence of red and green *Noctiluca* and our research also confirms that while green *Noctiluca* proliferates during the winter monsoon in the northern Arabian Sea (Parab et al. 2006, Gomes et al. 2008, 2009), red *Noctiluca* is seen mostly in summer, restricted to the coastal waters of western India especially in association with cold, hypoxic waters that upwell off the shelf (Padmakumar et al. 2009, 2016; Joseph et al. 2008, Vijayalakshmy et al. 2018, Shaju et al. 2018). Our preliminary studies with a laboratory culture of green *Noctiluca* indicates that both *Noctiluca* and the endosymbionts grow and proliferate only in a very narrow salinity range of 32-26 with the largest population of endosymbionts at the highest salinity of 36 (Figure 5a-d). In the Gulf of Thailand, (Sriwoon et al. 2008) found green *Noctiluca* in a wide salinity range of 14-35 but blooms only formed in a narrower salinity range of 25-30.

3. DESCRIPTION

Both the red and the green varieties of *Noctiluca* are large cells (often up to 1000 μm) (Figure 1, & 2a-b) with an oral pouch which is an invagination of the more or less spherical cell (Elbrachter and Qi 1998). Within this pouch is located a tentacle and a flagellum (Lucas 1982), a permanent cytostome, a rod organelle and a projecting tooth (Elbrachter and Qi 1998, Takayama 1983). Unlike the flagellum, the tentacle is highly visible, beating slowly and repeatedly to capture food (Figure 2a-b). Neither the flagellum nor the tentacle aid in vertical movement, which is mostly controlled by active ionic regulation of the specific gravity (Elbrachter and Qi 1998). In addition, large amounts of ammonia and lipids are concentrated within its cells (Gomes et al. 2018) which may aid in its buoyancy but accumulation of ammonia can also make *Noctiluca*, ichthyotoxic (Okaichi and Nishio 1976).

In contrast to the exclusively heterotrophic red *Noctiluca*, the green variety presents a highly interesting and specialized case of mixotrophy amongst the large and varied groups of mixotrophic protists recently classified by Mitra et al. (2016) and (Stoecker 2017) based on their mechanisms for energy and nutrient acquisition. The free-swimming green endosymbionts were first described by Subrahmanyan (1954) as *Protoeuglena noctilucae* because of their similarity to euglenoids in flagellar structure and the absence of a cellulose wall (Elbrachter and Qi 1998). Sweeney (1976) however reclassified it as a new species, *Pedinomonas noctilucae* based on its starch storage, dissimilarities in color, flagellum orientation and subcellular structures (Elbrachter and Qi 1998). More recently, Wang et al. (2016) used phylogenetics and ecological characteristics to reinstate it to the genus *Protoeuglena* and reclassify the endosymbiont as *Protoeuglena noctilucae*.

Vegetative cell division is by binary fission as is the case in most dinoflagellates, but, in addition to binary fission, *Noctiluca* also produces zoospores with one or more flagella (Elbrachter and Qi 1998). In the North Sea, red *Noctiluca* zoospore formation is common in spring and early summer, which Uhlig and Sahling (1990) ascribed to the cell maximal populations seen from March-June, but rare during population decline suggesting that it cannot be responsible for the decline of the bloom (Elbrachter and Qi 1998). Sexual reproduction has also been observed in the red *Noctiluca* (Schnepf and Drebes 1993, Fukuda and Endoh 2006). One of the first reports of both vegetative cell division and sexual reproduction in the mixotrophic green *Noctiluca* is by Furuya et al. (2006b) in cultures

isolated from the Gulf of Thailand and Manila Bay. They found that non-feeding strains of green *Noctiluca* that grow autotrophically for generations, (i.e., without an external food supply), form gametocytes only when food is supplied. A later field study by these authors in the Gulf of Thailand (Sriwoon et al. 2008) supported this interesting finding; gametocytes of green *Noctiluca* were frequently observed during the dense summer monsoonal blooms as well during the transition into the winter monsoon when food was still available, but not in winter when blooms are sparse, indicating that sexual reproduction occurred only under favorable food conditions.

Bioluminescence another distinctive feature of *Noctiluca* cells, originates from thousands of scintillons, which are organelles distributed throughout the peripheral cytoplasm. They contain luciferin and luciferase and are capable of emitting light flashes when triggered by mechanical stimulation of the cell. In both green and red *Noctiluca*, bioluminescence is a defense behavior against predators. It usually prevents the cells from being grazed upon, because the flash responses serve to startle predators that contact the cell as well as act as a light alarm to attract secondary predators of *Noctiluca*'s predators (Valiadi et al. 2019). Although most varieties of *Noctiluca* are bioluminescent, intriguingly the ones found on the west coast of USA are not, which Valiadi et al. (2019) have ascribed to a mutated gene and lack of luciferase.

4. GROWTH AND GRAZING

Red *Noctiluca* is known to be a voracious feeder, ingesting all particles, even those with no dietary value, all of which are engulfed using the tentacle that aids in both food uptake and the elimination of faeces (Elbrachter and Qi 1998). However, more focused studies now show that *Noctiluca* may have some food preferences that promote its growth over others (Buskey 1995), with a more recent study (Zhang et al. 2016) suggesting that while growth and feeding showed no significant relationship with cell size or swimming motility of the prey, over longer incubation times of approximately 2 days red *Noctiluca* preferred food mixtures that were nutritionally superior even if less abundant. In the natural environment, *Noctiluca* exerts significant pressure on phytoplankton, especially diatoms (Fonda-Umani et al. 2004, Baek and Kim 2011, Huang and Qi 1997), copepod eggs (Cabal et al. 2008, Quevedo et al. 1999), and even toxigenic *Dinophysis* and *Pseudo-nitzschia* suggesting that if consumed, *Noctiluca* could act as a vector for transfer of phycotoxins to higher trophic levels (Harrison et al. 2011). Escalera et al. (2007) found toxigenic microalgae of the genera *Dinophysis* and *Pseudo-nitzschia* in red *Noctiluca* food vacuoles from the Galician Rías Baixas (NW Spain). Although this was the first report of ingestion of toxigenic microalgae of these 2 genera by *Noctiluca*, no information is available on its role as a transvector of toxins to higher levels of the food web. However, Frangópulos et al. (2011), showed that although red *Noctiluca* actively fed on the toxic dinoflagellate *Alexandrium mintum*, the fed cells showed no detectable amounts of toxin, suggesting that the toxin was either bioconverted or excreted by *Noctiluca.* In the case of green *Noctiluca*, a study by Azanza et al. (2010) also oberved bioconveration/excretion of toxin of *Pyrodinium sp.* by *Noctiluca* as well as a preference for less toxic species of food.

Being mixotrophic, the green *Noctiluca*, can survive even without an external food supply, because it can sustain itself either by feeding on its endosymbionts (Figure 2b) or their photosynthetic products although mechanisms for both are currently unclear. External food, however significantly enhances growth and there is some evidence of food preferences. This has been shown in our recent study (Gomes et al. 2018) with a strain of the green *Noctiluca* isolated from the Arabian Sea, which showed its distinct preference for the dinoflagellate *Kryptoperidinium foliaceum* and the pennate diatom *Phaeodactylum tricornutum*, but not the chlorophyte *Pyramimonas* sp., nor the chain forming diatom *Thalassiosira weissflogii*. Growth rates of *K. foliaceum* fed green *Noctiluca* were double of those for unfed *Noctiluca*. However, irrespective of the food provided, adequate light was required for green *Noctiluca* to grow as evidenced by its maximum growth rates of 0.3 day^{-1} when fed the preferred dinoflagellate *K. foliaceum* and exposed to optimal irradiance of 250 µE m^{-2} s^{-1} versus growth rates of 0.13 day^{-1} with the same food but at a low irradiance of 10 µE m^{-2} s^{-1}. Similar growth rates were obtained by Hansen et al. (2004) for a strain isolated from Manila Bay where the preferred algae were two dinoflagellates, *Pyrodinium bahamense* and *Akashiwo sanguinea* growing at similar light intensities. The laboratory results of Gomes et al. (2018) are in agreement with those obtained for feeding experiments conducted during a winter *Noctiluca* bloom in the Arabian Sea to elucidate the interplay between the dependence of *Noctiluca* on its autotrophic endosymbionts and its facultative phagotrophy (Gomes et al. 2014). Growth rates were much lower in the dark even with addition of food as compared to the rates for cells grown in the light indicating the importance of autotrophic endosymbionts for growth. Even more interesting was the fact that growth rates in samples where light and nutrients were provided were comparable to those where light and food (but not nutrients) were provided. Growth rates did not increase when nutrients were added to flasks containing food and exposed to light, suggesting that when food is available, *Noctiluca* is capable of meeting a large fraction of its metabolic requirements via phagotrophy. Similar observations were made by (Sriwoon et al. 2008) who during four green *Noctiluca* bloom events in the Gulf of Thailand found a better correlation between the abundance of *Noctiluca* and the <20µm fraction of phytoplankton (food) than with nutrient concentrations.

One reason why green *Noctiluca* may not require extraneous nutrients is because it is known to accumulate ammonium and phosphate in its cells which the endosymbionts can utilize (Okaichi et al. 1991). Montani et al. (1998) found the intracellular concentrations of ammonium and phosphate in red *Noctiluca* to be 26–67 x 10^3 and 8.5–28 x 10^3 times higher respectively, than those in the extracellular environment while Okaichi et al. (1991) found lower concentrations of the same in green *Noctiluca*, which they attributed to uptake by the endosymbionts. Our measurements of the intracellular concentrations of NH_4^+ show similar results with increases and then decreases over the course of the experiment due to uptake by endosymbionts when light levels were conducive for photosynthesis and growth (Gomes et al. 2018). In contrast, intracellular NH_4^+ concentrations for low light exposed cells were almost an order higher than those exposed to optimal light levels indicating that nutrient uptake by endosymbionts was lower when photosynthesis was light limited.

Another interesting result from our laboratory study using the Arabian Sea isolated strain of *Noctiluca* is that parameters of the Photosynthesis-Light (P-E) curves derived using variable fluorescence measurements against a gradient of light (Gorbunov and Falkowski 2004) did not show any statistically significant differences between fed and unfed cells (Gomes et al. 2018). These parameters and variable fluorescence in general are sensitive to

changes in nutrient and trace metal concentrations (Falkowski and Kolber 1995, Behrenfeld and Kolber 1999) and their invariance indicates that endosymbionts did not experience nutrient variations whether they were fed or not. Similar results were obtained by Saito et al. (2006) who performed P-E curves using oxygen as a measure of photosynthesis. The fact that our culture of *Noctiluca* can survive for several months without food or replenishment of nutrients reinforces the idea of tight nutrient recycling by green *Noctiluca*, that allows its endosymbionts to grow while also supporting its own growth. This is also well demonstrated in the study by Zhang et al. (2017b) which shows that the heterotrophic red *Noctiluca* strictly regulates its stoichiometry to allow it to survive when resources are scarce. Total cellular elemental ratios of this red *Noctiluca* strain were nearly homeostatic, although its intracellular pools of $NH_4^+$and PO_4 were weakly regulated. Similar studies are required for green *Noctiluca* because like some other mixotrophs it appears to have the potential to mitigate variable or skewed inorganic nutrient levels that autotrophs face by using phagotrophy and internal nutrient cycling (Flynn et al. 2018).

In addition to being a significant predator that can influence carbon flow in marine food webs, red *Noctiluca* is also an important agent of nutrients to the environment it lives in, regenerating and excreting large amounts of dissolved inorganic nutrients (i.e., NH_4^+ and PO_4^{3-}) (Ara et al. 2013, Montani et al. 1998, Okaichi and Nishio 1976) and more complex organic substances (Zhang et al. 2017b). However, the study of Zhang et al. (2017) shows that both the internal dissolved pools and excretion rates of NH_4^+ and PO_4^- of red *Noctiluca* depend on its nutritional status and growth rate. Thus, the algal prey type of *Noctiluca* should have important consequences for marine ecosystems that are impacted by its blooms (Zhang et al. 2017b). Similar nutrient enhancement from excretion by *Noctiluca* has not been observed by us during the large-scale blooms of green *Noctiluca* that we sampled for several years in the Arabian Sea (Gomes et al. 2008, Parab et al. 2006) and no clear information is available for coastal waters and enclosed systems (Al-Azri et al. 2007, Sriwoon et al. 2008).

5. REMOTE SENSING OF *NOCTILUCA*

Recognizing that no other observational strategy can provide synoptic views of *Noctiluca* blooms with the spatial and temporal resolution required to better understand bloom dynamics and their expansion and proliferation, researchers have turned to satellite-based remote sensing. These methods involve the exploitation of optical properties of phytoplankton, such as absorption, scattering, and fluorescence excitation and emission spectra, to broadly separate *Noctiluca* from phytoplankton. One of the earliest attempts was by Balch and Haxo (1984) who compared the absorption spectra of the heterotrophic red *Noctiluca* from a bloom off the Scripps Pier in California with autotrophic dinoflagellates that served as its food. Although, the study showed that *Noctiluca* had a spectrum distinct from other dinoflagellates it was mostly from carotenoids from the *Lingulodinium* (*Gonyaulax) polyedrum* that it had ingested. Similar results were obtained by Astoreca et al. (2005) who found variable spectra related mostly to gut content and food availability. Using airborne hyperspectral images as well as *in situ* measured reflectance spectra and laboratory measurements of absorbance and reflectance from a thick and distinct *Noctiluca* bloom off the coast of Oostende, Belgium, Van Mol et al. (2007) were able to obtain spectra for *Noctiluca* distinct from phytoplankton

and non-algal particles. These spectra, which are highly reflective in the red and NIR wavelengths and have strong absorption in the blue-green wavelengths were exploited by Qi et al. (2019) who provided a proof-of-concept of the detection of red *Noctiluca* blooms using medium resolution MODIS and hyperspectral HICO data. Albeit with limitations cited in the paper, this optical signature allowed them to do a retrospective analysis of MODIS data from 2000-2017 over the East China Sea to reveal seasonal patterns and interannual changes as well as show an increase in red *Noctiluca* blooms possibly because of changes in nutrient stoichiometry caused by the construction of the Three Gorges Dam. Increased reflectance in the red and NIR wavelengths from dense blooms of *Noctiluca* was also exploited by Baliarsingh et al. (2017) guided by reports of *Noctiluca* blooms that had occurred in the Bay of Bengal (Baliarsingh et al. 2016, Mohanty et al. 2007, Gopakumar et al. 2009) and off the southwestern coast of India (Padmakumar et al. 2009, Sahayak et al. 2005).

Attempts have also been made to characterize green *Noctiluca* by taking advantage of the optical signature of the pigments of its endosymbiont *P. noctilucae*. One of the first attempts was by Thibodeau et al. (2014) who sampled the green *Noctiluca* bloom of 2011 in the northeastern Arabian Sea using a multichannel excitation chlorophyll *a* fluorescence (excitation at 440, 470 and 532nm and emission at 695nm) that was validated with microscopy, pigment composition, and spectral absorption. This effort provided them with a bio-optical proxy for the green *Noctiluca* based on the excitation and emission characteristics associated with pigment fluorescence. The fluorescence ratio proxy F_{532}:F_{440} could distinguish *Noctiluca* from diatoms when the numerical dominance of the former was above 10% of the total population. This was confirmed by both cell counts and pigment analysis. This study, which uses optical proxies to discern phytoplankton groups, offers great promise for identifying and monitoring green *Noctiluca* cheaply and rapidly by placing fluorescence probes on remote platforms such as moorings, floats and gliders and is ideally suited for countries like Oman, Thailand, Vietnam and India, which experience repeated *Noctiluca* bloom outbreaks.

The first study to show the temporal progression and spatial breadth of the distribution of *Noctiluca* in the Arabian Sea from space was that of Werdell et al. (2014) wherein they parameterized an ocean reflectance inversion model (ORM) to identify green *Noctiluca* in a mixed phytoplankton community using satellite ocean color data records that were collected in the northern Arabian Sea. Simply put, an ORM provides a common method for inverting the "color" of the water observed by a satellite into the spectral absorption and scattering characteristics of ocean water and its dissolved and particulate constituents all of which are known as marine inherent optical properties (IOPs). The ORM used by Werdell et al. (2014) discriminated between *Noctiluca* and other phytoplankton by producing estimates of spectral absorption for these two algal groups which were validated using measurements of spectral reflectance and spectral absorption coefficients during the field campaign described in Thibodeau et al. (2014). The ORM robustly detected whether or not *Noctiluca* appeared in the simulated water column but the authors advised caution when interpreting the absolute magnitudes of the retrievals. Incorporating reflectance data at additional wavelengths improved the quality of the ORM retrievals, underscoring the benefit of forthcoming satellite missions such as NASA's hyperspectral 'Pre-Aerosol Cloud and Ocean Ecosystem (PACE) which has a spectroradiometer that spans the ultraviolet to shortwave infrared region at 5 nm resolution. Other commendable efforts include that of Dwivedi et al. (2015) who used *in situ* reflectance spectra from a hyperspectral radiometer for the bloom and non-bloom waters in

several field campaigns in the northeastern Arabian Sea from 2001 to 2012. Shapes of the reflectance spectra for *Noctiluca* dominated waters, diatom dominated waters and non-bloom oceanic and non-bloom coastal waters were distinctly different. This classification based on the reflectance spectra was then applied to MODIS-derived remote sensing reflectance images for several wavebands as input images and a color-coded classified output image was generated. The latter was successfully validated with microscopically derived *Noctiluca* and diatom counts. However, an application of this method in Lotliker et al. (2018), showed that green *Noctiluca* could only be reliably detected at abundances > 1.5 x 10^3cells/L or approx. more than 50% of the total cells.

6. EMERGENCE AND EXPANSION OF *NOCTILUCA* BLOOMS

While the earliest documentation of green *Noctiluca* in the coastal waters of the Arabian Sea dates back to the 1950s (Subrahmanyan 1954), this early report showed it to be confined to episodic blooms in coastal waters and enclosed systems (Devassy and Sreekumaran Nair 1987, Eashwar et al. 2001). In the last decade and a half however, the northern Arabian Sea has witnessed a radical shift in the composition of winter phytoplankton blooms, with green *Noctiluca* emerging as the dominant bloom-forming organism every February through March with unprecedented regularity. Winter convective mixing; the primary mechanism for fertilizing the euphotic column of the northern Arabian Sea (Banse and English 2000) has been known to give rise to rich phytoplankton blooms that are dominated by diatoms. This was established by the British ARABESQUE study in 1994 (Tarran et al. 1999), the US JGOFS study from 1994 to 1996 (Garrison et al. 1998, Latasa and Bidigare 1998) and the Indian JGOFS effort (Sawant and Madhupratap 1996). None of these studies reported the presence of *Noctiluca* blooms either during the winter or summer monsoon. The study of Tarran et al. (1999) which forms part of the extensive JGOFS field campaigns in the Arabian Sea from 1990 to 1997 cites only diatoms as the dominant group of phytoplankton blooms fueled by nutrient inputs from winter convective mixing.

A large-scale, ongoing study conducted by the National Institute of Oceanography, India, from 2003 onward in support of India's ocean color program first documented the appearance of extensive blooms of green *Noctiluca* in late winter and early spring for several consecutive years (Gomes et al. 2008, 2009, Gomes et al. 2014, Goes et al. 2018, Goes and Gomes 2016, Dwivedi et al. 2012, 2015, Parab et al. 2006, Prakash et al. 2008, Madhu et al. 2012).

As mentioned in Section 2, a similar expansion of red *Noctiluca* has not been observed for the Arabian Sea and observations of its appearance suggest that it is confined to the coastal waters of western India especially in association with cold, hypoxic waters that upwell off the shelf (Joseph et al. 2008, Padmakumar et al. 2009, 2016, Shaju et al. 2018, Vijayalakshmy et al. 2018). Since its first appearance in in the Sea of Oman in 1999 (Thangaraja et al. 2007), dedicated sampling at two stations from 2005 onwards has shown the rapid increase in blooms of green *Noctiluca* both in the Sea of Oman (Figure 3a-b) and along the Arabian Sea coast of Oman, not only in early winter (November onwards) but in summer (July onward) as well, suggesting a likely temporal range expansion of these blooms (Al-Azri et al. 2007, 2012, Al-Hashmi et al. 2015, Gomes et al. 2008, 2009). Summer monsoonal *Noctiluca* blooms are however; largely confined to sheltered coastal embayments

where the water column is relatively stable compared to offshore, indicating that stable a water column is essential for *Noctiluca*'s growth. In the Arabian Sea too, green *Noctiluca* blooms are seen in Feb-March advantaged by both the stable water column and mixed diatom blooms that arises in early January (Gomes et al. 2008, 2009) and on which this mixotroph can feed.

In our quest towards understanding why *Noctiluca* blooms recur and proliferate annually we have conducted detailed studies using the available information on the environmental conditions prior to, and during the onset of blooms. During the winter monsoons of 2003 and 2004, green *Noctiluca* blooms were seen north of 17°N in the eastern Arabian Sea, in waters that were under-saturated with respect to oxygen, nutrient rich, and cooler (<25°C) (Gomes et al. 2008, 2014). This was also the case in the Gulf of Oman, during the winter monsoonal seasons from 2004 to 2006 when *Noctiluca* were also observed in association with cooler, nutrient rich, oxygen poor waters. Episodic coastal *Noctiluca* blooms seen off the west coast of India in earlier studies have also been in a water column of low oxygen content and cooler water temperatures, although generally in the summer monsoon when upwelling conditions prevail along this coast (Devassy and Sreekumaran Nair 1987, Naqvi et al. 1998, Nayar et al. 2001, Sahayak et al. 2005, Shetye et al. 1990). Onboard experiments (Gomes et al. 2014) conducted by us while sampling the *Noctiluca* blooms of 2010 and 2011 in the northern Arabian Sea provided us the first evidence that green *Noctiluca* is predisposed to hypoxic waters and is able to fix CO_2 more efficiently under hypoxic (~3.5 ml L^{-1}) rather than in norm-oxic (~6.5 ml L^{-1}) seawater (Gomes et al. 2014). In almost all the shipboard experiments, CO_2 fixation rates of the mixotrophic *Noctiluca* cells were consistently higher over ambient O_2 controls. In contrast, diatoms and other phytoplankton showed a >50% decrease in CO_2 fixation rates under low O_2. While it appears that green *Noctiluca* is pre-disposed to hypoxic waters, we do not know whether *Noctiluca* is capable of modulating its intracellular environment in order to maximize photosynthetic rates by its endosymbionts.

In Gomes et al. (2014), we posited that the advent of green *Noctiluca* blooms may be tied to a recent decrease in dissolved O_2 content of the upper water column of the Arabian Sea. Oxygen deficiency in the northeastern Arabian Sea is a unique mid-depth (>120–1,500 m) feature formed by the combined influences of monsoon-driven high surface productivity of the semi-annual phytoplankton blooms, sub- thermocline source waters that have naturally low dissolved O_2 content flowing from the Southern Ocean and poor ventilation of subsurface waters in the landlocked north (Morrison et al. 1999). While there are no known physical mechanisms to explain the appearance of O_2 deficient waters above 40m in the offshore region in winter, there is the possibility that the Arabian Sea's permanent oxygen minimum zone (OMZ) is expanding horizontally and vertically because of increased organic matter delivery to deeper depths (Gomes et al. 2014). Two possible sources of enhanced organic matter could be: 1) enhanced phytoplankton blooms in summer due to enhanced the land–sea pressure gradient, a consequence of the warming of the Eurasian continent and the systematic decrease in spring snow persistence over large parts of southwest Asia and the Himalayan–Tibetan Plateau region. This in turn has strengthened southwest monsoonal winds resulting in intensified wind-driven coastal upwelling favoring more intense phytoplankton blooms and greater export of carbon to depths (Goes et al. 2005)*;* 2) Domestic and industrial outfall from megacities bordering the Arabian Sea where waste water treatment plants have not kept pace with rapid population growth and urbanization of coastal cities (Al-Said et al. 2018, Gomes et al. 2014, Chaghtai and Saifullah 2006, Dhage et al. 2006). It is probable that *Noctiluca* not

only proliferates in low-oxygen waters but also exacerbates low oxygen conditions further by creating particulate and dissolved organic matter that decomposes through bacterial activity and utilizes oxygen in the process.

A more recent study (Lotliker et al. 2018) refutes the premise that *Noctiluca* blooms are tied to the vertical expansion of the OMZ, driven by cultural eutrophication from major coastal cities in western India. Their conclusions are however not backed up by experimental data of the type conducted in Gomes et al. (2014). They also cite data from oxygen sensors on BGC-ARGO from Feb 2013 to April 2016 to show that, regardless of the presence of a *Noctiluca* bloom, the dissolved oxygen in the photic zone was always >70% saturated, with an average oxygen saturation >90%. However, close inspection of the data shows that the ARGO floats were located south of the locations where Gomes et al. (2014) undertook their studies. Sensor calibration is also a contentious issue and Lotliker et al. (2018) provided only six calibration points for a dataset that spanned from Feb 2013 to April 2016. Additionally, they also provided dissolved oxygen concentrations for five cruises conducted in Feb.-Mar of 2009 and 2009 and measured by Winkler's titrimetric method, two of which were coincident with those in Gomes et al. (2014). These data too did not show the percentage saturation of oxygen approaching hypoxia observed by Gomes et al. (2014). Dissolved oxygen values in the latter study, however, were measured using Winkler method and the high-precision amperometric end-point of Langdon (2010) as opposed to titration employed in (Lotliker et al. 2018). Focused studies with data from sensors that have been accurately and repeatedly calibrated on ARGO floats in the northern Arabian Sea should provide more clarity on the disparate conclusions in the two studies. Another hypothesis for the sudden emergence of green *Noctluca* blooms is from Sarma et al. (2019) who attribute it to the deepening of the oxygen minimum zone (OMZ) and associated silicate limitation in the Arabian Sea during the winter monsoon. Shifts from diatoms to non-siliceous phytoplankton have been observed in several estuarine and coastal regions especially (Ittekkot et al. 2000), but there are no reports the appearance of *Noctiluca* when silicate is limiting. Additionally, the Sea of Oman (Emara 2010, Al-Azri et al. 2010), the coastal and open Gulf of Thailand (Sriwoon et al. 2008) and the coastal waters of Vietnam (Rochelle-Newall et al. 2011) where *Noctiluca* blooms have become common, are not silicate limited.

A comparison of the rise in green *Noctiluca* blooms in the Arabian Sea and the Gulf of Thailand by Goes et al. (2018) suggests some similarities although the latter is much smaller and very shallow. Both are monsoonal driven systems, with *Noctiluca* blooms seen in the upper Gulf of Thailand during the summer upwelling season and in the western half of the Gulf, where there is upward shoaling of nutrient rich, subsurface hypoxic nutrient-rich waters. However, because of the prevailing direction of flow of the currents, which is clockwise along the northern coastline, *Noctiluca* blooms accumulate downstream in the eastern half of the Gulf of Thailand where they are advantaged from a huge influx of nitrogenous nutrients from land runoff during the rainy season and the associated phytoplankton that serves as food. During the winter monsoon, currents reverse their direction and *Noctiluca* begins to accumulate in the western half of the upper Gulf of Thailand. Although winter *Noctiluca* blooms are smaller and sparser at this time of the year, they are still sustained by excess nutrients and consequently the plentiful prey phytoplankton throughout the water column (Goes et al. 2018). Increased frequency of intense *Noctiluca* blooms witnessed in recent years in the Gulf of Thailand, has been ascribed to excessive N-nutrient influxes from agricultural lands, urban waste water treatment plants, as well as shrimp farms located along the coast

(Cheevaporn and Menasveta 2003). However, Oman, which presently experiences the worst outbreaks of *Noctiluca* blooms (Figure 3a-b), has no long history of nitrogenous fertilizer use, and blooms are most intense and extensive during winter, a period when land runoff is low. In this case, the most likely N-nutrient-rich source could be from deeper depths, when cyclonic eddies that typically begin to populate the coast of Oman around the beginning of the winter monsoon season bring subsurface low-oxygen, nutrient-rich waters into the euphotic zone (Goes et al. 2018). This is based on earlier studies (Gomes et al. 2009, Harrison et al. 2017) using satellite altimetry data which suggested a strong coupling between green *Noctiluca* blooms and mesoscale cold-core eddies in this region. We are uncertain as to whether enhanced N nutrients promote growth simply through uptake and/or provide a large population of phytoplankton prey. A preliminary experiment with our Arabian Sea green *Noctiluca* isolate, with no supply of prey but optimal light conditions compared its growth rates in the presence of NO_3^-, NH_4^+, and urea (Goes et al. 2018). The results revealed that urea, a common ingredient in commercially available fertilizers, is the most preferred N source with cells grown in urea exhibiting the largest size (>1000 μm), the largest population of endosymbionts (measured as the concentration of pigment chlorophyll *a*) and highest photosynthetic competency (see Figure 17.3 in Goes et al. 2018).

Similar uncertainties also arise in ascribing a single causative factor to the emergence of red *Noctiluca.* In their review of the geographic distribution of red and green *Noctiluca,* (Harrison et al. 2011) also attempt to include some of the environmental features that lead to *Noctiluca* blooms. This included the recurring blooms of red *Noctiluca* covering much of the Adriatic from 1980 (Fonda-Umani et al. 2004) which decreased sharply when phosphorus in detergents was banned in 1988. This caused a shift to a mucilaginous phenomenon with low *Noctiluca* abundance (Degobbis et al. 1995) but since 1997, *Noctiluca* blooms have reappeared with highest abundances in areas near the Po River where plankton productivity and stratification are high (Fonda-Umani et al. 2004). Severe eutrophication has been linked to explosive development of *Noctiluca* in western Black Sea (Porumb 1992), while outbreaks in the Seto Inland Sea of Japan (Tada et al. 2004) and the Sagami Bay, near Tokyo (Baek et al. 2009) are more complex and depend on the water column stability, wind direction and rainfall.

Increasing incidents of the expansion of *Noctiluca* are now being reported elsewhere across the global oceans. Australia has seen the expansion of red *Noctiluca* along its entire coast from a rare bloom former in the 1980s, to one of the most prominent red-tide organisms in Sydney coastal waters, starting in 1993 (Hallegraeff et al. 2008) when 3 deep water ocean sewage outfalls were commissioned. This was followed by its appearance in March, 1994, in Tasmania when it was carried by the East Australian Current and it has persisted ever since by establishing permanent overwintering populations that thrive even in the winter months in colder Tasmanian waters of 10-12ºC. Hallegraeff et al. (2008) speculate that the gradual warming of East Coast Tasmanian waters (up 1.6ºC in the past 50 years and thrice the global average warming), associated with a greater influence of the East Australian Current (Ridgway and Dunn 2007), has paved the way for the apparent range extension of this warm-water dinoflagellate into Tasmanian waters. Of concern now is the recent report of its expansion into the Southern Ocean with a warm-core eddy moving southwards from Tasmania as the potential vector for its transport (McLeod et al. 2012).

7. SOCIOECONOMIC EFFECTS FROM BLOOMS OF *NOCTILUCA*

Although *Noctiluca* blooms are not known to be toxic, adverse effects on fisheries, aquaculture, human health and the coastal environment have been reported globally. In countries like Oman, where *Noctiluca* blooms are being seen throughout the year, effects are felt far beyond fisheries, and extend to tourism and recreation; aquaculture, oil refineries, ship repair, and a host of other coastal industries, including desalination plants threatening the supply of freshwater to major cities of Oman. The report by the Ministry of Fisheries, Oman (Thangaraja et al. 2007) cites several cases of fish mortality due to *Noctiluca* blooms starting from 1988. Toxicity appears to be primarily from the large amount of ammonia that it accumulates in its cells (Okaichi and Nishio 1976). Six incidences of fish mortality were reported from 1976-2001. Dissolved oxygen was depleted which increased the severity of fish kills, and in 1988 bacterial infection caused 'fin and tail rot' disease. Thangaraja et al. (2007) also noted that planktonic fish eggs collected from red *Noctiluca* bloom locations off the coast of Oman were also infected killing growing embryos before they hatched and possibly affecting forthcoming recruitments. There are reports (Chen et al. 2014) of red tides of *Noctiluca* causing heavy mortality of cultured prawn in the Northern China Sea in 1989-1990 and more recently in the summer of 2019 in the South China Sea with estimated losses win excess of US$ 100 million. The authors also found that the intensity and distribution range of *Noctiluca* have expanded from year to year, with a ten times increase from 1959 to 1993. A large bloom in March 2002 posed a significant threat to the salmonid aquaculture industry in Nubeena on the Tasman Peninsula (Hallegraeff et al. 2008). Instances of fish kill associated with the appearance of red *Noctiluca*, off the west coast of India and in association with summer monsoonal upwelling have been reported with a strong stench of ammonia, possibly causing the death of fish (Naqvi et al. 1998, Padmakumar et al. 2009). In this region, Padmakumar et al. (2009) observed that *Noctiluca* competes for food with the oil sardine (*Sardinella longiceps*) as both feed on diatoms like *Coscinodiscus, Nitzschia, Fragilaria* and *Thalassiosira* etc and this is now reflecting in the decreased oil sardine catch from this area. Although *Noctiluca* are nontoxic, thick blooms in the upper Gulf of Thailand often exacerbate oxygen loss and also cause the massive accumulation of $NH4^+$ in water column, both of which have been blamed for massive fish kills that follow (Wattayakorn 2006).

With their polyphagous feeding behavior, both red and green *Noctiluca* could severely affect and impair the food chain by competing with other grazers, such as copepods that are reliant on the same food source. It can replace copepods as primary grazers of phytoplankton. In the Bay of Biscay, blooms of the red *Noctiluca* have shown to exert heavy predation pressure on eggs of the copepod *Acartia clausi* (Cabal et al. 2008, Quevedo et al. 1999) while in the Sea of Marmara which connects the Mediterranean and the Black Sea, it plays an important role in the top-down control of the zooplankton community (Harrison et al., 2011). While some work suggests that *Noctiluca* can serve as a "biocontrol" by grazing on toxic algal blooms such as *Gymnodinium catenatum* (Rodrıguez et al. 2006) or the cysts of the genus *Alexandrium* (Drits et al. 2013), other work suggests that it might act as a vector of phycotoxins to higher trophic levels or transport it to natural shellfish beds (Escalera et al. 2007) as described earlier.

Although *Noctiluca*'s large size, its bioluminescence and intracellular ammonia concentrations preclude it from being grazed by large zooplankton (Yılmaz et al. 2005), it

appears that jellyfish and salps find *Noctiluca* highly palatable. These gelatinous, tubular zooplankton, distributed widely in the world ocean, are known to be highly efficient filter feeders exploiting particles over a wide size range, from bacteria to large diatoms and microzooplankton. This coupled with their rapid growth in suboxic waters, and unusual life history with alternation of asexual and sexual reproduction, enable them to form extensive swarms with hundreds of specimens per cubic meter (Batistić et al. 2018, Stone and Steinberg 2016). We have observed large jellyfish and salp populations associated with *Noctiluca* blooms during our field campaigns and we have conducted preliminary grazing experiments on board which estimated clearance rates of 200 to 800 ml per hour for the salp *Pegea confoederata*, suggesting that high concentrations of salps (up to hundreds of zooids per m^2) could rapidly consume dense aggregations of *Noctiluca*. Using pigments as a measure of food consumed in the first 3 h, we observed that a single salp can remove on average 71% of chl *a* and 78% of chl *b* from seawater that is dominated by *Noctiluca* with the deposition of large pellets (Gomes et al. 2014). A more recent study (Thomas et al. in press) shows the appearance of gelatinous zooplankton swarms during and after *Noctiluca* bloom events along eastern Arabian Sea with increased frequency of both events during the period from 2010 to 2017. Their feeding experiments with jellyfish and salps also showed rapid ingestion of *Noctiluca* although ingestion rates are not provided.

Although *Noctiluca* may not appear to contribute significantly to particulate organic carbon export, judging from the absence of any cells in shallow water sediment traps deployed during our cruises (Gomes et al. 2014), their contribution to carbon flux could be significant though the export of large, fast sinking salp pellets (Smith et al. 2014), found in floating particle intereceptor traps deployed by us during our Arabian Sea cruise of 2009 and 2010. Salps have also been found in association with high abundance of *Noctiluca* in the Adriatic Sea (Batistić et al. 2018) and in the coastal waters of Dubai (Murugesan et al. 2017). Although, not much is known of other predators of *Noctiluca* because its fragile nature does not allow it to persist in gut contents, its vegetative cells are grazed by large copepods (Petipa 1960), crab larvae (Lehto et al. 1998) and progametes are consumed by ciliates (Zhang et al. 2017a).

CONCLUSION

Findings from recent research synthesized in this review, provide an overview of the apparent expansion of both red and green *Noctiluca* and how anthropogenic activities may be contributing towards their recurrence and expansion. In a bid to gain a better understanding of the anthropogenic and climate-driven factors that have caused drastic changes in the biodiversity of winter blooms of the Arabian Sea and to circumvent the problems of piracy which hinders large, long-term multidisciplinary shipboard studies, our recent study (Yan et al. 2019) uses machine learning to investigate the onset and patterns of the *Noctiluca* blooms. In this study, a robust learning algorithm was developed, that uses sequences of ocean color remotely sensed data and drifter array data to understand the local-scale impact of the physico-chemical and physical oceanographic factors that contribute to green *Noctiluca* blooms that form in the Sea of Oman and along the Arabian Sea coast of Oman and their dispersal eastwards into the central and eastern Arabian Sea. It is suggested that the

mixotrophic green *Noctiluca* may be advantaged in a warming world where the upper layers of the ocean are experiencing diminishing nutrients from increased stratification and a weakened convective mixing (Gomes et al. 2014), a process that fertilizes the euphotic column during the winter monsoon of the Arabian Sea. We now seek to unravel the green *Noctiluca*'s flexible nutrient acquisition using a range of tools including implanting novel optical microchips inside the cells, to better understand how the mixotrophic mode of nutrition may play a significant role in *Noctiluca*'s ability to grow and establish itself as the dominant bloom forming organism even when the mixed layer stratifies and nutrients are limiting. In order to more rigorously test our earlier findings (Gomes et al. 2014) that the green *Noctiluca* can thrive under low O_2 conditions we plan to conduct studies that assess the transcriptome level response of the endosymbiont to hypoxic conditions. In other words, in order to ascertain whether the photosynthetic response of *Noctiluca* cells to different O_2 concentrations occurs at the genetic level we plan to identify the significantly expressed genes associated with photosynthesis and tolerance to hypoxia in *Noctiluca* bearing endosymbionts and exposed to different O_2 concentrations.

Another line of research that we are initiating is the identification of sterols and alkenones of green *Noctiluca* that are known to preserve well in sediment samples, and their use as paleo biomarkers (Sonja Schulte et al., 1999) of past *Noctiluca* blooms which may have coincided with wholesale fluctuations in productivity and expansion of mid-water oxygen depletion (from fully aerobic to anoxic) in the Arabian Sea as observed by Reichart et al. (1998); Schulz et al. (1998). In the case of red *Noctiluca*, researchers at the Guangdong Ocean University, China (Xie et al. 2015) recently launched three-dimensional observations from the air, onboard and from land to understand the horizontal and vertical distribution as well as environmental conditions that led to a large red *Noctiluca* bloom along the Guangdong coast and to possibly use the information in future modelling efforts.

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Chapter 150

PUTTING IT ALL TOGETHER: THE ARABIAN GULF DINOFLAGELLATES

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ABSTRACT

The Persian Gulf, a small hyper-saline semi-arid almost enclosed sea, is unique. The Gulf is subtropical, approximately 1000 km long, 200-300km wide; with a mean depth of 35 m. Indian Ocean Surface Water (IOSW) and water from the Gulf of Oman enter the Gulf via the Hormuz Strait during spring. Natural environmental stresses include the input of eolian dust, and the discharge from Tigris, Euphrates and Karun rivers. About 20,000 ships visit the Gulf annually for oil transportation also contribute pollutants. The Strait of Hormuz acts as a chokepoint for introduction of biota into the Gulf. Historical data showed an increase in the dinoflagellate xenobiodiversity with 509 species attributed to the incursion of the Central Indian Ocean Water (IOSW) and discharge of ballast water from ships' traffic. Dinoflagellate diversity is rich in the Hormuz-Oman waters and decreased towards the north, which coincides with the decreasing volume and dilution of Hormuz-Oman waters. Anthropogenic activities such as the discharge of effluents from slaughter houses, dairy plants, and brine from over 50 desalination plants, sewage wastewater, mariculture operations result in eutrophication of the Gulf. The prevailing high temperatures and high salinities probably limit the establishment and survival of many exotic dinoflagellates. There are 28 potentially bloom forming harmful species in the Gulf. Implementation of a long-term regional ecosystem-based "de-eutrophication strategy" is recommended to prevent further decline of this unique environment.

Keywords: semi arid waters, hypersaline, xenbobiodiversity, desalination plants, pollutants, de-eutrophication

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INTRODUCTION

The Persian Gulf (hereafter called the Gulf), is a small, nearly enclosed hyper-saline semi-arid sea. The Gulf is subtropical and measures approximately 1000 km long, 200-300km wide, with a mean depth of 35 m. About 20,000 ships visit the Gulf annually for oil transportation. Absence of seasons, lack of monsoons and classical upwelling characterize this body of water. The Gulf is referred to as the Regional Organization for Protection of the Marine Environment (ROPME). Based on the circulation pattern ROPME comprises of 4 regions: 1. The Northern Gulf (NAG) 2. Central region (Qatar to United Arab Emirates) 3. Iran 4. Strait of Hormuz –Gulf of Oman. From the northern part of Hormuz Strait, 0.23 Sv (Sverdrup = $0.001 km^3 s^{-1}$) of Indian Ocean Surface Water (IOSW) enters the Gulf during spring (Swift and Bower 2003) and skirts partly along the eastern Gulf (Iranian Coast) and forms an anticlockwise circulation front (Figure 1, Reynolds 1993) with 3 branches (Johns et al. 2003). In the central part, one of its branches (off Qatar) feeds a 0.21 Sv outflow through the southern part of the strait (Johns et al. 2003). The NAG region remains relatively stagnant.

With a total volume of approximately 8,600 km^3, the Gulf experiences several natural environmental stresses, including little precipitation (<100 mm y^{-1}), discharge from Tigris, Euphrates and Karun rivers (35-133 $km^3 y^{-1}$), 62.4 x 10^6 tons sediment y^{-1}, and input of 60-200 x10^6 tons y^{-1} eolian dust (Sheppard et al. 2010). Satellite observations have shown that eolian dust fertilizes the Gulf waters (Nezlin et al. 2010); in outdoor enrichment experiments conducted during summer, enrichment of trace elements via eolian dust sustained bloom levels (527 µg chlorophyll *a* l^{-1}) of phytoplankton (Subba Rao et al. 1999). The Iranian region experienced 156 dust storms since 2001, with a maximum of 9.36 mg m^{-3} (Rbbaniha et al. 2013). Also, anthropogenic activities contribute 160 x 10^6 t y^{-1} of oil The Strait of Hormuz acts as a chokepoint for introduction of dinoflagellates into the Gulf. spillage, effluents from slaughter houses and dairy plants, 0.023 x 10^6 $km^3 d^{-1}$ wastewater and 0.003 x 10^6 $km^3 d^{-1}$ sanitary waste water. Mariculture operations result in eutrophication of the environment. The present total seawater desalination in the Gulf is 5000 x 10^6 m^3 and to augment future needs is expected to increase by about 9000 x 10^6 m^3 in 2013 (Dawoud and Al Mulla 2012).

Light and nutrient levels do not seem to limit microalgal growth in the Gulf. Nutrient levels (µM) in the Gulf are on the increase; values for 1985-1998 are from Subba Rao and Al-Yamani (2000) and for 2000-2012 from Al-Said et al. 2017). In the offshore waters of Kuwait, nitrate levels ranged from 0.00-1.54 (1985-1993), 0.00-8.40 (1994-1998) and traces-32.79 (2000-2012). Corresponding phosphate vales were 0.00-0.30 (1985-1993), 0.00-2.1 (1994-1998) and traces -5.91 (2000-2012). Silicates were 0.11-11.60 (1985-1993), 0.84-17.77 (1994-1998) and 0.05-33.0 (2000-2012). Dissolved trace metals (nM) that sustain microalgal growth are also high: Fe 0.67-3.08, Cu 10.58-23.25, Ni 15.63-23.35, Co 0.53-1.03, Zn 6.32-25.33 (Al-Said et al. 2017b). A scaled critical depth model for Kuwait Bay based on the incident radiation, depth of mixed layer and scaled mixed layer showed availability of 83-275 W m^{-2} light near the bottom which is sufficient for microalgal growth (Subba Rao and Al-Yamani (1999).

Coupled with a severe decline in the freshwater runoff from the North, discharges of brine from over 50 desalination plants resulted in an increase in salinity (Al-Said et al., 2017) leading to shifts in functional groups of the plankton community.

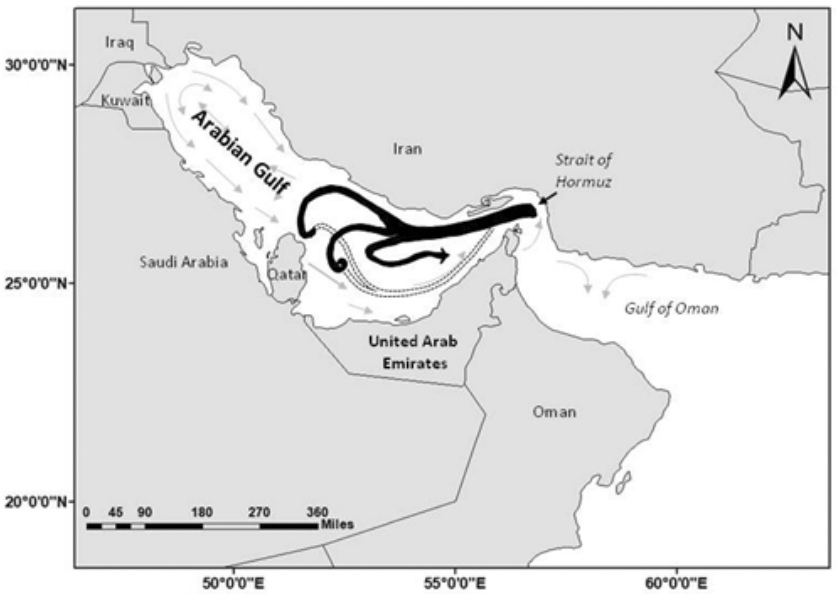

Figure 1. Conceptual circulation in the Arabian Gulf based on Reynolds (1993) and Johns et al. 2003). Inflow from IOSW is indicated in dark and the outflow from the Gulf in dashed line.

In the Gulf, long-term institutionalized phytoplankton studies are absent. Phytoplankton studies in the Gulf are reviewed (Subba Rao and Al-Faiza 1998, and Dorgham 2013). Dorgham (2013) listed 74 publications on phytoplankton studies mostly derived from one-time collections using 30-55 μm plankton nets. Thus, serious gaps exist due to sampling bias such as inadequacy in the spatial and temporal coverage of sampling sites and loss of biota. However, *massive* algal blooms (2906.75 – 4525.47 μg Chl *a* l^{-1}), dominated by diatoms and dinoflagellates were reported off Kuwait (Subba Rao et al. 2003).

Data gathered from various publications showed an increase in the abundance of dinoflagellate species in the Gulf (Subba Rao and Al-Yamani, 2000). Red tide episodes off Kuwait are frequent, ephemeral, massive (Subba Rao et al. 1999) and potentially harmful (Heil et al. 2001, Glibert et al. 2002, Al-Yamani et al. 2012). This review assembles the scattered observations on dinoflagellates in the Gulf from various journals. Shortcomings in the data suggest the need for research for a better understanding of the toxigenic harmful dinoflagellate blooms in the Gulf. Recommendations are made to better understand the structure and physiology of dinoflagellates thriving under unique conditions of high temperature and high salinity prevailing in the Gulf.

DINOFLAGELLATE DISTRIBUTION

A south to north gradient in the distribution of phytoplankton exists in the Gulf (Subba Rao and Al-Yamani, 1998). A maximum diversity of 509 dinoflagellate species exists in the Gulf of Oman and Straits of Hormuz (Dorgham and Moftah 1989). Diversity decreased to 148 species off Kuwait (Subba Rao and Faiza, 1998) and further to 116 species in the Shatt Al-Arab (Hadi et al. 1984).

Table 1. Occurrence (P) of dinoflagellate species in the Arabian Gulf

Ref & year	Bohm 1931	Dorgham and Moftah 1986	Dorgham et al. 1987			Dorgham and Moftah 1989				Darki and Karkmahmalnyi 2017	Al-Khandari et al 2009	Al-Yamani and Saburova 2019	Al-Harbi 2005	Saraji et al.2014
Total number of dinoflagellates recorded >>>	*45*	*143*	*39*	*56*	*71*	*75*	*76*	*92*	*106*	*108*	*102*	*203*	*45*	*100*
Region >>>	Iranian coast	Qatar	Kuwait	Saudi	Qatar	Qatar	UAE	Hormuz	Oman	Hormuz	Kuwait	Kuwait	ROPME	Iranian - Oman Sea
Season >>>		Seasonal				Sept. 1986				Jan, July 2015	Annual	Annual 2004-17	Dec. 1993	Jan & July 2015
Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
Abedinium dasypus												P		
Actiniscus pentasterias												P		
Achradina pulchra												P		
Akashiwo sanguinea											P			
A. insuetum											P			
A. leei											P	P		
A. minutum											P	P		
Alexandrium gaarderae												P		
Alexandrium hiranoi												P		
Alexandrium acatenella												P		
Alexandrium catenella												P		
Alexandrium sp. *1*											P			
A. halim										P	P			
Alexandrium ostenfeldii												P		
A. pacificum Litaker												P		
Alexandrium tamiyavanichii												P		
Alexandrium tamutum												P		
A. pseudogonyaulax												P		
A. tamarense										P		P		

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
Amphidinium											P			
Amphidinium sp. *1*											P	P		
Amphidinium pelagicum												P		
Amphisolenia bidentata											P			
A. palmata										P				P
Archaeperidinium minutum												P		
Azadinium caudatum												P		
Azadinium spinosum												P		
Balechina pachydermata												P		
Barrufeta resplendens												P		
Bernadinium sp.										P				
Blepharocysta splendor- maris	P						P	P				P		
Blepharocysta paulsenii												P		
Ceratium arietinum		P	P	P	P									
C. azoricum		P							P					
C. biceps									P					
C. belone						P	P	P	P					
C. boehmii		P		P	P					P				
C. breve	P		P	P	P							P		
C. breve var pulchellum						P	P	P	P		P			
C. breve var parallelum		P												
C. bigelowii		P			P	P	P	P	P	P				
C. candelabrum		P			P	P	P	P	P					
C. candelabrum var depressum		P								P			P	
C. compressum										P				
C. contortum										P				
C. contortum var karstenii									P					
C. contrarium										P				

Table 1. (Continued)

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
C. carriense										P			P	
C. declinatum		P		P						P				
C. declinatum var majus		P				P		P	P	P				
C. declinatum var noermale														
C. deflexum		P	P	P	P							P		
C. dens	P	P	P	P	P	P	P	P	P					
C. digitatum							P	P	P	P			P	
C. ehrenbergii		P											P	
C. euarctatum		P				P	P	P	P					
C. extensum					P									
C. falctum		P	P		P					P		P		
C. furca	P	P	P	P	P	P	P	P	P			P	P	
C. furca var bergii						P	P	P	P	P			P	
C. extensum	P	P												
C. furca eugrammum		P		P	P	P	P	P	P					P
C. fusus			P	P	P	P	P	P	P			P		P
C. gallicum				P	P	P	P	P	P				P	
C. geneculatum										P				
C. fusus var seta		P	P	P	P								P	
C. gibberum		P	P	P		P	P	P		P		P		
C. gibberum var dispar														
C. grvidum														
C. humile		P								P				
C. hexacanthum		P				P	P	P	P				P	
C. horridum		P	P	P	P					P		P		
C. hottidum var buceros							P		P	P				
C. horridum var claviger		P								P				
C. horridum var inclinatum		P												
C. horridum var molle		P												

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
C. horridum var tenue		P												
C. inflatum	P	P			P									
C. incisum		P												
C. karstenii										P				
C. lineatum		P			P					P				
C. longipes						P		P	P	P	P		P	
C. longirostrum		P								P				
C. lunula		P				P				P		P	P	
C. kofoidii		P	P	P	P					P		P		
C. macroceros		P			P					P		P		
C. masseliense	P	P	P	P	P	P	P	P	P	P		P	P	
C. maseliemse var armatum		P				P	P	P	P	P	P	P	P	
C. massliense var protruberans		P		P	P	P	P	P	P			P		
C. minutum		P			P	P	P	P						
C. mulleri var atlanticus												P		
C. pennatum		P	P	P	P	P	P	P		P				
C. pennatum var scapiforme		P												
C. pentagonum									P	P				
C. pentagonum var subrostrum									P	P				
C. pentagonum var tenerum		P								P				
C. praeolongum										P				
C. pulchellum										P				
C. pavillardii							P	P	P					
C. ranipes										P				
C. reflexum										P				
C. setaceum		P											P	
C. strictum		P												
C. sumatranum	P			P										
C. symmetricum														P
C. trichoceros	P	P	P	P	P				P			P	P	
C. tripos		P	P	P	P	P	P	P	P	P	P		P	P

Table 1. (Continued)

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
C.tripos var atlanticum		P				P	P	P	P	P	P		P	
C. trpos var balticum						P	P	P	P					
C. tripos var indicum						P	P	P	P					
C. tripos pulchellum		P				P	P	P	P					
C. vulture		P		P								P		
C. vultur var japonicum		P							P	P		P	P	
C. vultur var sumatranum												P		
C. wnula														
C. massliense var armatum													P	
C. schrankii														
C. schmidtii								P	P					
Ceratium sp. *1*								P	P					
Ceratocorys sp.		P						P	P		P			
Ceratocorys armata			P											
C. horrida						P		P	P					
C. magna								P	P	P				
Cladopyxis hemibrachiata										P				
Cladopyxis brachiolata											P			
C. setifera													P	
C. spinose													P	
Cochlodinium sp. *1*													P	
Cochlodinium clarissimum												P		
Cochlodinium convolutum												P		
Cochlodinium polykrikoides											P			*P*
Cochlodinium pulchellum												P		

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
Cochlodinium radiatum												P		
Cochlodinium tesselatum														
Cucumeridinium coeruleum												*P*		
Dicroerisma psilonereiella												P		
Dinophysis acuta											P			
D. acuminate		P									P	P		
D. caudata	P	P	P	P	P							P		
D. caudata var persica						P	P	P	P	P	P			
D. caudata var tripos			P	P	P									
D. cuneus														
D. diegensis		P							P					
D. homunculus		P					P							
D. miles Cleve	P											P		
D. miles var marisrubri						P	P	P	P	P	P		P	
D. mitra												P		
D. nasuta												P		
D. norvegica											P			
D. rotundata											P			
D. tripos											P			
D. truncate		P											P	
D. urocantha				P										
Dinophysis sp.												P		
Diploipelta bomba												P		
Diplopelta parva										P				
Diplopelta steinii										P	P	P		
Diplopsalis lenticula		P									P	P		P
Diplopsalopsis orbicularis										P	P			
Diplopsalis sp.										P	P			P
Dissodinium gerbaultii														

Table 1. (Continued)

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
Exuviella compressa	P	P		P	P								P	
E. marina		P				P	P	P	P					
Erythropsidinium agile												P		
Erythropsidinium sp. *aff. E. scarlatinum*												P		
Glenodinium danicum		P												
G. borges					P	P	P	P	P					
G. lenticula														
Goniodoma polyedricum	P								P			P		
G. catenate										P	P			
Gonyaulax conjuncta		P				P	P	P						
G. diacantha		P	P		P									
G. diegensis		P	P			P								
G. digitale	P	P	P	P	P	P	P	P	P			P		
G. fragilis						P	P	P	P	P				
G. hyaline													P	
Gonyaulax gabrielae												P		
Gonyaulax inflata												P		
G. Jolliffei	P								P					
G. kofoidii									P					
G. lingustica		P							P					
G. mimima		P												
G. minuta		P												
G. monocantha		P	P	P	P	P	P	P	P					
G. polyedra	P	P		P		P	P	P	P					
G. polygramma	P	P		P	P	P	P	P	P	P		P		
G. scrippsae		P				P	P	P	P	P	P	P		
G. spinifera		P								P		P		
Gonyaulax turbynei												P		
Gonyaulax verior Sournia												P		
Gonyaulax sp. *1*						P				P				

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
Gymnodinium spp. *cf. aff. Gracilentum*											*P*			
Gymnodinium arcticum		P									P			
Gymnodinium catenatum												P		
Gymnodinium endofasciculum												*P*		
G. fuscum					P									
Gymnodinium gracile												P		
Gymnodinium impudicum												P		
Gymnodinium ostenfeldii												*P*		
Gymnodinium sp.					P							P		
Gymnodinium sp. *2*												P		
Gymnodinium sp. *3*												P		
Gymnodinium sp. *4*												P		
Gymnodinium sp. *5*												P		
Gymnodinium sp. *6*												P		
Gymnodinium sp. *7*												P		
Gymnodinium sp. *8*												P		P
G. spirale												P		P
G. Splendens														
Gyrodinium britannicum												P		
Gyrodinium dominans												P		
Gyrodinium sp. *1*						P	P	P	P					
Gyrodinium sp. *2*											P		P	
Gyrodinium sp. *3*											P			
Gyrodinium fusiforme												P		
Gyrodinium guttula												P		
Gyrodinium heterogrammum												P		
Gyrodinium cf. maculatum												*P*		

Table 1. (Continued)

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
Gyrodinium moestrupii												P		
Gyrodinium pepo												P		
Gyrodinium cf. vesiculosum												P		
Gyrodiniellum shiwhaense											*P*			
Hemidinium nasutum		P	P	P	P									
Heterocapsa sp.*1*											P			P
Heterocapsa spp.												P		
Heteraulacus polyedricus														P
Histioneis costata	P										P	P		
Histioneis vouckii		P		P	P	P	P	P	P					
H. elongate					P									
Kapelodinium vestifici												P		
Karenia cf. brevis											P			
Karenia digitate												P		
Karenia mikimotoi												P		
Karenia selliformis											P	P		
Karenia papilionacea												P		
Karenia umbella												P		
Karlodinium australe												P		
Karlodinium decipiens												P		
Karlodinium gentienii												P		
Karlodinium veneficum												P		
Katodinium glaucum											P			
Kofoidinium pavillardii												P		
Kryptoperidinium foliaceum												P		

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
Lepidodinium chlorophorum												P		
Lepidodinium viride												P		
Lepidodinium spp.												P		
Lepidodinium sp. *1.*												P		
Lepidodinium sp. *2.*												P		
Lepidodinium sp. *3.*												P		
Leptodiscus medusoides														
Levanderina fissad												P		
Lingulodinium polyedra										P	P	P		
Margalefidinium fulvescens												*P*		
Margalefidinium polykrikoides												*P*		
Mesoporos perforatus												P		
Nematopsides vigilans												P		
Nematodinium armatum												P		P
Nematodinium torpedo												P		
Noctiluca scintillans										P	P		P	P
Noctiluca miliaris													P	
Oblea rotunda										P	P			
Ornithocercus magnificus	P	P								P	P	P		
O. splendicus					P									
O. steinii					P					P		P		
Ornithocercus thurnii		P	P	P	P	P	P	P	P	P				
O. quadratus												P	P	
Oxyrrhis marina											P			
Oxytoxum gracile											P			
Oxytoxum sceptrum		P										P		
Oxytoxum areolatum		P							P			P		
Oxytoxum crassum														
Oxy brunelli														

Table 1. (Continued)

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
O. curvatum		P	P	P										
O. elegans		P						P	P			P		
O. longiceps								P	P					
O. scolopax	P	P		P										
O. tesselatum		P									P			
O. variabile												P		
Oxytoxum sp. *1*														
Pentaplacodinium saltonense												P		
Peridinium lomnickii		P												
P. trochoideum														
P. brevipes														
P. brochi		P												
P. cerasus		P												
P. compressum		P												
P. conicoides		P												
P. biconicum var elgans	P													
P. conicum	P	P												
P. crassipes	P	P												
P. curvipes		P												
P. depressum var superba	P	P												
P. diabolus		P												
P. divergens	P	P												
P. fatulipes		P												
P. globulus	P	P												
P. grande		P												
P. granii		P												
P. granii var mite		P												
P. heteracanthum		P												
P. leonis		P												
P. longispinum		P												
P. minisculum		P												
P. minutum		P												

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
P. murrayi		P												
P. nipponicum		P												
P. oblongum		P												
p. oblogum var crassa														
P. obtusum		P												
P. oceanicum	P	P												
p. oceanicum var curvicorne														
P. okamurai	P	P												
P. orbiculare		P												
P. ovatum		P												
P. ovum		P												
P. pallidum	P	P												
P. pellucidum	P	P												
P. pendunculatum		P												
P. pentagonum		P												
P. pietschmanni	P													
p. punctulatum	P	P												
P. quaeneranse		P								P				
P. quinquecorne												P		
P. schilleri	P													
P. sphaericum		P												
P. sphaeroidea	P													
P. spiniferum		P												
P. steinii	P	P												
P. striatum	P													
P. subinerme	P	P												
P. trochoideum		P												
P. tenuissimum		P												
Peridinium sp.	P													
Peridinopsis minima												P		
Phalacroma acuta					P			P						
P. irregulare									P					
P. minutum									P					
P. mitra Schütt												P		
P. operculatum		P												
P. ovum		P												

Table 1. (Continued)

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
P. parvulum		P							P					
P. pulchellum		P												
P. rapa		P		P	P	P	P	P	P			P		
Phalacroma rudgei												P		
P. rotundatum		P			P							P		
Phalacroma whiteleggei												P		
P. elegans				P	P					P				
Podolampas bipes	P	P		P	P			P	P		P	P		
P. palmipes	P	P	P	P	P			P	P	P		P		
P. spinifera													P	
P. divergens													P	
P. elegans													P	
P. grandi													P	
P. sceabrve										P	P			
Polykrikos geminatum												P		
P. hartmanni												P		
Polykrikos kofoidii												P		
Polykrikos schwartzii												P		
Preperidinium meunieri						P	P	P	P		P			
Pronoctiluca pelagica											P			P
Prorocentrum balticum										P		P		P
Prorocentrum arabianum														
Proro. Belizeanum														P
Proto conicoides											P			P
Proroc. compressum												P	P	
P. magnum						P				P	P			
P. gracile	P	P				P	P	P	P		P	P	P	P
P. micans	P	P	P	P	P							P		P
P. micans var undulatum							P	P					P	

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
P. lenticulatum											P			
P. lima										P				P
Prorocentrum minimum												P		
P. norrisiamum									P					P
Prorocentrum nux												P		
P. pyriforme														
P. rocentrum											P			P
P. rhathymum												P		
P. rostratum		P												
P. scutellum					P						P			P
P. triestinum											P	P		P
Prorocentrum sp.*1*														
P sigmoides											P			P
Protoceratium reticulatum	P										P	P		
Protoceratium sp. *1*						P	P	P	P					
Protoperidinium abei								P		P		P		
P. acutipes										P	P			
Protoperidinium achromaticum												P		
P. biconicum											P	P		
P. bipes								P				P		
P. breve						P	P	P	P					
P. brochii								P	P	P	P			
P. cerasus					P					P	P			
P. claudicans							P	P	P	P	P	P		
Protoperidinium cf. compressum												P		
P. conicum			P	P	P	P	P	P	P			P		
P. conicum var asaumbusi					P	P	P	P	P	P	P			
P. crassipes											P			
P. curtipes						P				P		P		
P. curvipes						P	P	P	P		P	P	P	
P. depressum			P	P	P	P	P		P			P		
P. diabolus				P	P	P	P	P	P	P	P			
P. divergens			P	P	P		P	P	P	P	P	P		

Table 1. (Continued)

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
P. elegans										P		P		
P. equiquipes						P	P	P	P					
P. globulus					P	P	P	P	P		P			
P. grande				P	P	P	P	P	P					
P. granii			P	P	P				P			P		
P. heterocanthum					P					P				
p. inclinatum						P	P	P	P		P			
P. leonis			P	P	P				P					
P. minisculum										P	P			
P. marie-lebourae											P	P		
P. minutum			P				P	P	P					
P. murrayi				P	P	P	P	P	P					
Protoperidinium obtusum												P		
P. oceanicum			P	P	P			P				P		
P. orientale						P	P	P	P					
P. ovum					P			P	P					
P. oviforme						P	P	P	P					
P. orbiculare						P	P	P	P		P			
P. ovatum				P	P									
Protoperidinium pacificum												P		
P. pallidum					P		P	P	P					
P. pedunculatum						P	P		P	P	P			
P. pellucidum				P		P	P	P	P	P	P	P		
P. pentagonum										P	P	P		
P. pundunculatum			P	P		P				P		P		
P. pyriforme						P	P	P	P		P			
Protoperidinium quadrioblongum												P		
Protoperidinium quarnerense												P		
P. subinerme						P	P	P	P			P		P
P. subpyriforme														
P. spiniferum														

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
Protoperidinium steinii					P							P		P
P. triestnum										P				
P. inclinatum										P				
P. latissimum										P				
P. mite										P				
P. oblongum										P				
P. ovatum										P				
P. quarnerense										P				
P. sphericum										P				P
P. solidicorne										P				
P. spiniferum										P	P			
Protoperidinium steinii										P				
p. subpyriforme										P				
P. thorianum										P		P		
Protoperidinium thulesense												P		
Protoperidinium tohrui												P		
P. ventricum										P		P		
P. venustum						P	P	P	P			P		
Protoperidinium sourniai						P	P	P	P					
Protop tennuissimum									P		P			
P. thorianum											P			
Protoperidinium ventricum											P			
Protoperidinium sp. *1*											P			
Protoperidinium sp. *2*											P			
Protoperidinium sp. *3*											P			
Protoperidinium sp. *4*											P			
Protoperidinium sp. *5*											P			

Table 1. (Continued)

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
Protoperidinium sp. *6*											P			
Protoperidinium sp. *7*											P			
Protoperidinium sp. *8*											P			
Protoperidinium sp. *9*											P			
Protoperidinium sp. *10*											P			
Protoperidinium sp. *11*											P			
Protoperidinium sp. *12*											P			
Protoperidinium sp. *13*											P			
Pseudophalacroma nasatum	P	P			P			P	P	P	P		P	
Pyrocystis elegans												P		
Pyrocystis fusiformis		P	P	P	P							P	P	
P. hamulus										P				
P. lunula		P	P							P			P	
P. noctiluca		P		P				P	P	P	P	P		
P. obtusa		P	P		P			P	P			P		
P. pseudonoctiluca		P		P					P	P				
P. robusta										P	P			
Pyrodinium bahamense var. compressum						P	P	P	P	P	P	P	P	P
Pyrophacus horologicum	P	P	P	P	P							P	P	
P. ranaompoae						P	P	P	P	P	P		P	
P. steinii								P	P			P		P
Pyrodinium schilleri													P	
P. vancampoae						P	P	P						
Pytodinium bahamens										P				P

Nature of samples examined >>	Net	Water + 55 µm net	water+ 55 µm net			Water + 55 µm net				55 µm net	Water + 20 µm net	Water	35 µm net	55 µm net
Sclerodinium calyptroglyphe												P		
Scripsiella acuminate											P			
Scrippsiella irregularis												P		
Scrippsiella plana												P		
Scrippsiella precariat												P		
Scrippsiella spinifera												P		P
Scrippsiella trifida												P		P
S. trochoidea												P		P
S steinii														P
Scripsiella spp.												P		
Scaphodinium mirabile														
Torodinium robustum						P	P	P	P				P	
Triadinium sphaericum						P	P		P					
T. polyedricum		P		P	P									
Takayama acrotrocha												P		
Takayama cladochroma												P		
Takayama helix												P		
Takayama tasmanica												P		
Takayama tuberculata												P		
Takayama sp. *1*												P		
Takayama sp. *2 aff. T. xiamenensis*												P		
Torodinium robustum												P		
Torodinium teredo												P		
Warnowia polyphemus												P		
Note: The genus *Ceratium* is renamed *Tripos* (Gomez 2013)														

Historical analyses of dinoflagellate populations in the Gulf are limited and based on net collections; thus they underestimate breadth of species. Net collections from the Iranian coast (Bohm 1931) and Al-Harbi (2005) from the ROPME area yielded 45 species in each area. Net samples (55 μm net) from Iranian –Oman Sea contained 100 species (Saraji et al. 2014) and 108 species from the Hormuz Strait (Darki and Karkmahmalnyi 2017). However, water samples off Qatar contained 143 species (Dorgham and Moftah 1986), 106 species off Oman (Dorgham and Moftah 1989) and 102 dinoflagellate species off Kuwait, (Al-Kandari et al. 2009). When the analyses were extended to water samples collected from 2004 to 2017, 203 species were reported off Kuwait (Al-Yamani and Saburova 2019), underscoring the need for extended observations. In the Gulf, a high diversity of dinoflagellates- 509 species- exists (Table 1) and this could increase with enhanced sampling from sub-surface depths, throughout the year and over several years.

The waters between Hormuz and Oman represent a staging area for a mix of Gulf water with the incoming Indian Ocean Surface Water (IOSW) from the Gulf of Oman through the Straits of Hormuz, and an admixture of deeper water flow from Oman (Figure 1). Hormuz and Oman waters contain 198 dinoflagellate species (92 species off Hormuz and 106 off Oman) (Dorgham and Moftah 1989, Table 1). Compared to the total Gulf volume (~8,600 km^3), exchange of waters from the Western Indian Ocean (WIO) is high (7253 km^3 y-1) via the northern part of Hormuz Strait that flow into the Gulf (Johns et al. 2003). It is reasonable to assume that the Hormuz-Oman region serves as an exchange point for a number of dinoflagellates in the Gulf.

Utilizing 198 species of Hormuz-Oman area as a base, their contribution to Oman was 53%, Qatar 41%, UAE 38%, Kuwait 32%, Saudi Arabia 17% and Iran 8%. This pattern of distribution i.e., decreases of number of species from south to north is consistent with the decreasing volume and dilution of Hormuz-Oman waters as they flow into the Gulf. Observations of Thorrington-Smith (1971) corroborate this conclusion; components of the Western Indian Ocean (WIO) decreased from south to north. The number of such WIO dinoflagellate species in the Gulf of Oman was 61 (Dorghan and Moftah 1989), 17 in the Gulf in general, 12 off Qatar and 7 in Kuwait waters. North of Bushehr, Iran, probably due to lesser exchange with the open ocean, the number of species common to their southern waters is further reduced (Hulburt et al. 1981).

Based on samples collected during September 1986, Dorgham and Moftah (1989) recognized 6 patterns in the distribution of dinoflagellates: group 1. represents species common to the Gulf and the Gulf of Oman, 2. Species mainly found in the Gulf but extended to the straits of Hormuz and even to the Gulf of Oman, 3. Species restricted to the Gulf, 4. Species limited to Straits of Hormuz, 5. Species observed in the Gulf of Oman and in the Straits of Hormuz, and 6. Species restricted to the Gulf of Oman. Notably, sampling methods differed across the seasons. Analyses of Al-Kandari et al. (2009) off Kuwait (Table 1) are annual and based on whole water samples while those of Al-Harbi (2005) in ROPME area, and Darki and Karkmahmalnyi (2017) in Hormuz are net samples (35-55 μm). Thus, the spatial and temporal coverage of data do not permit a true comparison.

DISTRIBUTION LIMITATIONS

There were 55 dinoflagellate species present in the Gulf with no limitation to any region: *Ceratium arietinum, C. biceps, C. breve, C. breve* var *parallelum, C. deflexum, C. dens, C. falctum, C. furca, C. furca eugrammum, C. fusus, C. fusus var seta, C. humile, C. lineatum, C. macroceros, C. masseliense, C. masseliense* var *armatum, C. massliense* var *protruberans, C. minutum, C. trichoceros, C. tripos, C. tripos* var *atlanticum, D. caudata, D. miles, Exuviella compressa, Gonyaulax danicum, G. diegensis, G. digitale, G. minuta, G. monocantha, G. polyedra, G. polygramma, Histioneis vouckii, Ornithocercus thurnii, P. rotundatum P. palmipes, Pronoctiluca pelagica, P. micans, Protoperdinium conicum, P. crassipes, P. depressum, P. diabolus, P. divergens, P. globule, P. grande, P. granii, P. oceanicum, P. ovum,, P. ovatum, P. pellucidum, P. pentagomum, Pyrophacus horologicum, P. stenii, P. fusiformis, P. obtusa, Triadinium sphaericum,* and *T. polyedricum.*

As data on the Gulf dinoflagellates are scanty, many detailed studies will have to be carried out, which may necessitate revision of the present interpretation of the pattern of spatial and temporal distribution. The number of species exclusively present in Oman, Qatar, Saudi Arabia, UAE, Kuwait and Iran correspond to 24, 1, 1, 0, 119 and 11, respectively (Table 2). Analyses of more samples collected in Kuwait region during 2004 to 2017 emphasized the need for extended studies; they also account for the larger number of species (119 species) in Kuwait waters compared to other regions of the Gulf with limited sampling. Of interest is the dominance of Gymnodinians, *Peridinium* and *Protoperidinium* species in Kuwait waters. Parasitic dinoflagellate *Eduboscquella* sp. is recorded from the lorica of the tintinnid *Eutintinnus* sp. (Al-Yamani and Suburova, 2019).

XENOBIODIVERSITY AND PLAUSIBLE BALLAST WATER INTRODUCTIONS

Ecosystems of young enclosed brackish water seas such as the Caspian and Aral Sea have lower biological diversity, but major changes in structure and functioning do occur due to increased "foreign" or xenobiodiversity biological diversity (Leppäkoski and Olenin, 2000). A young Sea - the Arabian Gulf (Sheppard et al. 2010) - fits into this category. In this shallow Gulf, the diversity is ever changing due to increased anthropogenic stresses such as discharge of ballast waters from 20,000 ships that visit annually, dumping of sewage in the coastal waters and discharge of brine from the desalination plants that act synergistically; this is exacerbated by the Gulf's small size and slow circulation. The inadvertent introduction of alien biota via ballast water is sizeable. The Strait of Hormuz serves as a chokepoint for oil tankers that transport, in an opposite direction more than 17 million barrels of water from different regions of the world to the ROPME area every day. Introduction of non-indigenous species to the inner ROPME area and UAE and the Iranian waters is known (Hamza 2006, Jalili et al. 2008, Al-Yamani et al. 2015). The warm ROPME Sea Area receives a greater volume of ballast water discharges from crude oil tankers than any other area in the world, and is characterized as a high-risk area (Al-Yamani et al. 2015). The warm well-illuminated waters with high levels of nitrates and phosphates are conducive for the growth of

Gymnodinium sp. (Heil et al. 2001), *Gymnodinium catenatum*, *Gyrodinium impudicum*, and *Pyrodinium bahamense* var. *compressum* (Glibert et al. 2002).

Table 2. Species restricted in distribution

Region	Restricted/total	Species
Oman	24/ 106	*C. karstenii, C. pentagonum var subrostrum, C. praeolongum, C. ranipes, C. reflexum, C. schmidtii, C. magna, Cladopyxis hemibrachiata, Diplopelta parva, Phalacroma minutum, P. lima, P. triestnum, P. inclinatum, P. latissimum, P. mite, P. oblongum, P. ovatum, P. quarnerense, P. sphericum, P. solidicorne, P. spiniferum, Protoperidinium steinii, p. subpyriforme, Pyrodinium bahamens*
Qatar	1/75	*G. fuscum*
Saudi	1/56	*D. urocantha*
UAE	0/76	None
Kuwait	119/203	*Abedinium dasypus, Actiniscus pentasterias, Achradina pulchra, Akashiwo sanguine, A. insuetum, A. leei, A. minutum, Alexandrium gaarderae, A. hiranoi, A. acatenella, A. catenella, A. ostenfeldii, A. pacificum, A. tamiyavanichii, A. tamutum, A. pseudogonyaulax, Amphidinium pelagicum, Amphisolenia bidentata, Archaeperidinium minutum, Azadinium caudatum, A. spinosum, Balechina pachydermata, Barrufeta splendens, Ceratium mulleri var atlanticus, Ceratium vulture var sumatranum, Cochlodinium classimum, C. convolutum, C. pulchellum, C. radiatum, Cucumeridinium coeruleum, Dicroerisma psilonereiella, Dinophysis mitra, D. nasuta, Dinophysis sp. Diploipelta bomba, Erythropsidinium agile, E. sp. aff. scarlatinum, Gonyaulax gabrielae, G. inflata, G. turbynei G. verior, Gymnodinium catenatum, G. endofasciculum, G. gracile, G. impudicum, G. ostenfeldii, Gymnodium sps, G. britannicum, G. dominans, Gyrodinium fusiforme, G. guttula, G. heterogrammum, G. cf. maculatum, G. moestrupii, G. pepo, G.cf.vesiculosum, G. spirale, Heterocapsa sp., Kapelodinium vestifici, Karenia digitata, K.mikimotoi, K.papilionacea, K. umbella, Karlodinium australe, K.decipiens, K. gentienii, K. veneficum, Kofoidinium pavillardii, Kryptoperidinium foliaceum, Lepidodinium chlorophorum, Lepidodinium viride, Lepidodinium spp., Lepidodinium sp 1., Lepidodinium sp 2., Lepidodinium sp3., Levanderina fissa, Margalefidinium fulvescens, M. polykrikoides, Mesoporos perforates, Nematopsides vigilans, Nematodinium armatum, N. torpedo, Oxytoxum variabile, Pentaplacodinium saltonnse, P. quinquecorne, Peridinopsis minima, Phalcroma mitra, P. rudgei, P. whiteleggei Polykrikos geminatum, Polykrikos hartmannii, P. kofoidii, P. schwartzii, Prorocentrum compressum, P.minimum, P. nux, P. rhathymum P. triestinum, Protoperidinium achromaticum P. biconicum, P. compressum, P.pacificum, P. thulesense Protoperidinium tohrui, Sclerodinium calyptroglyphe, Scrippsiella irregularis, S. plana, S. precaria, S.spinifera, S. trifida, Takayama acrotrocha, T. cladochroma, T. helix, T. tasmanica, T. tuberculata, Takayama sp. 1, Takayama sp. 2 aff. T. xiamenensis, Torodinium robustum, T. teredo, Warnowia polyphemus.*
Iran	11/100	*Cochlodinium tesselatum, Dissodinium gerbaultii, G. splendens, Heteraulacus polyedricus, P. spiniferum, P.biconicum var elegans, P. pietschmanni, P. sphaeroidea, P. schilleri, Prorocentrum arabianum, P. belizeanum*

Table 3. Exotic species possibly introduced into the Arabian Gulf and the Sea of Oman

Species	Occurrence in the Gulf	Earlier records outside the Gulf	Reference
Achradina pulchra	Frequent	Atlantic, Pacific, Black Sea	Nival 1969 Konovalova 1998
Akashiwo sanguinea	Rare	Japan, New Zealand, Atlantic, Pacific	Hallegraeff 1991 Keifer and - Lasker 1975 Bockstahler and Coats 1993, Voltolina 1993
Corythodinium tesselatum	Rare	Atlantic	Wood 1968, Dodge 1985, Balech 1988
Gymnodinium catenatum	Freq	N. American waters, European, Australian and Japanese waters	Morey- Gaines 1982, Mee et al. 1986, Hallegraeff et al. 1995, Estrada et al. 1984
Peridinium quinquecorne	very rare	Japan, Malaysia, Philippines, S. Africa, Australia, Belige	Horiguchi and Pienaar 1991, Faust et al. 2005, Mohammad-Noor et al.2007
Pyrodinium bahamense var. compressa	Rare	Tropical Atlantic, Indian Ocean. Red Sea, Brunei, Philippines, New Guinea, Oman, Hormuz	Dodge 1985 Hallegraeff et al. 1995
Sinophysis ebriolum	very rare	North German, Japan, Russia	Selina and Hoppenrath 2004, Hoppenrath 2000

Data (1987 to 2019) based on analyses of water samples show an increase in the dinoflagellate populations off Kuwait from 39 species (Dorgham et al. 1987) to 108 (Al-Kandari et al. (2009) to 203 (Al-Yamani and Suburova 2019). This increase in the dinoflagellate diversity (119 to 203) in Kuwait waters (Table 2) suggests their possible introduction by ships visiting Kuwait from various geographic regions. It is possible that every species that could have been introduced has been introduced into the Gulf, evident from new records. Earlier it was suggested that the Strait of Hormuz acts as a chokepoint for ~20,000 ships that visit the Gulf annually. Occurrence of *Achradiona pulchr, Akashiwo sanguine, Corythodinium tesselatum, Gymnodinium catenatum, Peridinium quinquecorne, Pyrodinium bahamense var compressa* and *Sinophysis ebriolum* for the first time from the Gulf suggests their possible introduction from Malaysia, Brunei, Philippines, New Guinea, Japanese waters, Australia, Belige, Russia, and S. African seas by shipping traffic, similar to the occurrence of Indo-Pacific dinoflagellates in the Mediterranean (Gómez 2006). It is possible that the prevailing high temperatures and salinities in the Gulf may restrain the growth of potentially harmful and bloom forming species or the establishment of species introduced via shipping.

According to the "window of introduction theory" (Gollasch 1999), several physical and biological constraints determine the successful establishment of an exotic dinoflagellate. These include survival during the voyage, ballasting and deballasting cycle, predation and competition by native species. Also, of the invading species, only 10% have a significant impact (Williamson and Fitter 1996). It is to be noted that the species dominant in the blooms during different years differed (Table 4). Of interest is the change in the constituent dinoflagellate species from time to time (Table 4, Subba Rao D.V. et al. 2003) and the same

species did not recur which suggests that species newly introduced may have found the medium conducive for temporary growth. Usually recurrence of the same species is common as was the case with *Prorocentrum donghaiense* in the East China Sea (Zhou et al.2003, Zhou and Zhu 2006), *Karenia brevis* blooms off Florida (Vargo 2009) and *Alexandrium* blooms in the Gulf of Maine region in the U.S. (McGillicuddy et al. 2005). Our time series analysis of dinoflagellates from Kuwait Bay corroborates this; there were 103 species in 2000-2007 with a decrease to 58 species by 2012 – 2013. However in the offshore waters there were 48 species of dinoflagellates during 2005-2006 which increased to 67 by 2012-2013 (Al-Said et al. 2017).

A report on the winter phytoplankton distribution during a research cruise from February to March 2006 (Al-Yamani et al. (2008 and 2012) in the ROPME Sea Area (RSA) is of interest. These data are based on water samples collected from 2 and 4 meters, mid depth samples from 5 to 50m, and bottom samples (from 10 to 100m) from 101 stations spread over 23 transects. These stations were grouped into the Northeast Gulf (38 stations), 34 (South east) and the rest in the Gulf of Oman and Hormuz Strait which receives water from the Indian Ocean and the Arabian Sea which decrease from south to north. A total of 170 dinoflagellates were present and are categorized into ubiquitous, common, frequent, occasional, rare, and very rare. For survival and establishment of exotic species in a harsh environment, more cells than in the categories occasional, rare, and very rare will be required which can be possible only with a larger inoculum. Blanco (Ch.19, 2.1 this volume) discussed the role of resting cysts of recurrent species in initiating a dinoflagellate bloom. This is comparable to the observations of Bohutskyi et al. (2016) who reported increased effluent (inoculum) increased nutrient levels that supported higher growth rates , productivities and robustness in the micro algae *Chlorella* and *Scenedesmus.* There were marked differences in the dinoflagellate distribution between the Northeast Gulf, South east waters and the Hormuz Strait and Gulf of Oman. Although these data are based on one –time collections, suffice to say that dinoflagellate diversity is high in the RSA; of these a few were ubiquitous (*Gyrodinium fusiforme, Oxytoxum variable,* and *Torodinium robustum*); 16 species limited to Northwest area and 18 to the Southeast area.

However, of particular interest is the occurrence of 65 Species, common to the Central Indian Ocean (IOSW) and the Arabian Gulf; these include *Amphisolenia bidentata, Amphisolenia globifera, Balechina coerulea, Ceratium breve, Ceratium candelabrum, Ceratium dens, Ceratium extensum, Ceratium falcatum,, Ceratium furca, Ceratium fusus, Ceratium gibberum, Ceratium hexacanthum, Ceratium horridum, Ceratium lunula, Ceratium macroceros, Ceratium massiliense, Ceratium massiliense var. armata, Ceratium, Ceratium ranipes, Ceratium vultur v. sumatranum, Ceratocorys armata, Cochlodinium* sp., *Dicroerisma psilonereiella, Dinophysis caudata, Dinophysis miles, Dinophysis mitra, Diplopelta bomba, Diplopsalis lenticula, Goniodoma polyedricum, Gonyaulax fragilis, Gonyaulax hyalinum, Gonyaulax polygramma, Gonyaulax spinifera, Gyrodinium spirale), Histoneis costata, Noctiluca scintillans, Ornithocercus magnificus, Ornithocercus quadrates, Oxytoxum curvatum, Oxytoxum scolapax, Podalampas bipes, Polykrikos schwarzii, Pronoctiluca pelagic, Pronoctiluca spinifera, Prorocentrum compressum, Prorocentrum gracile, Prorocentrum micans, Prorocentrum minimum, Protoperedinium curtipes, Protoperidinium curvipes, Protoperidinium depressum, Protoperidinium divergens, Protoperidinium globules, Protoperidinium ovatum, Protoperidinium murrayi, Protoperidinium oceanicum, Protoperidinium steinii, Protoperidinium subinerme, Pyrocystis*

fusiformis, Pyrocystis noctiluca, Pyrodinium bahamense, Pyrophacus horologicum, Spiraulax jolliffei, Triposolenia bicornis and *Warnowia violesces.* Compared to the distribution of dinoflagellates in the Strait of Hormuz and the Gulf of Oman (groups iv, v and vi, Dorgham and Moftah 1989), the RAS had a different assemblage of 60 dinoflagellates; only *Ceratium vulture, Gonyaulax hyalinum, Oxytoxum scolapax, Podalampas bipes and Pyrocystis fusiformis* were common. More annual data based on systematic frequent sampling are needed to establish to what extent these differences are dependent on the incursion and the volume of Central Indian Ocean (IOSW) which decreases from south to north. The number of such IOSW dinoflagellate species in the Gulf of Oman was 61 (Dorgham and Moftah 1989), 17 in the Gulf in general, 12 off Qatar and 7 in Kuwait waters. North of Bushehr, Iran, probably due to lesser exchange with the open ocean, the number of species common to their southern waters is further reduced (Hulburt et al. 1981).

HARMFUL ALGAL BLOOMS IN THE GULF

During September to October 1999 the first fish kill in the Gulf, largely mullet *Liza macrolepis,* was reported (Heil et al. 2001). This was associated with *Gymnodinium* sp. that grew to >6 x 10^6 cells l^{-1} when the inorganic nitrogen increased by more than 20 fold and phosphate levels rose. Other dinoflagellates present were *Amphidinium carterae, Ceratium symmetriam, Ceratium fusus, Dinophysis caudata, Dinophysis miles, Gymnodinium galantum, Gymnodinium simplex, Gymnodinium* sp., *Gyrodinium fusiformis, Gyrodinium spirale, Gyrodinium* spp., *Polykrikos* sp, *Prorocentrum concavum, Prorocentrum emarginatum, Prorocentrum lima, Prorocentrum micans, Prorocentrum minimum, Protoperidinium minutum, Protoperidinium* spp., *Pyrophacus steinii and Scrippsiella troichiodea.* There was a massive kill of greater than 2500 metric tons of wild mullet (*Liza klunzingeri*), preceded by a small fish kill (100–1000 dead fish per day) of gilthead sea bream (*Sparus auratus*) in aquaculture net pens in August-September 2001 (Glibert et al. 2002). Causative harmful dinoflagellates included a set of dinoflagellates different from those of 1999 i.e., blooms of *Ceratium furca* and <10^6 cells l^{-1} *of Gymnodinium catenatum, Gyrodinium impudicum,* and *Pyrodinium bahamense var. compressum*. PSP and brevitoxin levels were below the detection limits and the fish kills were attributed to unusually warm temperatures (up to 35 ◦C) and growth of the bacterium *Streptococcus agalactiae*.

The ciguatera-related dinoflagellate *Gambierdiscus yasumotoi* is reported for the first time from two sampling sites on the southern Kuwait coast (Saburova et al. 2013).

Mortalities of marine biota associated with dinoflagellates from various regions in the Gulf (Table 3) show that *Prorocentrum micans* was common between 1999-2001 episodes in Kuwait, and in Oman in 2005 (Al-Busaidi et al. 2008). *Cochlodinium polykrikoides* was the causative dinoflagellate in the UAE (Richlen et al. 2010) and Iran (Saeedi et al. 2011) and seems to be persistent and spreading. Following the regular blooms of *Noctiluca scintillans*, massive blooms of *G. polykrikoides* (4.6×10^3 to 9×10^6 cells L^{-1} and chlorophyll *a* 78.0 μg L^{-1}) sustained over 8 months are associated with NH_4^+, urea, PO_4^{3-}, and organic nitrogen and phosphorus, many fold higher than observed earlier (Al-Azri et al 2014). The high nutrient levels are attributed to stronger than normal upwelling along the Iranian and northern Omani coasts.

Table 4. Harmful dinoflagellate blooms in the Gulf

Taxa	Region	Period	Mortality if any	Reference
Noctiluca scintillans	Oman	Oct 2005	Fish	Al Gheilani 2011
Cochlodinium polykrikoides		Oct 2008-Apr 2009	Fish and Shellfish	
Prorocentrum minimum, P. arabianum		Oct 2005		
Noctiluca scintillans	Muscat, Gulf of Oman	Mar 1988 Feb Apr 1995	Fish	Thangaraja et al. 2007
Prorocentrum arabianum	Gulf of Oman	May-June 1995		Morton et al. 2002
Alexandrium sp. *Dinophysis* sp. *Gymnodinium breve*	Qatar	Oct 1996		Al-Ansi et al. 2002
Prorocentrum sp.	UAE	2003		
Gymnodinium species	Kuwait	1999 September	Mullet *Liza macrolepis 2500 metric tons*	Heil et al. 2001
Gyrodinium impudicum, Pyrodinium bahamense.	Kuwait	Aug 2001	Wild mullet 2500 metric tons *Liza klunzingeri* Sparus auratus 100-1000 per day	Glibert et al. 2002
Prorocentrum micans Noctiluca scintillans	Oman	Oct 2005		Al-Busaidi et al. 2008
Cochlodinium polykrikoides	UAE	Aug 2008 May 2009	Wild and farmed fish	Richlen et al. 2010
Cochlodinium polykrikoides	Iran The Gulf	2008	Razor clam *Solen dactylus*	Saeedi et al. 2011
Prorocentrum sp.	Kuwait			Subba Rao et al 2003
Gonyaulax sp. *Noctiluca* sp.	Muscat	Feb and Oct 1976		Zahran, 1978
Ceratium fusus, C. macroceros		Sept 1988	Marine organisms	Thangaraja et al. 2007

Dinoflagellate species in the Gulf are on the increase; for example in Kuwait they numbered 39 in 1987 (Dorham et al. 1987), 102 in 2009 (Al-Kandari et al. 2009) and 203 in 2019 (Al-Yamani and Saburova 2019). Of these, only *Dinophysis caudata, Ceratium furca* and *C. fusus* are potentially harmful species (Al-Yamani et al. 2012) present during November 1984 (Dorgham et al. 1987). By 2009, 12 harmful species occurred and included *Alexandrium leei, A. minutum, Pyrodinium bahamense var. compressum, Dinophysis acuminata, D. acuta, D. miles, D. norvegica, D. tripos, Phalacroma mitra, Lingulodinium polyedrum, Protoceratium reticulatum, Noctiluca scintillans* (Al-Kandari et al. 2009). Harmful species *Alexandrium pseudogonyaulax, A. tamarense A. tamiyavanichiia, Alexandrium catenella, Karenia papilionacea, K. selliformis, Karenia mikimotoi, Scrippsiella trochoidea, Gonyaulax polygramma* and *Peridinium quinquecorne* were found in 2019 (Al-Yamani and Saburova 2019). It remains to be investigated whether these dinoflagellates were accidentally introduced and whether they have found a niche in this harsh environment to sustain bloom levels.

There have been several studies on the dinoflagellate cysts buried in the sediments of the Atlantic Ocean, Mediterranean Sea and adjacent basins (Marret and Zonneveld (2003), Zonneveld (1997), Zonneveld et al. (2013) and serve as cyst reservoirs. These cysts can remain viable in the sediment even for 100 years (Ribeiro et al. 2010) and when transported

to the warmer upper water column, excyst and play an important role in seeding recurrent annual blooms (Anderson and Wall 1978; Walsh et al. 2011a). However, in the Arabian Sea (northwestern Indian Ocean) occurrence of dinoflagellate cysts is affected by the SW and NE monsoons (Zonneveld 1997). In the Gulf of Aqaba qualitative and quantitative distribution of cysts varied between the sediments, the Gulf and the Red Sea. Phototrophic dinoflagellates *Brigantedinium* spp., *Votadinium calvum*, *Echinidinium* spp., *Lingulodinium machaerophorum*, *Spiniferites* spp., *Spiniferites bentorii*, *Spiniferites membranaceus* and *Spiniferites mirabilis* dominate the sediment while cysts of the heterotrophic dinoflagellates of *Impagidinium aculeatum*, *Impagidinium sphaericum*, *Operculodinium israelianum*, *Operculodinium longispinigerum*, *Operculodinium centrocarpum*, cysts of *Pentapharsodinium dalei*, and *Selenopemphix nephroides* are in the Gulf of Aqaba and the Red Sea (Elshanawany and Zonneveld (2016). Such comparative studies from the Arabian Gulf are absent. Availability of nutrients, light, suitable temperature and absence of predators dictate how well a species survives and finds it own niche. This may lead to expansion and their range of distribution. A case in point is the emergence and spread of *Cochlodinium polykrikoides* over 8 months in the Gulf and Gulf of Oman (Richelin et al. 2010) that coincides with an apparent global expansion of this taxon. Cultures of this organism were established in *f*/2 –Si medium and DNA analysis confirmed the *C. polykrikoides* isolates from the Gulf were identical to isolates from the northeastern USA, Puerto Rico, Mexico, and Malaysia, known as the ‘‘American/Malaysian’’ ribotype (Richelin et al 2010). The frequent dust storms during 2001 and 2012 in the Persian Gulf and Oman Sea coincided with 36 red tides and included the dinoflagellate *Cochlodinium polykrikoides* besides *Nitzschia*, *Noctiluca*, *Trichodesmium* and *Oscillatoria* (Rbbaniha et al. 2013).

CONCLUSION

The Gulf functions as a reverse estuary and receives waters from the Western Indian Ocean over the Hormuz Strait. The Gulf experiences several anthropogenic stressors and the hypereutrophic high-saline warm waters of the Gulf have a high xenobiodiversity, with 509 dinoflagellates. The number of dinoflagellate species, including potentially harmful species, has been on the increase. Of the 39 dinoflagellate species originally reported in 1987 (Dorgham et al. 1987), only *Ceratium tripos*, *Protoperidinium divergens* and *Pyrodinium bahamense* were present in 2009 (Al-Kandari et al. 2009); probably the rest have been replaced due to increased salinity, and taken over by those introduced via ballast waters. Dinoflagellate blooms in the Gulf are elusive; unique species neither recurred nor contributed to bloom formation in a defined region.

There are 28 potentially harmful dinoflagellate species from Kuwait waters *Alexandrium leei*, *A. minutum*, *A. pseudogonyaulax*, *A. tamarense*, *A. tamiyavanichii*, *Pyrodinium bahamense* var. *compressum* implicated elsewhere in Paralytic Shellish Poisoning, *Dinophysis acuminate*, *D. acuta*, *D. caudata*, *D. miles*, *D. norvegica*, *D. tripos*, *Phalacroma mitra*, *P. rapa*, *P. rotundatum*, *Prorocentrum lima* in Diarrhetic Shellfish Poisoning, *Karenia papilionacea*, *K. selliformis* in Neurotoxic Shellfish Poisoning, *Karenia mikimotoi*, *Scrippsiella trochoidea* in icthyotoxicity and *Lingulodinium polyedrum* in Yessotoxin. *Akashiwo sanguine*, *Alexandrium catenella*, *Ceratium furca*, *C. fusus*, *Gonyaulax*

polygramma, Noctiluca scintillans and *Peridinium quinquecorne* also occurred. So far these suspect species did not evolve into bloom proportions needed to deliver toxins but studies on their physiological ecology are required to elucidate their functioning in this unique environment.

RECOMMENDATIONS

The Gulf is the main water body for Gulf countries and is heavily used for oil transportation, desalination, recreation and mariculture. The Gulf receives enormous quantities of nutrients, such as inorganic nitrogen, phosphorous compounds and trace elements from many sources, such as urban wastewater, sewage treatment plant discharges, erosion of soil containing nutrients and eolian dust storms. Inorganic nitrogen, phosphorous compounds and carbon are involved in the eutrophication and human activities can accelerate their enrichment rate and impact ecosystems. Reduction of phosphorus loading - a means of 'de-eutrophication'- resulted in a decrease in dinoflagellate abundance in Thau Lagoon, France (Gowen et al. 2015). It is to be recognized that eutrophication in the Gulf countries is uninterrupted while blooming of most dinoflagellates is short lived with an exception of *Cochlodinium polykrikoides* that bloomed for 8 month in Oman waters. So, any models attempting to relate these and to explain their cause and effect should be based on comparable short –term spatial and temporal resolutions of the physical, meteorological nutritional and biological data.

Implementation of a regional ecosystem-based nutrient management strategy plan, the HELCOM Baltic Sea Action Plan, suggests long-term reduction of nutrient inputs to improve eutrophication status in the Baltic Marine environment (OSPAR, 2017; Murray et al. 2019). Environmentally, this young Arabian Gulf is on the decline (Sheppard et al. 2010), which should be prevented through mass environmental education at all levels and across multiple disciplines and policy makers. A central organization involving all Gulf nations should be set up to achieve a broader understanding of the problem.

Discharge of pollutants and brine from desalination plants should be abated, probably by pumping them to the desert. Ballast water discharge should be regulated adhering to the IMO mandate (2004). In recent years there has been an increase in the incidence of harmful algal blooms with impacts on human health, tourism and recreation and fishery. This problem has to be addressed. To mitigate these socio-economic impacts, a good monitoring program and strategies to reduce HAB episodes are to be planned. It is highly desirable to have an institutionalized phytoplankton program between the Gulf countries and to conduct synoptic spatio-temporal surveys on the origin, growth and progression of blooms. Also, aerial drone based sampling and underwater robots with autonomous capabilities are being developed to investigate the eelgrass beds in 36 different sites from San Diego to Juneau, Alaska and for water quality research in coastal and inland waters (Washburn et al. 2018). These can be utilized to map and track algal blooms in the Gulf.

Representative keystone dinoflagellate species from the Gulf should be brought into laboratory culture and utilized as analogues of natural blooms to obtain data on their growth response and production of toxins to multifactorial physical and nutrient conditions. Such data would enhance our understanding of the structure and functioning of the harmful algal

blooms. Microbial communities associated with HABS and "biomarkers" can be determined using metagenomics, with which progression of blooms can be predicted (Galuzzi et al. 2004 and Penna, and Galluzzi, 2013).

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Chapter 151

DINOFLAGELLATES AND GLOBAL ENVIRONMENTAL CHANGE

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ABSTRACT

The scarcity of sustained (>30 yrs) biological time series represents a major bottle neck in progressing our understanding of global plankton trends. Ecosystem responses to natural climate variability in the past can provide a glimpse into assessing the impact of anthropogenic stressors such as eutrophication or novel species introductions as well as climate-induced changes in the future. We can learn important lessons from the dinoflagellate cyst fossil record and from the few long-term data sets available such as Continuous Plankton Records (CPR) of morphologically distinctive dinoflagellate taxa. We can also learn from dinoflagellate responses to unusual climate events such as El Niño. Many aquaculture areas have accumulated long-term dinoflagellate plankton and seafood biotoxin time series which are now available to be carefully analysed for trends. eDNA approaches of environmental samples as well as ancient sediments offer considerable promise to define the environmental indicator value of keystone dinoflagellate taxa. Selected examples are explored and the increased use of metadata analyses is emphasized.

Keywords: eutrophication, introduced species, climate change, dinoflagellate cyst record, eDNA

1. INTRODUCTION

Dinoflagellates, being an ancient group of protists dating back to 330-400M years ago, exhibit a perplexing morphological, physiological and genetic diversity, which allowed them

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to explore a broad range of nutritional strategies (photosynthetic, mixotrophic, phagotrophic, symbiotic, parasitic) and thrive in a wide range of planktonic and benthic marine and freshwater habitats. Reliable taxonomic monographs on planktonic (Taylor 1976; Steidinger & Tangen 1996) and benthic dinoflagellates (Hoppenrath et al. 2014) and their fossil cyst stages (Fensome et al. 1993; Zonneveld and Pospelova 2015) are now available. Solid data bases have been generated on extant dinoflagellates (Ocean Biogeographic Information System OBIS; http://www. iobis.org) and harmful dinoflagellates (Harmful Algae Event Database HAEDAT; *http://haedat.iode. org*) as well as fossil dinoflagellate cysts (Zonneveld et al. 2013).

Coupled with human society's increasing interest in monitoring for harmful dinoflagellate blooms and seafood toxins, and the fact that a range of species produce fossilisable cysts, dinoflagellates have been widely explored as environmental indicators. Selected examples are explored from the dinoflagellate cyst record, of continuous plankton records, and preliminary time series of key HAB species. Examples are provided of dinoflagellates that track eutrophication trends, which reflect ocean currents, or point to recent ballast water introductions or climate driven range expansions. In many cases multiple environmental drivers may be at play, such as eutrophication or climate disturbed habitats are more prone to new species introductions, and high biomass blooms triggered by eutrophication have a better chance of expanding their range via changing ocean currents. Only with increased build-up of environmental data bases such as OBIS or HAEDAT can we start to resolve the precise mechanisms underpinning ever changing dinoflagellate species distributions.

2. Case Studies from the Oslofjord and Skagerrak Fossil Record

Some 80 out of 2000 extant dinoflagellate taxa produce fossilisable sporopollenin cysts which have been extensively explored as stratigraphic biomarkers in oil exploration, to reconstruct the impact of past climate on plankton communities, but also in assessing the suitability of new aquaculture sites with respect to toxic dinoflagellate blooms. Dale et al. (2009) used the dinoflagellate cyst record from the Oslofjord in the last 100 years to assess the role of local eutrophication. While spring phytoplankton in the Oslofjord has access to unlimited nutrients, late summer species operate in a nutrient limited system. Eutrophication thus benefitted summer species such as *Lingulodinium* whereas spring species such as *Pentapharsodinium* were relatively unaffected. Smaller punctuated peaks of increased *Lingulodinium* may reflect local oceanography such as the North Atlantic Oscillation, but the main signal was of increased amounts of cysts of mostly *Lingulodinium* tracking eutrophication (Figure 1).

Dale (2009) subsequently identified a second eutrophication signal in other sites in the Skagerrak coastal system marked by an increase in heterotrophic cyst species, and differentiated this from the Oslofjord signal.

In the Skagerrak, Thorsen et al. (1995), working with a much longer (860cm) sediment core, documented the immigration into the region 6000 Before Present (B.P.) of *Gymnodinium nolleri* (microreticulate cyst initially reported as *Gymnodinium catenatum*).

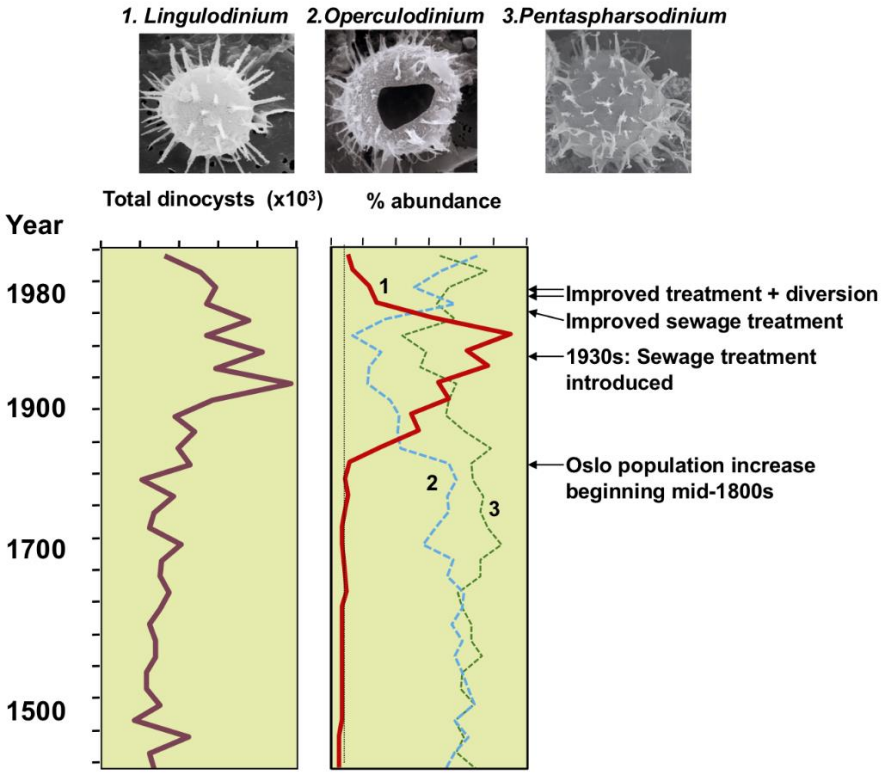

Figure 1. Dinoflagellate cyst data for the Oslofjord demonstrating increased total cyst abundance (left) towards the early 1900s, as well as (right) a shift from *Operculodinium* (2) and *Pentaspharsodinium* (3) towards the summer species *Lingulodinium* (redrawn after Dale et al. 2009).

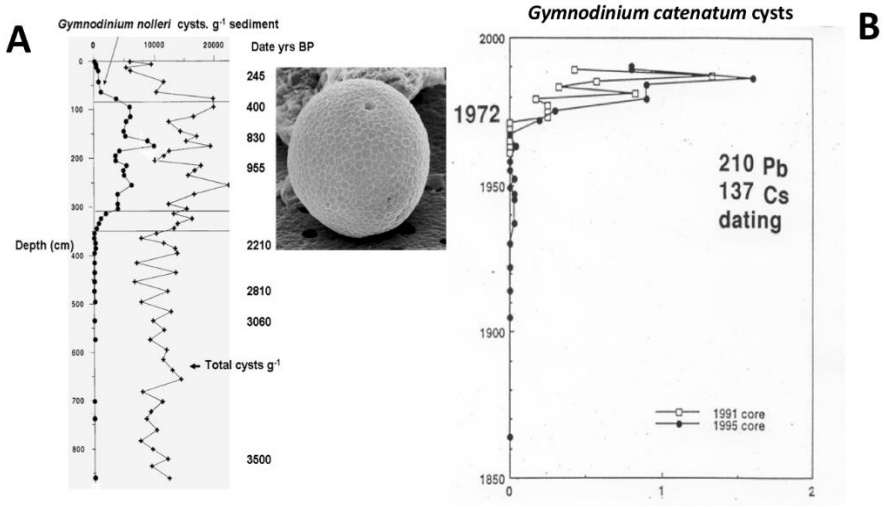

Figure 2A. Distribution of *Gymnodinium nolleri* cysts and total dinoflagellate cysts (cysts per gram dry sediment) in a 860cm long sediment core from the southern Kattegat (redrawn after Thorsen et al. 1995) demonstrating this species influx associated with a warming period 500 to 2000 years Before Present; 2B. Quantitative abundance of *Gymnodinium catenatum* cysts (as a percentage of total dinoflagellate cysts in 215cm and 80cm cores from Tasmania, Australia (after McMinn et al. 1997) demonstrating the new appearance of a putative ballast water introduction around 1972.

This species achieved bloom proportions from 2000 to 500 B.P. during a warming period, followed by a near extinction during the cooling period that commenced in 300 B.P. (Figure 2A). It is important to note that both cyst production and sediment input contribute to the amounts of cysts/g dry sediment, and therefore cysts may have also been produced in lower amounts but over a longer period of time rather than as a short-lived bloom.

The selectivity of the dinoflagellate cyst record complicates interpretation of microfossil sediment records. Promising novel approaches using ancient DNA technologies (Shaw et al. 2019) are now newly giving us access to fossil records also of non fossilisable cyst producing taxa such as *Alexandrium*, *Karlodinium*, *Noctiluca*, *Pfiesteria*. In the Bay of Brest (Brittany, France), the toxic dinoflagellate *Alexandrium minutum* had been detected since 1990 by the routine plankton monitoring network REPHY (Reseau de surveillance et d'observation du Phytoplankton et des Phycotoxines) but its abundance was low until 2012.

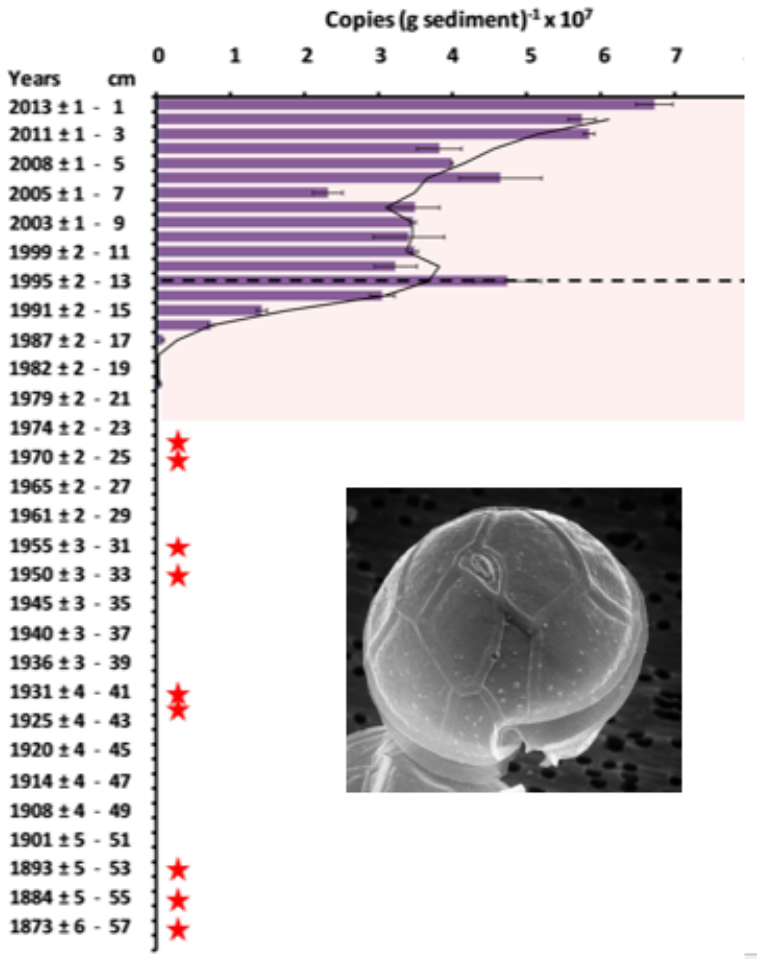

Figure 3. ITS1 rDNA copies g−1 sediment for *Alexandrium minutum* (purple) estimated by real-time PCR in a Bay of Brest sediment core. Colored shadows indicate the layers for which reliable rDNA quantifications were obtained. Stars indicate the layers where the presence of the species was detected but quantitative data were below detection limit of real-time PCR. The dashed lines indicate the limit layer of respective species germination (adapted from Klouch et al. 2016. *FEMS Microbiology Ecology*).

Working on a 60 cm sediment core from the Bay of Brest, France, Klouch et al. (2016) detected the first genetic traces of *Alexandrium minutum* dating back to 1873 (Figure 3), while quantitative real-time PCR approaches showed evidence for increasing abundance over time, corroborating observations from three decades of local plankton data.

3. Case Studies of Possible Anthropogenic Transport of Dinoflagellate Species via Ship's Ballast Water or Translocation of Shellfish Products

Whether new species occurrences represent new introductions, or the emergence of previously hidden flora remains an open debate in many dinoflagellate surveys. The potential for transport of non-indigenous marine microalgae via ship's ballast water (estimated to move 3 Billion tons per year) has been amply demonstrated. Nearly all known harmful dinoflagellate species have been documented in viable form from ship's ballast water, including *Alexandrium catenella, A. minutum, A. tamarense, Dinophysis* spp., *Gymnodinium catenatum, Karlodinium veneficum, Pfiesteria piscicida,* and *Pyrodinium bahamense* (Hallegraeff 2015). Ballast water uptake needs to be strongly discouraged during harmful algal bloom events. The adoption of the International Maritime Organisation's Ballast Water Management Convention (IMO) has served as an important catalyst for shipping industries to invest in significant research and development of treatment technologies to reduce this risk. This Convention came into force on 8 September 2017 after member states representing > 35% of shipping tonnage had signed off. The Convention requires ships to exchange ballast water at sea to a minimum of 95% volumetric exchange (Regulation D-1); or the discharge of ballast water so that the number of valuable organisms does not exceed specified limits (Regulation D-2). Available ballast water treatment technologies to achieve this have been reviewed by Murray and Hallegraeff (2018).

To prove that a dinoflagellate species has been introduced is complex. With *Gymnodinium catenatum* in South-Eastern Australia evidence focused on molecular markers to identify the suspected source population, combined with cyst studies in dated sediment depth cores that showed absence in historic times and appearance around 1972 coinciding with the start up of a woodchip mill attracting ships from known overseas source population (McMinn et al. 1997; Bolch and de Salas 2007; Figure 2B). *Alexandrium* do not tend to produce fossilisable cysts and the most convincing evidence for anthropogenic translocation has been the molecular detection in the Mediterranean ports of Sete and Barcelona of *Alexandrium pacificum* (as *A. catenella*) with a temperate Asian ribotype not found anywhere else in Europe (Lilly et al. 2002). Just like ballast water, the potential for transport of microalgae via the translocation of shellfish products has been well demonstrated (Shumway et al. 2004). The fine-scale genetic structure of *Alexandrium* populations in Japanese coastal waters thus strongly suggests a role for human-assisted dispersal associated with expansion of the aquaculture industry and translocation of shellfish stocks (Nagai et al. 2007).

In the past two decades, massive benthic *Ostreopsis* blooms, mostly *O.* cf. *ovata* , have newly occurred during summer along Mediterranean coasts associated with noxious aerosol effects on human health and mass mortalities of benthic marine invertebrates and/or macroalgae. Mortalities have been circumstantially attributed to the production of palytoxin-

like compounds. The first record of *Ostreopsis* bloom in this sea dates back to 1972, when *O. siamensis* (likely *O. ovata*) was reported within a mucilaginous matrix covering macroalgae in Villefranche-sur-Mer Bay (F.J.R. Taylor, pers. comm.). While this apparent bloom expansion (Figure 4) has often been attributed to tropicalisation of the Mediterranean, molecular studies revealed genetic homogeneity ("founder effect") of Mediterranean *Ostreopsis* contrasting with high genetic diversity of *Ostreopsis* along the West Pacific coast (Sato et al. 2011). This points to the fact that *Ostreopsis* may have been introduced into the Mediterranean from Japan for example with oyster spat.

4. DINOFLAGELLATES AS INDICATORS OF OCEAN CURRENTS AND BIOREGIONS

Marine dinoflagellates around the globe display the occurrence of the same morphospecies within broad latitudinal limits, the boundaries of which approximate to sea surface isotherms (Figure 6), a concept which Taylor (1976) referred to as latitudinal cosmopolitanism. The use of plankton communities to demarkate water masses dates back to Cleve (1900) and Schütt (1893). Dinoflagellates belonging to what used to be referred to as the genus *Ceratium*, now *Tripos*, are widespread in most marine plankton samples. The genus exhibits an amazing morphologically diversity, with over 77 species and numerous varieties and forms documented globally, and with numerous regional taxonomic monographs devoted to this dinoflagellate genus. All these studies hint at the potential of using *Tripos* species as water mass indicators, which has been quantitatively explored for the North Atlantic (Dodge and Marshall 1994) or to detect environmental change in the Mediterranean (Tunin-Ley et al. 2009). *Tripos* is best collected by phytoplankton or zooplankton nets, and Tunin-Ley et al. (2009) in the Mediterranean determined that a minimum sample volume of 70L was needed for a good estimate of *Tripos* species richness. The majority of *Tripos* species exhibit very broad temperate to subtropical to tropical temperature preferences from 10 to 25 (30)ºC (Dodge and Marshall 1994).

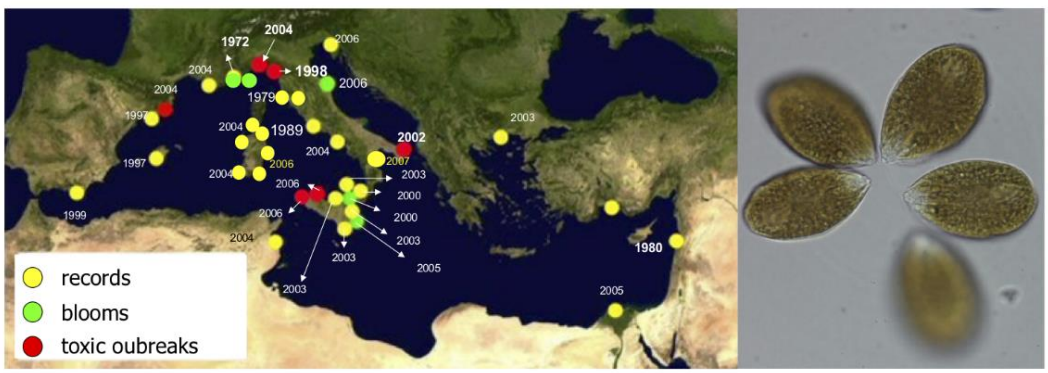

Figure 4. Expansion of the benthic dinoflagellate *Ostreopsis* in Mediterranean (map courtesy A. Zingone).

It is also noted that some species which are used as warm water indicators in the North Atlantic, e.g. *T. hexacanthus* (Figure 5 A; 7-30ºC, but "prefers higher temperature") are not necessarily diagnostic for warm-waters of the Australian region.

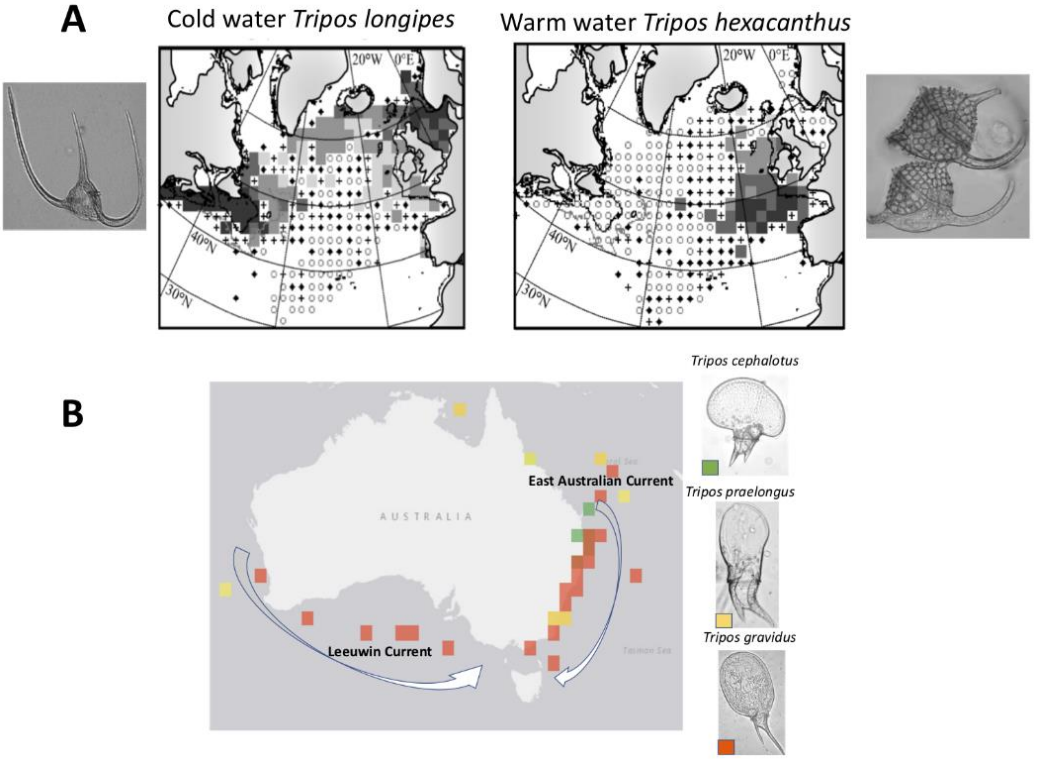

Figure 5A. Occurrence of the coldwater *Tripos longipes* and warm-water *T. hexacanthus* in North Atlantic Continuous Plankton Recorder data 1958-1999 (from Barnard et al. 2004 *Marine Ecology Progress Series*); 5B. Australia wide distribution of the warm-water marine dinoflagellate species *Tripos cephalotus, T. praelongus*, and *T. gravidus*. Southward transport of these taxa reflects the Leeuwin Current (in winter) or East Australian Current (in summer).

Similarly, Tunin-Ley et al. (2009) observed some strictly warm-water species such as *T. digitatus* in winter in the Mediterranean. Using a strict definition of stenothermal tropical species, in Australian waters we identified a narrow group of warm-water species including *T. belone, T. cephalotus, T. dens, T. digitatus, T. gravidus, T. incisus, T. paradoxides,* and *T. praelongus* which should receive careful attention in monitoring for future range expansions or upwelling/incursion of deep tropical waters (Hallegraeff et al. 2020). The power of this approach is well demonstrated by the early plankton studies by Wood (1954) who produced the first conclusive biological evidence for what is now called the Leeuwin Current occasionally transporting Indian Ocean dinoflagellates all the way to the west coast of Tasmania (Figure 5B), many decades before satellite images confirmed its existence (Cresswell and Domingues 2009).

5. DINOFLAGELLATES AND CLIMATE CHANGE

The dinoflagellate *Pyrodinium bahamense* is presently confined to tropical, mangrove-fringed coastal waters of the Atlantic and Indo-West Pacific. A survey of cyst fossils (named *Polysphaeridium zoharyii*) going back to the warmer Eocene 50M years ago indicates a much wider range of distribution in the past (Figure 6). For example, in the Australasian region at present, the alga is not found farther south than Papua New Guinea but, some 100 000 years ago in the Pleistocene, the species ranged as far south as Sydney Harbour. In the Philippines alone, *Pyrodinium* has been responsible in the period 1980 to 2010 for more than 2000 human illnesses and 100 deaths resulting from the consumption of contaminated shellfish as well as sardines and anchovies. There is concern that, with increased greenhouse warming of the oceans, this paralytic shellfish toxin-producing species may one day return to Australian waters. Comparable examples of spreading of dinoflagellate cyst species distributions with increasing temperatures, and shrinkage of biogeographical zones with decreasing temperatures, are known. The fossil dinocyst *Dapsilidinium pastielsii* became extinct in the Atlantic during cooling in the early Pleistocene, but a warm-water refuge for this taxon was recently discovered in the Indo-Pacific Warm Pool (Japan, Indonesia, Vietnam, Palau, Philippines) (Mertens et al. 2014).

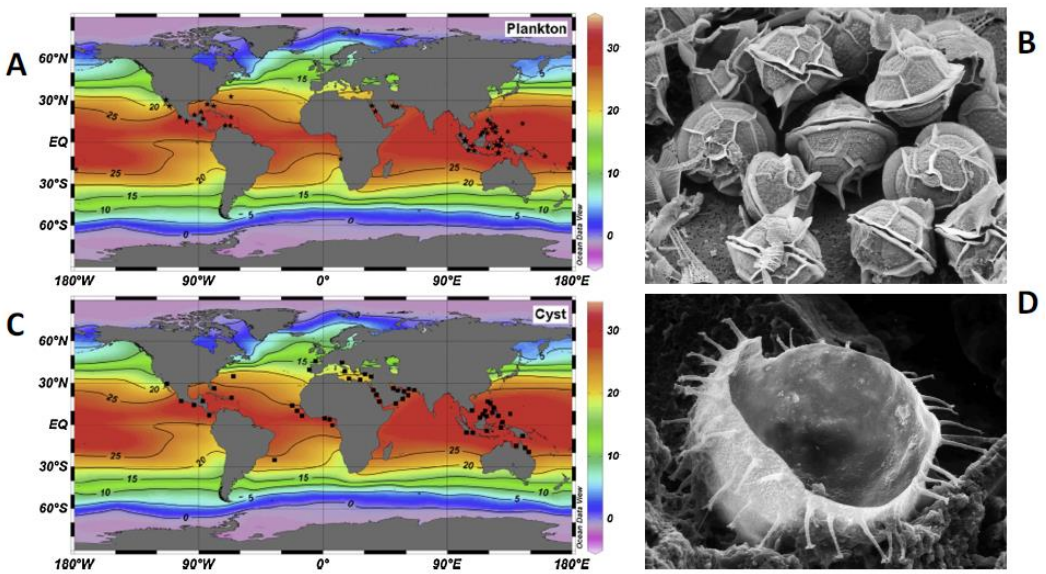

Figure 6. Global distribution of *Pyrodinium bahamense* in recent plankton (A) and much wider distribution in the fossil cyst record (C) (after Usup et al. 2012. *Harmful Algae*). Micrographs of *Pyrodinium* vegetative cells (B) from Bahia Phosphorescente (Puerto Rico) and hemispherical cyst with sulcal notch from Port Moresby, Papua New Guinea (D).

Azanza and Taylor (2001) presented strong evidence for a coincidence between *Pyrodinium bahamense* blooms and El Niño-Southern Oscillation (ENSO) climatic events in the tropical Indo-West Pacific. El Niño is caused by an imbalance in atmospheric pressure and sea temperature between the eastern and western parts of the tropical Pacific Ocean and results in a shoaling of the thermocline. The first *Pyrodinium* blooms became evident in 1972

in Papua New Guinea and coincided with the fatal food poisoning of three children. Since then *Pyrodinium* blooms have apparently spread to Brunei and Sabah (1976), the central (1983) and northern Philippines (1987) and Indonesia (North Mollucas). The pattern of initial *P. bahamense* blooms in Southeast Asia in the late 1970s to mid-1980s suggested that the cells or cysts were transported from place to place (reviewed by Usup et al. 2012). In the case of Manila Bay, *P. bahamense* might have inhabited the Bay since the 1950s and were present in Malampaya Sound, Palawan since the 1970s and Manila Bay since the 1920s, while in Indonesia, cysts were probably present in Hurun Bay since the 1860s and Ambon Bay since the 1850s. In Sabah, Malaysia the oldest occurrence of cysts was probably around 1966, about 10 years earlier than the first recorded bloom.

These results suggested that the expansion of *P. bahamense* blooms in Southeast Asia in the early 1980s might have been more the result of significant environmental changes in the region rather than the result the species being newly introduced to the area. Interestingly *Pyrodinium* blooms have declined in the Phillipines after 2000, and were replaced by less harmful blooms of green *Noctiluca*.

Ciguatera caused by benthic *Gambierdiscus* dinoflagellate species is a tropical seafood poisoning syndrome well-known in coral reef areas in the Caribbean, Australia, and especially French Polynesia. Whereas, in a strict sense, this is a completely natural phenomenon, from being a rare disease two centuries ago, ciguatera has now reached epidemic proportions in French Polynesia and the Caribbean. More than 500,000 Pacific Islanders suffer ciguatera during their life time, which means approximately 1 in every 4 persons in the Oceania region, which is about 2-fold higher than reported from the Caribbean. Evidence is accumulating that reef disturbance by hurricanes, military and tourist developments, as well as coral bleaching (linked to global warming) and perhaps in future increasing coral damage and changing macrophyte cover due to ocean acidification are increasing the risk of ciguatera. A recent game changer has been the elucidation via molecular tools that what was once thought to be a single causative dinoflagellate, is now considered to be a species complex of 19+ different morphospecies (Litaker et al. 2010). At least 4 *Gambierdiscus* species (*G. belizeanus*, *G. caribaeus*, *G. carolinianus* and *G. carpenteri*), are distributed globally, with the other 7 found only in the Pacific (*G. australes*, *G. pacificus*, *G. polynesiensis*, *G. toxicus*, *G. yasumotoi*), Indian (*G.toxicus*) or the Atlantic Oceans (*G. ruetzleri*, *G. excentricus*). This finding sheds new light on the previously recognised >100-fold variation in toxicity, but also suggests a much broader range of environmental tolerances. An apparent range expansion of human ciguatera poisonings has been claimed for the Mediterranean and the Canary Islands, the Caribbean and West Indies, and the East Coast of Australia (Farrell et al. 2016; Kohli et al. 2014; Litaker et al. 2010; Rodriguez et al. 2017) which needs to be rigorously monitored (Figure 7). It is not yet clear whether this is caused by range expansions of *Gambierdiscus* dinoflagellates, or migration of ciguatoxic fish, or both.

Several dinoflagellate species can exhibit invasive behavior that can disrupt ecosystems via strong phagotrophic feeding (red *Noctiluca*), mixotrophy (green *Noctiluca*, *Cochlodinium*), the killing of zooplankton or fish (*Karlodinium*), or the inability of being grazed. These phenomena have been referred to as Ecosystem Disturbing Algal Blooms (Sunda et al. 2006).

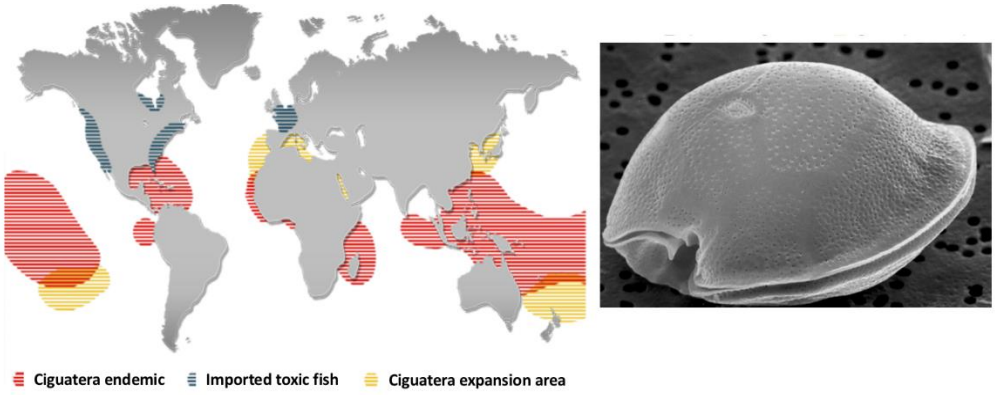

Figure 7. Left: Current global distribution of ciguatera food poisoning from fish, indicating ciguatera endemic areas, countries where cases have resulted from imported fish, and areas where ciguatera is considered to currently expand, either by expansion of the causative dinoflagellate and/or migration of fish (courtesy Louis Marde Institute, French Polynesia); Right: The causative benthic dinoflagellate *Gambierdiscus* from the Australian Great Barrier Reef (Map courtesy Ciguatera-Online@ ciguateraonline).

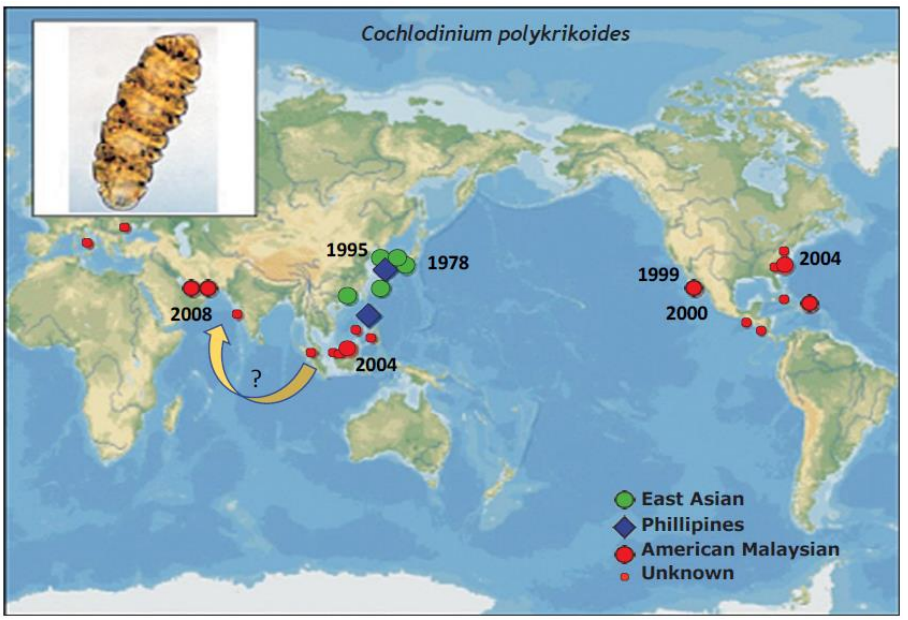

Figure 8. Expansion of global distribution of the fish-killer *Cochlodinium polykrikoides.* Redrawn after Iwataki et al. 2008. *Harmful Algae.*,

Noctiluca is an example of a HAB species that appears to have been increasing globally in the past two decades (Harrison et al. 2011, Gomes et al., this volume). Although in some regions this may be, at least in part, a function of increasing observations and awareness, in other regions such as southeast Asia this appears to reflect a real change in plankton dynamics associated with eutrophication and climate change. *Noctiluca* has sometimes been interpreted as a coastal or offshore manifestation of eutrophication, since an increase in nutrients provides an increase in phytoplankton, its main food supply as a grazer. Red *Noctiluca* has

dramatically increased along the east coast of Australia. While in the period 1860 - 1950 it was only known from Sydney where it has been responsible for tourist beach closures, from 1980-1993 it expanded along the New South Wales coast in response to eutrophication, during 1994 - 2005 the changing East Australian Current drove a range extension down to Tasmania (34ºS) where it impacted on salmon farms, and in 2008 - 2013 it expanded against prevailing current systems also implying a role for domestic ballast water dispersal (McLeod et al. 2012, Hallegraeff et al. 2019). Green *Noctiluca* has expanded in the last decade into the northern Arabian Sea where winter phytoplankton blooms were previously comprised mainly of diatoms, but now have been replaced by green *Noctiluca*. This organism combines carbon fixation from its green endosymbiont with ingestion of prey to the detriment of regional fisheries and the long-term health of an ecosystem supporting a coastal population of 120 million people (Gomes et al. 2014).

The fish-killing dinoflagellate *Cochlodinium polykrikoides* long known from Japan and Korean waters ($95M aquaculture kill in 1995) but also Mexico, California and British Columbia , exhibited apparent recent range expansions in 2004 into the Malaysian region and in 2008-2009 caused a major bloom in the Arabian Gulf region where it led to fish kills and even the closure of desalination plants (Figure 8). The latter was caused by the American/Malaysian ribotype, which is distinct from the East Asian and Philippines ribotypes (Iwataki et al. 2008).

We cannot yet resolve whether this Gulf bloom represents a ballast water introduction, a climate driven range expansion, or a response of a previously cryptic species to anthropogenic nutrient enrichment.

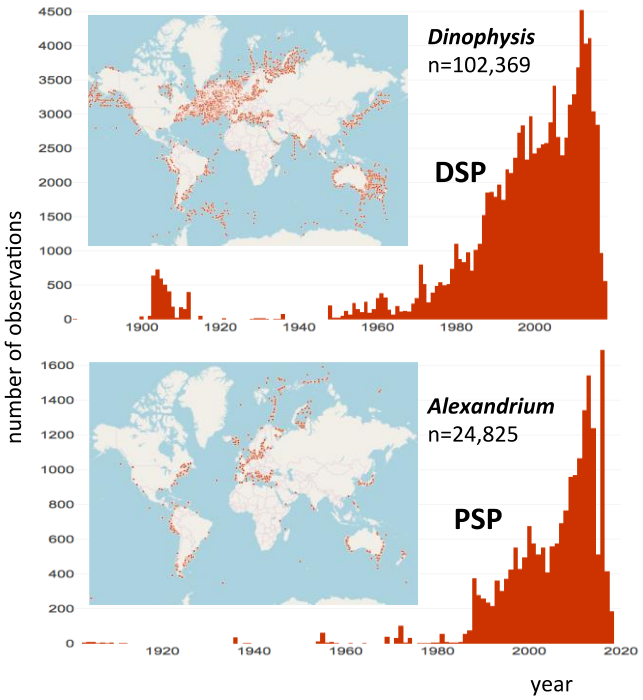

Figure 9. Time series of OBIS location records of the HAB genera *Dinophysis and Alexandrium* between 1900 and 2019, associated with the seafood poisoning syndromes DSP and PSP. Records are biased towards Europe (Adopted from Hallegraeff et al. 2017).

Dinoflagellate species responsible for shellfish toxicity syndromes such as *Alexandrium* (causative organism of Paralytic Shellfish Poisoning, PSP) and *Dinophysis* (causative organism of Diarrhetic Shellfish Poisoning, DSP) have been extensively monitored in many regions for >30 years (Figure 9).

PSP is the dominant seafood toxin syndrome in North America, and DSP in Europe. Ocean Biogeographic Information System (OBIS) Time series data for location records of the key target genera *Alexandrium* and *Dinophysis* exhibit an increase in frequency over the past 30 years, undoubtedly reflective of increased awareness and monitoring effort. Climate change is adding a new level of uncertainty to seafood safety and HAB monitoring programs**.** While we made considerable progress in understanding the physics of climate change, understanding of the impacts on biological communities is in its infancy. There will be winners and losers from climate change, but predicting how individual species will respond poses a formidable challenge.

Key environmental factors driving changes in HABs include: increased temperature, enhanced surface stratification (affecting nutrient and light availability), elevated CO_2 (stimulating photosynthesis and driving 'ocean acidification' biogeochemistry), and increased extreme precipitation and storm events. International Panel Climate Change (IPCC) physico-chemical models predict uneven geographic changes in temperature, ocean acidification and nutrient depletion hotspots (Bopp et al. 2013). Potentially emerging HAB climate responses include: range expansion of warm-water (e.g. *Gambierdiscus*) at the expense of cold-water species; changes in abundance and seasonal bloom window; and increased cellular toxin content of key HABs (*Alexandrium, Karlodinium*). Developing predictive capability on how HABs may respond has been frustrated by: contradictory species and especially strain-specific responses, lack of insights into evolutionary adaptation; and into how HABs interact with the broader phytoplankton and zooplankton grazer communities (Hallegraeff 2010; Wells et al. 2015). Indirect climate effects also need to be taken into account such as demise of coral reefs due to bleaching, which can increase macroalgal-dominated sea-beds and the risk of harmful benthic epiphyte blooms such as *Ostreopsis* and *Gambierdiscus*. Not all trophic levels are responding to the same extent, and where zooplankton or fish grazers are differentially impacted by ocean warming, this may have cascading impacts on the structure of marine food webs. Addressing these challenges calls for significant departures from traditional HAB experiments, such as studying multiple strains, with multifactorial experiments the norm, agreeing on best practices techniques, and making global extrapolations via modelling approaches. The analysis of long-term dinoflagellate data series, whether they be fossil cyst records, continuous plankton records, or seafood toxicity syndromes such as ciguatera, PSP and DSP, play an important role in improving our predictive capability of the impacts of global environmental change on ocean biology.

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Chapter 152

EVOLUTION OF DINOFLAGELLATE GENOMICS AND GENE EXPRESSION OF TOXINS

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ABSTRACT

Dinoflagellates are key species in marine environments but remain poorly understood due to their large, complex genomes, unique molecular biology, and unresolved in-group relationships. They are the source of "red tides" that cause fish killings and shellfish poisoning. Depending on the species, their toxins can trigger paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), diarrhetic shellfish poisoning (DSP) and Ciguatera poisoning (CP). Dinoflagellate toxins and bioactive compounds are of increasing interest because of their commercial impact, influence on safety of seafood and potential medical applications. Our understanding of the genes involved in toxin biosynthesis in dinoflagellates is currently limited due to the complex genomic features of these organisms. In evolutionary history, dinoflagellate genomes not only have undergone vertical evolution but have also been impacted by active horizontal gene transfer from their plastids, endosymbionts, as well as other organisms. The recent sequencing of various dinoflagellate transcriptomes has provided us with new valuable insights into the biosynthesis of paralytic shellfish toxins (PSTs) in dinoflagellate species. This chapter summarizes the genomics, biosynthesis and biotechnological applications of toxins and other bioactive molecules from dinoflagellates.

Keywords: dinoflagellates, genomics, transcriptomics, toxins, biosynthesis, bioactive applications

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1. DINOFLAGELLATES

Dinoflagellates are unicellular algae. Of the 2377 extant dinoflagellates, 116 are constitutive mixotrophs and 109 are non-constitutive mixotrophs (See chapter 7: Hansen and Tillman), ingesting other algae or dissolved organic matter [1], some of which can enslave ingested algal chloroplasts and perform photosynthesis (e.g., [2, 3]). These heterotrophic taxa are potentially important micrograzers in the microbial food web [4]. More than 60 taxa of dinoflagellates are known to form red tides, over 20 of which produce toxins that have profound impacts on fisheries, recreational values of coastal zones and public health [5].

Dinoflagellates possess a number of unusual features, e.g., unique plastids acquired from red algae via endosymbiosis [6-9], mitochondrial genomes encoding three proteins for the electron transport chain, and fragmented genes for rRNA [8, 9]. The nuclei of dinoflagellates referred to as dinokaryon [10] have unique features such as condensed chromosomes during interphase and lack of nucleosomes, thus the dinokaryons are distinctly different from typical eukaryotic nuclei (Figure 1) [11, 12]. In the dinokaryon the chromosomes exist in a condensed, liquid crystal state throughout the cell cycle. Instead of packaging DNA in nucleosomes, dinoflagellates appear to have replaced the bulk histone function with two non-eukaryotic proteins, the dino-viral like protein (DVNP) and a histone like protein (HLP) [13-16]. The chromosome interior is densely packed with DNA, with very low levels of protein, some RNA, and a large number of Mg^{2+} and Ca^{2+} ions [12]. The chromosomes appear to be too dense for transcription to occur on the interior; instead genes located on the extrachromosomal loops associated with HLPs are probably transcriptionally active (Figure 2) [17-19].

In addition to these structural anomalies of the nucleus, dinoflagellates further distinguish themselves from other alveolates (an ancient group of eukaryotes that occupy a diverse array of ecological niches, both free-living and parasitic) with high DNA content. Core dinoflagellates have a large number of chromosomes, with haploid DNA content ranging from 1.5 pg of DNA per cell in the coral symbiont *Symbiodinium* to 189 pg in the bioluminescent species *Lingulodinium polyedrum* [12, 20]. Due to a DNA content often orders of magnitude larger than the human genome (3 pg of DNA per haploid cell), full genome sequencing is very difficult. To date only draft genomes of *Symbiodinium* and the syndinean *Hematodinium* have been published [21-23]. Most sequencing work was focused on transcriptomes which, with recent advances in sequencing technologies linked to lower costs, could facilitate sequencing of more dinoflagellates.

These atypical features continue down to the most basic unit of information, DNA. In dinoflagellates up to 70% of thymine bases are replaced with 5 hydroxymethyl uracil, more commonly found in bacteria and bacteriophage and found in eukaryotic cells only under oxidative stress conditions [11, 24-26]. At the transcript level, a 22 nucleotide spliced leader (SL) sequence is added to the 5' end of all messenger RNA (mRNA) transcripts in dinoflagellates (Figure 2) [26-28]. This feature confirms that transcripts are complete and that they originate from dinoflagellate DNA rather than from contaminants or bacteria that may reside in the intracellular space or attached to the cell surface. Some highly expressed dinoflagellate genes are encoded in large tandem arrays with short intergenic spacers and are trans-spliced while moderately expressed genes tend to be intron rich and are encoded by a single gene [29, 30].

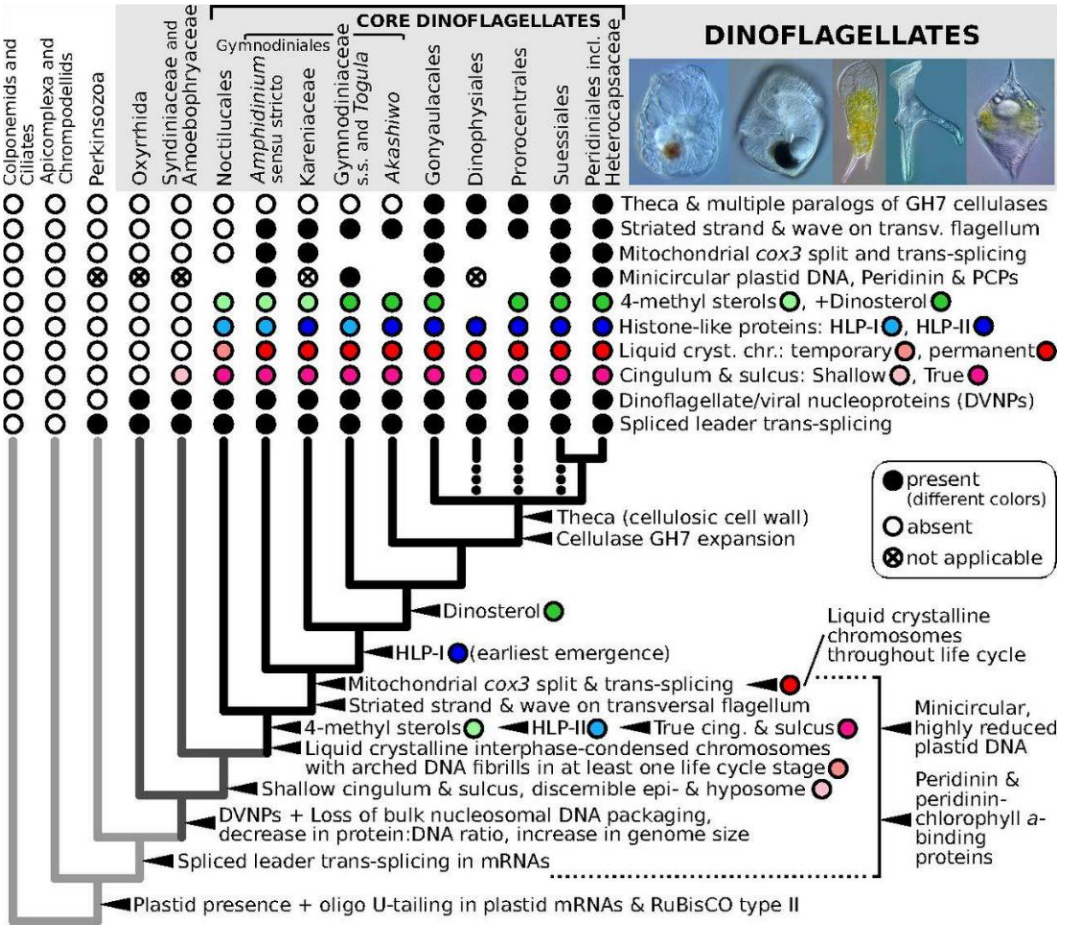


Figure 1. Model of evolutionary characteristics of dinoflagellates. Figure denotes character states for dinoflagellates mapped onto a phylogeny. Some of the key features seen in the core dinoflagellates are spliced leader trans-splicing of mRNA, loss of nucleosomes, presence of histone like proteins (HLP), liquid crystalline chromosomes, and the presence of 4-methyl sterols and dinosterol. Representative morphotypes are pictured in the upper right. Figure from Janouskovec et al. [31].

2. DINOFLAGELLATE GENOMICS

Genomes of dinoflagellates have undergone vertical evolution and were significantly impacted by horizontal gene transfer. Some dinoflagellates represent a photosynthetic organism with the most reduced plastid genome. The characteristic peridinin-containing lineages have plastid genomes broken into single-gene minicircles that encode only 16 of the typically 130-200 plastid proteins [32]. Transfer of the rest of the plastid genes to the nucleus has dramatically reconfigured the nuclear genome in dinoflagellates [33]. In addition, plant Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase) has been replaced by that of proteobacterial origin (form II) likely through lateral gene transfer [34, 35]. Dinoflagellates lack histone proteins with few exceptions (e.g., *Amoebophrya*) [36], but a histone H3-like protein [37] and a histone H2A.X [38] were reported recently in addition to findings of basic

and acidic nuclear proteins [38, 39]. Also, typical dinoflagellate cells divide with closed mitosis and extranuclear spindles and chromosomes remain permanently condensed. Other uncommon features for an eukaryote include the rarity of mRNA splicing and deviation from the universal GT/AG rule [40, 41], the extensive and novel mRNA editing in mitochondrial genes [42, 43] and widespread spliced leader RNA *trans*-splicing [44].

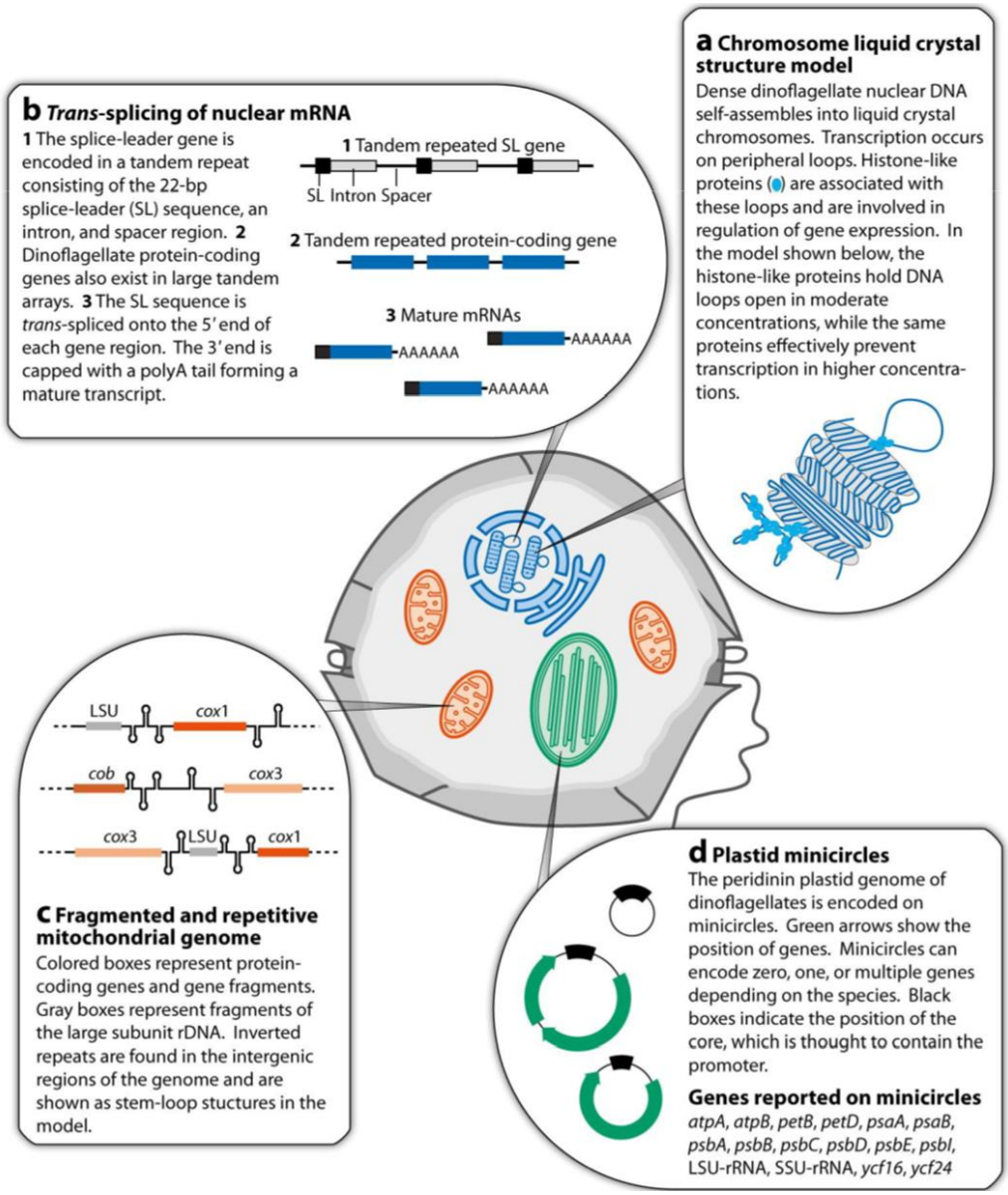


Figure 2. Distinct features of dinoflagellate nuclear, plastid, and mitochondrial genomes.
Model showing the liquid crystalline chromosome structure and peripheral loops on which transcription occurs. B) Model showing trans-splicing of mRNA messages as well as tandem gene arrays, and polyadenylation which completes the mature mRNA transcript. C) Model showing fragmented mitochondrial genome. D) Plastid minicircles found in dinoflagellate plastids and the genes associated with them. Figure from Wisecaver and Hackett [11].

In the past half century, the genome sizes of more than 30 dinoflagellates have been measured, ranging between 3 and 278 pg DNA per genome or cell [20, 45-47], which is about 1-80 times larger than that of the human haploid genome. Although smaller genomes may occur in some yet unrecognized dinoflagellates [48], dinoflagellate genomes are still huge considering their relatively small cell size and being "simple" organisms, another case of C-value enigma [49]. Equally striking is the wide range of genome size, which cannot be explained by differences in function or cell size. These peculiarities raise questions about the functions of the dinoflagellate genome in protein coding and other activities. Early work on biochemical properties has shown that a large fraction of the nuclear DNA in the dinoflagellate *Crypthecodinium cohnii* and *Prorocentrum cassubicum* is composed of repeated and interspersed DNA sequences [46, 50-52]. Hence, it has been suggested that significant portions of the dinoflagellate genomes are nonfunctional [53]. Recently, Lin [48] has detected potential binding targets of microRNA in dinoflagellate mRNA, suggesting existence of functional noncoding elements in dinoflagellate genomes.

The regulation of gene expression in these enigmatic organisms is currently being explored using a genomics approach. Several Expressed Sequence Tag (EST) projects have been conducted in recent years. For instance, Okamoto and Hastings [37] utilized microarrays and found that circadian clock-related genes in dinoflagellates were regulated at the transcriptional level. Erdner and Anderson [54] used Massively Parallel Signature Sequencing and found that only about one-fourth of the expressed gene pool was regulated transcriptionally while the remaining genes did not exhibit transcriptional regulation. Similarly, a microarray analysis on *Karenia brevis* revealed little transcriptional regulation of its genome [55]. Furthermore, several other studies using EST analyzed the evolution of plastid genomes in dinoflagellates [6, 38, 56, 57]. Taken together, these studies concluded that: 1) transcriptional regulation of dinoflagellate genes is rare and 2) most dinoflagellate plastid genes were transferred to the nuclear genome. It is still poorly understood what the dinoflagellate genomes consist of and which genes are commonly expressed under natural conditions ("Dino core genes") versus specific conditions. Lin et al. [58] have launched an EST project to investigate profiles of expressed genes in three trophically contrasting taxa: *Prorocentrum minimum* (photoautotrophic), *Karlodinium micrum* (mixotrophic), and *Pfiesteria piscicida* (heterotrophic). They observed distinct (largely undescribed) gene signatures expressed among the three species as well as changes in the profile of expressed genes in *Pfiesteria* from fed to starved conditions. Notably, the majority of these dinoflagellate genes produce no significant hits in the GenBank database. The traditional ESTs often do not provide sufficient information for prediction of the functions of these "novel" genes. To overcome this shortcoming, Lin et al. [58] took advantage of the recently discovered spliced leader at the 5' end of dinoflagellate nuclear-encoded mRNA and constructed cDNA libraries (e-cDNAs) from one marine and two freshwater plankton assemblages to sequence full-length cDNA. Small-scale sequencing of the e-cDNAs revealed functionally diverse transcriptomes proven to be of dinoflagellate origin. A set of dinoflagellate common genes and transcripts of dominant dinoflagellate species were identified. Further analyses of the dataset prompted them to delve into the existing, largely unannotated dinoflagellate EST (DinoEST) datasets. Consequently, all four nucleosome core histones, two histone modification proteins, and a nucleosome assembly protein were detected, clearly indicating the presence of nucleosome-like machinery long thought not to exist in dinoflagellates. The isolation of rhodopsin from taxonomically and ecotypically

diverse dinoflagellates and its structural similarity and phylogenetic affinity to xanthorhodopsin suggest a common genetic potential in dinoflagellates to use solar energy non-photosynthetically. Additionally, they found 55 cytoplasmic ribosomal proteins (RPs) from the e-cDNAs and 24 more from DinoEST, showing that the dinoflagellate phylum possesses all 79 eukaryotic RPs. Their results suggest that a sophisticated eukaryotic molecular machine operates in dinoflagellates that likely encodes many more unsuspected physiological capabilities and, meanwhile, demonstrate that unique spliced leaders are useful for profiling lineage-specific microbial transcriptomes *in situ*.

At present, very little is known about gene expression regulation in dinoflagellates. No canonical TATA box has been found in dinoflagellate genes, but TBP-like protein 2 (TBP-2), intermediate between TATA box binding proteins (TBPs) and TBP-like factors (TLFs), were found in *C. cohnii* [59]. In mammalian cells, TBP-2 has functions distinct from classical TBP that may be related to non-condensed chromatin conformation [60]. Homologs of TBP-2 and related proteins have been identified in recent dinoflagellate transcriptomes, such as the cDNA dataset in Lin et al., [58] and Toulza et al., [61]. Transcriptional regulation is quite limited in dinoflagellates. A microarray analysis on *Karenia brevis* revealed that only about 10% of genes were transcriptionally regulated [62]. Massively parallel signature sequencing studies of two *Alexandrium* species showed that 10%-27% of the detected cDNA pools exhibit transcriptional regulation [54, 63]. A metatranscriptomic analysis without normalization showed strikingly even levels of transcript abundance for the majority of the cDNAs detected, for all three (one marine and two freshwater) samples, with only a small number of genes showing markedly higher transcript levels [58]. The scarcity of transcriptional regulation is consistent with the lower abundance of histones in dinoflagellates given that histones play a key role in regulation of gene transcription in eukaryotes. Thus, the majority of genes in dinoflagellates are most likely regulated translationally or post-translationally. Post-translational modification of proteins, including phosphorylation, methylation, and sumoylation (by a small ubiquitin-like modifier), are widely recognized as important modification processes regulating protein activity in eukaryotes, yet their potential roles in dinoflagellate gene regulation remain largely unexplored.

Of the small proportion of genes that are transcriptionally regulated, those highly expressed are of particular interest but have not been given much attention. A small number of genes are highly represented in the EST datasets reported recently, e.g., *K. brevis* [62] and *Alexandrium catenella* [64]. In the latter case, the cDNA library was dominated by 17 genes, of which luciferin binding protein accounted for 15.6%, S-adenosyl-L-homocysteine hydrolase 4.9%, glyceraldehyde-3-phosphate dehydrogenase isoform 2 (GPDH) 3.7%, and S-adenosylmethionine (SAM) synthetase 3%.

Lin et al. [58] compared the sequenced cDNA libraries derived from two freshwater and one marine natural plankton assemblages along with the existing DinoEST dataset to identify commonly expressed genes. Five genes coding for major basic nuclear proteins, ubiquitin, centrin/caltractin, calmodulin and 14-3-3, were found to be commonly and highly expressed in both the cultured and wild dinoflagellate assemblages.

A literature survey shows that some of these and other genes are highly expressed, such as SAM synthetase, S-adenosyl-L-homocysteine hydrolase (¼S-adenosylhomocysteinase), glyceraldehyde-3-phosphate dehydrogenase, peridinin-chlorophyll-binding protein, heat shock proteins 90, 70, and 40, elongation factor 1a and calmodulin. These genes are important in methylation of DNA and toxin precursors, carbohydrate metabolism, light harvesting,

stress response, translation as well as signal transduction. In addition, the analysis of the environmental cDNA and DinoEST datasets yields a set of 79 ribosomal proteins that commonly occur in eukaryotes [58]. Some of these genes are highly represented in DinoEST. DinoESTs serve as markers for genes expressed under specific conditions and can be used as probes in the recovery of full-length cDNA or genomic sequences, recognition of exon and intron boundaries, delineation of protein families, and for the development of probes for genome wide expression profiling [38, 57, 62, 65]. Furthermore, transcriptomic surveys, in particular by way of next-generation sequencing (NGS) RNA-Seq, have illuminated our understanding of the unique biology, metabolism, and ecology of dinoflagellates [66-68].

3. DINOFLAGELLATE TOXINS

Dinoflagellates are the greatest contributors to harmful algal blooms and biotoxins in the ocean. Several types of toxins produced by dinoflagellates (reviewed by Wang, [69]) are responsible for all but one of the classical seafood poisoning syndromes, including paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), *ciguatera* fish poisoning (CFP)**,** amnesic shellfish poisoning (ASP) and azaspiracid shellfish poisoning (AZP) [70]. The only exception is amnesic shellfish poisoning (ASP), which is caused by the diatom *Pseudonitzschia* spp. Of these syndromes, PSP and DSP are most harmful to humans [71].

3.1. Paralytic Shellfish Poisoning (PSP) Toxin

Paralytic shellfish poisoning toxins (PSTs) are a group of naturally occurring small heterocyclic guanidinium alkaloids. The most common dinoflagellate toxin is saxitoxin (STX), a neurotoxin 100,000 times more potent than cyanide [72]. It has been found in North American shellfish from Alaska to Mexico, and from Newfoundland to Florida. The most notorious producers of saxitoxin are *Protogonyaulax catenella* on the west coast and *Gessnerium monilatum* on the east coast of North America. Both have been known to cause paralytic shellfish poisoning (PSP). The worst cases of PSP result in respiratory failure and death within 12 hours in humans. STX (Table 1) is the most common of at least 21 analogues of toxic cyclic guanidines that are known in shellfish. STXs exert their effects by directly binding to voltage-gated sodium channels in nerve and muscle cells blocking the influx of sodium and the generation of action potentials, leading to paralysis [73]. STX-producing Dinoflagellates belong to the three genera *Alexandrium*, *Gymnodinium* and *Pyrodinium*.

3.2. Neurotoxic Shellfish Poisoning (NSP) Toxin

Another toxin that accumulates in shellfish is brevetoxin (BTX), produced by the dinoflagellate *Karenia brevis* (Table 1). BTX is unique in that it becomes aerosolized when the aquatic dinoflagellates are transported to the surf zone from where it is blown onto the beach causing respiratory irritation in humans. Beachgoers on the Gulf coast of Florida who

notice asthma-like breathing symptoms, may be experiencing toxicity from an offshore *Karenia* bloom. A review on how BTX and its analogues can also affect finfish, aquatic mammals and birds has been published recently [74]. The symptoms of NSP include gastroenteritis and neurological problems [75]. The mode of action of BTXs is binding to sodium channels in nerve, muscle and cardiac tissue, arresting them in the "open" position thus enhancing sodium entry into the cell. This leads to incessant activation, which causes paralysis and fatigue of these excitatory cells [76]. Animal illnesses are also common during *Karenia* blooms with recorded deaths of bottlenose dolphins and manatees due to brevetoxin ingestion of contaminated organisms [74].

3.3. Diarrhetic Shellfish Poisoning (DSP) Toxin

Toxins produced by the dinoflagellate *Dinophysis* and *Prorocentrum* spp. cause diarrhetic shellfish poisoning (DSP), which results in digestive upset although it is not fatal. DSP toxins were originally divided into three different structural classes, (a) okadaic acid (OA) and its analogues, the dinophysistoxins (DTXs), (b) pectenotoxins (PTXs), and (c) yessotoxins (YTXs) (Table 1) [77]. However, YTXs have now been excluded from the DSP classification since they are not orally toxic and do not induce diarrhea [78, 79]. The mechanism of action of the OA group toxins is exerted via inhibition of the serine-threonine protein phosphatase 2A (PP2A) [80], which plays important roles in many regulatory processes within the cell. OA most likely causes diarrhea by stimulating phosphorylation of proteins that control sodium secretion in intestinal cells [81]. The production profiles of DSP toxins by dinoflagellates can vary within a single species [82, 83].

3.4. Ciguatera Fish Poisoning (CFP) Toxin

Ciguatera is another form of dinoflagellate toxicity in tropical areas caused by eating fish contaminated by toxins of *Gambierdiscus toxicus*. These toxins can accumulate in the food chain to larger carnivorous fish such as barracuda, snapper, and amberjacks to levels high enough to cause human illness [84]. Ciguatoxins are polycyclic polyether compounds that activate human voltage-gated sodium channels (VGSC), which leads to gastrointestinal, neurological, and cardiovascular disturbances [85-87]. Due to bioaccumulation in humans, ciguatera symptoms can persist from days to months depending on the amount of toxin ingested [84]. The number of congeners produced by dinoflagellates of not only ciguatoxin, but most dinoflagellate toxins, are difficult to quantify as new congeners are constantly being discovered with slightly different structures and varying potencies [88, 89]. For example, Pacific ciguatoxin PCTX-1 can cause illness at levels of 0.1 µg/kg in the flesh of fish while Caribbean ciguatoxin C-CTX-1 is 10 fold less toxic as it is more polar and therefore easier to detoxify [90]. In areas where ciguatoxins are prevalent, monitoring programs are in place to prevent contaminated fish from reaching markets for consumption [89, 90].

Table 1. Suite of toxins identified from marine dinoflagellate species. Table generated using information from Cembella [100] and Shimizu [101]

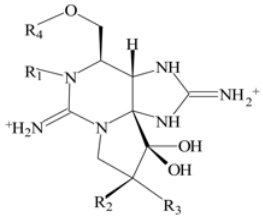
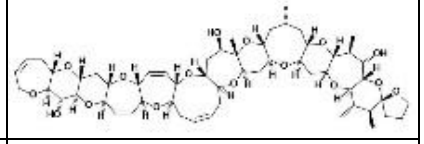
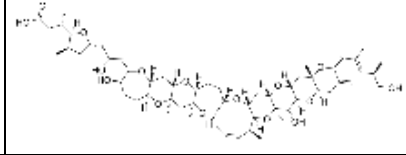
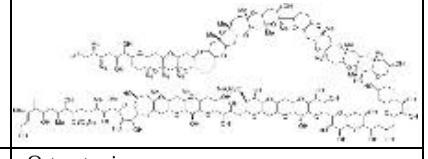
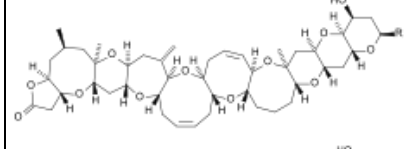
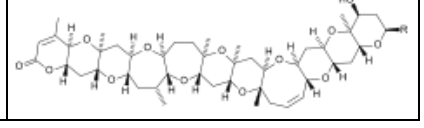
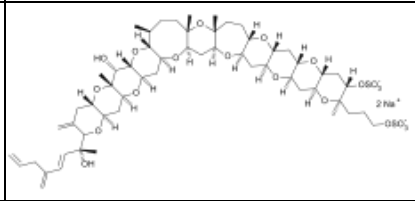
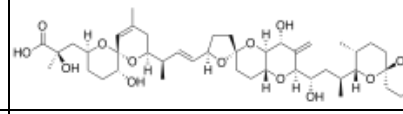
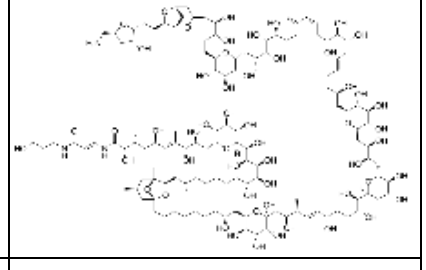
Toxin group	Toxin producing species	Class	Characteristics	Mode of action	Structure
Saxitoxins (neosaxitoxins, gonyautoxins)	*Alexandrium* spp., *Pyrodinium bahamense*, *Gymnodinium catenatum*	Dinophyceae	Hydrophilic N-toxin	Na^+-channel blocker (site 1)	
Ciguatoxin	*Gambierdiscus toxicus*	Dinophyceae	Ladder-frame polyether	Na^+-channel activator (site 5)	
Gambieric acid	*Gambierdiscus toxicus*	Dinophyceae	Ladder-frame polyether	?	
Maitotoxins	*Gambierdiscus toxicus*	Dinophyceae	Ladder-frame polyether	Ca^{2+}-channel effector	
Osterotoxin	*Ostreopsis lenticularis*	Dinophyceae	Ladder-frame polyether	Unknown	Osterotoxin
Brevetoxins	*Karenia brevis*, *K. brevi-sulcata*	Dinophyceae	Ladder-frame polyether	Na^+-channel activator (site 5)	
	Chatonella marina, *C. antiqua*, *C. cf. verruculosa*	Raphidophyceae			

Table 1. (Continued)

Toxin group	Toxin producing species	Class	Characteristics	Mode of action	Structure
Yessotoxins	*Protoceratium reticulatum*, *Lingulodinium polyedrum*	Dinophyceae	Ladder-frame polyether	Affects cyclic AMP, cytotoxic	
Okadaic acid and dinophysistoxins	*Dinophysis* spp., *Prorocentrum* spp.	Dinophyceae	Linear polyether	Protein phosphatase inhibitor	
Pectenotoxin	*Dinophysis fortii*, *D. acuta*	Dinophyceae	Macrocyclic polyether	Unknown, hepatotoxic	
Azaspriracids	*Protoperidinium crassipes*	Dinophyceae	Linear polyether	Unknown, neurotoxic	
Gymnodimine	*Karenia selliformis*	Dinophyceae	Macrolide	Unknown, potentially neurotoxic	
Spirolide	*Alexandrium ostenfeldii*	Dinophyceae	Macrocyclic polyether	Muscarinic receptor or cholinesterase inhibitor	
Ostreocin (palytoxin)	*Ostreopsis siamensis*	Dinophyceae	Linear polyether	Na^+/K^+ ATPase disruptor	
Amphidinolide, Caribenolide	*Amphidinium* spp.	Dinophyceae	Macrocyclic polyether	Cytotoxic	
Goniodomin	*Alexandrium* spp.	Dinophyceae	Macrocyclic polyether	?	
Prorocentrolide	*Prorocentrium lima*	Dinophyceae	Macrocyclic polyether	?	
Amphidinols, Amphiketide	*Amphidinium* spp.	Dinophyceae	Open-chainpolyketides	Antifungal	

3.5. Azaspiracid Poisoning (AZP) Toxin

AZP is the most recently discovered toxic syndrome from shellfish consumption and several analogues belonging to this new class of toxins have been identified in contaminated mussels [91-93]. More than 20 analogues of azaspiracid AZA1 have been described in shellfish [92-95], which complicates the regulatory control of these compounds as most have not yet been toxicologically evaluated. Cytotoxicity studies using neuroblastoma cells showed that AZA1 disrupts cytoskeletal structure, inducing a time- and dose-dependent decrease in F-actin pools. A link between F-actin changes and diarrhea has been suggested, which may explain the severe gastrointestinal disturbances during AZP outbreaks. AZPs have been identified in two dinoflagellates, *Protoperidinium crassipes* [94] and a new species, *Azadinium spinosum* [96].

Traditionally, the biosynthetic pathways for these complex compounds are elucidated by isotope labelled studies on cultured microorganisms [97, 98]. While these studies have not been able to comprehensively elucidate all involved biosynthetic pathways, they have provided great insights into the structural complexity of these compounds [98, 99].

4. DINOFLAGELLATE TOXIN SYNTHESIS

Toxin-producing taxa do not seem to show a phylogenetic trend [102], which suggests multiple independent evolutionary origins for this capability, such as horizontal gene transfer or multiple losses of the capacity in descendants of a toxin-producing ancestor.

Paralytic shellfish poisoning toxins (PSTs) are a group of naturally occurring small heterocyclic guanidinium alkaloids. Saxitoxin (STX), as the basic form, is the most researched PST to date, and since its discovery in 1957 58 other analogues have been described [103] [104]. The STX synthesis pathway in dinoflagellates is elusive. It is hypothesized that PSTs are synthesized via a pathway involving arginine, SAM and acetate, and that the methyl group of SAM is incorporated into the final PST molecule (reviewed by Shimizu [105]). Therefore, SAM synthetase is likely to play a key role in STX biosynthesis. The expression of SAM synthase in the heterotrophic dinoflagellate *Crypthecodinium cohnii* is slightly upregulated in the G1 phase of the cell cycle [106]. Interestingly, this coincidence with higher saxitoxin production in this phase in *Alexandrium fundyense* [107]. Using degenerate primer sets in PCRs, homologs of SAM synthetase gene were identified in 11 dinoflagellate species [108], not all of which are PST producers. Studies by John et al. [109] and Wang et al. [110] identified all three genes involved in the initiation-step of STX biosynthesis, sxtA, sxtG and sxtB, in *Alexandrium fundyense* and *Alexandrium pacificum*, which are phylogenetically close to the cyanobacteria (Table 2). Additional genes involved in STX biosynthesis were identified in an *A. pacifi*cum transcriptome study, including sxtD, sxtS, sxtU, sxtH/T, sxt I, sxtL, sxtN, sxtX, sxtF/M and sxtP (Table 2). In the PST-producing cyanobacterium *Cylindrospermopsis raciborskii*, the STX biosynthetic pathway is encoded by more than 35 kb, which contains 30 catalytic functions in 26 proteins [sxtA(sxtZ)] (Table 2) (Figure 3). STX biosynthesis is initiated with arginine, SAM and acetate by a new type of polyketide synthase, followed by three heterocyclizations and various tailoring reactions to yield the numerous isoforms of STX [111]. A comparative phylogenomic analysis for toxic

and non-toxic strains of *Anabaena circinalis* and *Cylindrospermopsis raciborskii* revealed that nine of the 26 putative STX genes (Table 2) were horizontally transferred from non-cyanobacterial sources. One key gene, sxtA, originated in STX þ cyanobacteria via two independent horizontal transfers followed by fusion [63]. Other evidence is provided through phylogenetic analysis, where the second key gene, sxtG, in dinoflagellates seems be similar to its ancestral gene in bacteria, actinobacteria and cyanobacteria [112]. Previous findings showed that the initial three genes, sxtA, sxtG and sxtB, have originated from proteobacteria before being transferred to cyanobacteria and further to dinoflagellates via HGT events [105, 113-115]. However, the toxin genes were modified significantly during this evolutionary process, resulting in higher GC content, conversion into monocistronic structure, generation of multiple copies, introduction of typical dinoflagellate spliced-leader sequences and eukaryotic poly (A) tails [112, 116].

Other dinoflagellate toxins are derivatives of polyketide. Essentially a polyether ring ladder (Table 1), the synthesis of polyketide requires sequential extension of the C backbone, which is catalyzed by a polyketide synthase (PKS) system. There are three types of PKSs (for review see Snyder et al. [117]; Monroe and Van Dolah [118]). Type I PKSs are large multifunctional enzymes in which several catalytic domains are located on a single protein (Fig. 4A). These functional domains may be used iteratively (fungal PKSs) or they can be arranged into modules with each module directing one round of chain extension (b-ketoacyl synthase [KS] domain) and post-condensation modifications (b-ketoacyl reductase [KR], dehydratase [DH], and enoyl reductase [ER]). Type II PKSs are multiprotein complexes of several individual enzymes, and are found only in bacteria that synthesize aromatic polyketides. Individual enzymes in type II PKSs are used repetitively for each cycle of chain extension. Type III PKSs are smaller PKSs (40-47 kDa) and are involved in flavonoid biosynthesis in plants and melanin in bacteria. Sequences of genes in several species of dinoflagellates that are similar to type I PKSs were first identified using PCR [117], for example *K. brevis*, the major producer of brevetoxin [119] and *Amphidinium* sp., which produces amphidinolides, a group of cytotoxins [120]. Subsequent screening of cDNA libraries revealed that while PKS sequences in *K. brevis* are similar to modular type I PKSs the organization of the genes resembles Type II PKSs, i.e., each functional 'domain' is present but is expressed as a separate protein [118] (Figure 4B). Using antibodies against peptides generated from deduced PKS amino acid sequences in *K. brevis*, Monroe *et al.* [121] found that protein abundance in the non-toxic substrain was 50-75% lower than in the toxic substrain although transcript abundance was similar between the two substrains. Furthermore, the authors found that the PKS proteins were localized to chloroplasts. This intracellular distribution is consistent with that of another polyketide toxin, okadaic acid (OA), in *Prorocentrum lima* and *Prorocentrum maculosum* [122]. This is particularly interesting, since the *Prorocentrum* spp. possesses peridinin plastids, whereas *K. brevis* possesses a haptophyte-type plastid. PKS genes from *P. lima* should be coded in the nuclear genome because the peridinin plastid only contains about 16 plastid genes, all related to photosynthesis. Presumably, PKS genes in *K. brevis* are also nuclear-encoded because their transcripts are SL trans-spliced [118]. These results will help determine the next directions to take to determine not only how brevetoxin is synthesized, but also its function within the cell.

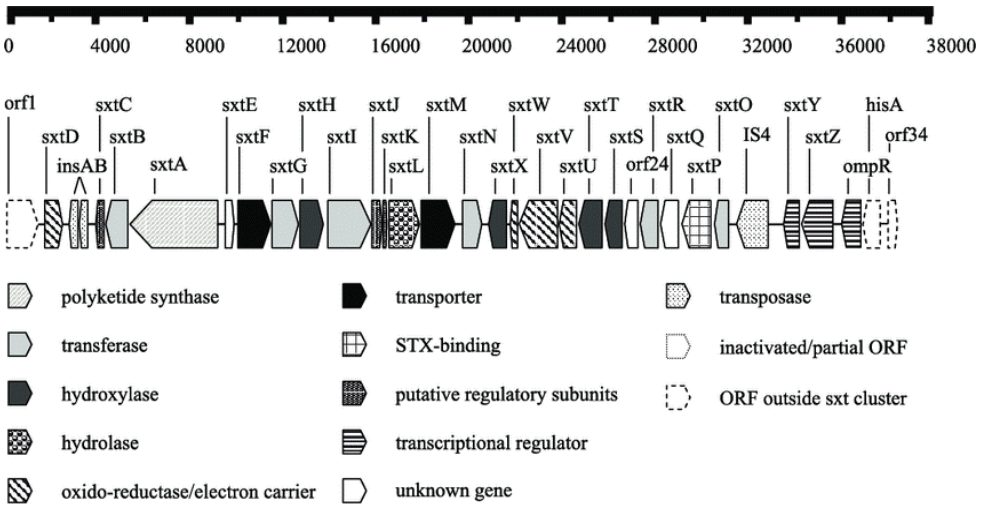


Figure 3. Structural organization of the sxt gene cluster from *Cylindrospermopsis raciborskii* T3. Abbreviations used are as follows: SXT, saxitoxin gene; IS4, insertion sequence 4; ompR, transcriptional regulator of ompR family; hisA, two-component histidine kinase; orf24, ORF 4. The scale indicates gene cluster lengths in base pairs. From Kellmann et al. [111] and Lin [57].

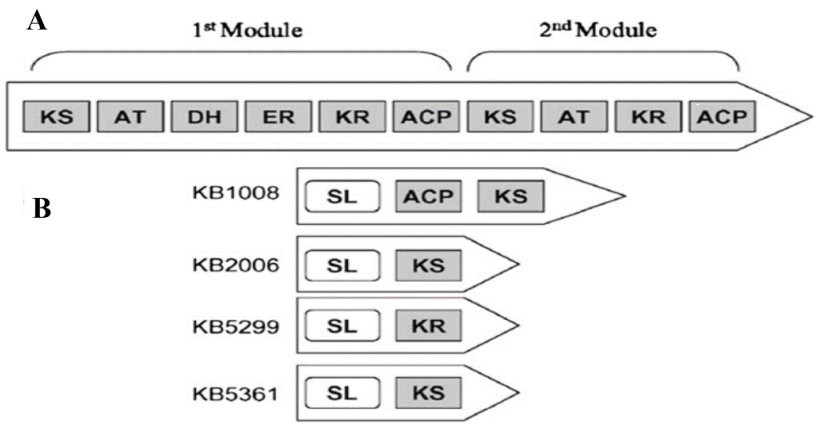


Figure 4. Model of PKS transcripts in *Karenia brevis*. A) Typical modular type I PKS transcript encoding multiple catalytic domains on a single transcript that form modules. B) K. brevis PKS transcripts with the SL sequence at the 50 end and 1e2 catalytic domains per transcript. KS: ketosynthase, AT: acyl transferase, DH: dehydratase, ER: enoyl reductase, KR: ketoreductase, ACP: acyl carrier protein. (BeC) are from Monroe and Van Dolah [118] and Lin [57].

5. Applications for Detection and Quantification of Toxic Dinoflagellate

The monitoring of toxic dinoflagellates is required by many countries to ensure the safety of fish and seafood intended for human consumption [130]. Microscopic identification and enumeration of dinoflagellate species are, however, very tedious and time-consuming. PST-producing *Alexandrium* spp. can be particularly difficult in this respect, due to the

morphological similarities to cryptic species that occur in the same habitat [109, 131]. To meet these challenges, numerous molecular tools are under development. These are mainly aimed to detect species with toxin-specific nucleic acids, using qualitative and quantitative PCR, and DNA-hybridization-based methods. Comparisons between methods for the determination of *Alexandrium* cell density indicated that these molecular methods facilitate the required sensitivity to monitor and discriminate between species in the field [132].

Several studies have shown that rRNA gene copy numbers can be highly variable [133-136], which may lead to over- or underestimation of cell densities [132, 137]. Characterization of SXT genes facilitated the development of qPCR assays that identify PST-producing strains [138-141]. Studies have shown that the copy numbers of SXT genes in genomes vary less when compared to rRNA genes, allowing for more accurate cell density estimates [128, 132, 140]. These assays have been successfully tested on toxic dinoflagellate strains in seawater [139-143], as well as in commercially harvested oysters [143].

The design of toxin-specific molecular assays for dinoflagellates was made possible by the identification of the first SXT genes utilizing EST data, and subsequent direct sequencing of the candidate genes [116, 124]. Qualitative screening for the presence of *sxtA1*, *sxtA4*, and *sxtG* genes in cultured dinoflagellate strains has revealed that they differ in the specificity of PST production. The results indicate that of these three target genes, *sxtA4* is the best predictor of toxin production [109, 116, 123, 125, 138]. Currently, there are five primer pairs available for qPCR and qRT-PCR assays as well as one locked nucleic acid (LNA)-based hydrolysis probe targeted for the dinoflagellate genes *sxtA1*, *sxtA4*, and *sxtG* in *Alexandrium* spp. and *G. catenatum*, respectively (Table 3). While the *sxtG* qPCR assay was 100% specific, a longer *sxtG* amplification product was obtained from four non-PST producing *Alexandrium* strains [144]. In the expression study by Perini *et al.* [145], only four *A. minutum* strains were analyzed, and assay specificity was not extensively addressed. The sensitivities of the different SXT assays cannot be directly compared, as they depend on the targeted nucleic acid, and the method of standard curve construction (Table 3).

The detection of toxic strains by sxt gene-based qPCR assays at low dinoflagellate concentrations in seawater, provides a means for early warning systems designed to identify developing harmful blooms. In the future, combining qPCR with other analysis methods such as meta-transcriptomics could provide even more information on the active metabolic processes related to toxin production throughout bloom events [146].

6. DINOFLAGELLATE BIOACTIVES: POTENTIAL APPLICATIONS

Dinoflagellates are able to produce bioactive compounds with distinctive chemical structures, with a wide range of functional groups possessing various toxicological and biological features; macrolides, cyclic polyethers, spirolides and purine alkaloids are a few examples of such categories [98, 149]. Due to their variable structures, these biocompounds form a heterogeneous group that has the potential to strongly affect a variety of biological receptors and metabolic processes [150]. Hence, their potential pharmacological activities hold the promise of beneficial applications in human or veterinary medicine, i.e., in analgesic, antitumor, anticholesterol, cytotoxic, anti-infective, immuno-suppressive and/or neurological disease therapeutics [151].

Table 2. Putative SXT genes in the dinoflagellates, adapted from Verma et al. [68]

Gene	Function*	Dinoflagellate Species	Reported from Transcriptome	Reported from Genome
Core SXT Genes				
sxtA (sxtA4)	Aspartate aminotransferase	*Alexandrium australiense*	na	Yes [116, 123]
		Alexandrium catenella	Yes [116, 124]	Yes [109, 116, 123]
		Alexandrium minutum	Yes [116]	Yes [116, 123]
		Alexandrium ostenfeldii	na	Yes [125]
		Alexandrium pacificum	Yes	Yes [116, 123]
		Alexandrium tamarense	[123, 124, 126, 127]	Yes [116]
		Gymnodinium catenatum	No [109, 124]	No [109, 123]
		Pyrodinium bahamense	Yes [123]	Yes [116, 128]
			Yes [123]	na
sxtG	Amidinotransferase	*Alexandrium affine*	No [129]	Yes [129]
		Alexandrium andersoni	No [129]	Yes [129]
		Alexandrium australiense	Yes [129]	Yes [129]
		Alexandrium catenella	Yes [116]	Yes [129]
		Alexandrium insuetum	No [129]	Yes [129]
		Alexandrium minutum	Yes [116]	Yes [129]
		Alexandrium pacificum	Yes [124, 126, 127, 129]	Yes [129]
		Gymnodinium catenatum	Yes [123, 124]	Yes [129]
		Pyrodinium bahamense	Yes [123, 124]	na
sxtB	Cytidine deaminase	*Alexandrium catenella*	Yes [116]	na
		Alexandrium minutum	Yes [116]	na
		Alexandrium pacificum	Yes [124, 126, 127]	na
sxtD	Sterole desaturase	*Alexandrium pacificum*	Yes [124, 126, 127]	na
sxtS	Phytanoyl-CoA dioxygenase	*Alexandrium catenella*	No [116]; Yes [124]	na
		Alexandrium minutum	Yes [116]	na
		Alexandrium pacificum	Yes [124]	na
		Gymnodinium catenatum	Yes [124]	na
		Pyrodinium bahamense	Yes [124]	na
sxtU	Alcohol dehydrogenase	*Alexandrium catenella*	Yes [116, 124]	na
		Alexandrium minutum	Yes [116]	na
		Alexandrium pacificum	Yes [124, 126, 127]	na
		Pyrodinium bahamense	Yes [124]	na
sxtH/T	Phenylpropionate dioxygenase		Yes [116, 124]	na
		Alexandrium catenella	Yes [116]	na
		Alexandrium minutum	Yes [124, 126]	na
		Alexandrium pacificum	Yes as H/T/DIOX [127]	na
sxtI	O-carbamoyl transferase	*Alexandrium catenella*	Yes [116]	na
		Alexandrium minutum	Yes [116]	na
		Alexandrium pacificum	Yes [124, 126, 127]	na
		Pyrodinium bahamense	Yes [124]	na
sxtF/M	Multidrug e_ux protein	*Alexandrium catenella*	Yes [116]	na
		Alexandrium minutum	Yes [116]	na
		Alexandrium pacificum	Yes [124, 126, 127]	na
sxtP	STX binding protein	*Alexandrium catenella*	Yes [124]	na
		Alexandrium pacificum	Yes [124, 127]	na
Other sxt Genes				
sxtL	GDSL lipase	*Alexandrium pacificum*	Yes [124]	na
sxtN	Sulfotransferase	*Alexandrium pacificum*	Yes [124]	na
sxtO	Adenylylsulfate kinase	*Alexandrium pacificum*	Yes [126, 127]	na

Table 2. (Continued)

Gene	Function*	Dinoflagellate Species	Reported from Transcriptome	Reported from Genome
sxtR	Acetyl CoA N-acyltransferase	*Alexandrium catenella*	Yes [116]	na
		Alexandrium minutum	Yes [116]	na
sxtW	Ferredoxin	*Alexandrium pacificum*	Yes [127]	
sxtX	Cephalosporine hydroxylase	*Alexandrium pacificum*	Yes [124, 126, 127]	na
		Pyrodinium bahamense	Yes [124]	na
sxtZ	Histidine kinase	*Alexandrium pacificum*	Yes [126, 127]	na
sxtPER	Permease	*Alexandrium pacificum*	Yes [127]	na
sxtACT	Acetylase	*Alexandrium pacificum*	Yes [127]	na

na: not available

Table 3. qPCR and qRT-PCR assays targeted at dinoflagellate *sxt* genes

Target	Detection chemistry	Detection range	Specificity	Reference
sxtA4, gDNA	EvaGreen	30–2600 *A. pacificum* cells reaction^{-1}, 4.1×10^{5}–4.1×10^{10} copies reaction^{-1}	94%	Murray et al. [138]
sxtA4, rDNA	rRNA probe	*A. catenella* for samples containing >5 cell equivalents qPCR reaction^{-1}	93–94%	Murray et al. [147]
sxtA4, gDNA	PicoGreen	6 copies reaction^{-1}, up to two orders of magnitude higher	95%	Savela et al. [148]
sxtA4, cDNA	LNA hydrolysis probe	160–1.6×10^{6} copies reaction^{-1}	100%	Stüken et al. [141]
sxtA1, cDNA	SYBR® Green	2 copies reaction^{-1}, 6 orders of magnitude	100%	Perini et al. [145]
sxtG, gDNA	SYBR® Green	0.63–10 ng *A. pacificum* gDNA reaction^{-1}	100%	Orr et al. [144]
sxtG, cDNA	SYBR® Green	2 copies reaction^{-1}, 6 orders of magnitude	50%	Perini et al. [145]

Since STX blocks at site 1 of the sodium ion channel, saxitoxin has therapeutic potential as a potent blocker of nerve conduction that can produce prolonged anesthesia without myo- and neurotoxic effects [152]. Diluted concentrations of the toxin produce a temporary paralytic state, which aids in the treatment of anal fissures and chronic tension headaches, which otherwise require longer durations of anesthesia. Studies have also concluded the synergistic potential of STX with other anesthetics. The synthesis of STX as a pharmaceutical could be lucrative, yet its potential systemic toxicity prevents the compound from passing clinical trials. BTX, a neuroagonist, increases the plasticity of neurons, revealing its potential for disease treatment such as apoplexy, neurodegeneration, and mucociliary dysfunction [153]. OA has inhibitory activities against serine/threonine protein phosphatase and can regulate intracellular signaling pathways, which opens up possible uses against Alzheimer's disease and other neurodegenerative disorders that are associated with memory impairment [154-156]. In addition, OA is a potent inhibitor of tumorigenesis, causing cell growth inhibition and apoptosis of lung and colon cancer cells, and may thus be an important candidate for anticancer drug screening [157, 158]. PTXs have demonstrated significant anti-tumor activities against human lung, colon, and breast cancer cells, and are considered potential chemotherapeutic molecules against p53-mutant type tumors [159-161].

Table 4. Selected patents related to biotoxins produced by dinoflagellates and potential therapeutic uses. Table adapted from Assunção et al. [149, 164]

Patent Name/Application	Biotoxin Used	Year	Reference
10',11'-modified saxitoxins useful for treatment of pain developing as consequence of chemotherapy	Modified saxitoxin	2017	US20170029431
Use of sodium channel blockers for treatment of neuropathic pain developing as consequence of chemotherapy	Tetrodotoxin, saxitoxin (analogues and derivatives	2017	US20170000797
Ladder-frame polyether conjugates	Brevetoxin, maitotoxin, yessotoxin, gambierol	2016	US20160128321
Sodium channel blocker for treatment of loss of superficial sensitivity Palytoxyn, medical use and process for its isolation Neosaxitoxin combination formulations for prolonged local anesthesia Treatment of loss of sense of touch with saxitoxin derivatives Using yessotoxin and its derivatives for treatment of gliomas	Gonyautoxin Palytoxin Neosaxitoxin Saxitoxin Yessotoxin and derivatives	2016 2014 2014 2014 2013	US20160000793 EP3087172 WO2014145580 EP2533785 ES2393696
Use of yessotoxin and analogues and derivatives thereof for treating and/or preventing neurodegenerative diseases linked to tau and beta amyloid	Yessotoxin (analogues and derivatives)	2013	US20130035302
Use of gambierol for treating and/or preventing neurodegenerative diseases related to tau and beta-amyloid	Gambierol	2012	US20120283321
Use of yessotoxins and derivatives thereof for treatment and/or prevention of metabolic diseases	Yessotoxin and derivatives	2012	WO2012140298
Use of gymnodimine, analogues and derivatives for treatment and/or prevention of neurodegenerative diseases associated with tau and beta-amyloid	Gymnodimine (analogues and derivatives)	2012	US20120245223
Therapeutic use of yessotoxin as human tumor cell growth inhibitor	Yessotoxin	2011	EP1875906
Methods and compositions for studying, imaging, and treating pain	Saxitoxin, gonyautoxin and other analogues	2011	WO2010129864
Use of sodium ion channel blocker in treating biology drug resistance of antibiotic	Tetrodotoxin, saxitoxin	2009	CN101450056
Polyether brevetoxin derivatives as treatment for cystic fibrosis, mucociliary dysfunction, and pulmonary diseases	Brevetoxins and derivatives	2008	US7399782
Use of sodium channel blockers for treatment of preterm labor	Tetrodotoxin, saxitoxin (analogues and derivatives)	2007	WO2007096170
Polyether brevetoxin derivatives as treatment for neurotoxic shellfish poisoning and ciguatera fish poisoning	Brevetoxin and derivatives	2005	WO2005027903
Methods of treating wounds with gonyautoxins	Gonyautoxin	2005	WO2005110275
Dinoflagellate karlotoxins, methods of isolation and uses thereof	Karlotoxin	2005	US2005/0209104
Use of yessotoxin in treatment of allergic and asthmatic processes	Yessotoxin	2003	EP1875907

Other interesting dinoflagellate-derived compounds, such as pigments (e.g., peridinin), fatty acids (e.g., PUFAs) and polysaccharides, may exhibit health benefits such as nutraceuticals, prevention of anti-proliferation and development of tumors, as well as anti-inflammatory and antiviral activities [162, 163].

Due to their biological potential, and despite several difficulties in obtaining the minimum amounts of biotoxins for testing, several studies and patents encompassing applications of dinoflagellates, directly associated with biotoxins, have been published or filed (Table 4).

CONCLUSION

Dinoflagellates are a diverse group of microalgae with numerous peculiar genetic features [165] that provide novel insights into their cellular processes [166]. Due to their immense sizes, no complete dinoflagellate genomes have yet been sequenced. Information on genomic structure mainly derives from analyses of the genomic sequences of individual genes. Coding sequences were largely obtained from cDNA and EST analyses. With the rapid advances in sequencing technology, the volume of dinoflagellate EST (transcriptomic) data has grown rapidly and can now span genome-wide, with affordable cost. Whole-dinoflagellate genome sequencing is also on the horizon. However, the challenge lies in the bioinformatical assembly of sequenced bits into genes or chromosomes. As second-generation sequencing platforms are improving their read lengths (e.g., 454 GSFLX titanium promising 1 kb read length) and third-generation sequencing technology (e.g., single molecule sequencing promising >10 kb read length) is emerging, these challenges may soon subside to some extent. In addition, advances in bioinformatics are continuously improving algorithms for genome assembly. The elucidation of genes involved in the biosynthesis of PSP toxin in dinoflagellates will provide new knowledge and a deeper understanding of the evolution and the origins of toxin-related genes in dinoflagellates. New insights on the genetic regulation of sxt gene expression will provide fundamental information about the STX biosynthesis pathway in dinoflagellates and cyanobacteria. In addition, the understanding of different environmental conditions that influence toxin production in dinoflagellates will also provide further insights into the ecophysiological aspects of the toxins. The development of targeted approaches, combined with advances in chemical identification of these toxic compounds, will allow us to address these issues and advance the discovery of the genetic basis and regulation of dinoflagellate toxins into the future. Dinoflagellate toxins and bioactives are potentially useful in many applications.

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Chapter 153

IMPACTS AND MANAGEMENT OF DINOFLAGELLATE HARMFUL BLOOMS

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ABSTRACT

This chapter summarizes the impacts caused by harmful blooms of some dinoflagellates that produce phycotoxins. Phycotoxins can contaminate seafood products causing poisonings in humans that consume them. To prevent human health diseases, harvesting and commercialization of the sea products is forbidden by law, resulting in important economic losses. Other species produce toxins that cause health problems due to direct contact with the water or inhalation of the aerosolized toxins. Wild and cultured fish and marine fauna may also be severely affected by some harmful blooms with subsequent economic problems. When blooms decay, bacterial degradation consumes oxygen and releases compounds, degrading water quality. Many blooms are natural processes; they cannot be completely avoided. However, some tools are available for the prevention, management and mitigation of the harmful blooms impacts. These include monitoring of the causative organisms and their toxins, sustainable use of the natural resources, and fundamental multidisciplinary research to predict occurrence of blooms. Information of the economic cost of impacts, prevention and mitigation strategies is a key element for the appropriate management of HABs. However, estimations of such economic studies are scarce and very difficult to obtain. In this document, economic data have been provided when available. A recent initiative to improve the understanding of the economic costs of harmful dinoflagellate blooms that could help to advance the comprehension of their costs and to mitigate their impacts is also presented.

Keywords: *Karenia*, *Dinophysis*, *Cochlodinium*, *Pyrodinium bahamense*, *Ostreopsis*, economic cost, paralytic shellfish poisoning, diarrheic shellfish poisoning, fish killing algal blooms

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1. INTRODUCTION

Harmful Algal Blooms (HABs) caused by dinoflagellates can occur in freshwater, estuarine and marine waters. Dinoflagellate blooms are a natural phenomenon known from ancient times in all cultures. The eminent ecologist Professor Ramon Margalef in a plenary session at the VIII International Conference on Harmful Algae (ICHA) in Vigo, Spain in 1997 (Margalef 1998), observed:

> "Alvar Núñez Cabeza de Vaca (1490?-1564), in his *Naufragios* (Ship-wrecks, Ferrando, 1984) composed between 1537 and 1549, referred to a place along the north coast of the Gulf of Mexico, probably not far from the present Apalachee Bay. He wrote that the indigenous people there did not know how to relate the passage of time to the movements of the Sun and the Moon, and used neither months nor years, but *they understand and know about the different periods in nature by observing when the fruits mature and when the fish die.* That is, native populations took as witness of the passage of time the return of the seasons marked by the death of fish, a mortality that could have been caused, then as nowadays, by the action of *Gymnodinium breve (=Karenia brevis)*."

Because the ICHA meeting was hosted by the Ría de Vigo (NW Iberian Peninsula), Professor Margalef referred to dinoflagellate blooms in this area that he had deeply studied in the 1950's (e.g., Margalef 1955; 1958):

> "To people making a living around the Galician Rías, red-tides ("purgas de mar") are familiar events, traditionally compared with menstruation, through which process the local waters would be cleansed from time to time, most notoriously in autumn. It was general knowledge that it is not safe to eat shellfish gathered when the water has the reddish-brownish hue. I suppose that when mass cultivation of mussels in the Galician Rías came to be in the hands of relatively inexperienced developers, regrettable events of dispersal of mussels became more frequent, with one particular instance of autumn of 1976.
>
> If red tides are compared with events of "acute illness," ciguatera may be described more appropriately as a "chronic situation," due to the persistence of benthic populations of active and toxic cells on well illuminated solid substrates, so long as nutrients are sufficient, under relatively steady conditions which prevail in warm seas. Species of *Gambierdiscus*, *Prorocentrum*, *Exuviella*, and *Ostreopsis*, occuring in such scenarios, are highly toxic. The fish contaminated by ingesting toxic algae are dangerous to humans who consume them. Serious consequences are perhaps less frequent than it could be feared. A considerable degree of familiarity with Caribbean fishermen convinced me that by observing subtle changes in aspect and behaviour they could recognize the individual fishes that had developed the condition of *ciguatos*, and discard them. Different fishermen did not always agreed exactly in the description and the relative importance assigned to the relevant criteria, but they tended to agree more about the list of fish species to which ciguatera appears locally to be restricted or, at least, most common. One is led to suspect that ichthyophagous species are also sensitive in some way to the condition of *ciguatos* in their potential prey."

Fukuyo et al. (2002) explained that

"In Japan, one of the old historical books entitled "The History of Great Japan" (Dai Nippon Shi), edited more than 300 years ago, described 16 cases of red tide, seven in freshwater and the rest in the marine environment. The oldest was an occurrence in 731 AD. A case that occurred in 1234 AD was reported to cause fish mass mortality and human fatalities after eating fishes.

In some fishing villages in northern Japan, warning about possible toxin contamination might be passed on from ancestors and known among elderly local fishermen. There are several traditional folk tales such as "Do not eat shellfish during snow water runoff into the sea," i.e., it warns that shellfish may become inedible in early spring. After modern aquaculture developed, fishermen tried to sell their products year round but closure of marketing happens often in the spring. Thus we notice the wisdom from the experience that led to the folk tale. Perhaps this indicates that toxin contamination of shellfish has repeatedly occurred almost every year over a long time, leading to many tragedies among the local people. (The Canadian fishermen's warning "not to eat shellfish harvested in the month with no R May to August)."

This illustrates examples of harmful dinoflagellate (and other microalgal) blooms with multiple human health, economic and ecological impacts. In fact, the problems associated to dinoflagellate blooms vary with the species and depend on the combination of i) the biomass level reached during the bloom, ii) the production of toxins directly affecting human health through different vectors (food-borne, water-borne, air-borne), iii) the effects on other aquatic organisms and iv) the duration and spatial dimension of the bloom. Thus, a priori, evaluating the human health, social, economic and ecological cost of dinoflagellate blooms will have species-specific and site-specific components. In this book, paradigmatic dinoflagellates causing blooms are described in detail and provide the necessary information to point out their negative effects (illustrated in Figure 1), namely:

1. *Karenia brevis* synthesises brevetoxin and analogues, and produces high biomass toxic blooms particularly in the Gulf of Mexico, East China Sea, and New Zealand waters. *K. brevis* blooms have a negative impact on:

- Human health, primarily via the consumption of shellfish contaminated with toxins causing neurotoxin shellfish poisoning (NSP), and also respiratory irritation by inhalation of aerosolized toxins;
- Macrofauna, due to brevetoxin bioaccumulation and transfer through the food webs causing massive mortalities of fish, marine mammals, birds, macroinvertebrates, etc.;
- Water quality through discoloration due to high *K. brevis* biomass, which limits light availability to aquatic photosynthetic organisms and thus alters O_2 and CO_2 levels with cascading impacts in the food webs;
- The overall ecosystem when the bloom decays, due to O_2 depletion (hypoxia) associated to the bacterial degradation;
- Economy, due to i) closures of shellfish extraction and losses on professional and recreational fishing activities or aquaculture, ii) tourism disturbance in the bloom affected areas due to unpleasant odour during and after the bloom decay, iii) overall damage in the environment.

2. *Dinophysis* spp. producing low biomass toxic blooms in general (Campbell et al. 2010, Alves and Mafra 2019), synthesize okadaic acid and analogues and pectenotoxins. *Dinophysis* spp. blooms occur worldwide and impact:

- Human health, via the consumption of contaminated shellfish causing diarrheic shellfish poisoning (DSP);
- Shellfish industry, due to closures of shellfish extraction.

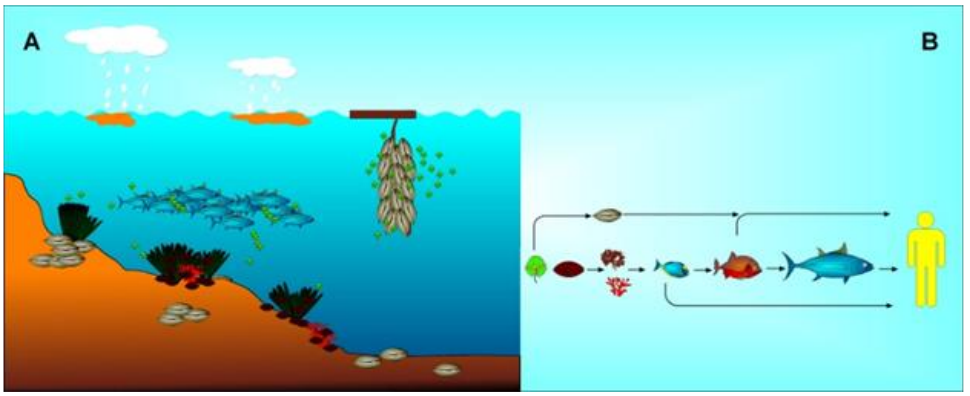

Figure 1. Multiple impacts on the environment and human health caused by dinoflagellate blooms. A. Massive fish kills by ichthyotoxic species, decrease in water quality (light availability, oxygen levels), respiratory problems by aerosolized toxins, contamination of food products by toxins produced by plankton and benthic species. B. Biotoxins bioaccumulation and transfer through the food webs causing seafood borne poisonings in humans. DSP, PSP and ASP occur due to consumption of contaminated shellfish, while different fish species are involved on CFP and palytoxicosis.

3. *Pyrodinium bahamense* var. *compressum* a tropical organism that synthesises saxitoxin and analogues and produces high biomass toxic blooms with impacts on:

- Human health, via the consumption of contaminated shellfish (also some crustaceans and fish) causing paralytic shellfish poisoning (PSP);
- Economy, due to closures of shellfish extraction.
- Macrofauna, by cuasing mortalities on marine mammals, sea turtles, etc.

4. *Gambierdiscus* spp. tropical benthic dinoflagellates (grown attached to live and dead corals, macroalgae, sand, rocks) producing ciguatoxins, whose blooms do not always reach high cell numbers, having impacts on:

- Human health, via the consumption of coral reef fish and some shellfish, contaminated with ciguatoxins causing ciguatera fish poisoning (CFP);
- Economic impacts on personal incomes due to chronic neurologic symptoms that persist for months, impairing working activities;
- Social disruption of fish eating habits to avoid local fish consumption, including loss of traditional fish related jobs.

5. *Noctiluca* spp. this non-toxic heterotrophic dinoflagellate (feeds on microorganisms) causes high biomass blooms resulting in:

- Decrease in water quality due to i) discolorations that limit light availability to aquatic photosynthetic organisms and thus alter O_2 and CO_2 concentrations with cascading impacts in the food webs, and ii) to subsequent reduction of O_2 by bacterial degradation of the organic matter (BOD) when the bloom decays;
- Economic losses for the tourist industry that avoids low beach water quality (affected also by bad odours during the senescent phase of the blooms), although *Noctiluca* natural bioluminescence may also constitute a touristic attraction in some places.

Following a description of the various impacts of harmful dinoflagellate blooms, key tools available for prevention of occurrence of HABs and for management and mitigating their impacts, including their cost when possible, are discussed. The chapter details information on the economic cost of impacts, prevention and mitigation, which are the key elements for the appropriate management of HABs. However, these data are scarce and difficult to obtain. A final section briefly presents a recent initiative to improve the understanding of the economic costs of harmful dinoflagellate blooms that could help to advance the comprehension of their costs and mitigate their impacts.

2. IMPACTS OF HARMFUL DINOFLAGELLATE BLOOMS

This section summarizes the main impacts caused by harmful dinoflagellate blooms and, when possible, information on their economic costs. Note that the monetary numbers are copied from the original citations, without any conversion to the current year.

2.1. Direct Impacts on Human Health

Toxic dinoflagellate blooms can impact human health mainly through:

I. Consumption of seafood contaminated with toxins that have bioaccumulated through the food webs (Figure 1). For example, *Dinophysis*, *Alexandrium*, *Gymnodinium catenatum* or *Karenia* are ingested by cultured or naturally growing filter feeders (mainly shellfish) that bioaccumulate the phycotoxins, causing diarrheic- (DSP), paralytic- (PSP) or neurotoxic (NSP) shellfish poisonings in humans (Table 1). *Gambierdiscus* and *Ostreopsis* grow attached to corals or macroalgae (mainly in the tropics), that are grazed by small fish which, in turn, are consumed by other carnivorous fish; by eating different fish species considered to be within the (not well understood) food webs, humans can suffer ciguatera fish poisoning (CFP) or palytoxicosis (intestinal injuries) (Table 1).

II. inhaling aerosolized biotoxins produced by *Karenia* and *Ostreopsis* (Figure 1, Table 1).

Table 1. Main poisoning syndromes, toxins involved, dinoflagellates toxin producing taxa, main vector and a few selected references that contain more detailed and specific information

Syndrome	Main toxins	Taxa	Symptoms	Vector	Selected references
AZP	Azaspiracids	Amphidomataceae: *Amphidoma*, *Azadinium*	Severe diarrhoea, nausea, vomiting, stomach cramps	Shellfish	Tillmann et al. 2009 Twiner et al. 2008
CFP	Ciguatoxins	*Gambierdiscus*, *Fukuyoa*	Tingling, itching, numbness of mouth and extremities; vomiting, diarrhoea, nausea; neurological symptoms may persist for several months	Coral reef fish, *Tectus niloticus*	Chinain et al. 2010a, b; Friedman et al. 2017 Gatti et al. 2018 Tester et al. 2014
DSP	Okadaic acid and derivatives (*Dinophysis* toxins)	*Dinophysis*, benthic *Prorocentrum* species	Nausea, vomiting, severe diarrhoea, abdominal cramps, respiratory distress	Shellfish	Blanco et al. 2019 Reguera et al. 2014
NSP	Brevetoxins	*Karenia brevis*	By seafood poisoning: nausea, temperature sensation reversals, muscle weakness, and vertigo. Aerosol-borne: respiratory and eye irritation, particularly for asthmatics	Consumption of shellfish (and fish at least for marine mammals); inhalation of marine aerosols during active blooms	Fleming et al. 2007 Fleming et al. 2011 Kirkpatrick et al. 2004
PLTX	Palytoxin analogues: Ostreocin, Ovatoxin, isobaric PLTX	*Ostreopsis* spp.	Food-borne: nausea, vomiting, severe diarrhoea, abdominal cramps, lethargy, tingling of lips, mouth, face and neck, lowered heart rate, muscle spasms and pain, myalgia, etc. Exposure to aerosols: eye and nose irritation, rhinorrhoea, general malaise, fever. Cutaneous irritations in beach swimmers	Certain fish. Marine aerosols	Tubaro et al. 2011 Vila et al. 2016
PSP	Saxitoxins	*Alexandrium catenella*, *A. minutum*, *Gymnodinium catenatum*, *Pyrodinium bahamense* var. *compressum*	Nausea, vomiting, diarrhea, numbness and tingling of the lips, mouth, face and neck. Severe cases: paralysis of chest and abdomen muscles possibly leading to death	Seafood: shellfish, crustacean, fish	Hégaret et al. 2012 Munday et al. 2013 Usup et al. 2012

Humans exposed to dinoflagellate-produced phycotoxins can experience different clinical syndromes characterized by non-specific acute symptomatic modes (Table 1). In developed countries, health infrastructures are ready to effectively assist the most common HAB-related

poisonings, i.e., PSP, DSP, NSP, Azaspiracid Shellfish Poisoning (AZP) and Amnesic Shellfish Poisoning (produced by some species of the diatom genus *Pseudo-nitzschia*). Given the efficient monitoring of toxins and toxic organisms implemented there, the real incidence of poisoning is relatively low, although unexpected intoxications from new and emergent toxins can occur. From 1970 to 2010, these four shellfish poisonings accounted for about 4000 cases reported worldwide (Visciano et al. 2016). These data are still to be updated. This is partly due to the non-specificity of the symptoms related to phycotoxin poisonings, which can be misdiagnosed with viral or bacterial food poisonings, and thus underreported except during exceptional outbreaks and when testing specifically for okadaic acid or dinophysistoxin (e.g., Taylor et al. 2013). Detailed and updated information on dinoflagellate toxins and the associated human poisonings can be found for instance in Vilariño et al. 2018. This overview also indicates potential, but still poorly known, threats due to chronic exposure to phycotoxins. It also highlights the variety of toxin analogues lacking standards and the current limitations of existing data to improve consumer´s protection from a toxicological point of view.

K. brevis blooms along the Florida coast of the Gulf of Mexico are one of the best studied cases concerning their associated health costs. A review of the intensive and costly interdisciplinary and inter-agency research conducted over 10 years in the US to understand the ecology, toxicology, early warning detection, modelling, impacts on public health, economic cost, etc. of *K. brevis* blooms can be found in Fleming et al. 2011. Direct medical care costs associated with the respiratory and digestive illnesses ranged from 0.1 to 0.7 million USD annually (Hoagland et al. 2009; 2014) and up to 1.0 million USD per year for large, long-lasting blooms (such as one occurred during 2005-2006).

CFP is a threat to public health in most tropical areas, and it is the most widespread and best-described (non-bacterial nor viral) seafood poisoning in the Pacific and the Caribbean (e.g., Friedman et al. 2017, and references therein). During 1973-83, in the 17 Pacific Island Countries and Territories, the Health and Fisheries Authorities (Skinner et al. 2011) estimated a mean annual incidence of 104 cases per 100,000 people across the region. In Moorea, the second most populated island in French Polynesia, with a high number of regular fishermen, the mean incidence rate was 8 cases per 10,000 inhabitants on 2007–2013 with a 54% average of underreporting rate (Morin et al. 2016). Taking into account hospitalization and medication fees, and loss of productive days, the health-related costs due to CFP were estimated to be USD $1613 and $749 for each reported and unreported case, respectively, with an overall cost of USD $241,847 for the study period.

Indeed, the real extent of CFP is also unknown due to under-reporting and misdiagnosis (Radke et al. 2015). The most important challenge to cope with CFP is developing a reliable, cost-efficient and easy to use method to detect ciguatoxins in seafood products to be applied by the local people in the tropical areas, because CFP has associated economic and social impact, and because the monitoring of benthic dinoflagellates and their toxins is complex (see the Monitoring 3.1. section). Furthermore, in recent years, CFP is becoming an emerging issue in non-tropical, non-endemic areas, given the increase in trade and tourism (Epelboin et al. 2014; Mattei et al. 2014). For instance in Canada, CFP cost was estimated at about 2,470 CAD per case, with about 1,000 cases per year related to tourism and food imports in 1990 (Todd, 1995; Fleming et al. 2002). More recently, CFP cases have been reported in subtropical zones, such as the Canary Islands (Pérez-Arellano et al. 2005), where this illness had not been previously documented. The presence of *Gambierdiscus* species seems to be

spreading to temperate waters, including the Mediterranean, likely due to global warming (Bravo et al. 2019; Fernández-Zabala et al. 2019; Tudó et al. 2018).

2.2. Losses in Shellfish and Finfish Aquaculture

The contamination of seafood products by dinoflagellate toxins results in closures of commercial and recreational harvesting or growing areas in order to prevent risks to human health. In many countries, law establishes bans on harvesting the toxic species and/or when their biotoxins are detected above species-specific alert levels in the production areas. Closures have negative impacts on the producers as well as related industries (processing, distribution, product shipping, and selling) (e.g., see details in Guillotreau et al. 2017; Adams et al. 2018), at least over a short term. For instance, in Japan, temporary workers employed to ship bivalves, such as scallops, actually lose their jobs during the period of quarantine (Imai et al. 2014). Fish killing algae (FKA) including dinoflagellates (*Cochlodinium, Karenia, Heterocapsa, Alexandrium catenella*, *Karlodinium veneficum*, *K. australe*) and other microalgal taxa (*Pseudochattonella verruculosa, Chattonella marina, Prymnesium / Chrysochromulina, Pseudochattonella / Vicicitus, Heterosigma*) can massively affect natural and cultured fish causing important negative impacts. Economic loss is also due to reluctance by consumers to buy seafood products during the HAB events.

Although estimating the economic cost of HABs is difficult, in part for confidentiality reasons, scientific collaboration with aquaculture and fishing companies is facilitating a better understanding of the problem. As an example, the Workshop on Economic Costs of Harmful Algal Blooms on Fisheries and Aquaculture, hold during the North Pacific Marine Science Association (PICES) 2013 Annual Meeting in Nanaimo, Canada (Yoshida and Trainer 2014), detailed the information about the economic and social impacts of HABs in the eastern and western Pacific by PICES and UN-NOWPAP (Northwest Pacific Action Plan) researchers. A summary of the main reports of economic cost of harmful dinoflagellates is included next (Table 2), but the readers are invited to download the Report freely available from the PICES website https://meetings.pices.int/publications/scientific-reports). Detailed information is given on potential impacts in the Russian east coasts (Orlova et al. 2014) and impacts on shellfish closures due to *Pseudo-nitzschia* blooms in the Pacific coast of the US, Washington State (Huppert and Trainer 2014).

Haigh and Esenkulova (2014) discussed that in 1999 the first recorded bloom of *Cochlodinium* sp. caused fish mortalities with approximately $2 million CAD in losses in British Columbia, Canada (Whyte et al. 2001). Microalgal cells produce direct physical damage or irritation of gill tissue or are ichthyotoxic, and the massive blooms result in oxygen limitation (Rensel and Whyte 2003). However, finfish flesh does not become toxic to humans. The impacts on farmed fish constitute an indicator of how HABs could also affect the health of wild fish, much more difficult to monitor, and the environmental conditions.

In Japan, PSP and DSP events caused the complete closure of shellfish harvesting in the 1980s, resulting in dramatic economic impacts (more than 100 milion JPY) (Fukuyo et al. 2002). Concerning fish kills (Itakura and Imai 2014), the most severe events from 1972 to 2012 were due to *Chattonella* (*C. antiqua/marina/ovata* complex) blooms, with economic impacts of 7.1 billion JPY (approximately 73 million USD that year). Also, intense fish kills were caused by dinoflagellates such as *Karenia mikimotoi* in 1984 (4.6 billion JPY), 1991

(1.5 billion JPY) and 2012 (1.3 billion JPY), *Herocapsa circularisquama* in 1992 (3.0 billion JPY) and *Cochlodinium polykrikoides* in 2000 (4.0 billion JPY).

Table 2. Some of the most negative impacts of dinoflagellate blooms mentioned in this document, documented in detail in Trainer, and Yoshida (2014). Note that, for comparison and given their magnitude, non-dinoflagellate HAB events have been included

Area	Year	Causing dinoflagellate	Impact	Economic cost	Reference
British Columbia, Canada	1999	*Cochlodinium* sp.	Farmed fish kills	$2 million CAD	Haigh and Esenkulova 2014
Japan	1980's	*Alexandrium* catenella, *A. tamarense*, *Dinophysis*	PSP, DSP events, shellfish extraction bans	>100 milion JPY	Fukuyo et al. 2002
Japan, Seto Island	1972 - 2012	*Chattonella* (*C. antiqua / marina / ovata*)	Farmed fish kills	7.1 billion JPY (about 73 million USD)	Itakura and Imai 2014
Japan, Seto Island	1984	*Karenia mikimotoi*	Farmed fish kills	4.6 billion JPY	Itakura and Imai 2014
Japan, Seto Island	1991	*Karenia mikimotoi*	Farmed fish kills	1.5 billion JPY	Itakura and Imai 2014
Japan, Seto Island	2012	*Karenia mikimotoi*	Farmed fish kills	1.3 billion JPY	Itakura and Imai 2014
Japan, Seto Island	1992	*Herocapsa circularisquama*	Farmed fish kills	3.0 billion JPY	Itakura and Imai 2014
Japan, Seto Island	2000	*Cochlodinium polykrikoides*	Farmed fish kills	4.0 billion JPY	Itakura and Imai 2014
China, overall	2008- 2012	All HAB species	Various	2.23 billion CNY (364 million USD)	Guo et al. 2014
China	Since 2006	*Cochlodinium* spp.	Farmed fish kills	Up to 3.2 million CNY (0.52 million USD) per event	Guo et al. 2014
China	2012	*Karenia mikimotoi*	Farmed fish kills	2 billion CNY (330 million USD)	Guo et al. 2014
Korea	1981	*Karenia mikimotoi*	Farmed fish kills	1.7 million USD	Lee et al. 2014
Korea	1992	*Gyrodinium* sp.	Farmed fish kills	5 million USD	Lee et al. 2014
Korea	1993	*Cochlodinium polykrikoides*	Farmed fish kills	7 million USD	Lee et al. 2014
Washington State, US	2008	*Pseudo-nitzschia*	Recreational shellfish collection	110 thousand to 10.57 million USD	Huppert and Trianer 2014

In China, more than 330 HAB events were reported over approximately 52,777 km^2 annually from 2008 to 2012, with a total direct economic loss of nearly 2.23 billion CNY (364 million USD) (Guo et al. 2014). The dinoflagellates involved in harmful events include *Karenia mikimotoi* and *Cochlodinium geminatum*, *Noctiluca scintillans* (non toxic, high biomass, NH_4 production impacting in the ecosystem), and *Prorocentrum donghaiense* (non toxic, high biomass, fish kills). Since 2006, *Cochlodinium* blooms are increasingly affecting

the coast of China with high impacts on aquaculture fish up to 3.2 million CNY (0.52 million USD). In 2012, *K. mikimotoi* caused massive abalone kills with a direct economic cost of more than 2 billion CNY (330 million USD).

In Korea (Lee et al. 2014, and references there in), HABs have become more frequent since the 1990s. Fish kills have been caused by blooms of *Karenia mikimotoi* (1981) and *Gyrodinium* sp. (1992) with fisheries losses of 1.7 and 5 million USD, respectively. *Cochlodinium polykrikoides* recurrent and annual blooms caused fish kills with fisheries losses of 7 million USD in 1993 and 60 million USD in 1995 (the largest fish kill). Through the 2000s and 2010s, *C. polykrikoides* was the single most important fish killing species causing damage to aquaculture farms in Korea.

In the highly productive Galician (NW Iberian Peninsula) ecosystem, shellfish production accounting for about 0.2-0.3 million tonnes of mussels (*Mytilus galloprovincialis*) per year is mainly threatened by DSP, and to a lesser extent by PSP producing dinoflagellates and domoic acid produced by diatoms (*Pseudo-nitzschia* spp.). *Dinophysis* blooms may result in harvesting bans up to nine months in hot spot areas, whenever DSP biotoxin levels exceed European Union regulatory thresholds. Similar situations occur in western Ireland, western Scotland and Atlantic coast of France (see e.g., Blanco et al. 2005; 2013).

In Southern Chile, the worst reported bloom of *Alexandrium catenella* in terms of geographical extension and affected species, occurred in the late summer of 2016 (Guzmán et al. 2016, Álvarez et al. 2019). The event spread along the Pacific Ocean coastal zone from the southernmost of the Chiloé Archipelago (43º50' S) to Mehuín, Los Ríos Region (39º25' S). The bloom, occurred in the late summer, caused human intoxications (12 people were affected) and dramatic socioeconomic impacts for more than a thousand fishermen in the affected area because of the banning of shellfish harvesting from the involved natural beds. The bloom massively affected marine fauna (see section 2.5).

2.3. Societal Losses: Tourism and Culture

Coastal tourism may be specially affected by high biomass algal blooms, either toxigenic or innocuous. Beaches can be unpleasant to tourists due to high accumulations of foam (consisting on microalgal cells and excreted carbohydrates and proteins) at the surface, water colour changes due to the microalgal pigments (red, brown, green), noxious odors from compounds released by the microalgae (in particular Dimethylsulfoniopropionate - DMSP) or from bacterial decomposition when the bloom decays. Exploitation of coastal resources includes recreational shellfish (oysters, scallops, clams, mussels, crabs, or marine snails) collection and fishing, which can be forbidden when microalgal toxic blooms or massive fish kills occur. In the tropical and subtropical areas, expansion of CFP may constitute a problem for touristic activities in the future.

However, nowadays data on the economic costs of HABs on tourism, most of them just rough approximations, are rare so that conclusions cannot be drawn yet. A brief discussion of the challenges to conduct these studies can be found in Berdalet et al. (2015) and more technical details in Adams et al. (2018). Here, as an example, the research conducted on *Karenia brevis* blooms impacts in Florida (Morgan et al. 2010) is summarized. The authors studied how and why participation in marine-based activities (beach-going, fishing, and coastal restaurant patronage) could be affected during a red tide. Using a sample of residents

in Southwest Florida, participant choice models for each activity were estimated to determine the likelihood of alternate behavioral decisions during a red tide event. Factors influenced by extension activities appeared to have a larger impact than socioeconomic factors commonly hypothesized to affect individual response behavior. Overall, the survey suggested that while some local activities may have direct negative impacts, change in behaviour of the tourists and residents may benefit others.

Furthermore, Morgan et al. (2009) estimated monetary losses that some businesses may have suffered due to *K. brevis* blooms. From three beachfront restaurants, daily proprietary data along 7 years were combined with environmental data from nearby weather stations. Using statistical models, the study revealed that reductions in daily sales ranged from $868 to $3734 (13.7%–15.3% on average) during bloom conditions, which were similar to losses due to other environmental events. The economic incidences occurred when *K. brevis* cell concentrations were ca. $181x10^3$ cells/liter within 6 miles near the restaurants. The authors concluded that this value could constitute a threshold cell count for future localized economic losses projections.

Socio-cultural changes can also occur due to HABs. A paradigmatic case is CFP. To avoid CFP, Pacific islanders have been reducing traditional fishing and consumption of coral reef fish (Dewailly et al. 2008) and increased eating imported, canned fish or red meat. In Rarotonga (Cook Islands), with the highest CFP incidence the per-capita fresh fish consumption decreased from 149 g/day in 1989 to 75 g/day in 2006, concurrent to the increase in obtention of alternative proteins, particularly imported meat. The direct loss in value of marketable goods from commercial fisheries amounted to 0.8 million NZD (about 0.5 million USD) per year, and the approximate costs associated with dietary shifts to 1.0 million NZD (about 0.7 million USD) per year. After a decline in cases of CFP cases in recent years, fresh fish has returned to Rarotonga diet, and per-capita fresh fish consumption increased to 104 g/day in 2011.

2.4. Losses on Desalination Plants

In 2008 and 2009, a dense bloom of the ichthyotoxic dinoflagellate *Cochlodinium polykrikoides* in the Arabian Gulf and Gulf of Oman that lasted for more than eight months forced the halt of operation of at least five seawater desalination plants in the United Arab Emirates (UAE) (Richlen et al. 2010; Anderson and McCarthy 2012). Algal biomass clogged the filtration systems and reverse membranes in the desalinization process. In this case, the high biomass bloom resulted in the disruption of water production and significant economic losses.

An additional risk is posed by the removal of small (300 - 900 Da) HAB biotoxins (saxitoxins, brevetoxins and domoic acid). Laboratory tests and a 5-year monitoring of an operational plant in California (Seubert et al. 2012) confirmed that the osmosis membranes can efficiently remove these small toxins, provided they had no micro-fissures such as those produced by high pressures when they are clogged.

The increase in drinking water supply from desalination systems worldwide requires to carefully studying the potential risks posed by HABs and at least, conduct preventive monitoring around the plants.

2.5. Impacts on Marine Organisms

Section 2.2 considered only the economic impacts of fish mortalities and shellfish extraction bans. The direct effects of HABs on fish and shellfish and marine fauna with non-commercial value are briefly presented in this section.

Fish killing algae (FKA) produce lytic compounds that damage fish gill tissues causing death from suffocation (e.g., Deeds et al. 2002, Lim et al. 2014). However, the particular impairment mechanisms are still poorly understood. For instance, *Cochlodinium polykrikoides* ichthyotoxicity depends on viability of cells and direct physical contact between fish and cells is not required to cause mortality (Tang and Gobler 2008). Shin et al. (2019) found significantly elevated levels of DNA damage in gill tissue exposed to 1,000 and 3,000 *C. polykrikoides* cells/mL. The study suggested that one of the major mechanisms mediating *C. polykrikoides* induced devastation in aquaculture and fisheries would consist on increases in the activity of the antioxidant defense system and DNA damage. Still, more work should be done using the latest technologies available to understand key questions on FKA blooms. With the aim to progress in the subject, an Advanced International Colloquium and Technical Workshop was held in Puerto Varas, Chile, 8 – 11 October, 2019, under the auspices of IOC-IPHAB and GlobalHAB (http://www.globalhab.info/activities/globalhab-activities), with the support of the government of Chile through CORFO and collaboration of CREAN-IFOP. Participants reviewed the state-of-knowledge, main gaps and challenges to develop strategies for technological and scientific approaches to mitigate impacts. Publication of the main conclusions and a road map for research in the coming years will be available soon.

Marine organisms and ecosystems may experience lethal or harmful effects when exposed to certain HABs as well. Massive mortalities associated to blooms of FKA are the most known events, because they often affect commercial aquaculture sites. However, effects on wild fauna and ecosystems are more difficult to be detected, especially at low trophic levels and plankton food webs (e.g., Li et al. 2017). When visible, these impacts are perceived as a degradation of ecosystem services potentially leading to passive value losses.

In the natural ecosystems, high biomass and toxic microalgal blooms could be involved in about 50% of the mass mortalities of wild fauna (turtles, dolphins, manatees, whales, birds (e.g., Scholin et al. 2000), according to the US National Oceanic and Atmospheric Administration (NOAA). For instance, mortalities of sand eels (*Ammodytes* spp.) and an estimated 80% of the breeding population of shag (*Phalacrocorax aristotelis*) in 1968 coincided with a bloom of *Alexandrium tamarense* off the northeast coast of the United Kingdom (Adams et al. 1968; Coulson et al. 1968). Saxitoxins (White 1984, references in Amaya et al. 2018) were associated to four cases of fish kills, deaths of 19 humpback whales and likely to the deaths of pygmy, dwarf sperm, and North Atlantic right whales off Cape Cod (USA), as well as Southern right whale mortalities in Peninsula Valdés (Argentina). In the coast of El Salvador, PSP events are recurrent and have been associated to the death of turtles reported since 2005 (Amaya et al. 2018), based on combined analysis of paralytic shellfish toxins (PST) in the animals and phytoplankton monitoring *in situ* and by MODIS-Aqua satellite. Extensive marine bird mortality by hypothermia in the Pacific Northwest (Monterey Bay, California, USA) in winter 2007 was caused by a bloom of the non-toxic dinoflagellate *Akashiwo sanguinea* that produced high amount of foam containing surfactant-like proteins

accumulated at water surface and destroying the waterproofing and insulative characteristics of the bird feathers (Jessup et al. 2009).

In Southern Chile, the exceptional bloom of *Alexandrium catenella* occurred in 2016 caused the mortality of vertebrates, including sea lions (*Otaria flavescens*), seagulls (*Larus dominicanus*) and dogs. The toxic episode resulted in the largest invertebrate massive mortalities ever recorded in Chile (Álvarez et al. 2019). Organisms mostly affected were crustaceans (*Austromegabalanus psittacus* and *Romaleon polyodon*) and mollusks (*Mesodesma donacium* and *Gari solida*). High and variable levels of PST were found in different tissues of *M. donacium* that also exhibited different degree of histopathologies. It was hypothesized that the mortality of the surf clam was an indirect effect of PST-induced paralysis hampering clams to burrow and thus leading to death by desiccation. In this case, massive and sudden mortality suggested that clams had not been previously in contact with PST, so that toxin resistance mechanisms had not been developed. Monitoring records indicating that previous and recurrent PST events had not occurred in the area supported this hypothesis.

Studies in molecular technology are rapidly shedding light on the mechanisms of detoxification, resistance and biotransformation of exogenous compounds activated in bivalves after being exposed to HAB toxins (e.g., Rolton et al. 2016). As an example, Prego-Faraldo et al. (2019) recently described the transcriptome and gene expression profiles of the mussel *Mytilus galloprovincialis* digestive gland and gills exposed to *Prorocentrum lima* (okadaic acid -OA- producer) cell concentrations corresponding to the early stages of the blooms. The first and fast responses of the mussel tissues involved defense, immunity and metabolism genes. The expressed genes were to some extent unspecific, which can indicate the rapid protective response of the mussels to OA. Similarly, Chi et al. (2019) presented the inmune- and detoxification-associated genes involved on the resistance to OA exposure by bay scallop. These studies can explain lack of lethal or acutely toxic effects of *A. catenella* to Northern scallops (Hégaret et al. 2012). However, combined to other stressors, relatively low *A. catenella* cell density could modify the susceptibility of scallops, to both parasites and predators. In turn, impacts on scallops could have effects in the benthic communities, as scallops are a preferred food source for sea stars and crabs.

Since the end of the 20th century, blooms of the benthic dinoflagellate *Ostreopsis* have been linked to damage to marine fauna (i.e., mussel mortalities, loss of spines and death of sea urchins, loss of one or more arms in sea stars, and coral bleaching) and subsequent alterations of the coastal ecosystems (e.g., in the Mediterranean, Brazilian -Rio de Janeiro -, and New Zealand - norteastern shallow reefs- coasts) (Simoni et al. 2003; Shears and Ross 2009) in temperate waters. Oxygen depletion seems to be the most likely cause of the observed effects given that rusty-brown coloured mucilaginous films containing high *Ostreopsis* cell densities covered the benthos. The fact that palytoxin analogues (i.e., ostreocin and ovatoxin) produced by *Ostreopsis* have been isolated from certain macrofauna in certain Mediterranean sites, suggests a plausible direct toxicity to the fauna and potentially to humans (Aligizaki et al. 2008; 2011; Amzil et al. 2011; Biré et al. 2013).

3. PREVENTION, MANAGEMENT AND MITIGATION

3.1. Prevention: Monitoring and Scientific Research

As already mentioned, monitoring programmes on harmful species and their toxins in seafood and in the production areas have been successful in protecting human health (e.g., Davidson and Bresnan 2009) and reducing economic costs by e.g., limiting any shellfish harvesting closures to the minimum time needed. As an example, in the Pacific coast of the Washington State, US, phytoplankton monitoring provided by programs such as ORHAB and SoundToxins helped reduce the number of beach closures to shellfish harvesting (Huppert and Trainer 2014). The economic balance of less closures exceeded the 150 thousand USD cost of monitoring HABs. Also, the study revealed that razor clam diggers were willing to pay 625 thousand USD to avoid frequent closures of the recreational fishery. Thus, the established monitoring systems should be maintained, reinforced and improved with new technologies. In developing countries and in transition economies, monitoring is very rudimentary, or even absent concerning goods consumed by the local population, but monitoring efforts focus on exportation to other countries.

Monitoring tools include:

- Light microscopy. It is the most widespread monitoring tool for harmful algal species. Although it is time consuming and requires specialized training and expertise, it is relatively cheap and effective as an early warning of the likely occurrence of a harmful event. Thus, training of microscopists in the identification and quantification of biotoxin-producing species should be maintained and supported worldwide.
- Quantitative biomolecular tools can facilitate monitoring, and progress have been achieved in the last years in order to track and enumerate the long and diverse list of harmful microalgae. Still, molecular tools are incipient and expensive for most monitoring agencies.
- Biotoxins monitoring in seafood or in the environment is necessary because the presence of harmful microalgal species in the water is not always linked to toxicity in seafood. For example, toxic and non-toxic species can occur simultaneously (e.g., Touzet et al. 2010), or some toxic organisms are not always efficiently detected by microscopy due to their fragility when preserved or their small size (as in the case of *Azadinium* species e.g., Tillmann et al. 2009). Also, their patchy distribution difficults their sampling (Escalera et al. 2012). However, toxin analysis is limited in itself, partly because some biotoxins may be present in complex mixtures and matrices (animal tissues), associated to unexpected vectors, below detection levels for common analytical instruments, or difficult to get quantified due to the lack of pure toxin standards. This is especially problematic in the case of ciguatoxins or of some emergent toxins, such as tetrodotoxin, fast acting cyclic amines (gymnodimine, spirolides, pinnatoxins) (FAT toxins), and ß-N-methylamino-L-alanine (BMAA). For these technical reasons, mouse bioassays have been a fast tool for years and the standard method to detect the presence of toxins in potentially contaminated seafood, although the method has other technical and ethical issues (Hess et al. 2006). New

approaches are nowadays being explored and implemented including non-animal alternatives, i.e., cell tissue cultures (Van Dolah et al. 2012), biosensor systems (Campbell et al. 2014, and references therein; Dechraoui-Bottein and Clausing 2017), and passive capture of biotoxins by resins (MacKenzie et al. 2004). As discussed in Berdalet et al. 2015 "*The large number of compounds that need to be detected and an inability to validate new tools for detection mean that many monitoring agencies will need to continue to rely on either insensitive (and ethically questionable) mouse bioassays or on comparatively complex and expensive LC-MS/MS detection methods, neither of which can be implemented in situ.*"

- Automatic devices developed to facilitate frequent sampling of microalgae and biotoxins. Two successfully tested instruments are the Environmental Sample Processor (ESP) (Scholin et al. 2009) and the Imaging Flow Cytobot (IFCB) (Olson and Sosik 2007). The ESP collects and processes water samples *in situ*, using sensitive and specific molecular assays to detect target HAB cells and toxins, and transmitting the data to the laboratory in near real time. The IFCB is an imaging flow cytometer that captures the high-resolution images and fluorescence characteristics of all plankton cells (in the 5-150 μm size range) at a high frequency (3 samples per hour); the data can also be sent to a laboratory in near real time (e.g., Campbell et al. 2010). Nowadays, these instruments are improving the scientific understanding of HABs dynamics, but are still too expensive to be widely used in monitoring programs.
- Artificial substrates for a low cost, efficient and standardized sampling method for benthic harmful species (e.g., *Gambierdiscus, Ostreopsis, Prorocentrum lima*) are under development and being tested by different researchers (Fernández-Zabala et al. 2019; Jauzein et al. 2016; Tester et al. 2014), which will help to detect CFP causing organisms.
- In addition to monitoring for microalgae and their toxins, it is necessary to sample physical (temperature, salinity) and chemical (nutrients, oxygen) variables to better understand the dynamics of the harmful blooms and develop site-specific forecast models. This knowledge constitutes an added value of monitoring as early warning systems and helps in the adaptation of each particular monitoring to detect natural and antropogenic changes in the aquatic environment, including those attributed to climate change. A successful example constitutes the ASIMUTH (Applied Simulations and Integrated Modelling for the Understanding of Toxic and Harmful algal blooms) project (e.g., Maguire et al. 2016) aimed to develop short-term HAB alert systems for Atlantic Europe. ASIMUTH used information on the most current marine conditions (weather, water characteristics, toxicity, harmful algal presence etc.) combined with high-resolution local numerical predictions (Aleynik et al. 2016). As noted by Maguire et al. 2016, on one side, "*the integrated, multidisciplinary, trans-boundary approach to the study of HABs developed during ASIMUTH led to a better understanding of the physical, chemical and ecological factors controlling these blooms, as well as their impact on human activities.*" Multidisciplinary data combined with modelling allowed the development of an alert system that it is, nowadays, facilitating an effective management of areas usually associated with HAB events and diminish their negative impact on human activities.

Nowadays, weekly prediction maps on the occurrence of HABs enables aquaculture professionals to adapt their working practices in time to prevent mortalities in finfish farms and/or manage their shellfish harvest more effectively.

The costs of monitoring should be evaluated in each area. Bernard et al. (2014) estimated the total cost of monitoring worldwide on the order of 1 billion USD annually. This value would correspond to 10% of the overall costs of HABs, estimated to be at 10 billion USD annually for marine and freshwaters. Satellites, which are used to detect and track high biomass blooms such as those of *Karenia brevis*, could also help monitoring in other areas. International cooperation in the collection, interpretation, and sharing of Earth observation information, as conceived by the Global Earth Observation System of Systems (GEOSS) (Fritz et al. 2008) is necessary. As an example, efforts are undertaken nowadays to integrate the HABs monitoring to the wide Ocean Observing Systems (Anderson et al. 2019).

3.2. Management and Mitigation

Because HABs are natural phenomena, they cannot be completely avoided. However, a good understanding of their dynamics can help to predict their occurrence and thus prevent or mitigate their impacts. This understanding, coordinated with efficient monitoring of the harmful organisms and their toxins is, so far, the most proven tool to manage the risks posed by HABs occurrence. In addition, certain anthropogenic forcings on coastal ecosystems (eutrophication, harbour and artificial beach construction, fishing intensity) and global warning are also contributing to the observed increased trend of some HAB events intensity and frequency (Wells et al. 2019). An efficient management is essential to decrease the risk of HAB occurrences and minimize their impacts. See Anderson et al. (2012) for a review.

Management strategies or programmes should:

i. Address the control of factors that increase the risk of HAB occurrence, such as coastal eutrophication (Glibert and Burford 2017); excessive use of the coastal zone that destroy the natural habitat (e.g., Airoldi and Beck 2007); ballast water discharges in harbors and coastal areas that contribute to dispersion of harmful organisms among areas (Hallegraeff and Bolch 1999), etc. These initiatives will have a cost to be compared with the potential benefits in each area, i.e., monitoring systems should be cost-effective.
ii. Implement microalgal monitoring to detect harmful organisms as discussed in the previous section.
iii. Implement biotoxin monitoring programmes, such as those carried out in Europe by Food Hygiene Regulations (EC) No 853/2004 and (EC) No 854/2004, which require Member States to monitor both for biotoxin concentrations in shellfish tissues and the presence of marine biotoxin-producing phytoplankton in coastal waters.
iv. Limit shellfish harvesting closures to the minimum possible by varying the timing of shellfish or finfish harvesting to reduce the impacts of HABs, although these actions will also have associated costs in terms of product marketability at least in a short term. However, mandatory seafood inspection programs can effectively restore the consumer's confidence and expand the demand (Wessels et al. 1995).

v. Develop closure strategies in shellfish production areas that can be beneficial over the long term, because the organisms and stocks can recuperate from exploitation, as it has been proven in Japan (Imai et al. 2014).
vi. Develop efficient strategies for fishermen in tropical waters affected by CFP by avoiding their access to areas where finfish may have bioaccumulated ciguatoxin, which could diseminate CFP.
vii. In aquaculture sites, protect finfish cages from HABs by moving the fish cages to unaffected zones, using barrier curtains, pumping of deep water into cages or water oxygenation/aeration. Cost of maintaining equipment and fuel is estimated to be about $1 to 2 million CAD annually per company in British Columbia (Haigh and Esenkulova 2014).
viii. Explore and improve strategies to stop HABs, for example by means of modified clay flocculation (Louzao et al. 2015; Seger et al. 2017) of algal biomass and/or mopping up of ichthyotoxins.
ix. Offer aquaculture insurance (mutual aid system), as it is done in Japan by the Fishery Damage Indemnification System, to mitigate the economic losses due to HABs. The System is both a mutual aid system and an important part of the national government's fishery damage assistance policy (Imai et al. 2014). However, risk perception varies with countries, professional activities and cultured seaproducts and requires detailed studies as well (e.g., Le Bihan et al. 2013).
x. Increase the knowledge of medical professionals to detect symptoms in patients exposed to marine biotoxins. In general, there is a need to improve coordination between marine biotoxin monitoring and public health surveillance and epidemiology activities. This will facilitate both the correct diagnosis and reporting. Examples of successful initiatives are the Harmful Algal Bloom-related Illness Surveillance System (HABISS) (http://www.cdc.gov/hab/surveillance.htm in the USA, the Antipoison Centers in France, and the Accord RAMOGE (www.ramoge.org) at Mediterranean region level (mainly on the coasts of Italy, Monaco, France and Spain).
xi. Develop diagnostic tools for biotoxins, such as those that have been achieved for the detection of saxitoxins in human urine (Johnson et al. 2009).
xii. Coordinate monitoring with operational oceanography and modelling, to provide forecasts and early warnings and ultimately predictions of HAB events (e.g., GEOHAB, 2011; Aleynik et al. 2016).
xiii. Sustain fundamental research, needed for gaining a better understanding of HAB dynamics, to inform and improve monitoring programmes, and to design methods to mitigate the impacts of HABs on human health and well-being.

4. How to Progress on the Estimation of the Economic Costs of Harmful Dinoflagellate Blooms?

Estimating the economic losses and impacts of HABs and dinoflagellate blooms is challenging. The cost of the impacts described in earlier sections vary markedly, depending on the kind of biotoxin and produced illness on humans, the duration, extension and

frequency of the toxic bloom, the affected geographic region, the kind of contaminated or lost seafood products, etc. Consistent data on market sectors are lacking and there are very different methods to determine the economic costs of HABs. Recently, a Workshop "GlobalHAB: Evaluating, Reducing and Mitigating the Cost of Harmful Algal Blooms: A Compendium of Case Studies" was held in Victoria, BC, Canada within the PICES Annual Meeting (https://meetings.pices.int/meetings/annual/2019/PICES/Program).

The Workshop brought together expertise in the science of HABs and economics to review and analyze case studies to better estimate the economic impacts of HABs on fisheries and aquaculture. The Workshop was structured in three parts:

1) Overview of Economics Analysis of HABs impacts. The discussion was focused on types of economic assessment that guided the discussions of case studies on workshop day 2.
2) Case studies: examples of HAB impacts to wild fisheries, recreational fisheries and aquaculture worldwide. The discussions focused on what economic studies can be done in the future and identifying gaps in the data.
3) Mitigation strategies, Value of Information. The discussions focused on the value of HAB forecasts; wrap up and writing assignments were taken.

The output of this workshop will be a compendium of examples describing economic approaches used to estimate the costs of HABs and their mitigation, focusing on establishing connections between HAB scientists and economists. A shorter version of the compendium may be prepared for submission to a journal. In addition, the workshop will (1) propose priorities for research and effective management in the future, (2) develop partnerships between economists and HAB researchers to develop transdisciplinary projects, and (3) attract resources to the field. Outcome of the Workshop will improve the understanding of the impacts of HABs and manage and mitigate them. When entering the UN Decade of the Sustainable Development Goals, this activity should provide tools to implement them.

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Chapter 154

MITIGATION OF THE EFFECTS OF HARMFUL ALGAL BLOOMS ORIGINATED BY DINOFLAGELLATES

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ABSTRACT

Harmful algal blooms (HABs) originated by dinoflagellates can produce multiple damages to aquaculture of shellfisheries. A number of methods have been devised to mitigate their effects some of which are in use and others are promising alternatives to be used in the future. In this work, possible ways of mitigation and the methods that can be used in each way are reviewed.

Keywords: mitigation, clay, algicidal, bacteria, virus, flocculation, chemicals

1. INTRODUCTION

Phytoplankton is the basis for the biological production of aquatic ecosystems and dinoflagellates are an important part of it, especially in marine environments. Blooms of most of the species constitute a valuable food source for filter-feeding organisms, as some bivalves, which can be used in aquaculture or whose natural populations can be exploited. In some cases, notwithstanding, blooms can be harmful to the organisms or for humans that consume them, acting through several ways: a) by modifying the characteristics of seawater; b) by producing substances that affect the cultured or exploited population; or c) by producing toxins that affect humans and that, consequently, render the cultured of fished product useless as food. Additionally to public health, several human activities can be affected by the blooms,

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mainly, aquaculture, fisheries, tourism, and water desalination, having large social and economic impact.

Very dense blooms can modify the characteristics of the seawater making it unsuitable for the life of different organisms, including some which are important for aquaculture as bivalves or fishes. Massive blooms of photosynthetic species can produce hyperoxia during the day –due to the production of oxygen in photosynthesis– and hypoxia or even anoxia at night, when photosynthesis does not take place and respiration can deplete the oxygen dissolved in the seawater. Hyperoxia, especially when bubbles are formed, can produce different alterations but anoxia (or hypoxia) is a frequent cause of mortality in aquaculture facilities and even in relatively confined areas [1-4]. The effects of low oxygen concentration can be amplified by the production of polymers [5] that increase water viscosity, thus reducing the oxygen exchange with an environment that is already poor in it [6].

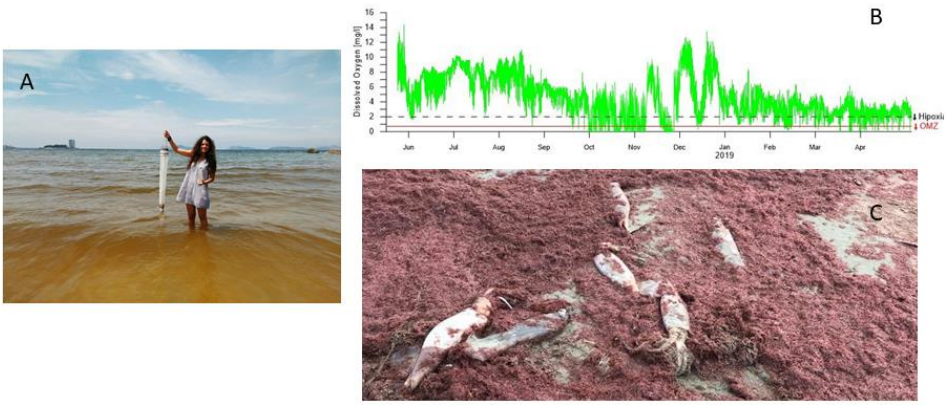

Figure 1. Sampling a HAB of *Alexandrium minutum* in the ría de Vigo (Spain) (courtesy of María García Portela) (A), time course of dissolved oxygen concentration (B) and resultant squid mortality in Bahía Tongoy, Chile (courtesy of Gonzalo Álvarez, project FONDEF IT17F10002) (C).

Some dinoflagellate species produce or release noxious compounds for commercially important marine organisms, being or not also toxic for humans. Large mortalities have been caused by (or associated with) intense blooms of several dinoflagellate species. *Karenia brevis*, which produces brevetoxins, *K. mikimotoi* and other species of the genus produced, in different oceans, large kills of both, fish [7-21] and bivalves [7, 8, 22, 23] or their larvae [23, 24, 17, 25, 20, 26]. The genus *Karlodinium* also includes some species whose blooms are known to produce fish and shellfish kills, as *K. veneficum* [23, 27], *K. armiger* [28, 29], *K. micrum* [30] and *K. australe* [31]. Some species of the genus *Alexandrium* have also been involved in marine fauna mortalities [32-39] and, as recently reviewed by Costa [40], they can also have a number of effects on fish, mainly due to the presence of the PSP (Paralytic shellfish poisoning) toxins they produce, but also by the combination of reactive oxygen species (ROS) and fatty acids [41-43], and by the action of other biologically active compounds [44-46]. Some yessotoxin producers, as *Protoceratium reticulatum* (*Gonyaulax grindley*), *Gonyaulax spinifera* and *Lingulodinium polyedrum*, have been associated with mortalities of abalone and other marine organism in South Africa [47, 48]. *Heterocapsa circularisquama* produces mass mortalities of bivalves in Japan [49-55] and China (Hong Kong) [56]. *Cochlodinium polykrikoides* can also produce problems in bivalves but it mostly

affects fishes in different areas of Japan [57], Korea [58-60], Malaysia [61], Philippines [62], and several other countries [63-68]. Other dinoflagellate species can also be involved in damages to populations of marine organisms, but, in general, with less importance.

Additionally to these direct effects, noxious substances can produce alterations in the organisms, as changes in their optimal commercial aspect or their organoleptic characteristics, with a consequent total or partial loss of value [69, 70].

When the algae produce toxins that affect humans, the situation becomes more complex. First, because it is necessary to regulate the allowable levels of the compounds in the products used as food. Second, because systems to monitor the toxin levels in the organisms should be designed, implemented and ran. Third, because the marketing of the products must be stopped while their toxin content is above the regulated allowable levels.

To minimize the effects of the harmful algal blooms, several strategies have been followed and different methods are under development. In this chapter, the most important are reviewed. Basically, it is possible to act at four points of the problem: 1) directly on the phytoplankton populations, 2) on the alterations they produce in the environment, 3) on the accumulation of toxic or noxious substances in fish and shellfish, and 4) on the regulation and monitoring of toxic substances.

2. ACTIONS ON PHYTOPLANKTON POPULATIONS

2.1. Inoculum Reduction

The development of phytoplankton populations in any area requires an inoculum. Some cells are required to start a bloom. The characteristics of the inoculum vary with the species involved and the area. Some dinoflagellate species have a life cycle that includes resting cysts, that can be in the sediment until they are exposed to favourable environmental conditions and germinate releasing vegetative cells that start planktonic populations. Other species do not produce those cysts and the blooms have to be started by vegetative cells or temporary cysts. In enclosed or semi-enclosed area, advection of vegetative cells from other areas can be very limited and, in such cases, the start of the blooms can depend on resting cysts. In those areas, in which blooms of resting cyst-producing species are usually recurring, reducing the number of cysts by dredging [71] or by any treatment that kills them, for example, hydrogen peroxide [72], can be useful to reduce the number or intensity of HABs. An additional possibility would be to add new sediment to bury the viable cysts and reduce its germination capability (complete suppression of germination would be impossible in natural conditions because bioturbation and sediment reworking should affect cyst resuspension). When the areas are not enclosed, then, advection would be important, whether or not the initial populations are originated by cyst germination, and this kind of measure would very likely be ineffective.

Especially for the dinoflagellates that form resting cysts (but also for other dinoflagellates) the inoculum can come from very distant areas through human activities. Viable cysts or cells can be present in ship ballast water, which is released to the sea supplying and inoculum, even of species that were not previously present in an area [73-75]. This can be also the case with shellfish transplantation [76]. Managing and reducing these

possible sources of cells can contribute to reduce the expansion of HABs, but it is not an easy task. Several methods have been devised and developed [77-80, 75], but some adverse effects of the needed treatments have been identified [81], and the maritime traffic and the behaviour of the individual vessels are very difficult to control.

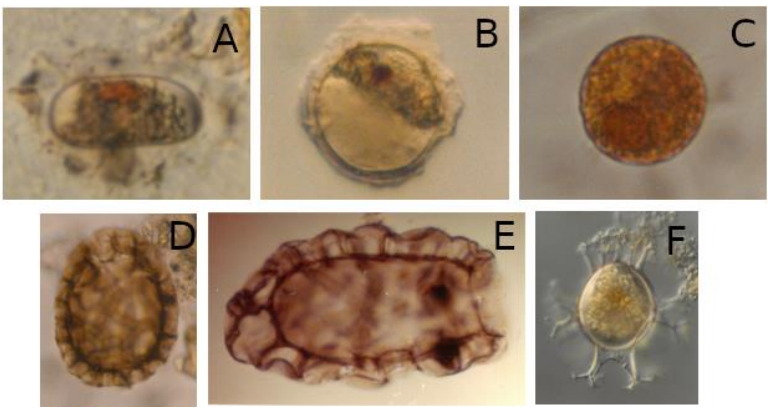

Figure 2. Some dinoflagellate cysts. *Alexandrium tamarense* (A), *A. minutum* (B), *Gymnodinium catenatum* (C), *Polykrikos kofoidi* (D), *P.swartzii* (E) and *Gonyaulax scrippsae* (F).

2.2. Flocculation/Sedimentation of Algae from the Water Column

Once the populations start their development, different strategies can be used. Currently, the most common one is the use of flocculant clays. When some clays are dispersed in the water, they form aggregates with the dinoflagellate cells of the bloom, increasing their sedimentation thus removing them from the water column. This method, obviously, can be used to protect relatively small areas, usually surrounding aquaculture facilities, as fish cages or shellfish rafts, but has noticeable problems to be used in large extensions. The first attempts to control HABs with flocculant clays were made in Japan by Shirota [82-84] and in Korea by Kim [85] with good results. Currently, yellow clay (mostly composed by montmorillonite and kaolinite [86]) is used in Korea to protect fish aquaculture cages from several species but mainly from *Cochlodinium polykrikoides* [84]. Numerous studies have also been carried out using other clays unmodified or chemically modified, to increase the removal capability of cells but also of dissolved toxins [87-98].

The removal efficiency of this technique is, in general, high, attaining 90% or even more (depending on the size of the clay particles and other characteristics) and the cost of the clay is low. Notwithstanding, the operating costs are much higher and protecting an area of approximately 1.5 km^2 can reach 70,000 USD per day, without including labour costs [84], which makes this kind of methodology useless for species that do not have a high economic value.

In addition to the above, using clays as a way to remove cells from the water has some problems. One is that large amounts of clay have to be dispersed, which complicates the operational procedures. To minimize this problem, a number of physical or chemical modifications of diverse clays have been tried, in several cases attaining a noticeable increase of the removal efficiency [89, 92, 94, 95, 99, 84, 96, 97, 100, 98]. A second problem is the

impact of the formed aggregates on marine fauna, especially on filter-feeding organisms. In this sense, Shumway et al. [101] demonstrated the adverse effects of yellow clay on several molluscs and hydroids and [102] found that they can produce important damages to the benthic community. The same kind of clay (montmorillonite yellow clay) was shown to reduce drastically the growth of the clam *Mercenaria mercenaria* when it is suspended, as it happens in high energy environments, but not when it quickly sediments (low energy environments) [103]. The recovery of the settled dinoflagellate populations can represent a problem when dealing with some species. Sun and Choi [90] found that *Alexandrium* and *Cochlodinium* cells were nearly completely destroyed after being settled with clays, but that, when *Scripssiella* was treated, cells were less affected and additionally a large amount of resting cysts were formed in the settled clay, reaching 87,000 cysts per gram of clay. Obviously, this can contribute to the persistence of the species in the area where the clay is dispersed. Another problem is the release by the clay of different substances that can be toxic. Finally, the concentration of cells in the water has to be high for the treatment to be effective. Sengco [104], for example, found reduced removal efficiencies (70%) for blooms of *Karenia brevis* below 1000 cells/mL, and also for other dinoflagellate species.

Figure 3. Clay dispersion from a ship in Korea (a) and aerial view of clay dispersion around the fish cages (b). Reprinted from [105] with permission (Springer-Nature).

2.3. Chemical Methods to Kill Algae

Several chemical treatments have been tried to eliminate or reduce toxic algae blooms, with some success in very precise situations. Copper sulphate was used to eliminate the HABs that usually take place in Florida (*Karenia brevis*), but was found to be too expensive and to have toxic effects on the marine biota [106]. Posteriorly, diverse surfactants were tried with little success also on *K. brevis* [107]. The same group found aponin, a compound produced by the bluegreen algae *Gomphosphaeria aponina*, to kill *K. brevis* [108, 109] and tried to apply both, the algae and the compound, to control the populations of *K. brevis* [110] finding that cellular lysis can increase the ichtyotoxicity. Sophorolipids, other surfactants, have been shown to have some effect on *Cochlodinium polykrikoides* and have been used in Korea to increase the effectivity of the yellow loess, but requires concentrations of 5 g/L [111, 112, 92]. Oxidizing agents have also been applied. Sodium hypochlorite, electrolytically obtained from seawater, has been used in Korea [113, 114] and more recently, in a channel in The Netherlands, hydrogen peroxide was utilized to terminate a bloom of *A. ostenfeldii* [115].

Ozone has been shown to be useful in eliminating *K. brevis* toxins [116-119]. Some other compounds (mostly of biological origin) have also found to affect the growth of some dinoflagellate species but have not been used to mitigate blooms (reviewed in Sellner and Rensel [120]).

Except for some special cases of very noxious events in restricted areas, the effectivity of chemical methods to reduce dinoflagellate populations seems to have limited applicability because mainly of their cost and the side effects on the local biota. Some compounds, with selective effect on harmful algae are being studied [121].

2.4. Biological Methods

Predation, parasitism, and allelopathy can, theoretically, be used to reduce or eliminate populations of harmful dinoflagellates and other algae (reviewed in Salomon and Imai [122]).

2.4.1. Predation

Adding predators, as tintinids or copepods, for example, can be expected to contribute to bloom termination or impede the bloom initiation [123-132]. The practical use of this process, notwithstanding, is very difficult because massive cultures of the predators are expensive and difficult to synchronize with the blooms.

2.4.2. Parasitism

Many parasites of dinoflagellates probably exist and have some potential for mitigation [133-137]. Some bacteria have been shown to be algicidal [138-141], but it is sometimes difficult to discriminate if they are parasitic of if they produce algicidal substances.

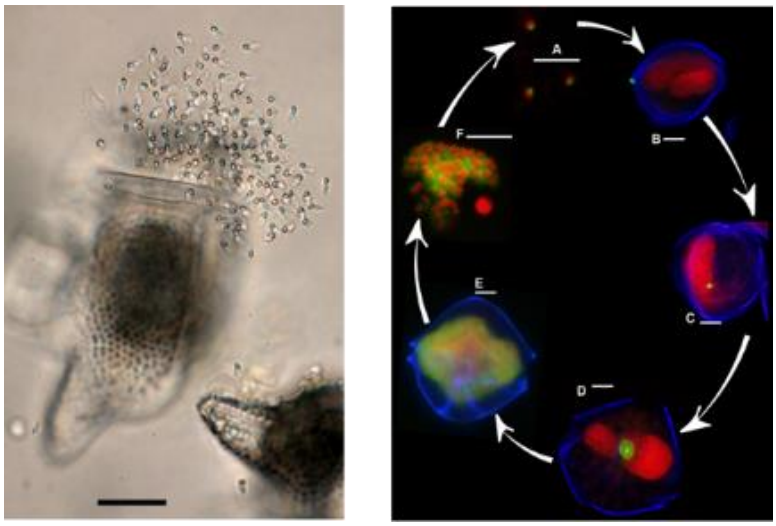

Figure 4. Sporozoites of *Parvilucifera* emerging from an infected cell of *Dinophysis caudata* (left) (courtesy of Isabel Bravo), and cycle of the infection of *Alexandrium fundyense* by *Amoebophrya* (right) (green color indicates the presence of the parasite) (reprinted from [142]).

Among protozoans, the dinoflagellate genus *Amoebophrya* [143-148, 134, 149-152, 135, 153, 154, 136, 155, 27, 142, 156-158] and the Perkinsidae genus *Parvilucifera* [159-171], have been shown to affect, to a different degree, the development of the proliferation of several species. Even when these parasites can contribute substantially to bloom termination, that does not happen in all cases (e.g., Velo-Suárez et al. [172]) and their use presents the same problems than the predators, mainly the difficulty of producing cultures to add a significant amount of parasites to the blooms and additionally, the risk that other dinoflagellate or phytoplanktonic species can be affected, producing damages to the ecosystem. The use of *Parvilucifera infectans* as a way to mitigate harmful algal blooms is, nevertheless, the object of a currently active patent [173].

3. ACTIONS ON THE ENVIRONMENT

The most important environmental effect of HABs is decreasing the concentration of dissolved oxygen to hypoxia or anoxia levels. This mainly occurs because the oxygen consumption by microalgae or bacteria is not balanced by the photosynthesis. This can happen mainly during the night when HABs are very intense or during the decay phase of the blooms, due to the respiration by the bacteria that are consuming the organic matter released by cell breakdown of the phytoplanktonic populations. The two obvious ways to mitigate this problem are: a) restoring the balance between photosynthesis and respiration; and b) supplying oxygen to the water. The first way is quite difficult to implement because it would require lighting the area during the night, which would be very expensive and relatively ineffective if light transmission is reduced because of the high plankton biomass. The second way is more feasible [120] and some methods trying to implementing it exist, as aeration with porous stones or use of airlifts, for example. Some of them are already in use in fish farms to minimize the problems associated with high fish densities (which also deplete oxygen) and can be easily used to mitigate HAB-induced anoxia or hyperoxia. Some of the possible approaches can be simultaneously used, as hydrodynamic barriers (e.g. air curtains at the walls of the cages) or to replace surface water –usually rich in HAB organisms– by bottom water, with smaller concentrations (as airlifts) [120].

4. ACTIONS ON THE ACCUMULATION OF TOXIC OR NOXIOUS SUBSTANCES IN FISH AND SHELLFISH

4.1. Actions on the Accessibility of the Harmful Algae to the Cultured Organisms

There are three possibilities of action: a) moving the cultured organisms or the culture facilities; b) isolate the cultured organisms; and c) reduce the rate at which the harmful cells reach the affected organisms.

Spatially segregating the HABs and the exploited organisms, by means of moving the culture facilities or the organisms themselves, is a useful action (1) when the blooms are not extensive (leaving some areas unaffected), (2) when the culture facilities or the organisms can

be easily transported, and (3) when the price of the product is high enough to balance the costs of the required operations with its loss of value due to the effect of the HAB. Relocating fish cages or even farms to areas with little or no exposition to a bloom has been suggested by several authors [174-178, 84] and used to avoid the fish mortalities produced by HABs in many places as, for example, New Zealand [179], Canada [180], USA [181] and Norway [182]. This approach can be used also with shellfish, but, in general, the technical difficulties involved are much higher. Long lines, for example, are very difficult to relocate and, in all culture devices in which bivalves are attached to a vertical rope (as usual in mussel culture in rafts and long lines), detachment, with the consequent loss of product, can be easily produced by movements during relocation. In some countries, moreover, as in Chile, for example, moving mollusc culture facilities is not allowed and this strategy cannot be used.

Changing the depth at which the fish cages or shellfish culture systems are placed can help to avoid blooms that develop at the sea surface. However, fishes can suffer several problems depending on the species. Some of them, that tend to swim to the surface, can become stressed, and some others can undergo problems to regulate the contents of the gas bladders they use to attain neutral buoyancy. Theoretically, shellfish should have fewer problems with this method, but their cultures are usually located in areas of high plankton concentration, which usually are relatively shallow, and consequently, there is no room to deepen the culture devices. Additionally, the food reduction at depth can originate substantial weight losses, with two main consequences: reduction of the amount of product, and–in the case that some toxins had already been accumulated– an increase of their concentration (as toxins are usually not lost with biomass). Notwithstanding, this method has been successfully used in mussel cultures in Canada [183], Italy [184] and Venezuela [185].

Isolating the cultured organisms is also a possibility to avoid their contact with blooms and, therefore, minimize the effects of HABs in terms of damages to the organisms or toxin accumulation. *In situ* isolation methods are possible (plastic envelopes, for example) but are difficult to deploy and require equipment to maintain oxygen levels and to avoid high concentrations of toxic excretions. Using recirculating aquaculture systems (RAS) [186], are probably the most effective solution, but they are expensive and have to be optimized and equilibrated previously. When the organisms are already being cultured in RAS, the typical water renewal rates of the system can be reduced, or even temporally suppressed, when blooms are detected in the area. In other cases, using this kind of systems can be much more difficult or ineffective, because increasing suddenly the charge of the system will, very likely, led to the impossibility to regulate the water quality.

Reducing the rate at which the harmful cells reach the affected organisms can also be employed to mitigate the consequences of HABs, especially when the impact derives from the ingestion of cells and not from the exposure to dissolved toxins. These methods, consequently, are expected to be more effective in suspension feeders, like bivalves, than in fishes. Blanco, Martín, and Mariño [187], during a *Dinophysis* bloom (okadaic acid producer) used an envelope made of fishing net (a net cylinder) to limit the water flow to the mussel ropes in a raft, managing to reduce the toxin concentration to a half and obtained a similar result by grouping ropes, thus reducing the amount of toxic cells that reach the inner part of the groups. The method can be fine-tuned by choosing the net mesh but also presents some drawbacks. The first one is that, in some occasions (if the phytoplankton concentration is low or the flow reduction too high), shellfish can receive less food than the maintenance ration, therefore losing biomass and concentrating the toxin that has accumulated. The second is that,

when the phytoplankton concentration is higher than the optimum for ingestion, a reduction can place it near the optimum level producing, consequently, an increase in the ingestion of toxic cells [188, 189].

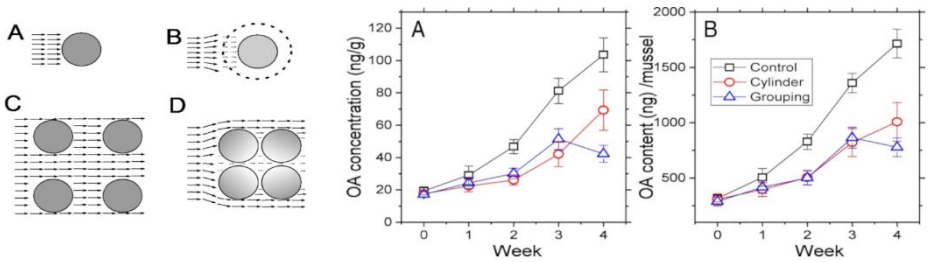

Figure 5. Expected effect of hydrodynamic barriers (A and B left panel) and rope clustering (A and B right panel) on the water flow to mussel ropes in a raft and its effect on okadaic acid concentration during a *Dinophysis* bloom. From Blanco, Martín, and Mariño [187] (with permission (Elsevier)).

Selecting individuals which ingest low amounts of toxic cells can also be a feasible approach, especially for cultures. Bricelj et al. [36], for example, found strong differences in PSP toxin uptake between *Mya arenaria* strains, and Pino-Querido et al. [190] found that the accumulation of DSP toxins by the mussel *Mytilus galloprovincialis* is a trait with a high heritability (>33%), both during the toxin incorporation and elimination phases. In some cases, as in the one of *M. arenaria*, notwithstanding, the reduction of toxin uptake can be due to the toxicity of the algae, more than to an active rejection, and consequently, that selection can pose a noticeable risk for the survival of the selected populations.

4.2. Actions on Toxin Accumulation

Toxin accumulation results from the balance between absorption and elimination. As, in most toxins, nearly no breakdown has been observed, from a practical point of view, elimination is equivalent to the efflux of the toxins from the body tissues. Absorption is, in general, made from ingested toxic particles or toxin-producing organisms – as it happens with bivalves and other filter feeders – or from other organisms that have accumulated the toxins through the food web, which is the case of some fishes – for example, barracuda in the Caribbean [191, 192] or amberjack in West Africa [193, 194] – that can accumulate ciguatoxins, primarily produced by the benthic dinoflagellate genus *Gambierdiscus*. Typical planktonic HABs affect mainly to filter feeders, even when other animals that consume them can also be affected, as crabs or lobsters [195, 196, 197 and references therein].

No method has been devised to reduce the accumulation in fishes, but some possibilities exist for filter feeders.

Absorption efficiency of organic matter (including toxins) is mainly regulated by the time that the ingested materials remain in the digestive system (GPT, gut passage time), which, in turn, depends on the volume of those materials. It is, therefore, dependent on the ratio between the toxic compound and the volume of the ingested materials (Moroño et al. [198] and reviewed in Blanco [189]). Sampayo et al. [199] showed that mussels accumulate less DSP toxins when the populations of the causative species (*Dinophysis* spp.) co-occurred with

other non-toxic populations. Using this knowledge to reduce accumulation in cultured or natural population of bivalves is, notwithstanding, very difficult because large amounts of microalgae –that are expensive to produce and dispense– should be supplied to large areas.

Several approaches have been used to try to accelerate the depuration of the accumulated toxins from bivalves, in general, with little or no success [200]. Changes in temperature, salinity, pH, as well as chlorination, ozonation, electric discharges, emersion, and other treatments have been tested [201-204, 177 and references therein], but none of them is currently in use. Supplying non-toxic food has been shown to accelerate the reduction of toxicity in some bivalves [200] but this is probably due to an increase of body weight more than to a decrease of toxin content, and consequently, this method cannot be expected to increase substantially the depuration rate. Moreover, the cost of supplying that additional food can be substantial, therefore limiting the possible use to very high-valued bivalve species. Some attempts to reduce toxin accumulation have been also made by adding N-acetylcysteine [205], emulsifiers [206], activated charcoal [207], synthetic resins [189], a sucrose polyester [189], or chitosan [208] in some cases with apparent success, but additional studies about their economic viability and about the combination of toxins, species and phases of toxin accumulation in which these compound can be used are still needed. Toxin accumulation has three main components that have different importance during the time-course of the intoxication-depuration process. During the early steps, toxins are mainly accumulated in the lumen of the digestive system and start to be accumulated inside the cells of the digestive gland. During the subsequent steps, the toxin is, first, mainly accumulated into the digestive gland cells and finally accumulated in other organs or tissues [189]. In the first phase, compounds that bind the toxins and that make them less efficiently absorbed, or compounds that accelerate the digestive system evacuation, can be effective but might have no effect on the toxin accumulation during other phases (probiotic bacteria, for example, can be used at this step [209]). During the second accumulation step, compounds that favour the efflux of the toxins by facilitating the activity of the involved membrane transporters or by activating the vesicular transport (with the formation or excretion spherules [210]) can be used, but those compounds or materials should be absorbed by the digestive cells and not digested by them (as probably is the case with the resin Diaion HP20 and Olestra (Procter & Gamble), a polyester of sucrose that have been shown to be effective in reducing okadaic acid accumulation [189]). In the third phase, probably, compounds with the capability to mobilize resources between organs or that increase the transfer from other organs to the two main excretory organs of bivalves (digestive gland and kidney) should be used.

As already commented in the previous section, the selection of strains with low rates of accumulation can be possible, at least for DSP toxins in mussels, as the heritability of this trait is higher than 33%.

4.3. Actions on the Final Product

4.3.1. Selection of Individuals

Once the organisms have been extracted from the sea a number of actions can be taken in order to avoid risk for consumers while minimizing economic losses.

When the organisms are large and high-valued, individual selection is possible. This method is being used, for example, for ciguatoxin-contaminated fishes in Canary Islands

(Spain). There, the current regulation establishes that fishes of certain species which exceed a species-dependent weight, have to be analysed for their ciguatoxin concentration and they are allowed to be commercialized only when the toxins do not exceed the established level [211].

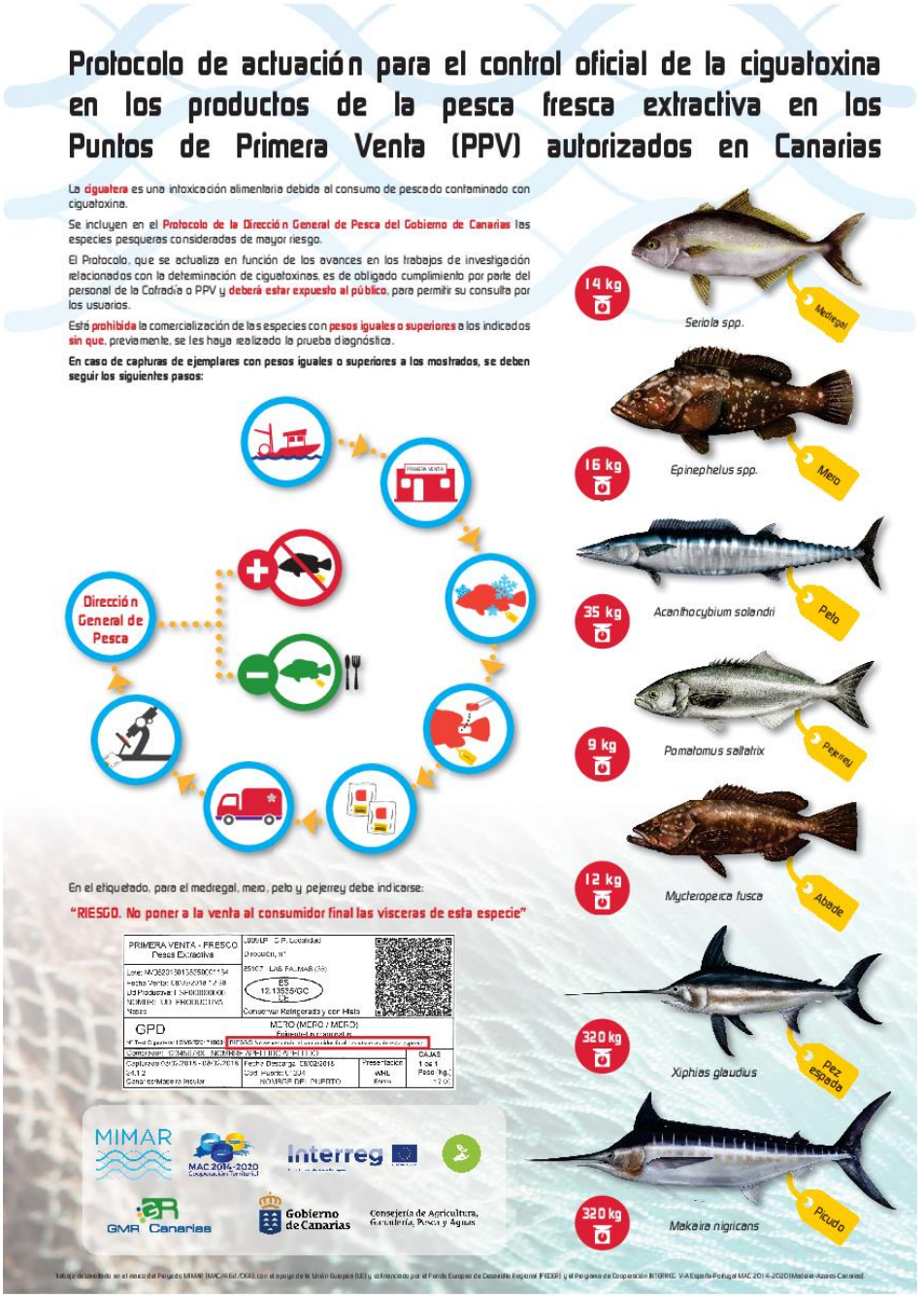

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Figure 6. Poster describing the procedure used in Canary Islands to select fishes to be analysed to determine their contents in ciguatoxins.

4.3.2. Selective Evisceration

Removing the most toxic organs or tissues from bivalves or fishes is another possibility to reduce the impact of toxins. For some fishes contaminated with ciguatoxins, removing viscera is, in some regulations, mandatory. The king scallop *Pecten maximus* which accumulates domoic acid (ASP toxin) for a long time, mostly (more than 90%) in the digestive gland can only be marketed, in Europe, after selectively removing that organ (and checking the levels in the remaining tissues) [212]. The possibility of effectively use this method strongly depends on the anatomical distribution of the toxin and the commercial value of the bivalves, as the processing step and the mandatory post-process analysis introduce a noticeable cost to the final product.

4.3.3. Industrial Processing

If the organisms are to be industrially transformed, some steps with the capability of reducing the concentration of some toxins can be included. PSP toxins in *Acanthocardia tuberculatum* are completely or nearly completely destroyed by some canning procedures [213] and this has led the European Union to implement an exception to the general regulation to allow the collection of this species, contaminated with PSP toxins, to be processed in that way [214]. Other cooking methods, especially in alkaline conditions, have been found experimentally to be effective with this group of toxins in other species [215]. Some bacteria can degrade PSP toxins but they have not been used in industrial processing [216, 217].

Thermal processes, like those used in cooking and canning, do not have relevant effects on toxins of the okadaic acid group [215, 218, 219]. Although some degradation of those toxins takes place at high temperatures it is compensated by the increase of toxicity due to dehydration [218, 219].

The toxins in this group can be extracted from bivalves using supercritical fluid extraction [220] but the organisms should be dehydrated, which limits the possible use of the procedure, and the economic aspects have not been evaluated.

5. REGULATION AND MONITORING

When human health is at risk because the blooming organism produces toxins, the allowable levels of these compounds in the products that are going to be consumed, have to be regulated, and how such products have to be monitored should be defined and implemented.

Establishing the allowable limits for the toxins is a difficult task for several reasons. When a toxin first appears, producing a human intoxication, it is necessary to identify and quantify the toxin in the food which caused the intoxication. Frequently, the precise product consumed is not available and the same organisms gathered from the same or nearby areas, at the same time or sometime later (usually days), should be used, thus adding uncertainty to the estimations. Additionally, nor optimized methods for the determination of the toxins neither reference materials are usually available. These drawbacks make the estimates of the LOAEL (lowest observed adverse effect level), and other parameters needed to establish a safe level, very imprecise. Reasonably, Health Authorities lower the allowable levels to include this

uncertainty (and others, as body weight, sensitivity or impairment of some metabolic functions) in order to protect the consumers. For producers, notwithstanding, this means that the periods in which marketing of their products is closed can be longer than necessary. When analogues of the known toxins or other products which are shown to be toxic in the usual assays, have to be regulated, the situation is even more complex because no data about their actual effect on humans exist (but they are expected to have one) and consequently, their toxicity has to be indirectly estimated.

The effects of differences in regulated levels can be important because, in most cases, toxin depuration takes place following an exponential decay. A reduction from 800 µg/kg of PSP toxins to approximately 400, for example, would extend the closure period in some mussel cultures of Galicia, for even more than 200% [221].

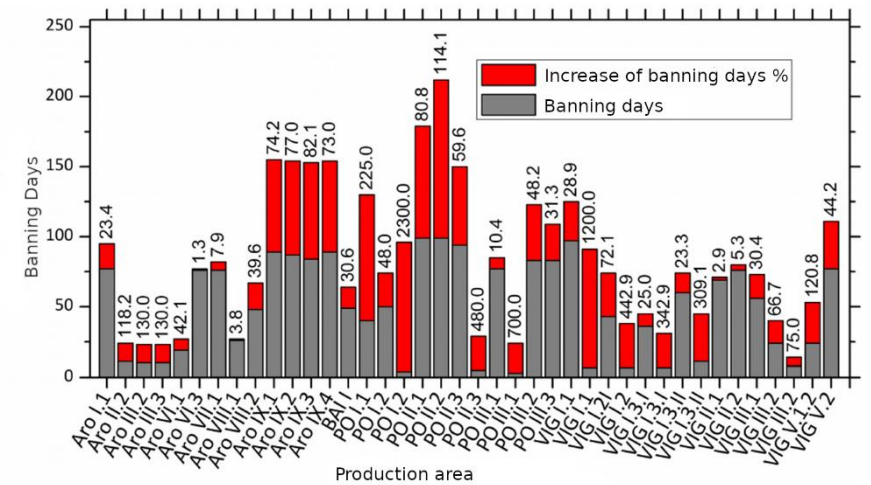

Figure 7. Extension of the banning period if the limit of quantification of bioassay, instead of the current regulatory limit, was taking as allowable level, during a *Gymnodinium catenatum* bloom in 2005 in Galicia. The numbers above the bars are the percentage of extension. The production areas are coded as Ría-Zone-Subzone (ARO= Arousa, BAI= Baiona, PO= Pontevedra, VIG= Vigo). [Modified from 221].

Detailed studies of the actual toxicity and the strictly required allowable toxin levels are, therefore, a tool to mitigate the impact of toxic blooms.

To ensure that the products which reach the market do not surpass the levels established in the regulation, different methods are used. In Europe (and in all countries that sell their products in Europe) several control steps have been regulated, which can be grouped into two categories [222]: pre-harvest and post-harvest. The first one is in charge of the competent authorities (CA), and the second one is in charge of each intermediary between the sea and the final consumer ("from sea to fork"). If the product at any step is shown to surpass the allowed levels, it should be destroyed or re-installed in the aquaculture facilities or the production areas.

The pre-harvest controls (used in Europe and some countries outside Europe) require the implementation of monitoring systems, which frequently (always in Europe) include periodic (usually weekly) determinations of the concentration of dangerous phytoplankton in water and of toxins in bivalves (or fishes, in some cases) from the harvested areas. The costs of

these monitoring systems are not low and frequently lay between 3 and 5% of the product's first-sale value and their structure is usually defined by the cost/benefit ratio. Developing and using cost-effective methodologies to determine phytoplankton and toxin concentration in the organisms can considerably reduce the cost of running the monitoring. Optimizing the sampling design and strategies, mainly by means of risk analysis and predictive modelling, can reduce the number of samples to be processed, reducing the cost, or allow to subdivide the monitored areas, maintaining the cost but increasing the benefit for the producers, and therefore, in both cases, reducing the cost/benefit ratio and contributing in that way to mitigate the consequences of toxic blooms.

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Chapter 155

U.S. OFFSHORE AQUACULTURE REGULATION AND DEVELOPMENT*

Harold F. Upton

ABSTRACT

Regulatory uncertainty has been identified as one of the main barriers to offshore aquaculture development in the United States. Many industry observers have emphasized that congressional action may be necessary to provide statutory authority to develop aquaculture in offshore areas. Offshore aquaculture is generally defined as the rearing of marine organisms in ocean waters beyond significant coastal influence, primarily in the federal waters of the exclusive economic zone (EEZ). Establishing an offshore aquaculture operation is contingent on obtaining several federal permits and fulfilling a number of additional consultation and review requirements from different federal agencies responsible for various general authorities that apply to aquaculture. However, there is no explicit statutory authority for permitting and developing aquaculture in federal waters. The aquaculture permit and consultation process in federal waters has been described as complex, time consuming, and difficult to navigate.

Supporters of aquaculture have asserted that development of the industry, especially in offshore areas, has significant potential to increase U.S. seafood production and provide economic opportunities for coastal communities. Currently, marine aquaculture facilities are located in nearshore state waters. Although there are some research-focused and proposed commercial offshore facilities, no commercial facilities are currently operating in U.S. federal waters. Aquaculture supporters note that the extensive U.S. coastline and adjacent U.S. ocean waters provide potential sites for offshore aquaculture development. They reason that by moving offshore, aquaculturalists can avoid many user conflicts they have encountered in inshore areas. Offshore areas also are considered to be less prone to pollution and fish diseases.

Environmental organizations and fishermen generally have opposed development of offshore aquaculture. They assert that poorly regulated aquaculture development in inshore areas has degraded the environment and harmed wild fish populations and ecosystems. Those who oppose aquaculture development generally advocate for new

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authorities to regulate offshore aquaculture and to safeguard the environment and other uses of offshore waters. Some segments of the commercial fishing industry also have expressed concerns with potential development of aquaculture on fishing grounds and competition between cultured and wild products in domestic markets.

Proponents of aquaculture counter that in many parts of the world a combination of farming experiences, technological advances, proper siting, and industry regulation has decreased environmental impacts and improved efficiency of marine aquaculture. They argue that many who oppose marine aquaculture lack an understanding of the benefits and risks of aquaculture and that opposition persists despite research that contradicts the extent or existence of these risks.

Generally, the outcomes associated with aquaculture development depend on a variety of factors, such as the characteristics of aquaculture sites, species, technology, and facility management. Regardless of potential environmental harm, it remains to be seen whether moving to offshore areas would be profitable and if offshore aquaculture could compete with inshore aquaculture development and lower costs in other countries.

Comprehensive offshore aquaculture bills were introduced in the 109th, 110th, 111th, 112th, and 115th Congresses, but none were enacted. In the 115th Congress, the Advancing the Quality and Understanding of American Aquaculture Act (AQUAA; S. 3138 and H.R. 6966) was introduced; AQUAA would have established a regulatory framework for aquaculture development in federal waters. It also would have provided National Oceanic and Atmospheric Administration (NOAA) Fisheries with the authority to issue aquaculture permits and coordinate with other federal agencies that have permitting and consultative responsibilities. Conversely, since the 109th Congress, bills have been introduced that would constrain or prohibit the permitting of aquaculture in the EEZ. The Keep Finfish Free Act of 2019 (H.R. 2467), introduced in the 116th Congress, would prohibit the issuance of permits to conduct finfish aquaculture in the EEZ until a law is enacted that allows such action. It remains an open question whether legislation could be crafted that would provide the regulatory framework desired by potential commercial developers of offshore aquaculture and avoid or minimize risks of environmental harm to the satisfaction of those currently opposed to offshore aquaculture development.

INTRODUCTION

Offshore aquaculture is generally defined as the rearing of marine organisms in ocean waters beyond significant coastal influence, primarily in the federal waters of the exclusive economic zone (EEZ).[1] Currently, marine aquaculture facilities are located in nearshore state waters, but no commercial facilities operate in U.S. federal waters. Some aquaculture advocates contend that developing such offshore aquaculture facilities could increase U.S. seafood production and provide economic opportunities for coastal communities; opponents counter that doing so could harm the environment and have negative impacts on other coastal activities, such as fishing.

Offshore aquaculture development will likely depend on several interrelated legal and institutional requisites, such as establishing a regulatory framework, minimizing environmental harm, and developing the capacity to manage and support the industry. Regulatory uncertainty has been identified as one of the main barriers to developing offshore

[1] Proclamation 5030, "Exclusive Economic Zone of the United States of America," March 10, 1983.

aquaculture in federal waters of the United States.[2] According to the U.S. Commission on Ocean Policy, "aquaculture operations in offshore waters lack a clear regulatory regime, and questions about exclusive access have created an environment of uncertainty that is detrimental to investment in the industry."[3] Some observers have concluded that "offshore aquaculture will not fully develop unless governments create a supportive political climate and resulting regulatory conditions."[4] A framework also may be needed to assure environmentalists, fishermen, and other stakeholders that coastal and fisheries managers would have the authority to address potential threats to the environment and other impacts.

According to most observers, congressional action may be necessary to develop a comprehensive regulatory framework for offshore aquaculture. Comprehensive legislation has been introduced a number of times since the 109th Congress, but none of the bills have been enacted. Controversy has stemmed from different perspectives of aquaculturalists, environmentalists, fishermen, and others. Some environmental organizations and fishermen have asserted that poorly regulated aquaculture development has degraded the environment and harmed wild fish populations and ecosystems.[5] Some segments of the commercial fishing industry are opposed to marine aquaculture because of potential development on fishing grounds, environmental effects on fish populations, and competition of cultured products with wild products in domestic markets. Offshore aquaculture advocates counter that a combination of farming experiences, technological advances, proper siting, and industry regulation has decreased environmental impacts and improved the efficiency of marine aquaculture. It appears that renewed efforts have emerged in the 116th Congress to meet current challenges by attempting to improve regulatory efficiency, minimize environmental degradation, and avoid impacts on existing ocean uses.

Additional related factors, such as technical advances, economic feasibility, and the level of government support, also are likely to affect future growth of the U.S. aquaculture industry. Although a regulatory framework appears to be necessary for establishing offshore aquaculture in federal waters, it may not be sufficient for significant development of the industry. Sometimes overlooked are the services that may be needed to establish a new industry, such as program administration, research, and other services (e.g., disaster assistance, insurance). Technical uncertainties related to harsher offshore environmental conditions and higher costs of operating farther from shore may slow extensive offshore development, especially in the immediate future.

This report examines issues and challenges related to the development of offshore aquaculture in federal waters.[6] It introduces the topic with background information that

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[2] U.S. Congress, Senate Committee on Commerce, Science, and Transportation, Ocean Policy Study, *Statement of William T. Hogarth, Assistance Administrator, National Marine Fisheries Service*, Hearing on Offshore Aquaculture, 109th Cong., 2nd sess., April 6, 2006.

[3] U.S. Commission on Ocean Policy, *An Ocean Blueprint for the 21st Century*: *Final Report*, p. 330. Hereinafter cited as Oceans Commission, *Ocean Blueprint.*

[4] John S. Corbin, John Holmyard, and Scott Lindell, "Aquaculture Perspectives of Multi-use Sites in the Open Ocean," in *Regulation and Permitting of Standalone and Co-located Open Ocean Aquaculture Facilities* (Springer, 2017), pp. 187-229.

[5] Center for Food Safety, Fishing and Public Interest Groups File Challenge to Fed's Unprecedented Decision to Establish Aquaculture in Offshore U.S. Waters, February 16, 2016, at https://www.centerforfoodsafety.org/press-releases/4229/fishing-and-public-interest-groups-file-challenge-to-feds-unprecedented-decision-to-establish-aquaculture-in-offshore-us-waters.

[6] Comparisons and references are made to inshore and land-based aquaculture, but the focus of this report is offshore aquaculture.

covers aquaculture production and methods, federal agencies involved in aquaculture, and potential congressional interest in the topic. It then focuses on three of the main challenges faced by the industry, including the current regulatory framework, environmental concerns, and economic viability. The report concludes with issues related to regulatory and institutional development that have been identified by researchers and stakeholders, potential issues for Congress, and a summary of legislation that has been introduced in recent Congresses.

BACKGROUND

Seafood Production

Global aquaculture production is nearly equal to the volume of seafood produced for human consumption by wild fisheries.[7] From 1997 to 2016, world seafood production from wild sources (capture fisheries) leveled off at a range of 89 million metric tons (mmt) to 96 mmt.[8] According to the United Nations Food and Agriculture Organization, further growth of global wild fisheries production is unlikely, because approximately 93% of marine stocks are now either fished unsustainably or fished at maximum sustainable levels.[9] During the same period, world aquaculture production increased from 28.3 mmt to 80.0 mmt; it now makes up 47% of global fish production.[10] It is likely that aquaculture production will continue to expand with advances in aquaculture technologies and the need to satisfy the demand of the world's growing population.[11] Figure 1 illustrates the growth in global aquaculture production and relatively constant wild fisheries production. Nearly all of global marine aquaculture production is from inshore areas, such as estuaries and coastal areas, not from offshore areas.

Wild fisheries in the United States are limited by the productive capacity of U.S. waters. Most U.S. stocks are now fished at their maximum sustainable levels. However, unlike worldwide trends, U.S. aquaculture production has generally stagnated and makes up a relatively small portion of total U.S. seafood production. In 2016, the United States ranked fifth in global seafood production at 5.36 mmt; 0.44 mmt (8.2%) of this total was produced by aquaculture.[12] Figure 2 illustrates the relatively constant domestic production of aquaculture and wild fisheries. Most U.S. aquaculture production consists of freshwater species, such as catfish, trout, and crawfish. Growth in U.S. seafood consumption has depended on imports, which provide approximately 80% to 90% of the seafood consumed in the United States.[13]

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[7] Global aquaculture totals include both freshwater and marine aquaculture.

[8] Food and Agriculture Organization (FAO), *The State of World Fisheries and Aquaculture 2018*: *Meeting the Sustainable Development Goals*, 2018. Hereinafter cited as FAO, *State of World Fisheries*. The total includes all fresh and marine landings, which was 90.9 mmt in 2016.

[9] FAO, *State of World Fisheries*, p. 40.

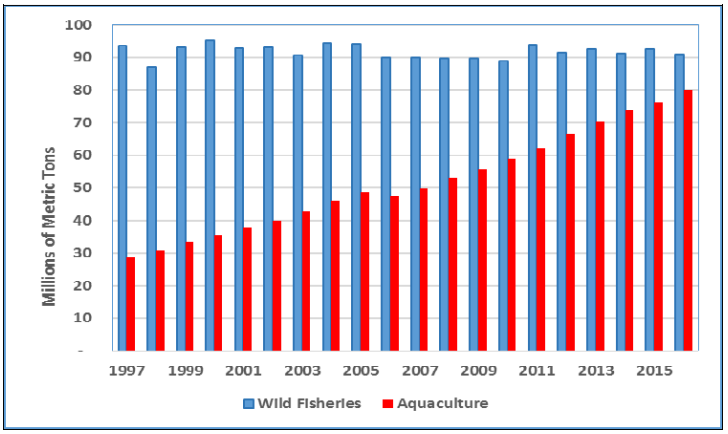
[10] Aquaculture represents 47% of all fish landings, including fish used for purposes other than direct human consumption such as fish meal and fish oil for animal feeds, and 53% of fish landed for direct human consumption. The term *fish* includes harvest of invertebrates such as crustaceans, mollusks, and echinoderms.

[11] FAO, *State of World Fisheries*, p. 182.

[12] U.S. total and aquaculture production reported to FAO includes shell weight of oysters and clams. The National Marine Fisheries Service (NMFS) reported U.S. aquaculture production in meat weight without shells. Thus, the NMFS reported figure was 0.29 million metric tons (mmt).

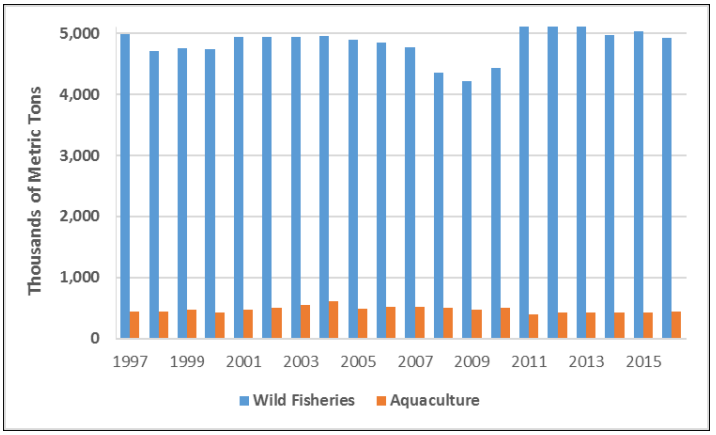
[13] NMFS, Office of Science and Technology, *Fisheries of the United States, 2017 Report*, Current Fishery Statistics No. 2017, September 2018, p. 114. Hereinafter cited as NMFS, *Fisheries of the United States*. A portion of

Approximately 50% of seafood imports, such as shrimp from Southeast Asia and salmon from Norway or Chile, are produced by aquaculture in ponds and nearshore areas. According to some observers, U.S. reliance on seafood imports will continue to increase without changes to current policies and regulatory obstacles that currently impede expansion of aquaculture.[14]



Source: National Marine Fisheries Service, *Fisheries of the United States* reports, 1999-2017.
Notes: Fisheries and aquaculture totals include marine and freshwater sources.

Figure 1. Global Wild Fisheries and Aquaculture Production (1997-2016).



Source: National Marine Fisheries Service, *Fisheries of the United States* reports, 1999-2017.
Notes: Aquaculture totals include marine and freshwater production.

Figure 2. U.S. Wild Fisheries and Aquaculture Production (1997-2016).

Aquaculture Overview

Aquaculture is broadly defined as the propagation and rearing of aquatic species in controlled or selected environments.[15] Aquaculture is difficult to characterize because of the

imports include domestic catch that was exported for further processing and returned to the United States as an import in processed form.

[14] Hauke L. Kite-Powell, Michael C. Rubino, and Bruce Morehead, "The Future of U.S. Seafood Supply," *Aquaculture Economics & Management*, vol. 17, no. 3 (August 2013), p. 229.

[15] This definition of aquaculture is from the Aquaculture Act of 1980 (16 U.S.C. §1802(1)).

diverse nature of facilities, methods, technologies, and species that are cultured. Organisms are cultured in freshwater environments, land-based closed systems, coastal and estuarine areas, and offshore areas.[16] Often, hatcheries are used to spawn fish and shellfish to produce eggs that are hatched and grown to specific stages; these organisms are then transferred to facilities where they are grown to marketable size.

Aquaculture operations range from systems where there is only minimal control over the organism's environment to intensive operations where there is complete control at each stage of the organism's life history. For example, an intensive system would include freshwater species such as catfish that are often raised in shallow earthen ponds; production relies on control of inputs. Water, feed, and disease treatment are controlled to maximize growth while minimizing costs. Farming of finfish, such as salmon, also requires stocking at high densities and relies on extensive feeding. Commercial salmon aquaculture facilities often employ net pens (Figure 3), which are moored to the bottom and located in protected inshore marine areas, such as bays and fjords.[17]

Bivalves such as oysters and clams are grown in estuaries and inshore areas, feeding on a diet of plankton and detritus that they filter from seawater. Bivalve aquaculture may employ varying degrees of control. In some cases, they are suspended on lines, in wire cages, and on rafts. Oyster larvae are grown in hatcheries and transferred to these structures as oyster spat or seed and grown to market size. Some oyster production is less intensive and depends on enhancement of the benthic (ocean bottom) environment by placing oyster shells on the bottom to facilitate attachment of wild oyster larvae.[18]

In Alaska, hatcheries are used to enhance the production of salmon fry, which are released to the wild to feed and grow until they are caught by fishermen as adults. These programs are run as nonprofit cooperatives overseen by Alaska fishermen.[19] Most states and the U.S. Fish and Wildlife Service run public stocking programs, which often address a variety of objectives such as enhancing recreational fisheries and restoring depleted populations. Each strategy requires different inputs and interacts with the environment to differing degrees. Nevertheless, a common factor is to control some aspect(s) of the organism's life to enhance survival and growth.

Over the last decade, catfish aquaculture has accounted for most food fish production by volume and revenue in the United States (Table 1). However, catfish production has declined by nearly 44% over this period due to a variety of factors, including competition from Asian imports. For freshwater species, only crawfish production (78.0%) and revenue (66.2%) increased significantly. During the same period, production of salmon and oysters increased in both volume and revenue. Cultured oysters exhibited the largest increases in production (66.0%) and revenue (86.5%), which is likely related to greater demand for high quality raw

[16] Most production is from freshwater and coastal areas, whereas offshore and closed systems account for only a small portion of global and domestic production.

[17] Most commercial salmon aquaculture facilities use net pens, floating enclosures that are anchored to the ocean bottom. The enclosures are separated from the environment by netting which allows for the free exchange of water and fish wastes between the enclosure and the environment. Salmon aquaculture also can be conducted in land-based tanks and raceways.

[18] In other cases, small oysters (spat) may already be attached to the shell when it is placed on the bottom of the estuary.

[19] Alaska Department of Fish and Game, Hatcheries at https://www.adfg.alaska.gov/index.cfm? adfg=fishing Hatcheries.main.

oysters.[20] However, except for cultured oysters, production of most domestic marine seafood products is from wild marine fisheries.[21]

Table 1. U.S. Aquaculture production and revenue (2006 and 2016)

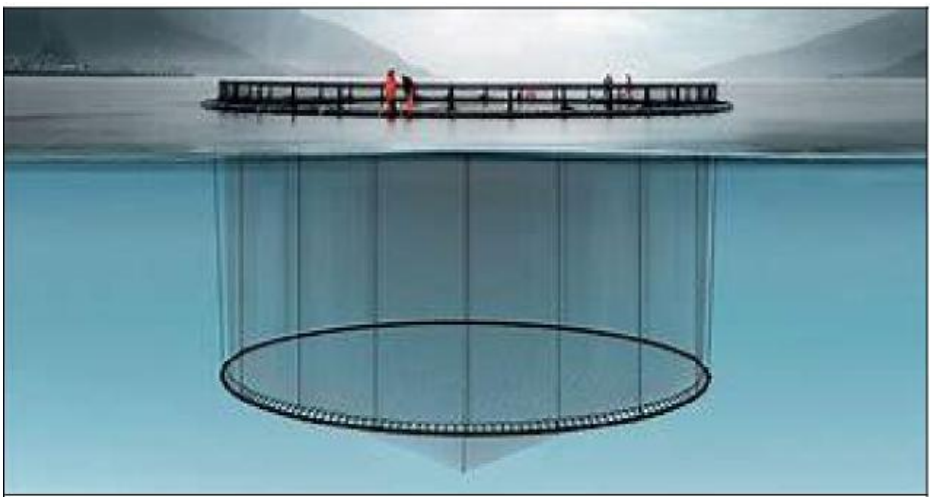
Species	Production (thousands of pounds)		Production (metric tons)		Revenue ($ in thousands)	
	2006a	2016	2006	2016	2006b	2016
Freshwater						
Catfish	568,900	320,174	258,049	145,230	$519,015	$363,075
Crawfish	83,714	149,015	37,972	67,593	118,356	196,695
Trout	49,659	48,451	22,525	21,977	67,824	79,558
Tilapia	20,000	18,999	9,072	8,618	40,441	42,745
Striped Bass	11,925	10,322	5,409	4,682	35,360	37,737
Total Freshwater	*734,198*	*546,961*	*333,027*	*248,100*	*$780,996*	*$719,810*
Marine						
Salmon	23,115	35,682	10,485	16,185	$50,070	$67,654
Oysters	22,046	36,601	10,000	16,602	103,103	192,328
Clams	11,307	9.722	5,129	4,410	88,635	137,793
Shrimp	7.800	3,600	3,538	1,633	19,226	10,075
Mussels	1,008	859	457	325	8,382	10,201
Total Marine	*65,276*	*86,499*	*29,609*	*43,790*	*$269,416*	*$393,998*
Miscellaneous/ Otherc	—	—	—	—	404,265	315,944
Totals	*799,474*	*633,460*	*362,636*	*287,336*	*$1,454,677*	*$1,454,080*

Sources: NMFS, Office of Science and Technology, *Fisheries of the United States, 2017*, Current Fishery Statistics. No. 2017, September 2018, and NMFS, Office of Science and Technology, *Fisheries of the United States, 2012*, Current Fishery Statistics No. 2012, September 2013.

[a]Clams, oysters, and mussels are reported as meat weight, whereas all other species are reported as whole live weight.

[b]Aquaculture revenue in 2006 is provided in real 2016 dollars as calculated using U.S. Bureau of Economic Analysis, Interactive Data Application, Table 1.1.9 Implicit Price Deflators for GDP, at https://www.bea.gov/ itable/.

[c]The miscellaneous category was only reported by value and includes baitfish, ornamental/tropical fish, alligators, algae, aquatic plants, and others.



Source: AKVA Group, https://www.akvagroup.com/news/image-gallery.

Figure 3. Example of a Salmon Net Pen.

[20] Cultured oysters provide a consistent and aesthetically pleasing product for the raw oyster market.

[21] NMFS, *Fisheries of the United States*.

Offshore Aquaculture

As stated above, offshore aquaculture is the rearing of marine organisms in ocean waters beyond significant coastal influence, primarily in the federal waters of the EEZ. Aquaculturalists, the Department of Commerce, several task force and commission reports, and some academics have identified offshore aquaculture as a potential alternative to some land-based and nearshore aquaculture. Supporters of aquaculture have asserted that development of the industry, especially in offshore areas, has significant potential to increase U.S. seafood production and provide economic opportunities for coastal communities. The potential of offshore aquaculture in the United States is likely to differ by species, region, and technology.[22]

Despite plans for several offshore operations, no commercial offshore aquaculture facilities are currently operating in the U.S. EEZ. Some marine aquaculture facilities are located in nearshore state waters, however. In the future, inshore marine production is likely to be constrained by the availability of suitable sites, poor water quality, high coastal land values, and competition with other ocean uses.[23] Potential aquaculture development in offshore areas has received increasing attention because of these limitations.

The cost of working offshore may be greater than the costs of working in inshore and land-based areas, in part because offshore aquaculture in the EEZ would be subject to relatively high-energy offshore environments caused by high and variable winds and storms.[24] However, research and technical advances have demonstrated that operating in these environments is feasible. Expansion of offshore aquaculture into clean, well-flushed waters appears to have nearly unlimited potential, although major technological and operational challenges remain.[25] For example, further development will require structures and materials that will contain stocks under harsh oceanic conditions and keep costs low enough to remain profitable.[26]

It is likely that offshore aquaculture, at least initially, would employ species with established markets and production systems that are similar to those used in inshore areas.[27] Examples of marine species that are candidates for offshore areas may include Atlantic salmon (*Salmo salar*), white sea bass (*Atractoscion nobils*), cobia (*Rachycentron canadum*),

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[22] Gunnar Knapp, "Economic Potential for U.S. Offshore Aquaculture: An Analytical Approach," in *Offshore Aquaculture in the United States: Economic Considerations, Implications, and Opportunities*, NOAA, NOAA Technical Memorandum NMFS F/SPO-103, July 2008, pp. 15-50. Hereinafter cited as Knapp, "Economic Potential."

[23] B. Cicin-Sain et al., *An Operational Framework for Offshore Marine Aquaculture in Federal Waters*, Center for Marine Policy, University of Delaware, 2005.

[24] This report uses the terms *open ocean* and *offshore* interchangeably to refer to aquaculture in federal waters in the exclusive economic zone (EEZ). *Federal waters* and *EEZ* are also used interchangeably to refer to water ranging from 3 nautical miles (nm) to 200 nm from shore.

[25] Peter Edwards, "Aquaculture Environment Interactions: Past, Present and Likely Future Trends," *Aquaculture*, vol. 447 (2015), pp. 2-14. Hereinafter cited as Edwards, "Aquaculture Environment Interactions."

[26] John Forster, *Emerging Technologies in Marine Aquaculture*, ed. NOAA Aquaculture Program (Silver Spring, MD: National Marine Fisheries Service, 2008), pp. 51-71. Hereinafter cited as Forster, *Emerging Technologies*.

[27] James McDaid Kapetsky, Jose Aguilar-Manjarrez, and Jeff Jenness, *A Global Assessment of Offshore Mariculture Potential from a Spatial Perspective*, FAO, FAO Fisheries and Aquaculture Technical Paper 549, 2013.

and blue mussel (*Mytilus edulis*).[28] Currently, salmon net pen facilities operate in protected inshore waters of Maine and Washington. Several other net pen aquaculture facilities have operated in exposed state waters of Hawaii and Puerto Rico that have characteristics similar to those of offshore areas.[29] Over the last two decades, permits have been issued to conduct research and limited commercial aquaculture in the EEZ.[30] Recently, three mussel farms received permits from the U.S. Army Corps of Engineers (USACE) to operate in offshore waters. Several other ventures have been proposed;[31] including proposals to operate commercial facilities in several regions.

Researchers are developing systems to adapt facilities used in inshore areas to the unique needs of offshore aquaculture. Offshore systems (e.g., submersible cages, net pens, longline arrays) may be free-floating, secured to a structure, moored to the ocean bottom, or towed by a vessel. Systems have been developed to overcome problems associated with harsh open ocean conditions, including submersible cage designs that do not deform under strong currents and waves, and single-point moorings. Cage-mounted autonomous feeding systems have been developed that can operate both at the surface and submerged. Other components under development include mechanized and remote systems that can be controlled from land-based facilities; for example, universities and private-sector research interests are developing automated buoys that can monitor the condition of stock and feed fish on a regular basis for weeks at a time.[32]

FEDERAL GOVERNMENT INVOLVEMENT IN AQUACULTURE

Federal aquaculture, regulation, research, and support are conducted by a number of federal agencies. Their roles vary widely depending on the agency's statutory responsibilities, which may be related directly or indirectly to aquaculture. Congress enacted the National Aquaculture Act of 1980 to encourage development of the aquaculture industry and coordinate federal activities.[33] The act established the Subcommittee on Aquaculture (SCA) to provide opportunities to exchange information and enhance cooperation among federal agencies.[34] SCA's main functions include the following:

[28] There are many potential candidates, and this list includes only selected species commonly considered in the press or aquaculture trade literature.

[29] It appears that only one of these facilities is currently operating in the United States. See State of Hawaii, Animal Industry Division, "Open Ocean Fish Farming," at http://hdoa.hawaii.gov/ai/aquaculture-and-livestock-supportservices-branch/open-ocean-fish-farming/.

[30] No commercial production statistics are available for these cases, and production has not been significant. Examples include blue mussel and scallop culture off New England. In the Southeast, permits have been issued for live rock aquaculture that provides material for use in aquaria.

[31] NMFS, "NOAA Expands Opportunities for U.S. Aquaculture," press release, January 11, 2016, https://www.fisheries.noaa.gov/media-release/noaa-expands-opportunities-us-aquaculture. Proposals have included mussel and seaweed aquaculture off California and striped bass net pen culture off Long Island, NY. According to the National Oceanic and Atmospheric Administration's (NOAA's) aquaculture website, as of January 2016 there were no commercial aquaculture facilities operating in the EEZ.

[32] Forster, *Emerging Technologies*.

[33] 16 U.S.C. §§2801 et seq.

[34] The Subcommittee on Aquaculture was known previously as the Interagency Working Group on Aquaculture and initially as the Joint Subcommittee on Aquaculture.

- reviewing national needs for aquaculture research, technology transfer, and technology assistance programs;
- supporting coordination and communication among federal agencies engaged in the science, engineering, and technology of aquaculture;
- collecting and disseminating information on aquaculture;
- encouraging joint programs among federal agencies in areas of mutual interest relating to aquaculture; and
- recommending specific actions on issues, problems, plans, and programs in aquaculture.[35]

SCA operates under the Committee on Environment of the National Science and Technology Council in the Executive Office of the President. SCA is chaired by the Secretary of Agriculture, in consultation with the Secretaries of Commerce and the Interior. In addition to the three main departments, SCA includes nine additional departments and agencies with an interest in aquaculture.[36] SCA meets quarterly and has provided information on topics such as fish disease, aquaculture regulation, and other areas of interest.

Most federal aquaculture activities and programs that are specific to aquaculture are carried out by the Department of the Interior (DOI), Department of Commerce (DOC), and the Department of Agriculture (USDA). Other federal agencies have roles that are indirectly related to aquaculture, such as regulatory programs that apply to a variety of aquatic or marine activities, including aquaculture. Examples include USACE for activities in navigable waters, the Environmental Protection Agency (EPA) for protection of environmental quality, and the Food and Drug Administration for regulation of drugs used to treat fish diseases.

U.S. Department of Agriculture

USDA plays a lead role in support of freshwater aquaculture for species such as catfish that are raised on private property in fishponds. USDA is authorized to conduct cooperative research and extension: it funds five aquaculture regional research centers. Work at aquaculture centers complements other USDA research and education programs undertaken at state land-grant universities. The USDA National Agricultural Statistics Service periodically conducts the national aquaculture census and collects and publishes other related statistical information. The Animal and Plant Inspection Service provides animal health certifications for exports of live species and products; assistance for producers experiencing losses from predators; and veterinary biologics for preventing and treating animal diseases, including those affecting aquatic species. The Farm Service Agency administers farm lending programs, including ownership, operating, and emergency disaster loans. Under certain circumstances, aquaculture operations may be eligible for disaster assistance under the Noninsured Crop Disaster Assistance Program and the Emergency Assistance for Livestock,

[35] National Institute of Food and Agriculture, USDA, "Notice of Public Meeting for the IWGA of the Committee on Science of the National Science and Technology Council," 82 *Federal Register* 4026-4027, January 29, 2018.

[36] Department of Energy, Department of Health and Human Services, Environmental Protection Agency, Agency for International Development, Small Business Administration, National Science Foundation, Farm Credit Administration, Tennessee Valley Authority, and U.S. Army Corps of Engineers.

Honeybees, and Farm-Raised Fish Program.[37] It appears that some of USDA's programs and experiences that focus on land-based agriculture, such as finance, research, disaster assistance, marketing, and extension, may be adapted and applied to marine aquaculture development.

Department of the Interior

DOI's U.S. Fish and Wildlife Service (FWS) focuses on support of public efforts, such as stocking programs, that benefit recreational fishing of freshwater and anadromous species. FWS operates the National Fish Hatchery System, which consists of more than 60 facilities used to enhance fish stocks, restore fish populations, and mitigate fish losses. The system includes fish production and distribution facilities, fish health centers, fish passage facilities, and technology centers. FWS research programs indirectly benefit the private sector through research and applications that control fish disease and regulation of potentially invasive species. FWS and NMFS are responsible for regulating potential interactions between aquaculture activities and endangered species and marine mammals under the Endangered Species Act (ESA) and the Marine Mammal Protection Act (MMPA).[38]

Department of Commerce

The NMFS Office of Aquaculture in DOC focuses on regulatory, technical, and scientific services related to marine aquaculture. NOAA headquarters provides general direction for the program and coordinates with other NOAA offices, federal agencies, and the general public. The program includes five regional aquaculture coordinators, who coordinate regulatory and permitting activities, serve as liaisons with the state and local government and stockholders, and assist with grant management. Aquaculture in federal waters is regulated as a regional fishery under the Magnuson Stevens Fishery Conservation and Management Act (MSA).[39] NOAA's efforts to regulate offshore aquaculture are discussed in the following section concerning federal agency regulatory responsibilities (see Current Regulatory Framework).

In October 2015, NOAA released its five-year strategic plan (2016-2020) for marine aquaculture.[40] NOAA's vision is a "robust U.S. marine aquaculture sector that creates jobs, provides sustainable seafood, and supports a healthy ocean." The plan provides a blueprint of NOAA's involvement in marine aquaculture, including program impact, goals and strategies, deliverables, and crosscutting strategies. To increase aquaculture production, the program's four main goals are to

- develop coordinated, consistent, and efficient regulatory processes for the marine aquaculture sector;

[37] For further discussion, see CRS Report RS21212, *Agricultural Disaster Assistance*, by Megan Stubbs.
[38] 16 U.S.C. §§1531 et seq. (ESA) and 16 U.S.C. §§1361 et seq. (MMPA).
[39] 16 U.S.C. §§1801 et seq.
[40] Office of Aquaculture, *Marine Aquaculture Strategic Plan FY2016-2020*, NMFS, February 2015, https://www.fisheries.noaa.gov/aquaculture-library.

- encourage environmentally responsible marine aquaculture using the best available science;
- develop technologies and provide extension services for the aquaculture sector; and
- improve public understanding of marine aquaculture.

The plan also includes four crosscutting strategies to achieve these goals and objectives:

- strengthen government, academic, industry, and other partnerships;
- improve communications within NOAA;
- build agency infrastructure within NOAA; and
- develop sound and consistent management within NOAA.

Various NOAA programs may support aquaculture both directly and indirectly. The National Sea Grant Marine Aquaculture Grant Program is the only U.S. government grant program that funds marine aquaculture exclusively.[41] These grants focus on industry challenges, such as improving aquaculture feeds, enhancing seafood safety and quality, refining culture methods, and diversifying aquaculture species.[42] Other NOAA offices or programs that may contribute to or become involved in aquaculture development include inspections provided by the NOAA Seafood Inspection Program, research conducted at NOAA regional fisheries science centers, and awards funded by the Saltonstall-Kennedy Grant Program.[43]

OFFSHORE AQUACULTURE CHALLENGES

A broad array of challenges is associated with offshore aquaculture development and expansion. These challenges pertain to evolving production technology, uncertain economic costs and benefits, and potential environmental and social impacts. Generalizations about how to address these challenges are difficult to make because of the variety of candidate species, different technologies, and potential scales of operation.

Major categories of concerns related to offshore aquaculture development include (1) the legal and regulatory environment; (2) potential environmental harm; (3) economic, trade, and stakeholder concerns related to development of a new industry; and (4) business and institutional support.[44]

[41] The general mission of the Sea Grant College Program is to enhance the practical use and conservation of coastal, marine, and Great Lakes resources to provide for a sustainable economy and environment.

[42] NOAA Sea Grant, "Sea Grant in Aquaculture," at https://seagrant.noaa.gov/Our-Work/ Aquaculture.

[43] NMFS, "Saltonstall-Kennedy Grant Program," at https://www.fisheries.noaa.gov/grant/ saltonstall-kennedy-grantprogram.

[44] Detailed discussions of many of the issues discussed in this section are available in *Development of a Policy Framework for Offshore Marine Aquaculture in the 3-200 Mile U.S. Ocean Zone* (2001) by the University of Delaware's Center for the Study of Marine Policy, at http://darc.cms.udel.edu/sgeez/sgeez1final.pdf; and *Recommendations for an Operational Framework for Offshore Aquaculture in U.S. Federal Waters* (October 2005) by the University of Delaware's Gerard J. Mangone Center for Marine Policy, at http://darc.cms.udel.edu/sgeez/ sgeez2final.pdf.

Current Regulatory Framework[45]

One of the main issues associated with marine offshore aquaculture is the concept of ownership and individuals' rights to use the marine environment for economic gain (in contrast to, for example, the catfish industry, where fishponds are constructed and operated on private land). Some envision development and management as a partnership, where the government's role is one of both enabler and steward.[46] This partnership could provide for property rights and regulatory clarity, certainty, and stability. For example, the government already provides specific rights to businesses that extract or use resources of the continental shelf, such as oil and gas and wind energy development.

Aquaculture regulation depends primarily on the geographic location and characteristics of aquaculture facilities. In state waters, in accordance with the federal Submerged Lands Act of 1953, coastal states exercise jurisdiction over an area extending 3 nautical miles (nm) from their officially recognized coast (or *baseline*).[47] States also have jurisdiction over internal waters, areas inside the baseline in bays and estuaries, such as the Chesapeake Bay or Puget Sound. States may impose restrictions or requirements as they see fit, subject to any applicable federal laws. If located in federal waters, in waters from 3 nm to 200 nm from the baseline, aquaculture facilities are regulated primarily by federal agencies under a number of federal statutes and regulatory requirements (Figure 4). Some federal laws apply to marine aquaculture and waters of the United States generally and include facilities located in both state and federal marine waters.

Marine Jurisdictional Zones

Federal management of marine fisheries generally extends from 3 nautical miles (nm) to 200 nm from shore (baseline). State waters are measured from the baseline to 3 nm offshore. Exceptions include the west coast of Florida, Texas, and the Commonwealth of Puerto Rico, where state and commonwealth waters extend out to 9 nm. Under international law, internal waters include those areas landward of the baseline, territorial waters include those waters from 0 nm to 12 nm seaward of the baseline, and the exclusive economic zone (EEZ) includes waters from 12 nm to 200 nm from the baseline. In the United States, fisheries in the territorial sea beyond state waters and in the EEZ are managed by the federal government under the MSA.

Internal Waters – waters landward of the baseline from which the territorial sea is measured. States manage internal waters.

Baseline – generally measured as the low-water line along the coast; also accounts for features such as bays, river mouths, and fringing reefs.

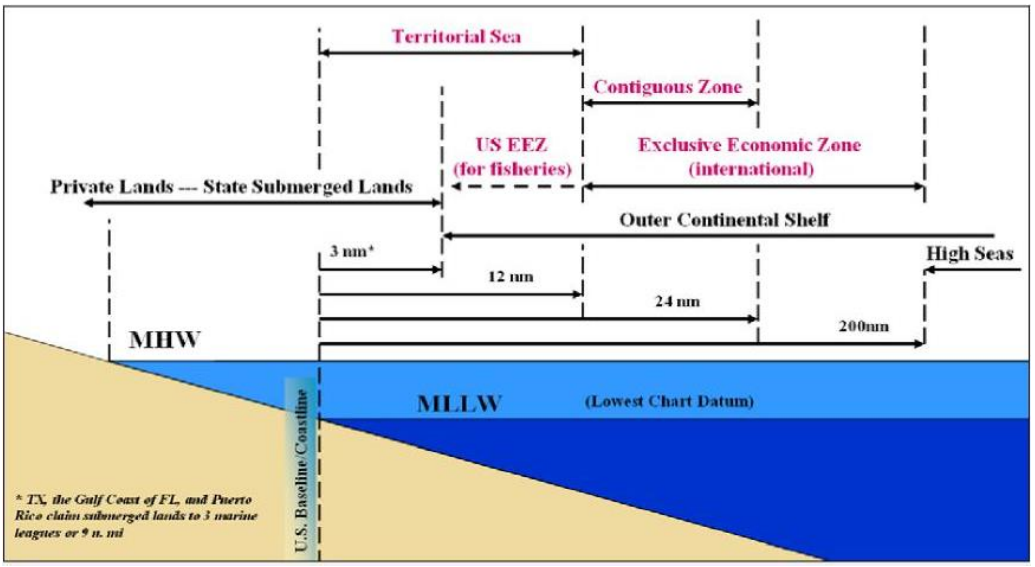
Territorial Sea – the coastal state (nation) may claim sovereignty over the territorial sea, the airspace above it, and the seabed and subsoil below it from 0 nm to 12 nm seaward of the baseline. In 1988, the United States claimed a territorial sea (Presidential Proclamation 5928), which includes both state waters (generally 0 nm to 3 nm and federal waters (generally from 3 nm to 12 nm).

Exclusive Economic Zone – the coastal state (nation) may claim sovereign rights for the purpose of exploring, exploiting, conserving, and managing natural resources, either living or nonliving, in the EEZ.

[45] Adam Vann of the CRS American Law Division contributed to this section.

[46] John Forster, "Broader Issues in the Offshore Fish Farming Debate," in *Offshore Aquaculture in the United States: Economic Considerations, Implications, and Opportunities*, NOAA, NOAA Technical Memorandum NMFS F/SPO103, July 2008, pp. 245-263. Hereinafter cited as Forster, "Broader Issues."

[47] 43 U.S.C. §1301(b).



Source: Meredith A. Westington and Matthew J. Slagel, *U.S. Maritime Zones and the Determination of the National Baseline*, NOAA, Office of the Coastal Survey, at http://ushydro.thsoa.org/hy07/11_01.pdf.

Notes: EEZ = exclusive economic zone; MHW = mean high water; MLLW = mean lower low water. The tidal datum is a standard elevation defined by a certain phase of the tide.

Figure 4. U.S. Maritime zones.

Currently, no single federal agency is authorized to approve or permit offshore aquaculture facilities in federal waters, generally the EEZ. USACE, NMFS (NOAA Fisheries), and EPA are separately authorized to regulate certain activities that are required to establish and operate aquaculture facilities.[48] Federal agencies that issue permits are required to consult with other regulatory agencies concerning the potential effects of each application. The permitting process also involves consultation and other requirements that are incorporated into the review of these applications. The following sections summarize the required federal permits, consultation, and review requirements.

Federal Permits to Conduct Aquaculture in the Federal Waters

Section 10 Permits

Section 10 of the Rivers and Harbors Act of 1899 (hereinafter referred to as Section 10) prohibits the unauthorized obstruction or alteration of any navigable water of the United States.[49] Authorization by the Secretary of the Army, through USACE, must be provided before construction is initiated. Construction may include any structure or work in or affecting the course, condition, or capacity of navigable waters, excavation or fill, including aquaculture facilities, in or over any navigable waters of the United States within 3 nm from

[48] Stephanie S. Otts and Terra Bowling, *Offshore Mussel Culture Operations: Current Legal Framework and Regulatory Authority*, National Sea Grant Law Center, April 2012.

[49] 33 U.S.C. §403.

shore. Because aquaculture facilities may be located in and may affect navigable waters, the developer of the facility may be required to obtain authorization from USACE under Section 10. USACE's role is to regulate the use of the navigable water (not to regulate aquaculture per se).

The Outer Continental Shelf Lands Act extends USACE authority over all artificial islands and all installations and other devices permanently or temporarily attached to the seabed, which may be erected for the purpose of exploring for, developing, or producing resources.[50] Therefore, a Section 10 permit is also required prior to construction or placement of installations—such as aquaculture facilities—in federal waters from the seaward limit of state waters to the seaward limit of the outer continental shelf.[51] The decision to issue a permit is based on the effects on navigation and the proposed activity's probable impacts on the public interest. The public interest is assessed by comparing the benefits that may be expected to accrue from the proposed activity and the reasonably foreseeable harm that reflects the national concern for the protection and use of important resources.[52] Offshore aquaculture permits would be required for structures such as cages, net pens, or lines that are anchored or attached to the sea floor.

Section 10 permit requirements for aquaculture development beyond 3 nm may differ from those within 3 nm, because installations or other devices that are not temporarily or permanently attached to the seabed do not appear to be included. Examples of facilities beyond 3 nm that may not require Section 10 permits include bottom shellfish culture or unmoored floating aquaculture facilities if they do not impede navigation.

National Pollutant Discharge Elimination System Permit

EPA protects water quality by regulating the discharges of pollutants into U.S. waters under the Clean Water Act (CWA).[53] Under the CWA, a National Pollutant Discharge Elimination System (NPDES) permit is required to discharge pollutants from point sources into federal ocean waters.[54] A *point source* is defined as "any discernable, confined and discrete conveyance, including but not limited to any pipe, ditch, channel, tunnel, conduit, well, discrete fissure, container, rolling stock, concentrated animal feeding operation, or vessel or other floating craft, from which pollutants are or may be discharged."[55]

Aquaculture facilities may discharge materials such as fecal matter; excess feed; antifoulants; and therapeutic agents, such as antibiotics. EPA currently regulates aquaculture facilities as a point source if the activity qualifies as a Concentrated Aquatic Animal Production (CAAP) facility;[56] CAAPs are defined according to discharge frequency and production level or as designated by EPA on a case-by-case basis if they are significant contributors of pollution.[57] Commercial scale aquaculture operations in federal waters would be likely to trigger the CAAPs threshold and require a NPDES permit.[58]

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[50] 43 U.S.C. §1333(a)(1).
[51] 33 C.F.R. §320.2(b).
[52] 33 C.F.R. §320.4(a)(1). The processing of the permits is addressed in 33 C.F.R. §325.
[53] 33 U.S.C. §§1251 et seq.
[54] 33 U.S.C. §1342.
[55] 33 U.S.C. §1362(14).
[56] 40 C.F.R. §122.24(a).
[57] 40 C.F.R. §122.24(a), 40 C.F.R. 122 Appendix C. Concentrated Aquatic Animal Production facilities include cold-water facilities that discharge at least 30 days per year, produce more than 20,000 pounds of fish per year,

Fishing (Aquaculture) Permit

NMFS is the only federal agency that claims explicit management authority over offshore aquaculture. Currently, NMFS manages federal fisheries under authority of the MSA. The MSA regulates fishing in the EEZ through development and implementation of federal fishery management plans (FMPs). The MSA "does not expressly address whether aquaculture falls within the purview of the act."[59] The MSA defines a *fishery* as "one or more stocks of fish ... and any fishing for such stocks" and *fishing* as the "catching, taking, or harvesting of fish."[60]

> The Magnuson-Stevens Act does not expressly address whether aquaculture falls within the purview of the Act. However, the Magnuson-Stevens Act's assertion of exclusive fishery management authority over all fish within the EEZ, its direction to fishery management councils to prepare fishery management plans for any "fishery" needing conservation and management, together with the statutory definitions of "fishery" and "fishing," provide a sound basis for interpreting the Act as providing authority to regulate aquaculture in the EEZ.[61]

Under the MSA's authority, several regional fishery management councils and NMFS have exercised regulatory oversight over offshore aquaculture.[62] In some cases, NMFS authorized offshore aquaculture in federal waters for research and experimental purposes under an exempted fishing permit.[63] These permits are of limited duration and not intended to apply to development of permanent commercial operations.

The Gulf of Mexico Fishery Management Council (GMFMC) has been particularly active on aquaculture issues.[64] In 2009, an aquaculture FMP was approved by the GMFMC; NMFS issued its final rule to implement that FMP in 2016.[65] The aquaculture plan establishes a regional permitting process for regulating aquaculture in the Gulf of Mexico EEZ. The regulations authorize permits for up to 20 facilities that are limited to combined total production of 64 million pounds annually of species that are native to the Gulf of Mexico. Applicants are required to acquire other federal permits before NMFS can issue a Gulf aquaculture permit. NMFS also has developed a memorandum of understanding to coordinate federal agency actions and outline the permitting responsibilities of each agency in the Gulf.[66]

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and use 5,000 pounds or more of feed per month and warm-water facilities that discharge at least 30 days per year and produce at least 100,000 pounds of fish per year.

[58] Section 404 of the Clean Water Act regulates discharges of dredged or fill material into the waters of the United States, which does not include federal waters beyond 3 nm. This permit is often required for shellfish aquaculture in state waters.

[59] Memorandum from Constance Sathre, Office of the General Counsel, to Lois Schiffer, NOAA General Counsel, June 9, 2011. Hereinafter cited as Sathre, 2011.

[60] Sathre, 2011.

[61] Sathre, 2011.

[62] Regional fishery management councils were established by Congress under the Fishery Conservation and Management Act (P.L. 94-265).

[63] 50 C.F.R. §600.745(b).

[64] Eight Regional Fishery Management Councils were established under the Magnuson Stevens Fishery Conservation and Management Act (16 U.S.C. §§1801 et seq.) to develop fishery management plans for fisheries in each of the eight regions.

[65] NMFS, "Fisheries of the Caribbean, Gulf, and South Atlantic; Aquaculture," 81 *Federal Register* 1762-1800, January 13, 2016.

[66] NOAA, *Memorandum of Understanding for Permitting Offshore Aquaculture Activities in Federal Waters of the Gulf of Mexico*, 2016, at http://sero.nmfs.noaa.gov/sustainable_ fisheries/gulf_fisheries/aquaculture/documents/pdfs/final_offshore_aquaculture_mou_020617.pdf.

However, a recent legal decision has cast doubt on NMFS's authority to regulate aquaculture under the MSA. In *Gulf Fisherman's Association v. National Marine Fisheries Service,*[67] the U.S. District Court for the Eastern District of Louisiana held that NMFS exceeded its authority under the MSA when it adopted a regulatory scheme for aquaculture operations in the Gulf of Mexico. The court found that the MSA's grant of authority to regulate "fishing" and "harvesting" did not include aquaculture, noting that "[h]ad Congress intended to give [NMFS] the authority to create an entirely new regulatory permitting scheme for aquaculture operations, it would have said more than 'harvesting.' The MSA is a conservation statute, aimed at the conservation and management of natural resources. Fish farmed in aquaculture are neither 'found' off the coasts of the United States nor are they 'natural resources.'"[68]

Some are concerned that regional management of offshore aquaculture under the MSA may add another additional administrative requirements, especially if several regional fishery management councils develop their own, possibly contradictory, open ocean aquaculture management policies.[69] Currently, commercial aquaculture is less likely to occur in federal waters under the jurisdiction of other regional fishery management councils because they have not prepared aquaculture FMPs or generic aquaculture amendments to the appropriate FMPs for species that could be cultured. In addition, it is unclear what regulatory authority NMFS and the regional councils might have over species, such as mussels, that are not managed under a federal FMP.

Federal Consultation and Review Requirements

Consultation and review requirements are often triggered by federal permitting programs. Some crosscutting environmental requirements are entirely procedural, because they require that the federal agency implement certain procedures to ensure the agency identifies and analyzes potential impacts the proposal would have on certain resources before deciding whether to issue the permit. Other environmental requirements may prohibit the agency from permitting the action, as proposed, unless the level of adverse impacts can be minimized or mitigated.

Coastal Zone Management Act

Under Section 306 of the Coastal Zone Management Act (CZMA),[70] states may develop and implement a coastal management program (CMP) pursuant to federal guidance. State CMPs "describe the uses subject to the management program, the authorities and enforceable policies of the management program, the boundaries of the state's coastal zone, the organization of the management program, and related state coastal management concerns."[71]

Arguably the main feature of the CZMA is federal consistency.[72] Federal agency activities that have reasonably foreseeable effects on a state's coastal zone resources and uses

[67] No. 16-1271, 2018 U.S. Dist. LEXIS 163685 (E.D. La. Sept. 25, 2018).
[68] No. 16-1271, 2018 U.S. Dist. LEXIS 163685 (E.D. La. Sept. 25, 2018).
[69] Gulf of Mexico Fishery Management Council, *Fishery Management Plan for Regulating Aquaculture in the Gulf of Mexico* (Tampa, FL: January 2009).
[70] 16 U.S.C. §1455.
[71] NOAA, *CZMA Federal Consistency Overview*, February 20, 2009 (revised January 4, 2016), p. 3.
[72] 16 U.S.C. §§1451 et seq.

should be consistent with the enforceable policies of the state's coastal management plan.[73] Section 307 of the CZMA requires

> any applicant for a required Federal license or permit to conduct an activity, in or outside of the coastal zone, affecting any land or water use or natural resource of the coastal zone of that state" to "provide in the application to the licensing or permitting agency a certification that the proposed activity complies with the enforceable policies of the state's approved program and that such activity will be conducted in a manner consistent with the program.[74]

Enforceable policies are legally binding state policies, such as constitutional provisions, laws, regulations, land use plans, or judicial or administrative decisions.[75]

Federal licensing and permitting (such as aquaculture permit requirements) is one of four general categories of federal activities that may be reviewed for consistency.[76] The state lists federal licenses and permits that affect coastal uses and resources in its federally approved CMP. For listed activities, the applicant submits related data and information and a consistency certification that the proposed activity will be conducted in a manner consistent with the state's approved management program.[77] For a listed activity outside the coastal zone (such as in federal waters), the state also must describe the geographic location or area in its CMP.[78]

If a license, permit, or geographic location in federal waters is not listed in the state's CMP, the activity is treated as unlisted. To review an unlisted activity, the state notifies the applicant, federal agency, and NOAA Office of Coastal Management (OCM) that it intends to review the activity. OCM decides whether to approve the request, generally based on whether the activity will have reasonably foreseeable effects on the state's coastal zone. If approved, the consistency review proceeds as in the case of a listed activity.

The state may object to the applicant's consistency certification and stop the federal agency from authorizing the activity or issue a conditional concurrence to the applicant. The permit is issued for the activity if (1) the state concurs with the consistency determination; (2) the state fails to act, resulting in a presumption of consistency; or (3) the Secretary of Commerce overrules the state on appeal and concludes that the activity is consistent with CZMA objectives or is otherwise necessary for national security.[79] In the vast majority of federal actions, states concur with the applicant's self-certification, often resolving any disputes collaboratively.

National Environmental Policy Act

The National Environmental Policy Act (NEPA) requires federal agencies to consider the potential environmental consequences of proposed federal actions but does not compel

[73] NOAA, *CZMA Federal Consistency Overview*, February 20, 2009 (revised January 4, 2016).
[74] 16 U.S.C. §1456(c)(3)(A).
[75] NOAA, *CZMA Federal Consistency Overview*, February 20, 2009 (revised January 4, 2016).
[76] Other activities that may be subject to review include direct federal agency activities; outer continental shelf exploration, development, and production plans; and federal assistance to state and local governments.
[77] 30 C.F.R. §930.50.
[78] For example, the state may identify the area from the seaward boundary of state waters to 20 miles beyond state waters.
[79] 16 U.S.C. §1456 (c)(3)(B).

agencies to choose a particular course of action.[80] If an agency anticipates that an action would significantly affect the quality of the human environment, the agency must document its consideration of those impacts in an environmental impact statement (EIS).[81] If the impacts are uncertain, an agency may prepare an environmental assessment (EA) to determine whether a finding of no significant impact could be made or whether an EIS is necessary.[82] NEPA creates procedural requirements but does not mandate specific outcomes.[83]

Endangered Species Act and Marine Mammal Protection Act

NMFS and FWS have responsibilities under the ESA and the MMPA to review project proposals that may affect marine mammals or threatened and endangered species.[84] If issuance of a federal permit may adversely affect a species listed under the ESA, consultation may be required under Section 7 of the ESA.[85] Through consultation with either FWS or NMFS, federal agencies must ensure that their actions are not likely to jeopardize the continued existence of any endangered or threatened species or adversely modify critical habitat. If the appropriate Secretary judges that the proposed activity jeopardizes the listed species or adversely modifies critical habitat, then the Secretary must suggest reasonable and prudent alternatives that would avoid harm to the species.[86] If reasonable and prudent measures are adopted, the federal action is allowed to go forward.

The MMPA prohibits the harassment, hunting, capturing, killing (or *taking*) of marine mammals without a permit from the Secretary of the Interior or the Secretary of Commerce.[87] If marine mammals are likely to interact with aquaculture facilities and this interaction is likely to result in the taking of marine mammals, a marine mammal exemption would be required.[88] To be eligible for an exemption, the aquaculture facility would need to obtain a Marine Mammal Authorization Program certificate from NMFS.[89]

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[80] See 42 U.S.C. §4332.

[81] 40 C.F.R. §1502.

[82] 40 C.F.R. §1508.9.

[83] CRS Report RL33152, *The National Environmental Policy Act (NEPA): Background and Implementation*, by Linda Luther.

[84] Endangered Species Act (ESA; 16 U.S.C. §§1531 et seq.) and Marine Mammal Protection Act (MMPA; 16 U.S.C. §§1361 et seq.).

[85] For further information on the ESA, see CRS Report RL31654, *The Endangered Species Act: A Primer*, by Pervaze A. Sheikh.

[86] The Secretary of Commerce is generally responsible for listing and ESA-related activities for marine species, and the Secretary of the Interior is responsible for all other species.

[87] Under the MMPA, the Secretary of Commerce, acting through NMFS is responsible for the conservation and management of whales, dolphins, and porpoises (cetaceans), as well as seals and sea lions (pinnipeds). The Secretary of the Interior, acting through the Fish and Wildlife Service, is responsible for walruses, sea and marine otters, polar bears, manatees, and dugongs. This division of authority derives from agency responsibilities as they existed when the MMPA was enacted.

[88] A Marine Mammal Authorization Program certificate is issued when marine mammals may be taken incidentally in marine fisheries. If aquaculture is not defined as fishing, an incidental take authorization may be required, as in the case of nonfishing activities that take marine mammals such as construction projects and oil and gas development. See NMFS, "Incidental Take Authorizations Under the Marine Mammal Protection Act," at https://www.fisheries.noaa.gov/node/23111.

[89] NMFS, *A Guide to the Application Process for Offshore Aquaculture in U.S. Federal Waters of the Gulf of Mexico*, Southeast Regional Office, August 2017, at http://sero.nmfs. noaa.gov/sustainable_fisheries/gulf_fisheries/aquaculture/documents/pdfs/permit_applicant_guide_updated_aug2017.pdf.

MSA Essential Fish Habitat

The MSA also requires the federal permitting agency (e.g., USACE) for any aquaculture facility to consult with NMFS if the activity has the potential to harm essential fish habitat (EFH). EFH is designated for all marine species for which there is an FMP and may include habitat in both state and federal waters.[90]

National Marine Sanctuary Act

NOAA manages national marine sanctuaries established under the National Marine Sanctuary Act (NMSA).[91] Federal agencies are required to consult with the Secretary of Commerce when federal actions within or outside a national marine sanctuary, including activities that are authorized by licenses, leases, and permits, are likely to harm sanctuary resources.[92] If the Secretary finds that the activity is likely to injure a sanctuary resource, the Secretary recommends reasonable and prudent measures that the federal agency can take to avoid harm to the sanctuary resource. If the measures are not followed and sanctuary resources are destroyed or injured, the NMSA requires the federal agency that issued the permit to restore or replace the damaged resources.

National Historic Preservation Act

The National Historic Preservation Act (NHPA) is another procedural statute.[93] Under Section 106 of NHPA,[94] federal agencies must determine whether actions they may permit or license will have adverse effects on properties listed or eligible for listing in the National Register of Historic Places. Such sites could include shipwrecks, prehistoric sites, or other cultural resources. Federal agencies must determine whether such resources may be affected in consultation with state and/or tribal historic preservation officers.[95]

Fish and Wildlife Coordination Act

The Fish and Wildlife Coordination Act requires federal agencies to consult with FWS, NMFS, and state wildlife agencies when activities that are authorized, permitted, or funded by the federal government affect, control, or modify waters of any stream or bodies of water.[96] Consultation generally is incorporated into the process of complying with other federal permit requirements, such as the NEPA and CWA.

Other Authorizations and Approvals

The Coast Guard has authority to control private aids to navigation in U.S. waters.[97] Regulations require structures such as aquaculture facilities be marked with lights and signals for protection of maritime navigation.[98] To establish a private aid to navigation, the applicant would need formal authorization from the appropriate U.S. Coast Guard district.

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[90] 16 U.S.C. §1855(b).
[91] 16 U.S.C. §§1431 et seq.
[92] 16 U.S.C. §1434(d).
[93] 16 U.S.C. 470 et seq.
[94] 54 U.S.C. §306108.
[95] CRS Report R45800, *The Federal Role in Historic Preservation: An Overview*, by Mark K. DeSantis.
[96] 16 U.S.C. §661.
[97] 14 U.S.C. §83.
[98] 33 C.F.R. §§66.01 and 64.21.

The Bureau of Safety and Environmental Enforcement (BSEE) has regulatory responsibility for the offshore energy industry on the outer continental shelf.[99] BSEE would review aquaculture applications and provide comments regarding potential conflicts, interactions, or effects on mineral exploration, development, and production operations. The Bureau of Ocean Energy Management (BOEM) manages development of the outer continental shelf energy and mineral resources. BOEM would require a right-of-use easement for any offshore aquaculture operations that uses or tethers to an existing oil and gas facility.[100]

Environmental Concerns

One of the main features of many previous aquaculture bills has been consideration of environmental protection and monitoring of offshore aquaculture facilities. Critics of offshore aquaculture have expressed concern with potential environmental degradation and conflicts with existing uses of marine areas. They cite historic problems in inshore areas—such as escapes of cultured organisms, the introduction of disease and invasive species, pollution in areas adjacent to net pens, and habitat loss—which have created a negative perception of aquaculture.[101]

Aquaculture supporters assert that those who oppose marine aquaculture lack an understanding of aquaculture's benefits and risks and that "these perceptions persist despite significant scientific literature that contradicts the extent or existence of risk to the values that these groups want to protect."[102] Supporters contend that, in many parts of the world, a combination of farming experiences, technological advances, proper siting, and industry regulation has decreased environmental impacts and improved efficiency of marine aquaculture. Some researchers suggest that by moving operations offshore and selecting appropriate sites, the remaining impacts can be further reduced.[103] Others add that offshore waters would be less prone to environmental impacts than inshore waters because fish wastes and other pollutants would dissipate more rapidly in the deeper and better-flushed offshore areas.

A present lack of knowledge—owing to limited experience and few studies focusing specifically on offshore aquaculture—limits understanding of potential harm to the environment from offshore aquaculture. Most information has been collected from inshore areas, where salmon net pens and other types of aquaculture farms have been established. Some characteristics of inshore operations are similar to those that would be established offshore (e.g., both are open to the surrounding environment); however, other characteristics of offshore operations, such as offshore currents, wind and waves, water quality, and depth, are likely to differ from inshore areas. Generally, the outcomes associated with offshore

[99] 43 U.S.C. §§1331 et seq.
[100] 30 C.F.R. §585.
[101] Carol S. Price and Jessica Beck-Stimpert, *Best Management Practices for Marine Cage Culture Operations in the U.S. Caribbean*, NOAA, GCFI Special Publication Series Number 4, 2014.
[102] Gunnar Knapp and Michael C. Rubino, "The Political Economics of Marine Aquaculture in the United States," *Reviews in Fisheries Science & Aquaculture*, vol. 24, no. 3 (2016), pp. 213-229. Hereinafter cited as Knapp and Rubino, "Political Economics of Marine Aquaculture."
[103] Rebecca R. Gentry et al., "Mapping the Global Potential for Marine Aquaculture," *Nature Ecology & Evolution*, vol. 1 (September 2017), pp. 1317-1324.

aquaculture development depend on characteristics of aquaculture sites and how technology is employed and managed.

Over the years, researchers have identified several issues related to marine aquaculture and the use of net pens in inshore areas. These issues include water pollution from uneaten feed and waste products (including drugs, chemicals, and other inputs); habitat degradation, such as alteration of benthic habitat from settling wastes; sustainability of fish used in aquaculture feeds; use of antibiotics and other animal drugs; introduction of invasive species; escape of cultured organisms; and the spread of waterborne disease from cultured to wild fish. During the last two decades, technical advances and farming practices have reduced these impacts in nearshore areas.[104] Existing laws and regulations also have established performance standards and addressed many of the potential adverse environmental effects of net pen aquaculture.[105]

Fish Waste

Fish feed is the main source of waste from aquaculture and contributes to most environmental impacts associated with aquaculture.[106] The discharge of wastes, such as unused feed, and metabolic fish wastes, such as nitrogen (ammonia and urea), has been an ongoing concern because of potential effects on water quality and degradation of the seafloor environment under net pens. Treatment of effluent is not feasible because wastes are discharged directly into the ocean through net enclosures. Impacts on the environment depend on a variety of factors, such as feed quality, digestion and metabolism, feeding rate, biomass of fish, and species. Site characteristics such as cage design, depth, currents, existing water quality or nutrient levels, and benthic features also influence nutrient dispersion and impacts.

Impacts on water quality in the water column adjacent to net pens are often related to a combination of increases in nitrogen, phosphorus, lipids, and turbidity and depletion of oxygen.[107] Eutrophication may occur when net pens are placed at high densities and flushing of semi-enclosed water bodies is poor.[108] According to studies, aquaculture's contribution to nitrogen in areas adjacent to net pens ranged broadly from none to significant levels depending on a variety of factors, including environmental characteristics and species.[109] In some cases, it appears that nutrients are flushed away from the immediate cage area to the surrounding water body. Management practices such as choosing sites with adequate current and depth are likely to improve circulation and dissipation of waste products.[110]

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[104] Michael B. Rust et al., "Environmental Performance of Marine Net-Pen Aquaculture in the United States," *Fisheries*, vol. 39, no. 11 (November 2014), pp. 508-524. Hereinafter cited as Rust et al., "Environmental Performance."

[105] Rust et al., "Environmental Performance," p. 519.

[106] Stefanie M. Hixson, "Fish Nutrition and Current Issues in Aquaculture: The Balance in Providing Safe and Nutritious Seafood in an Environmentally Sustainable Manner," *Journal of Aquaculture Research and Development*, 2014, pp. 1-10. Hereinafter cited as Hixson, "Fish Nutrition."

[107] Lipids are a large group of organic compounds composed of fats and fatty compounds that are insoluble in water. They are a source of stored energy and a component of cell membranes.

[108] Eutrophication is the process by which a water body or coastal area is overly enriched with nutrients that stimulate excessive growth of algae. When algae die, they are decomposed by bacteria that use and deplete oxygen.

[109] Carol Seals Price and James A. Morris Jr., *Marine Cage Culture and the Environment*, NOAA, NOAA Technical Memorandum NOS NCCOS 164, December 2013. Hereinafter cited as Price and Morris, *Marine Cage Culture*.

[110] Rust et al., "Environmental Performance," p. 513.

Solid feed and fish waste descend through the water column and may accumulate on the bottom below and around aquaculture facilities. In some cases, wastes accumulate at rates greater than the assimilative capacity of the environment, and the increase of respiration from microbial decomposition decreases oxygen levels (hypoxia) and changes sediment chemistry. This may cause hypoxia in sediments and the water overlying the bottom, which may in turn affect the abundance and diversity of marine organisms in the area. Reviews have identified changes to sediment chemistry as one of the primary impacts of marine aquaculture in the United States.[111]

Over the last several decades, harmful environmental impacts have been reduced because of advances in technology, improved facility siting, better feed management, and stricter regulatory requirements.[112] Feed formulations have been modified to improve digestibility without losses in growth. When feed is more fully digested, the amount of waste (nutrient) outputs per unit of fish produced is reduced and fewer solid wastes and nutrients are released to the environment. Modifying feeding practices also has reduced the loss of uneaten food.[113] Some facilities now use underwater devices to monitor feeding to avoid overfeeding and waste. Environmental monitoring also informs farmers and regulators of the need to leave a site fallow or to adjust feeding.

Some researchers and aquaculturalists have proposed the use of multi-tropic aquaculture by adding other organisms such as invertebrates and seaweeds to the aquaculture system. The system would mimic natural tropic relationships, where wastes from cultured organisms are food for other organisms, such as shellfish, and supply nutrients for seaweed.[114] These additions could lessen environmental impacts from nutrients and increase the efficiency of feed utilization.[115]

Proponents suggest that offshore aquaculture may produce fewer and less severe environmental impacts than those caused in nearshore areas. They hold that open ocean waters are normally nutrient deficient, and nutrients released from offshore aquaculture operations would likely dissipate. Critics question whether experiences with experimental facilities are relevant to future commercial operations, which may need to operate at larger scales to be profitable. Generally, environmental impacts are likely to vary depending on management and culture techniques, location, size and scale, and species.

Fish Diseases

Fish diseases are caused by bacteria, viruses, and parasites that commonly occur in wild populations. Aquaculture production is vulnerable to mortality associated with fish diseases, and serious losses have occurred.[116] Disease outbreaks cost the global aquaculture industry an

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[111] Price and Morris, *Marine Cage Culture*, p. 22.
[112] Rust et al., "Environmental Performance," p. 519.
[113] Rust et al., "Environmental Performance," p. 512.
[114] B. H. Buck et al., "Offshore and Multi-Use Aquaculture with Extractive Species: Seaweeds and Bivalves," in *Aquaculture Perspective of Multi-Use Sites in the Open Ocean*, ed. B. H. Buck and R. Langan (Cham, Switzerland: Springer International, 2017), pp. 23-70.
[115] In nature, different tropic levels generally refer to plants (algae and seaweed), herbivores (organisms that graze on plants), and carnivores (predators of herbivores).
[116] Frank Asche et al., "The Salmon Disease Crisis in Chile," *Marine Resource Economics*, vol. 24, no. 4 (2009), pp. 405-411. Hereinafter cited as Asche et al., "Salmon Disease Crisis."

estimated $6 billion per year.[117] Starting in 2007, the Chilean aquaculture industry suffered the worst disease outbreak ever observed in salmon aquaculture.[118] The outbreak of infectious salmon anemia virus cost the industry 350,000-400,000 mt of production and $2 billion.[119]

Net pens are open to the marine environment, so pathogens may pass freely as water moves through net pen enclosures. Cultured organisms are often more susceptible to diseases because fish are kept at higher densities, which increases the rate of contact among fish and may induce stress. Research suggests that fish pathogens may be transferred from farmed to wild fish and that nonnative pathogens may be introduced when fish are moved from different areas.[120] Some fish farmers counter that more disease problems originate in wild populations, where reservoirs of disease naturally exist and are subsequently transferred to cultured organisms.

For example, some researchers have identified sea lice as a serious problem for Atlantic salmon farming because of lost production and the costs of disease management.[121] Studies demonstrate that high host densities in net pens promote transmission and growth of the parasite.[122] It has been hypothesized that sea lice may be spread from salmon in net pens to wild counterparts that are passing in adjacent waters. Some assert that sea lice have harmed wild salmon populations migrating near infested salmon farms. Studies have shown that transmission is initiated from wild to cultured fish, and then the lice are transmitted back to wild salmon hosts.[123] The extent of the impact on wild salmon is a matter of debate, because many different factors affect salmon population abundance. However, a recent study concluded that "Atlantic salmon populations are already under pressure from reductions in marine survival and the addition of significant lice-related mortality during the coastal stage of smolt out-migration could be critical."[124] Sea lice control and prevention strategies have included the use of approved therapeutants (aquaculture drugs) and fallowing of sites between production cycles.

Drugs and Other Chemicals

Various drugs have been used to treat and prevent the occurrence of disease, including disinfectants, such as hydrogen peroxide and malachite green; antibiotics, such as sulfonamides and tetracyclines; and anthelmintic agents, such as pyrethroid insecticides and avermectins.[125] Antibiotics are used to control bacterial diseases and are sometimes introduced to cultured fish in their feed. Drugs also are used to aid in spawning, to treat

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[117] World Bank, *Reducing Disease Risk in Aquaculture*, Agriculture and Environmental Services Discussion Paper 09, June 2014.

[118] Asche et al., "Salmon Disease Crisis," p. 405.

[119] Asche et al., "Salmon Disease Crisis," p. 408.

[120] Rust et al., "Environmental Performance," p. 514.

[121] O. Torrissen, "Salmon Lice—Impact on Wild Salmonids and Salmon Aquaculture," *Journal of Fish Diseases*, vol. 36, no. 3 (January 2013), pp. 171-194.

[122] Sea lice is a parasite that lives in the water column as planktonic larvae. It attaches to fish and feeds on the host's mucus, skin, and blood.

[123] Rust et al., "Environmental Performance."

[124] Samuel Shepard and Patrick Gargan, "Quantifying the Contribution of Sea Lice from Aquaculture to Declining Annual Returns of a Wild Atlantic Salmon Population," *Aquaculture Environmental Interactions*, vol. 9 (May 5, 2017), pp. 181-192.

[125] Priyadarshini Pandiyan et al., "Probiotics in Aquaculture," *Drug Prevention Today*, 2013, pp. 55-59. Anthelmintics are a type of medicine that kills helminths, worm-like parasites such as flukes, roundworms, and tapeworms. The medicine is selectively toxic to the parasite and not the host, in this case salmon.

infections, to remove parasites, and to sedate fish for transport or handling. Viral diseases are managed by monitoring and focusing on management practices, such as lowering stress, selecting organisms with greater resistance, and providing feed with proper nutrients. However, in some cases it is necessary to depopulate farms to stop the spread of the disease.

The Food and Drug Administration (FDA) is responsible for approving drugs used in aquaculture. The drug must be shown to be safe and effective for a specific use in a specific species.[126] Only drugs approved by the FDA Center for Veterinary Medicine may be administered to aquatic animals. Drug withdrawal periods and testing are required to prevent the sale of fish that contain drug residues. The USDA Animal and Plant Health Inspection Service is responsible for controlling the spread of infectious diseases and requires an import permit and health certificate for certain fish species.[127] Many states also have animal health regulations to prevent disease introductions and manage disease outbreaks.

Aquaculture drugs such as antibiotics that are used to treat marine finfish may be transferred to open water environments when unconsumed feed or fish wastes pass through net pen enclosures. Extensive use of these agents may result in the development and spread of bacteria that are resistant to antibiotics.[128] The use of many of these drugs reportedly is declining, as vaccines eliminate the need to treat bacterial diseases with antibiotics and other drugs.[129] Examples include salmon farming in Norway, where antibiotic use has decreased by 95%, and in Maine, where antibiotics are now rarely used.[130] Proponents of offshore aquaculture suggest that, because of the more pristine and better oxygenated water conditions offshore as compared to many inshore areas, the occurrence of fish diseases could be lower for offshore aquaculture.

Escapes, Genetic Concerns, and Invasive Species

The escape of organisms from aquaculture facilities, especially nonnative species, is another environmental concern related to aquaculture. This issue might arise if genetically selected or nonnative fish escape and persist in the wild. Historically, nonnative species have been used in aquaculture, sometimes resulting in long-term environmental harm. For example, Asian carp such as silver, bighead, and grass carp were introduced to the United States from Asia to improve water quality of freshwater aquaculture ponds and waste treatment ponds. These species are now found in most of the Mississippi drainage area, and they have affected the basin's aquatic ecology and harmed species such as freshwater mussels and native fish.[131]

Genetic diversity could be affected if hatchery-raised fish spawn with wild conspecifics (wild fish of the same species). Interbreeding could result in the loss of fitness in the

[126] Letter from FDA, Center for Veterinary Medicine to Aquaculture Professionals, October 2015, at https://www.fda.gov/animal-veterinary/product-safety-information/letter-aquaculture-professionals.

[127] Animal Health Inspection Service, *Import Live Fish*, U.S. Department of Agriculture, February 15, 2017, at https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-and-animal-product-import-information/import-liveanimals/sa_marine_life/ct_marine_import_fish.

[128] Jaime Romero, Carmen Gloria Feijoo, and Paola Navarrete, "Health and Environment in Aquaculture," in *Antibiotics in Aquaculture -Use, Abuse and Alternatives*, ed. Edmir Carvalho (Rijeka, Croatia: In InTech Europe, 2012), pp. 159-198.

[129] Carol Seals Price et al., *Protected Species and Marine Aquaculture Interactions*, NOAA, NOAA Technical Memorandum, January 2017. Hereinafter cited as Price et al., *Protected Species*.

[130] Rust et al., "Environmental Performance," p. 515.

[131] U.S. Geological Survey, *Nonindigenous Aquatic Species*, Data Queries and Species Lists, at https://nas.er.usgs.gov/ taxgroup/fish/default.aspx.

population due in part to the loss of genetic diversity. Genetic risks would depend on the number of escapes relative to the number of wild fish, the genetic differences between wild and escaped fish, and the ability of escaped fish to successfully spawn in the wild.[132] There are also concerns that nonnative fish could become established in the wild and compete with wild fish for food, habitat, mates, and other resources.

Experiences with farmed Atlantic salmon may provide some insight regarding escape of farmed fish both within and outside their native ranges.[133] Atlantic salmon have escaped from farms in the Pacific Northwest (outside their native range) and have been recaptured in Alaskan commercial fisheries. In 2017, over 100,000 Atlantic salmon escaped from facilities owned by Cook Aquaculture off Cypress Island, WA.[134] Many of the escaped fish were recovered, and fishery managers assumed the remaining fish were unable to make the transition to a natural diet. In British Columbia, escaped Atlantic salmon have spawned and produced wild-spawned juvenile Atlantic salmon, but it is uncertain whether they have established self-reproducing breeding populations.[135]

Within the range of Atlantic salmon, farmed salmon have been found on spawning grounds during the period when wild Atlantic salmon spawning occurs. Domestication of farmed salmon has changed their genetic composition and reduced genetic variation. These changes have occurred because limited numbers of brood fish are used for spawning farmed fish and farmers select for specific traits.[136] Much present-day farm production of Atlantic salmon is based on five Norwegian strains. Farmed and wild hybrids and backcrossing of hybrids in subsequent generations may change genetic variability and the frequency and type of alleles present in wild populations.[137] The extent and nature of these changes to genetic variability may affect survival (fitness) of these populations.[138] Changes in the genetic profiles of wild populations have been found in several rivers in Norway and Ireland, where interbreeding of wild and farmed fish is common.[139] Large-scale experiments in Norway and Ireland show highly reduced survival and lifetime success rates of farmed and hybrid salmon compared to wild salmon.[140] Some researchers have concluded that further measures are needed to reduce the escape of salmon from aquaculture farms and their spawning with wild populations.[141]

Researchers and managers have made several recommendations to decrease the risk of invasive species introductions and the loss of genetic diversity. There appears to be common agreement, as in the case of the Gulf of Mexico FMP, that only native species should be

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[132] Price et al., *Protected Species*.

[133] Eva B. Thorstad, Ian A. Fleming, and Philip McGinnity, *Incidence and Impacts of Escaped Farmed Atlantic Salmon Salmo Salar in Nature*, Norwegian Institute for Nature Research, 2008. Hereinafter cited as Thorstad et al., *Incidence and Impacts*.

[134] Lynda V. Mapes, "Escaped Atlantic salmon have disappeared from Puget Sound but legal fight begins," *Seattle Times*, November 14, 2017.

[135] Thorstad et al., *Incidence and Impacts,* p. 67.

[136] Oystein Skaala, Vidar Wennevik, and Kevin A. Glover, "Evidence of temporal genetic change in wild Atlantic salmon, *Salmo salar*, populations affected by farm escapees," *ICES Journal of Marine Science*, vol. 63 (2006), pp. 1224-1233.

[137] An allele is one of two or more versions of a gene occupying a specific spot on a chromosome that controls a specific trait.

[138] Fitness can be generally described as the ability to survive and reproduce.

[139] Thorstad et al., *Incidence and Impacts,* p. 60.

[140] Thorstad et al., *Incidence and Impacts,* p. 60.

[141] Kjetil Hindar et al., "Genetic and Ecological Effects of Salmon Farming on Wild Salmon: Modeling from Experimental Results," *ICES Journal of Marine Science*, vol. 63, no. 7 (January 2006), pp. 1234-1247.

farmed. To decrease genetic risks associated with escapes, farmers might be required to use wild broodstock with a genetic makeup that is similar to local wild populations. However, by using this approach, farmers may forgo benefits of selective breeding. Another approach might involve the use of sterile fish created through techniques such as hybridization, chemical sterilization, polyploidy, and others.[142] However, these methods are not always 100% effective and the approach may increase costs of production.[143]

Interactions with Other Species

Interactions between aquaculture operations and marine wildlife may occur when predators in search of food are attracted to aquaculture facilities or if aquaculture sites overlap with the ranges or migration of marine species. These interactions are common in Chile, British Columbia, and Norway, where marine mammals and birds often are attracted to salmon farms.[144] Most interactions are seasonal and involve sea lions, seals, and otters, as well as seabirds such as sea gulls and cormorants. Predation can result in loss of fish, damage to equipment, and stress to fish. Deterrence measures seek to address these concerns; for example, predator nets may be placed outside the main net to stop marine mammals directly accessing the net pen. Some farms also install bird nets over net pens to protect fish from bird predation. When nonlethal measures fail, sea mammals are sometime culled.

Offshore facilities could affect some endangered species as they migrate or alter essential habitat for feeding, breeding, and nursing. Information on incidental entanglement and mortality is limited, because of the small number of facilities working in offshore areas. NOAA recently investigated longline aquaculture gear that might be used for mussel culture and found that interactions are rare.[145] However, researchers questioned whether the small number of interactions indicates that this type of aquaculture is benign or is due to the failure to detect and report interactions. Minimizing impacts on protected species may require monitoring and research into natural interactions between predators and prey. Management strategies might involve preventive measures, such as spatial planning and aquaculture gear modifications.

Wild fish also are sometimes attracted to net pens to consume feed that has fallen through net pen enclosures.[146] The attraction of wild fish may provide a benefit, because their consumption of feed may lessen environmental impacts such as the release of nutrients or deposits of feed near net pens. At the same time, it could have negative impacts, such as the transfer of diseases from farmed to wild fish or from wild to farmed fish. Impacts related to changes in wild fish physiology from the ingestion of feed and changes in the distribution of wild fish are unknown.

Aquaculture Feeds and Related Issues

Fish feed is a critical input, because it must provide all of the essential nutrients and energy needed to meet the cultured organism's physiological requirements. The supply and use of aquaculture feed are directly related to the economic viability of aquaculture

[142] Polypliody occurs in organisms with cells containing more than two paired sets of chromosomes.
[143] Price et al., *Protected Species*, p. 12.
[144] Cermaq, *Marine Mammals and Birds*, Fact Sheet, October 18, 2012.
[145] Price et al., *Protected Species*, p. 8.
[146] Ingebrigt Uglem, "Impacts of wild fishes attracted to open-cage salmonid farms in Norway," *Aquaculture Environment Interactions*, vol. 6 (2014), pp. 91-103.

operations, fish growth and health, environmental quality, ecological concerns, and human nutritional benefits from aquaculture products.[147] Fish meal and oil are used to produce feed for carnivorous species such as salmon, because these ingredients provide nutritional requirements that are similar to those found in the wild. Aquaculture feeds must have a composition that maintains growth and fish health while balancing the costs of feed components against the value of outputs associated with fish growth. Researchers note that future aquaculture production is likely to be constrained if feeds are limited to sources of fish meal and oil, which require wild fish production and fish processing wastes. Research efforts have focused on the use of fish meal and oil substitutes that are derived from terrestrial plants.[148] Plant meal and oils now supply the bulk of feed ingredients, but they are not a perfect substitute and, in many cases, fish meal and oil are still an important component of most fish feeds.

Feed Production and Use

Nutritional requirements and feed composition vary according to species, the life stage of the organism (e.g., larvae, fry, fingerlings, adults), and management objectives.[149] Fish feeds are formulated to provide a mixture of ingredients, such as proteins, lipids, carbohydrates, vitamins, and minerals, which provide the greatest growth at the lowest cost. Historically, fish meal and oil have been principal ingredients of many aquaculture feeds, because these ingredients have been a cost-effective means of providing the nutritional requirements of many cultured species. Fish meal and oil are obtained from reduction fisheries that target small pelagic species such as anchovies, capelin, herring, and menhaden and from fish processing wastes of wild and aquaculture products.[150] Reduction fisheries target species that are generally less valuable than those used for human consumption.[151] The fish are heated and pressed to obtain fish oil and milled and dried to produce fish meal. Since 2006, the annual world supply of fish meal has ranged from 4.49 mmt to 5.86 mmt and the supply of fish oil has ranged from 0.86 mmt to 1.08 mmt.[152] In 2016, the United States produced 253,600 metric tons (mt) of fish meal and 80,500 mt of fish oil, approximately 5% and 8% of global production, respectively.[153]

Reduction fisheries supply approximately 70% of fish meal and fish oil, with the remainder obtained from fish processing wastes.[154] In the last 20 years, global production of fish meal and oil has declined in part because of increasing use of fish from reduction fisheries for direct human consumption and tighter quotas and controls on unregulated

[147] Hixson, "Fish Nutrition," p. 1.

[148] Michael B. Rust, *The Future of Aquafeeds*, NOAA and USDA, December 2011.

[149] Production of food fish is one of many potential objectives of aquaculture. Other examples of aquaculture objectives may involve enhancement of recreational fishing or restoration of aquatic populations.

[150] A reduction fishery uses fish to produce fish oil and fish meal for animal feeds, including those used for aquaculture.

[151] Many oppose the use of these species for animal feeds, because they assert that the protein should be available for direct human consumption. In many cases, such as U.S. menhaden fisheries, fish are not marketed as food items because of the taste and texture of their flesh. However, in some parts of the world, direct human consumption of other forage fisrubionh (often small pelagic species) is increasing.

[152] Seafish, *Fishmeal and Fish Oil Facts and Figures*, December 2016. Hereinafter cited as Seafish, *Fishmeal and Fish Oil*.

[153] NMFS, *Fisheries of the United States, 2016*, NOAA Current Fishery Statistics No. 2016, Silver Spring, MD, 2017, at https://www.fisheries.noaa.gov/feature-story/fisheries-united-states-2016.

[154] Rust et al., "Environmental Performance," p. 511.

fishing.[155] The global decrease in total fish meal production has occurred despite increasing production of meal and oil from fish processing wastes.

Conversion of Aquaculture Feed to Fish Flesh

Researchers have found that fish meal (protein) and fish oil (lipids) are important ingredients for fish growth.[156] Most feeds are formulated to increase efficiency by using high-energy lipid to allow for greater conversion of dietary protein into fish muscle. In addition to fish protein and oil, fish feeds may include plant proteins, terrestrial animal protein, carbohydrates, moisture, ash, vitamins, and minerals. In comparison to other animals, fish are relatively efficient in converting fish feed to flesh.[157] For example, feed conversion ratios for Atlantic salmon are approximately 1.15 (approximately 1.15 kilograms [kg] of dry feed are used to produce 1.0 kg of salmon flesh [wet]).[158] In 2013, salmon fish feed used on Norwegian farms consisted of approximately 18% fish meal and 11% fish oil.

The amount of marine fish protein and oil needed to produce a unit measure of seafood such as salmon has been decreasing with the use of plant-based substitutes. The "fish in fish out" ratio is the amount of wild fish needed to produce the fish meal and fish oil required to produce one kilogram of farmed fish. The ratio of "fish in to fish out" varies according to the nutritional requirements of different species, with higher ratios for carnivorous fish such as eels (1.75) that are fed higher fish protein and fish oil diets and lower ratios for omnivorous fish such as tilapia (0.18).[159] When aggregated across species, worldwide aquaculture is a net producer of fish protein, with estimates ranging from 0.22 kg to 0.5 kg of wild marine fish used to produce a kilogram of farmed seafood.[160]

Substitutes for Fish Meal and Oil

Over the last two decades, research on fish dietary requirements has contributed to progress in developing substitutes for fish meal and oil from terrestrial plant ingredients and other potential sources, such as marine algae.[161] This has led to reductions in the use of fish meal and oil as ingredients in fish food. Terrestrial plant meal and oils now supply the bulk of feed ingredients for most fish species.[162] The focus of research has been on plant protein and oil sources such as soy, canola, sunflower, cottonseed, and others. For example, the Norwegian salmon industry has reduced the content of fish meal and oil in fish feed from over 60% to less than 25% by using plant proteins and oils.[163]

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[155] Seafish, *Fishmeal and Fish Oil*.

[156] Hixson, "Fish Nutrition," p. 1.

[157] Knapp and Rubino, "Political Economics of Marine Aquaculture."

[158] Trine Ytrestoyl, Turid Synnove Aas, and Torbjorn Asgard, "Utilization of Feed Resources in Production of Atlantic Salmon," *Aquaculture*, vol. 448 (2015), pp. 365-374.

[159] Marine Ingredients Organization (IFFO), "Fish In: Fish Out (FIFO) Ratios for the Conversion of Wild Feed to Farmed Fish Including Salmon," at http://www.iffo.net/fish-fish-out-fifo-ratios-conversion-wild-feed (hereinafter IFFO, "Fish In: Fish Out"). These estimates attempt to provide a ratio that includes fish meal and fish oil.

[160] NOAA Fisheries, Office of Aquaculture, *Feeds for Aquaculture*, at http://www.nmfs.noaa. gov/aquaculture/faqs/faq_feeds.html. (hereinafter cited as NOAA, *Feeds for Aquaculture*) and IFFO, "Fish In: Fish Out."

[161] Fish oils are produced by marine algae, and in nature algae are consumed by fish that feed at relatively low tropic levels.

[162] Michael B. Rust, *The Future of Aquafeeds*, NOAA and USDA, December 2011.

[163] Rust et al., "Environmental Performance," p. 512.

In spite of decreasing global production of fish oil and meal, use of plant-based substitutes has allowed production of feeds for all aquaculture to expand at 6% to 8% per year.[164] Increasing demand and a limited supply of fish meal and oil have caused prices to triple for these ingredients in recent years.[165] These price increases are likely to continue, because production is generally limited to supplies from wild sources.[166] The cost of aquaculture feeds accounts for approximately 50% of net pen aquaculture operating costs. Limited wild supplies and rising feed costs have encouraged researchers and aquaculturalists to improve feeding techniques to reduce waste, modify feed formulations, use alternatives such as waste from fish-processing plants, and investigate new sources. Substitution has become more attractive, as the prices of fish meal and oil have risen faster than the prices of plant proteins and oils. Fish can be cultured with substitutes for fish meal and oil, but the commercial use of substitutes depend on whether the lower costs of the substitute can offset losses associated with lower growth rates, less disease resistance, and inferior nutritional value of aquaculture products.[167]

Although significant progress has been made in using plant protein and oil substitutes for fish feeds, there are still limitations to their use. In the near future, some fish meal and oil will still be needed in feed formulations. Plant meals are deficient in certain essential amino acids and contain fiber, carbohydrates, and certain antinutritional factors, which can adversely affect absorption, digestion, and growth.[168] Nutritional quality of plant proteins can be improved through chemical and mechanical processing, which can reduce certain antinutrients and concentrate protein. Plant oils are an excellent source of energy, but they do not contain omega-3 fatty acids (eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]). These fish oils have been found to improve immune responses and fish health generally.[169] Fish species have differing tolerances to diets without certain fatty acids, which appear to be related to their natural diet. The use and substitution of plant protein and oils is likely to increase with further research into alternatives and as prices of fish meal and oil increase.

Fish Health

Proper feed formulations also are essential to promote fish health and prevent disease outbreaks. When fish are farmed at high densities, good nutrition tends to reduce stress, decrease the incidence of disease, and boost immune systems. A deficiency in any required nutrient may impair health by affecting the organism's metabolism and increasing susceptibility to disease. Research has shown that the use of plant oils and the ratio of different fatty acids can affect the immune response in fish. Dietary additives of immunostimulants, probiotics, and prebiotics have been found to increase immunity, feed efficiency, and growth.[170] An ongoing challenge is to improve knowledge and commercial application of feed formulations, especially for nutrimental requirements of newly domesticated species.

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[164] NOAA, *Feeds for Aquaculture*.
[165] Knapp and Rubino, "Political Economics of Marine Aquaculture," p.220.
[166] Wild sources include both forage species and wastes from processing wild and farmed fish.
[167] Nutritional value to consumers (humans) of fish.
[168] Hixson, "Fish Nutrition," p. 3.
[169] Hixson, "Fish Nutrition," p. 5.
[170] Hixson, "Fish Nutrition," p. 5.

Human Health and Preferences

The human health benefits of seafood are widely recognized because fish species contain high-quality protein, oils, minerals, and vitamins. Some research has found that diets that include omega-3 fatty acids enhance early brain and eye development and reduce heart disease and cognitive decline later in life. Feeds with plant-based substitutes can affect the quality of seafood products because these alternatives lack the fatty acids that are beneficial to human health. Farmed fish products that have been fed plant substitutes for fish oil may have lower concentrations of beneficial fish oils in their flesh. Two potential ways to reduce the use of fish oils in feed while maintaining high levels of omega-3 fatty acids in fish are (1) to develop genetically modified plants, fungi, or microbes to produce DHA and EPA for use in fish feeds or (2) to grow fish on low fish oil diets in the beginning of the production cycle and boost the omega-3 fatty acids in fish diets to raise their levels at the end of the production cycle.

There also are growing public health concerns about persistent organic pollutants, such as polychlorinated biphenyls (PCBs), and inorganic contaminants, such as heavy metals, in farmed fish. The accumulation of contaminants varies by location and associated sources of pollutants. It can occur in both wild and farmed fish.[171] Fish fed with fish meal and oils may accumulate contaminants from marine sources. Several studies have reported elevated levels of contaminants in feeds and farmed Atlantic salmon flesh. An advantage of using plant protein and oil is the potentially lower contaminant levels than those found in some wild seafood products. Several studies have found that replacing fish protein and oil with plant-derived material lowered the level of contaminants significantly.[172]

Consumer perceptions of changes in the quality of fish raised with substitute feeds also may affect acceptance of aquaculture products. There are widely held beliefs regarding the composition and health benefits of farmed and wild fish. Studies have shown that there are differences in taste and texture of fish farmed with alternative proteins and oils, but consumer preference studies have yielded mixed results.[173] Public perceptions of aquaculture products also include concerns with the use of therapeutants such as antibiotics and the crowding and industrial nature of fish farming.

Sustainability Concerns

Some stakeholders have described the use of fish meal and oils for aquaculture feeds as an issue related to the sustainability of forage species and marine ecosystems. More than 30% of global fish production and a large portion of fish meal and oil used for aquaculture feeds (75%) is derived from the harvest of forage species, such as herring, anchovies, capelin, and menhaden. Fatty acids are produced by marine algae (phytoplankton), consumed and concentrated in fish that consume algae, and transferred to organisms higher in the food chain that consume forage species. As stated earlier, forage species have a relatively low economic value, and most are not marketed for direct human consumption. However, their biomass is relatively large because they feed at somewhat low tropic levels, and they can be caught fairly

[171] Hixson, "Fish Nutrition," p. 7.
[172] Hixson, "Fish Nutrition," p. 8.
[173] Hixson, "Fish Nutrition," p. 7.

easily in large volumes because they are schooling species.[174] Forage species serve as prey for higher tropic level fish species such as tuna, cod, and striped bass, marine mammals, and marine birds.[175]

Aquatic ecologists question whether aquaculture demand and increasing prices may encourage higher levels of fishing pressure and cause or continue overfishing of forage fish populations. Management of wild fish stocks is improving in many parts of the world, and many stocks are now considered to be well managed. However, some researchers have concluded that fishing for forage species should be limited to relatively low levels, because forage species are needed to support production of other marine species.[176] Research using ecosystem models suggests that forage fish should be fished at lower rates to benefit the ecosystem rather than at rates that would provide long-term maximum yield.[177] One report recommended that catch rates should be reduced by half and biomass of forage fish should be doubled.[178] However, other researchers have questioned whether there is a strong connection between forage fish abundance and the abundance of their predators;[179] they conclude that harvest policies for forage species need to be guided by a variety of factors that recognize the complexities of fisheries and ecosystems.

Economics, International Conditions, and Stakeholder Concerns

Increasing demand for seafood, advances in aquaculture methods, and increases in global aquaculture production have led many observers to take an optimistic view of potential offshore aquaculture development in the United States. Nevertheless, the future of offshore development is uncertain because of the paucity of experiences in establishing and managing U.S. offshore aquaculture facilities. Greater regulatory certainty may encourage U.S. offshore development, but economic viability will determine whether the industry expands and produces significant quantities of seafood.

The viability of offshore aquaculture in the United States is likely to depend on future developments, such as further technical advances, economic conditions, and social and political acceptance. Another economic consideration for policymakers is how to integrate policies that recognize the potential costs (externalities) of environmental harm that may be caused by offshore aquaculture and are not captured by markets.[180] In addition to economics, user conflicts and related political factors are likely to play a role in the potential development of an offshore industry.

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[174] *Tropic levels* generally refer to organisms in an ecosystem that occupy a similar level in the food chain. Prey items, such as sardines, occupy a lower tropic level with relatively higher levels of biomass than predators at higher tropic levels and lower biomass, such as tuna.

[175] Timothy E. Essington and Stephen B. Munch, "Trade-Offs Between Supportive and Provisioning Ecosystem Services of Forage Species in Marine Food Webs," *Ecological Applications*, vol. 24, no. 6 (September 2014).

[176] E. Pikitch et al., *Little Fish, Big Impact: Managing a Crucial Link in Ocean Food Webs*, Lenfest Ocean Program, 2012. Hereinafter cited as Pikitch et al., *Little Fish, Big Impact*.

[177] Anthony D. M. Smith et al., "Impacts of Fishing Low Trophic Level Species on Marine Ecosystems," *Science*, vol. 333 (August 2011), pp. 1147-1150.

[178] Pikitch et al., *Little Fish, Big Impact*.

[179] Ray Hilborn et al., "When Does Fishing Forage Species Affect Their Predators?" *Fisheries Research*, vol. 191 (2017), pp. 211-221.

[180] Externalities are defined as spillover costs or benefits, which are unintended consequences or side effects associated with an economic activity.

Factors Related to the Economic Viability of Offshore Aquaculture

The economic potential of offshore aquaculture will depend on the prices of seafood products and the cost to produce them. The following discussion identifies some of the factors that will determine whether offshore aquaculture may be profitable.

Demand

The quantity demanded for an aquaculture product is a function of price—each point along the demand curve is the quantity that consumers are willing to buy at a specific price. Consumers are generally willing to buy less product at higher prices and more product for lower prices. A change in demand, a shift of the demand curve, depends on a variety of factors, such as changes in income, prices of substitutes (domestic wild fish) and complements, and consumer tastes and preferences.[181]

Offshore aquaculture production will compete with a variety of other protein products, such as imported seafood; domestically produced wild fish; and agriculture sources such as chicken, pork, and beef. Generally, demand for seafood products is rising both globally and domestically because of increasing population levels and incomes. The health benefits of seafood are also influencing changes in consumer preferences, with general movement away from traditional protein sources such as beef. Other types of domestic marine aquaculture production, such as land-based and inshore aquaculture, may compete with offshore aquaculture, but currently these activities provide a relatively small portion of the seafood consumed in the United States.[182] Domestic sources of seafood may increase marginally as some overfished stocks recover, but most domestic fisheries are already at or near their natural limits.

Some have reported that offshore aquaculture could produce a higher-quality product because of the constant flow of clean water through net pens. If it can be shown that offshore products contain fewer toxin residues or if offshore products can be raised without aquaculture drugs, these products may become more attractive to health-conscious consumers. The FDA Seafood Safety Program and the NOAA Seafood Inspection Program also may reassure U.S. consumers of the safety and quality of domestic seafood, including seafood produced by offshore aquaculture.[183] These factors may allow offshore producers to differentiate their products and receive higher prices relative to imports or other domestic seafood, especially in niche markets.

Supply

The amount of seafood that aquaculturalists will be willing to produce at a given price depends on production costs. Economic conditions determine the costs of labor, hatchery supplies for stocking, feed, maintenance, and other inputs. For most aquaculture operations, the bulk of costs are for feed and stocking of early life stages, such as finfish fingerlings or

[181] A change in demand results in a shift of the demand curve rather than a change in quantity demanded, movement along the demand curve.

[182] Advances in more intensive land-based culture techniques, such as recirculating systems, are another means to increase production with minimal environmental impacts, but the viability of these operations is still uncertain.

[183] The FDA program also inspects imports and may be found at https://www.fda. gov/food/resources-you-food/seafood. The Department of Commerce program can be found at https://www.fisheries.noaa.gov/insight/noaas-seafoodinspection-program.

oyster seed.[184] Fixed costs include equipment depreciation, insurance, taxes, and lease payments. Shifts in supply result from changes in input prices, which also may be affected by technology, weather conditions, and other influences. At the level of individual farms or facilities, most costs are not set and often depend on short-run and long-run choices of the aquaculturalist. For example, in the short run, the producer may change feed quality and quantity, harvest intervals, or stocking rates, while in the longer term she may change species, location, technology, and scale.[185]

Costs to produce seafood in offshore aquaculture facilities are likely to be higher than costs in inshore areas, because of the need for more resilient cage materials and construction, shore-side infrastructure, specialized vessels, and automation of facility systems. The location of offshore aquaculture facilities also is likely to increase costs for fuel, monitoring, harvest, and security.[186] According to the Food and Agriculture Organization, offshore facilities operating at distances of greater than 25 nm from shore are unlikely to be profitable, because costs increase with distance from shore.[187]

Some have speculated that offshore facilities will need to take advantage of economies of scale because of the relatively high costs of transporting materials between inshore and offshore facilities. Operating large-scale operations will require new coastal facilities and networks to supply and transport feed, construction materials, fingerlings, and harvested fish. Logistics networks to supply these inputs would need to be developed in coastal areas, where "working waterfronts" are already threatened due to competing uses and the relatively high cost of coastal real estate. These startup costs may exclude smaller producers who may not have access to the capital and resources needed to establish large-scale operations.

Financial risk, generally the probability of losing money, is another factor that is related to potential viability of offshore aquaculture and may affect the availability of capital and insurance. *Risk* is defined as uncertain consequences, usually unfavorable outcomes, due to imperfect knowledge.[188] Assessing risk for offshore aquaculture is complicated by different species, technologies, site characteristics, and the lack of experience working in offshore areas. Risks may be greater in offshore than inshore areas because of the threat of severe weather conditions and exposed offshore environments. Attracting investment may be difficult because offshore aquaculture is a new industry with limited experiences for investors to evaluate. As risk and uncertainty increase, generally, a greater revenue stream is required to justify the same level of investment.[189] Known risks can be reduced by decreasing the probability of adverse outcomes, such as by using stronger materials to build more resilient structures. The cost of reducing risks must be weighed against the probability and magnitude of potential losses.

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[184] Early stages of marine organisms are often raised in hatcheries and subsequently transferred to larger enclosures to be grown to adult size.

[185] Knapp, "Economic Potential."

[186] California Environmental Associates (CEA), *Offshore Finfish Aquaculture Global Review and U.S. Prospects*, The David and Lucile Packard Foundation, 2018. Hereinafter cited as CEA, *Offshore Finfish Aquaculture*.

[187] FAO, *A Global Assessment of Offshore Mariculture Potential from a Spatial Perspective,* Technical Paper 549 2013.

[188] Lotus E. Kam and Pingsum Leung, "Financial Risk Analysis in Aquaculture" in *Understanding and Applying Risk Analysis in Aquaculture*, FAO, 2008.

[189] Di Jin, "Economic Models of Potential U.S. Offshore Aquaculture Operations," in *Offshore Aquaculture in the United States: Economic Considerations, Implications, and Opportunities,* NOAA, NOAA Technical Memorandum NMFS F/SPO-103, July 2008, pp. 117-140.

Another approach to reducing risk is through insurance. Insurance transfers risk from the producer to the insurance underwriter through payments of insurance premiums. The cost of insurance premiums may be higher for offshore than inshore areas because of greater uncertainty and potentially higher risks of losses for offshore facilities.

Private Benefits and Externalities

The previous discussion of supply and demand considers private costs of production that are borne by the producer. Policymakers are concerned with a broader definition of costs that may affect individuals who are not involved in the aquaculture business—often referred to as *externalities*. Externalities are defined as spillover costs or benefits, which are unintended consequences or side effects associated with an economic activity.[190] For example, commercial fishermen may be harmed by habitat degradation caused by pollution from aquaculture because of associated declines of wild populations. When externalities are not considered, markets become inefficient because more of a good or service is produced than when the externality is fully considered.

The recognition of externalities is another way in which policymakers can examine the tradeoffs related to the private benefits from aquaculture production and the environmental harm caused by the activity. In the case of offshore aquaculture, external costs may be associated with environmental harm from pollution, escaped organisms, disease transmission, and other effects. The existence of externalities means that policymakers may need to consider whether and to what degree the government should intervene to account for these costs. Intervention may involve regulatory measures that minimize externalities while maximizing benefits associated with the industry (e.g., fish production). Decisions related to site selection, technology, and facility operations are likely to be some of the main factors that determine the level of offshore aquaculture externalities.

International Factors and Domestic Experiences

Trade

DOC has expressed concern with increasing U.S. imports of seafood products. According to NMFS, 80%-90% of the seafood consumed in the United States is imported.[191] International trade in seafood has grown over the last several decades. The value of seafood trade is now more than twice the trade of meat and poultry combined.[192] Relatively high-value seafood from wild fisheries and aquaculture dominates imports. In 2017, the United States imported approximately 2.7 mmt of edible seafood valued at $21.5 billion.[193] After accounting for exports valued at $5.7 billion, the value of imports was $15.8 billion greater than exports of edible seafood products. Approximately half of seafood imports are cultured.

[190] Externalities may be related to costs or harm related to pollution or benefits, such as the utility gained from observing flowers planted in roadside areas.

[191] NMFS, 2018. A portion of imports include domestic catch that was exported for further processing and returned to the United States as an import in processed form.

[192] James L. Anderson and Gina Shamshak, "Future Markets for Aquaculture Products," in *Offshore Aquaculture in the United States: Economic Considerations, Implications, and Opportunities*, NOAA, NOAA Technical Memorandum NMFS F/SPO-103, July 2008, pp. 231-244.

[193] NMFS, *Imports and Exports of Fishery Products Annual Summary, 2016*, Current Fishery Statistics No. 2016-2, Silver Spring, MD, July 19, 2017, at https://www.st.nmfs.noaa.gov/ Assets/commercial/trade/Trade2016.pdf.

The two main imported products are farmed shrimp and salmon. In 2017, shrimp accounted for $6.5 billion and salmon accounted for $3.5 billion of U.S. seafood imports.[194]

Supporters of offshore aquaculture assert that development of offshore areas and associated increases in seafood production could reduce the U.S. deficit in seafood trade. The Department of Commerce Strategic Plan states that, "a strong U.S. marine aquaculture industry will serve a key role in U.S. food security and improve our trade balance with other nations."[195]

Some may counter that the seafood trade deficit is not a good reason to support development of the aquaculture industry. Cultured salmon and shrimp imports have lowered prices and, therefore, the profits of domestic wild fisheries and aquaculture producers, but U.S. consumers have benefited from lower salmon and shrimp prices. According to economic theory, countries gain from trade when they specialize in products that they are best at producing. If other countries have an absolute or comparative advantage in aquaculture, the United States would likely benefit from supporting other industries.[196] Advocates of aquaculture note that the United States has advantages compared to other countries because of its extensive coastline and EEZ, skilled labor, technology, domestic feed production, stable government and economy, and large seafood market.[197] Others counter that U.S. federal waters are exposed to high winds and wave action for large parts of the year, whereas other parts of the world have readily available inshore areas and calmer offshore waters that could be developed, as well as lower labor costs.[198]

Overall operating costs and environmental standards for aquaculture in other countries are often lower than in the United States. Some have speculated that costs of inputs such as labor and less strict regulations provide producers outside the United States with an insurmountable competitive advantage. Other observers stress that costs may be lower in other countries, but if prices are high enough, U.S. producers may still be able to operate profitably.[199] Domestic producers also have some advantages, such as a large and relatively wealthy market and lower shipping costs than those for imports.

The government sometimes provides government-sponsored trade protections such as tariffs or import quotas to new industries. Protection may be rationalized by an infant industry that claims it requires time to overcome short-term cost disadvantages.[200] Cost disadvantages may be related to the need to become more efficient by constructing new facilities, training workers, and installing new equipment. In these cases, tariffs would act as a subsidy that increases the domestic price of the good. When the industry becomes more efficient, the tariff

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[194] An unknown portion of seafood imports, including salmon, was harvested in U.S. wild fisheries and exported to other countries for processing. Some of these products are then exported back into the United States.

[195] U.S. Department of Commerce, *U.S. Department of Commerce Strategic Plan 2018-2022*: *Helping the American Economy Grow*, 2018, p. 9, at https://www.commerce.gov/about/ strategic-plan.

[196] A country has an absolute advantage if its production costs for a good are lower than those of other countries at prevailing prices and exchange rates. According to the concept of comparative advantage, a country should import goods when the international price is less than the domestic opportunity cost (the potential benefit foregone) of producing an additional unit domestically. The opportunity cost is the cost of producing additional units of the product in terms of the reduction in the output of another product.

[197] Knapp and Rubino, "Political Economics of Marine Aquaculture," p. 213.

[198] CEA, *Offshore Finfish Aquaculture*, p. 4.

[199] Knapp, "Economic Potential."

[200] James P. Houck, *Elements of Agricultural Trade Policies* (University of Minnesota: Waveland Press Inc., 1992), p. 21. Hereinafter cited as Houck, *Elements of Agricultural Trade*.

would expire. However, as the industry becomes larger and more politically powerful, it may become difficult to remove the tariff.[201]

U.S. Experiences

U.S. aquaculture production from inshore marine areas and freshwater ponds and raceways is small relative to global production levels. The bulk of U.S. aquaculture production is from freshwater catfish, crayfish, and trout. Catfish production increased from 62,256 mt in 1983 to its peak of 300,056 mt in 2003. Factors that have supported the industry's development include research and development, marketing efforts, industry leadership, and vertical integration.[202] However, production decreased from 215,888 mt in 2009 to 145,230 mt in 2016. An increase of pangasius (an Asian catfish) and tilapia imports has contributed to lower prices, which have contributed to decisions by less profitable catfish farms to take acreage out of production.[203]

Salmon is the only marine finfish with significant U.S. marine aquaculture production, but it has struggled to compete with relatively inexpensive imports from Norway, Chile, and Canada.[204] These countries are endowed with protected coastal areas such as fjords or bays where net pens may be deployed. Although environmental regulations and limitations on inshore leases may have affected U.S. salmon aquaculture production, stagnant prices and competitive imports also appear to have played a role. There is room for expansion of inshore net pen salmon aquaculture in areas of Maine, Washington, and Alaska.[205] However, many residents in these areas do not support establishing or expanding net pen aquaculture because of environmental concerns and potential impacts on existing fishing industries. The ban on finfish aquaculture in Alaska and regulatory constraints in other states reflect these concerns.[206]

Offshore Development in Other Countries

Currently, nearly all worldwide marine aquaculture production is from relatively well-protected inshore waters. Countries in the forefront of efforts to move offshore have experience with inshore aquaculture and with aquaculture industries that are characterized by relatively large investments in vertically integrated firms.[207] Norway and China are the two largest investors in offshore aquaculture development, but neither country has facilities that are operating commercially. Their efforts have focused on developing structures that can withstand harsh offshore conditions and operate at scales that may offset the higher costs of offshore areas as compared to inshore areas.[208]

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[201] Houck, *Elements of Agricultural Trade*, p. 21.

[202] James L. Anderson and Gina Shamshak, "Lessons from the Development of the U.S. Broiler and Catfish Industries: Implications for Offshore Aquaculture in the United States," in *Offshore Aquaculture in the United States: Economic Considerations, Implications, and Opportunities*, NOAA, NOAA Technical Memorandum NMFS F/SPO-103, July 2008, pp. 97-116. Hereinafter cited as Anderson and Shamshak, "Lessons."

[203] The increase in fish feed prices is another factor that has affected U.S. catfish businesses.

[204] Shrimp are the largest seafood import by value and volume, but shrimp are not considered to be a viable candidate for offshore aquaculture because they are raised in topical coastal ponds and do not appear to be suited for offshore aquaculture.

[205] Atlantic salmon net pen aquaculture is not currently allowed in Alaska. Washington and Maine allow net pen salmon aquaculture but have limited its expansion because of environmental concerns.

[206] Knapp, "Economic Potential," p. 183.

[207] CEA, *Offshore Finfish Aquaculture*, p. 5.

[208] CEA, *Offshore Finfish Aquaculture*, p. 4.

Norway's industry already has extensive experience with inshore salmon aquaculture industry and is a leader in developing technology needed to move farther offshore.[209] Norway has granted development licenses in offshore waters, and Norwegian companies are experimenting with different offshore concepts. Although there has been significant investment in offshore aquaculture in Norway, it is unclear whether these concepts will be profitable. It appears that long-term business strategies are still focused on inshore waters.[210] Offshore aquaculture facilities are also under development in other countries, including Mexico, Panama, and Turkey.[211]

The characteristics of specific regions also may offer advantages, as some believe future development will occur in the calm water tropical belt between 10°N and 10°S.[212] One former offshore aquaculture farmer believes future investment will focus on new species in tropical and subtropical regions.[213] It appears that growth of marine aquaculture may take different approaches in different parts of the world, with further increases in production from proven nearshore areas and research and development of potential land-based and offshore areas. Generally, movement offshore is likely to occur if seafood demand continues to increase and suitable nearshore areas are occupied or constrained by other factors.[214]

Stakeholder Concerns and Aquaculture Development

Some stakeholders have expressed concerns about offshore aquaculture that include environmental degradation, competition for ocean space, and market interactions between wild fishery and aquaculture products. Historically, user conflicts associated with aquaculture have occurred in inshore areas where oceans activity and use are more intensive. For example, some fishermen oppose aquaculture and perceive it as competition that lowers prices and fishing revenues. Most interactions are characterized as conflicts, but in some cases synergistic relationships may emerge.[215]

Environmental concerns have been among the most controversial elements of the aquaculture debate, including expansion of aquaculture into offshore waters. Generally, environmental and commercial fishing interests have been opposed to plans for offshore aquaculture development because of potential harm to marine resources. They have asserted that poorly regulated inshore aquaculture development has degraded the environment and harmed wild fish populations and ecosystems.[216] Concerns identified by these stakeholders

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[209] The Alaska shoreline may include suitable areas for salmon net pens, but the state does not allow net pen aquaculture and many salmon fishermen believe this activity is not compatible with wild salmon fisheries.

[210] CEA, *Offshore Finfish Aquaculture*, p. 21.

[211] The definition of offshore aquaculture varies across countries. For example, offshore aquaculture facilities in Turkey are reported have characteristics that more closely resemble inshore aquaculture. CEA, *Offshore Finfish Aquaculture*.

[212] Edwards, "Aquaculture Environment Interactions," p. 11.

[213] U.S. Congress, Senate Committee on Commerce, Science, and Transportation, Ocean Policy Study, *Written Statement by John R. Cates*, Hearing on Offshore Aquaculture, 109th Cong., 2nd sess., April 6, 2006.

[214] CEA, *Offshore Finfish Aquaculture*, p. 4.

[215] Diego Valderrama and James Anderson, "Interactions between Capture Fisheries and Aquaculture," in *Offshore Aquaculture in the United States: Economic Considerations, Implications, and Opportunities*, NOAA, NOAA Technical Memorandum NMFS F/SPO-103, July 2008, pp. 189-206.

[216] Center for Food Safety, "Fishing and Public Interest Groups File Challenge to Fed's Unprecedented Decision to Establish Aquaculture in Offshore U.S. Waters," press release, February 16, 2016, at https://www.centerforfoodsafety.org/press-releases/4229/fishing-and-public-interest-groups-file-challenge-to-fedsunprecedented-decision-to-establish-aquaculture-in-offshore-us-waters. Hereinafter cited as Center for Food Safety, "Fishing and Public Interest Groups."

include pollution, the use of wild species for fishmeal, fish escapement, threat of disease and parasites, harm to marine wildlife, and general impacts on marine ecosystems.[217] Most commercial fishing and environmental interests advocate a precautionary approach.

Industry supporters and aquaculturalists respond that research, innovation, and management practices have reduced or eliminated environmental risks.[218] Generally, aquaculturalists assert that many previous environmental concerns have been addressed and that long-term aquaculture production relies on maintaining a clean and productive environment, an objective that environmental and fishing industry advocates also hold. Some also view offshore aquaculture as an additional means to support the domestic seafood industry, which has decreasing levels of employment in many regions. Some have noted that synergistic effects might support infrastructure and services such as docks, cold storage, and processing facilities that benefit both wild fishing and aquaculture.

Seafood imports from aquaculture production have affected seafood markets and coastal communities, such as salmon fishermen in Alaska and shrimp fishermen in the Gulf of Mexico.[219] Prices fell during the 1990s, as global salmon and shrimp aquaculture production and associated imports increased. This shift caused significant economic difficulties for Alaska salmon fishermen, processors, and communities.[220] Wild salmon prices have recovered to some extent, likely due to growing consumer differentiation between wild and cultured products. Some have responded that competition will occur with or without domestic growth in aquaculture because imports of farmed products are likely to continue and grow.[221] Other changes that have been attributed to aquaculture include accelerated globalization of the seafood industry, increased industry concentration and vertical integration, and introduction of new product forms.[222]

Marine aquaculture, especially the offshore aquaculture industry, is a small and new industry with few committed supporters and relatively little money and political influence.[223] One observer noted that, "marine aquaculture will become politically stronger as it grows—but it is difficult to grow without becoming politically stronger."[224] The industry also faces opposition from environmental and commercial fishing interests. Several developments will need to take place if offshore aquaculture can be expected to become established and grow into a viable commercial industry; these developments are discussed in the next section.

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[217] Center for Food Safety, "Fishing and Public Interest Groups."
[218] Knapp and Rubino, "Political Economics of Marine Aquaculture," p. 219.
[219] Written statement of Mark Vinsel, *Hearing on Offshore Aquaculture*, before the U.S. Senate, Committee on Commerce, Science, and Transportation, National Ocean Policy Study (April 6, 2006).
[220] Knapp, "Economic Potential," p. 175.
[221] Knapp, "Economic Potential," p. 175.
[222] Diego Valderrama and James Anderson, "Interactions Between Capture Fisheries and Aquaculture," in *Offshore Aquaculture in the United States: Economic Considerations, Implications, and Opportunities*, NOAA, NOAA Technical Memorandum NMFS F/SPO-103, July 2008, pp. 189-206.
[223] Knapp and Rubino, "Political Economics of Marine Aquaculture," p. 215.
[224] Knapp and Rubino, "Political Economics of Marine Aquaculture," p. 216.

INSTITUTIONAL NEEDS AND INDUSTRY SUPPORT

Regulatory Framework for Offshore Aquaculture

Most stakeholders agree that a regulatory framework likely needs to be developed before establishing offshore aquaculture in U.S. federal waters. A potential framework would need to fulfill the government's public trust responsibilities while remaining flexible enough to take advantage of evolving technology and markets.[225] Many of the basic elements of the framework would depend on legislation providing statutory authority and requirements for leasing offshore areas, agency leadership and interagency coordination, and environmental protection. A regulatory framework could provide the industry with clear and understandable requirements for aquaculture facilities while minimizing potential environmental harm. Supporters of offshore aquaculture have advocated for a permitting and consultation process that is more timely, efficient, and orderly than the existing process. Most also agree that the regulatory process should be transparent and support public involvement.

Lead Agency

NMFS has been the lead federal agency for marine aquaculture in inshore areas and for the potential development of offshore aquaculture.[226] According to a 2008 U.S. Government Accountability Office (GAO) study, "there is no lead federal agency for regulating offshore aquaculture, and no comprehensive law that directly addresses how it should be administered, regulated, and monitored."[227] Stakeholders also have supported NOAA's role in managing federal aquaculture research, including research and development of offshore aquaculture technologies.[228]

Since publication of the GAO report, NMFS has attempted to regulate offshore aquaculture under the MSA. A recent court decision, however, cast doubt on whether NOAA has the authority under MSA to regulate offshore aquaculture. Several studies have recommended that NOAA should be granted clear authority to regulate offshore aquaculture.[229] They point out that NOAA already has authority to evaluate proposed marine activities and projects to ensure the protection of marine mammals, endangered species, and marine sanctuaries. Furthermore, NOAA is responsible for federal management of marine fisheries and essential fish habitat.

Permits and Leases

One of the needs for offshore aquaculture development is permitting or leasing of discrete ocean areas.[230] Within the EEZ, the United States has sovereign rights for the purpose

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[225] Oceans Commission, *Ocean Blueprint*, p. 332.

[226] NOAA has played a supportive role in state waters but does not have jurisdiction to lease or regulate aquaculture in state waters.

[227] U.S. Government Accountability Office (GAO), *Offshore Marine Aquaculture*, GAO—08-594, May 2008. p. 2. Hereinafter cited as GAO, *Offshore Marine Aquaculture*.

[228] GAO, *Offshore Marine Aquaculture*.

[229] B. Cicin-Sain et al., *An Operational Framework for Offshore Marine Aquaculture in Federal Waters*, Center for Marnie Policy, University of Delaware, 2005; Oceans Commission, *Ocean Blueprint*.

[230] The terms *permits* and *leases* are used interchangeably in this report. The rights to discrete areas associated with permits or leases define their general meaning. Whether considered a lease or permit, the most important concerns are related to the rights and responsibilities granted to the offshore aquaculture developer.

of exploring, exploiting, conserving, and managing natural resources, whether living and nonliving, of the seabed and subsoil and superjacent waters. The federal government grants rights to develop specific areas for specific activities in the EEZ are granted. Currently, no permitting or leasing program is specific to offshore aquaculture and leases depend on permits and consultation requirements under different laws and agencies that apply to marine activities generally.

Observers generally agree that aquaculture developers will need assurances that they will have exclusive rights via leases or permits to use specific ocean areas for agreed-upon periods.[231] A leasing system could provide aquaculturalists with clearly defined rights to ocean space including the water surface, water column, and ocean bottom. Other characteristics of a leasing system might include transferability of the lease or permit, which would allow the aquaculturalist to transfer the permit or lease and benefit from its sale or use. Stakeholders told GAO that clear rights to use specific ocean areas would be needed to obtain loans.[232] Proponents of offshore aquaculture development stress that, without some form of long-term (at least 25 years) permitting or leasing, offshore aquaculture will have problems securing capital from traditional funding sources and obtaining suitable insurance on the capital investment and stock.[233]

The Gulf of Mexico Aquaculture Fishery Management Plan (Gulf FMP) provides a 10-year site permit and 5-year permit renewals.[234] Aquaculture industry representatives have expressed concern that these intervals are too short because of the time it will take their businesses to become profitable. Environmentalists would prefer "shorter timeframes to ensure more frequent reviews and closer scrutiny of environmental impacts during the lease or permit renewal process."[235] In state waters, Maine grants 10-year leases for salmon net pen aquaculture. Hawaii grants 20-year leases for permits in its waters.

The public's primary concerns are likely to include minimizing harmful effects on environmental quality and conflicts among ocean uses. Most recognize that a leasing framework will require review of potential environmental impacts of offshore aquaculture. This review likely would require the preparation of a programmatic environmental impact statement (PEIS) with a follow-up site-specific environmental review before a facility might be established.[236] A PEIS could review potential environmental impacts of offshore aquaculture over broad areas of the ocean. Aquaculturalists generally agree that this approach would be useful if it reduced the need for facility-specific reviews.[237]

Some have suggested that permits should be issued on a case-by-case basis by determining whether a specific site is appropriate for the proposed aquaculture facility. Others oppose this approach, because it could lead to an approval process that is less consistent and it

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[231] Anderson and Shamshak, "Lessons."

[232] GAO, *Offshore Marine Aquaculture*, p. 5.

[233] Some nations (e.g., Canada) lease nearshore areas with implied automatic renewal of tenure as long as the lessee meets current licensing requirements.

[234] NMFS, "Fisheries of the Caribbean, Gulf, and South Atlantic; Aquaculture," 81 *Federal Register* 1762-1800, January 13, 2016.

[235] GAO, *Offshore Marine Aquaculture*, p. 5.

[236] Bureau of Reclamation, *What is the Difference Between a Programmatic and a Project-Level Environmental Impact Statement?*, Yakima River Basin Integrated Water Resource Management Plan, November 2013, at https://www.usbr.gov/pn/programs/eis/kkc/ scoping/progsite.pdf. A programmatic environmental impact statement evaluates the effects of broad proposals for planning-level decisions that may include a wide variety of individual projects.

[237] GAO, *Offshore Marine Aquaculture*.

could make it more difficult for regulators to assess cumulative impacts of different facilities within a region.

Still others have suggested that ocean planning should identify both appropriate and prohibited areas for aquaculture. Regulators could assess potential sites for permitting aquaculture before and independently of individual permit applications. Some believe that this would make permitting more predictable and consistent. For example, the likelihood of harm to marine mammals might be decreased by limiting permits for aquaculture facilities to areas with a low risk of interactions.[238] However, some aquaculturalists question whether regulators will choose the most viable sites for aquaculture.

Conditions of Use

A regulatory framework is likely to require specific conditions on the use of a site. These requirements likely will vary depending on the species and technology employed. Nevertheless, some basic requirements related to environmental quality, inspections, and other public concerns are likely to be common to many offshore aquaculture operations. The Gulf FMP includes specific requirements that could be applicable to managing offshore aquaculture in other regions. A partial list of operational requirements under the Gulf FMP includes the following:

- placing at least 25% of the facility in the water at the site within two years of issuance of the permit;
- marking each system placed in the water with an electronic locating device;
- obtaining juveniles for stocking from certified hatcheries within the United States;
- providing a health certificate prior to stocking fish at the aquaculture facility;
- complying with all FDA requirements when using drugs or other chemicals;
- monitoring and reporting environmental survey parameters consistent with NMFS guidelines,
- inspecting for interactions or entanglements of protected species; and
- allowing access to facilities to conduct inspections.

Some have recommended requirements for aquaculture facility plans to address potential contingencies, such as fish escapes from aquaculture facilities. Some representatives of fishery management councils supported marking or tagging hatchery fish as a potential means of tracking escaped organisms. However, some have questioned whether the added costs of marking fish are warranted and contend that tagging requirements should depend on the level of associated risk to natural resources.

Monitoring could also be required to track interactions with marine life and other changes to the environment. State regulators in Maine and Washington have developed monitoring requirements for net pen salmon aquaculture, such as monitoring the benthic community under net pens. Both states also require notification of disease outbreaks and can require specific mitigation measures depending on the severity of the outbreak. Federal monitoring requirements could be informed by state experiences and modified as better information becomes available. The Gulf FMP includes reporting requirements for stocking, major

[238] GAO, *Offshore Marine Aquaculture*, p. 24.

escapement, pathogen episodes (disease), harvest, change of hatchery, marine mammal and sea bird entanglement, and other activities or events.

Aquaculture facilities in offshore areas would occupy areas that may be used for other ocean uses, such as oil and gas development, wind and tidal energy, maritime transportation, and commercial and recreational fisheries. Some have recommended that "development of a national aquaculture management framework must be considered within the context of overall ocean policy development, taking into account other traditional, existing, and proposed uses of the nation's ocean resources."[239] If conflicts develop over access to particular areas, a process would need to be developed to identify suitable areas in federal waters for aquaculture development and/or to mediate disputes. For example, commercial and recreational fishermen may have concerns regarding access to areas they have fished historically and potential interactions of cultured and wild fish. Some ocean managers have suggested that overlaying maps of different jurisdictions, ocean uses, and conditions favorable to aquaculture would be useful in avoiding user conflicts.[240]

Other Management Entities

As a regulatory framework for offshore aquaculture is developed, it could be enhanced by improving coordination and cooperation among federal, state, territorial, and tribal entities. Existing groups, such as the Subcommittee on Aquaculture, have provided a means for communication among federal agencies that might be used to enhance federal coordination of offshore aquaculture development and management.

The fishery management councils established under the MSA likely would have a role in offshore aquaculture development. Each of the eight regional councils develops FMPs for wild marine fisheries within its particular region. These plans are then sent to NMFS for approval and implementation. Historically, fishery management councils have had a role in considering whether to support offshore aquaculture in federal waters. In addition to the Gulf of Mexico FMP for aquaculture, several exempted fishing permits were issued for limited periods to investigate potential aquaculture development in federal waters off New England. Potential interactions with wild fisheries and harm to essential fish habitat and wild fish populations are likely to be fishery management councils' main concerns.

In addition to consultation requirements under the Coastal Zone Management Act, the state role in developing a regulatory framework for offshore aquaculture may deserve additional consideration. Some stakeholders support an opt-out provision allowing states to refuse development in federal waters adjacent to state waters. Others suggest that the opt-out provision should apply only within a certain distance of shore (such as 12 nm).[241] In response to earlier proposed legislation, NOAA supported a 12 nm distance to provide states with a buffer zone and simplify the difficulties of projecting state boundaries out to 200 nm.[242] Harmonizing aquaculture regulations with adjacent states could provide an advantage to future development, because states would be in a position to limit or promote offshore aquaculture development.

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[239] Oceans Commission, *Ocean Blueprint*, p. 333.

[240] Carol S. Price and Jessica Beck-Stimpert, *Best Management Practices for Marine Cage Culture Operations in the U.S. Caribbean*, NOAA, GCFI Special Publication Series Number 4, 2014.

[241] GAO, *Offshore Marine Aquaculture*.

[242] Projecting state zones in the northeastern United States could be problematic because of the number and geography of coastal states in the region.

Federal Support for Offshore Aquaculture

Some assert that federal government assistance would be needed to promote the initial development of a U.S. offshore aquaculture industry. Assistance could range from general support of research to direct support of industry needs, such as finance. One argument in support of government assistance is that, in comparison to relatively well-known agriculture sectors such as animal husbandry, there are more uncertainties associated with offshore aquaculture.[243] With the exception of Atlantic salmon, culture of most marine finfish is still at a relatively early stage of development. Development of offshore aquaculture is likely to require new culture techniques for rearing species not presently cultured. For this reason, the U.S. Oceans Commission recommended more assistance for aquaculture generally and an active government role to foster industry development.

Stakeholders identified federal research needs in four areas:[244]

- developing fish feeds that do not rely on harvesting wild fish;
- developing best management practices;
- exploring how escaped offshore aquaculture-raised fish might impact wild fish populations; and
- developing strategies to breed and raise fish while effectively managing disease.

In addition to improving culture techniques, further research of interactions between aquaculture and the environment and potential harm to specific species and ecosystems could inform decisions related to site selection and monitoring needs.

A remaining question is which agency or agencies will provide the support needed for offshore aquaculture development. Some may question whether NOAA has adequate institutional experience with aquaculture or whether additional resources are needed to provide adequate program management and services. Some NOAA programs support the fishing industry, but none focus specifically on offshore aquaculture. Similarly, USDA administers a number of programs that support agriculture in areas such as finance, research, extension, market development, and disaster assistance, but none are specifically focused on offshore aquaculture. Legislation in the 116th Congress to support offshore aquaculture may address whether and how NOAA and/or USDA programs could be adapted to the needs of offshore aquaculture, which is the appropriate agency to manage specific programs, and what level of federal support is appropriate.

POTENTIAL ISSUES FOR CONGRESS

Currently, development of offshore aquaculture appears unlikely because of regulatory, technical, and economic uncertainties. One of the main issues for Congress is whether legislation can be developed that could provide the industry with greater regulatory certainty while assuring other stakeholders that environmental quality can be maintained and other potential conflicts minimized. Research and development of inshore facilities have shown that

[243] Some would argue that this is also true for most inshore and freshwater species. Only freshwater finfish, such as catfish, salmon, and trout, and oysters in estuarine waters have been cultured extensively in the United States.
[244] GAO, *Offshore Marine Aquaculture*.

offshore aquaculture is technically feasible but have not shown whether moving facilities to offshore areas would be profitable. It is likely that the investment required for commercial development of offshore aquaculture facilities will depend to some degree on greater regulatory certainty. For example, one business that was developing offshore aquaculture in Puerto Rico has moved its operations to Panama; according to the owner, U.S. regulations made expansion impossible.[245]

Aquaculturalists and investors are likely to require secure property or leasing rights and clear regulatory requirements before investing in large-scale operations. Stakeholders with concerns that aquaculture will degrade the environment also may need assurances that adequate regulation, inspections, and enforcement will be required features of a regulatory program. These concerns have been reflected in several aquaculture bills that would prohibit offshore development until comprehensive legislation is enacted.

Previous congressional actions, such as hearings and bills, have concentrated on several areas, which include

- providing institutional support for aquaculture, such as planning, research, and technology transfer;
- identifying a lead agency to administer and coordinate aquaculture development and regulation;
- establishing and streamlining permit and consultation requirements to improve the efficiency of the permitting process;
- developing processes to consult and communicate with other stakeholders to reduce user conflicts; and
- minimizing environmental harm and addressing environmental concerns through planning and monitoring.

If aquaculture is developed in the EEZ, most stakeholders likely would agree that there is a need for better coordination, clear regulation, and focused agency leadership. Some assert that congressional action will be necessary to support both commercial development and environmental protection.

CONGRESSIONAL ACTIONS

Congress has made several attempts to pass offshore aquaculture legislation, including bills in the 109th, 110th, 111th, 112th, and 115th Congresses, but none of these bills were enacted. Bills also were introduced that would have prevented aquaculture development in federal waters until statutory authority for offshore aquaculture development is enacted. While many stakeholders continue to call for federal legislation, it has been difficult to find a common vision among them for future development of an offshore aquaculture industry.

[245] Eva Tallaksen, "Deep-Sea Cobia Producer Gears up for Full-Scale Launch," *Under Current News*, March 22, 2013, at https://www.undercurrentnews.com/2013/03/22/deep-sea-cobia-producer-gears-up-for-full-scale-launch/.

116th Congress

In the 116th Congress, no comprehensive offshore aquaculture legislation has been introduced, but several bills have been introduced that are related to offshore aquaculture and aquaculture generally. The Keep Finfish Free Act of 2019 (H.R. 2467) would prohibit the issuance of permits to conduct finfish aquaculture in the EEZ until a law is enacted that allows such action. The Commercial Fishing and Aquaculture Protection Act of 2019 (S. 2209) would amend the MSA to provide assistance to eligible commercial fishermen and aquaculture producers.[246] Assistance could be provided when an eligible loss occurs due to an algal bloom, freshwater intrusion, adverse weather, bird depredation, disease, or another condition determined by the Secretary of Commerce. Other bills include the Prevention of Escapement of Genetically Altered Salmon to the United States Act (H.R. 1105) and the Shellfish Aquaculture Improvement Act of 2019 (H.R. 2425).

115th Congress

In the 115th Congress, the Advancing the Quality and Understanding of American Aquaculture Act (AQUAA Act; S. 3138 and H.R. 6966) would have established a regulatory framework for aquaculture development in federal waters.[247] The bills would have provided NMFS with the authority to issue aquaculture permits and to coordinate with other federal agencies that have permitting and consultative responsibilities. They also would have identified NOAA as the lead federal agency for providing information on federal permitting requirements in federal waters.

S. 3138 and H.R. 6966 would have required the Secretary of Commerce to develop programmatic environmental impact statements for areas determined to be favorable for marine aquaculture and compatible with other ocean uses. Section 9 of the bills stated that it would not supersede the requirements of the National Environmental Policy Act of 1969 (NEPA) and that individual projects may require additional review pursuant to NEPA.[248] The bills would have required the Secretary of Commerce to consult with other federal agencies, coastal states, and fishery management councils to identify the environmental and management requirements and standards that apply to offshore aquaculture under existing federal and state laws. The bills also identified 10 standards that should be considered for offshore aquaculture and applied when issuing permits conducting programmatic environmental impact statements. These standards included other ocean uses, conservation and management of fisheries under the MSA, and minimizing adverse impacts on the marine environment, among others.

S. 3138 and H.R. 6966 would have provided institutional support of offshore aquaculture by establishing the Office of Marine Aquaculture within NOAA. The Office of Marine Aquaculture would have been responsible for coordinating NOAA activities related to

[246] An eligible commercial fisherman and farm-raised fish producer generally are described as an individual or entity that assumes production and market risks associated with harvesting fish (fisherman) or production of fish in a controlled environment (farm-raised fish producer) for commerce. The term *fish* would include shellfish, finfish, and other aquatic organisms harvested with the intent of entering commerce.

[247] H.R. 6966 is nearly identical to S. 3138 and was introduced in the House near the end of the 115th Congress.

[248] 42 U.S.C. §§4321 et seq.

regulation, scientific research, outreach, and international issues. The Office of Marine Aquaculture would have replaced the current Office of Aquaculture, which conducts activities that are similar to those proposed by the bills.[249] The bills also would have made NOAA the lead agency for establishing and coordinating a research and development aquaculture grant program.

A bill was also introduced (H.R. 223) that would have prohibited the issuance of permits to conduct finfish aquaculture in the EEZ except in accordance with a law authorizing such action. Similar bills also were introduced in earlier Congresses to stop offshore aquaculture development in the EEZ.

Congressional Actions Prior to the 115th Congress

Offshore aquaculture bills also were introduced in the 109th, 110th, 111th, and 112th Congresses.[250] Generally, these bills focused on establishing a regulatory framework to develop offshore aquaculture in federal waters of the EEZ. The bills varied to some degree on the balance between the potential rights and responsibilities of aquaculturalists, especially between aquaculture development and environmental protection. For example, S. 1195 (109th Congress), and H.R. 2010 and S. 1609 (110th Congress) would have supported production of food, encouraged development, established a permitting process, and promoted research and development of offshore aquaculture. In contrast, H.R. 4363 (111th Congress) and H.R. 2373 (112th Congress) would have focused to a greater degree on potential impacts of offshore aquaculture. These bills stressed elements such as determining appropriate locations, issuing regulations to prevent impacts on marine ecosystems and fisheries, and supporting research to guide precautionary development of offshore aquaculture.

Other bills that would have constrained offshore aquaculture development were introduced in the 108th, 109th, 110th, 112th, 113th, and 114th Congresses. Most of these bills would have prohibited the issuance of permits for marine aquaculture facilities in the EEZ until requirements for issuing aquaculture permits are enacted into law.[251]

CONCLUSION

The United States is the largest importer of seafood products in the world, and nearly half of domestic seafood imports are produced by aquaculture. Aquaculture development and production in the United States have lagged behind other countries due to a variety of factors, such as relatively inexpensive imports, regulatory policies, user conflicts, and higher costs of production. Some have speculated that marine aquaculture facilities could be developed farther offshore in federal waters, where they would be subject to fewer user conflicts and have space to operate in relatively clean ocean waters. However, movement to offshore areas also would involve several significant challenges, such as establishing a regulatory

[249] NMFS, NOAA Office of Aquaculture, at https://www.fisheries.noaa.gov/about/office-aquaculture.

[250] Bills included S. 1195 (109th Congress), S. 1609 and H.R. 2010 (110th Congress), H.R. 4363 (111th Congress), and H.R. 2373 (112th Congress).

[251] Examples include S. 2859 (108th Congress), S. 796 (109th Congress), S. 533 and H.R. 7109 (110th Congress), H.R. 574 (112th Congress), H.R. 753 (113th Congress), and H.R. 331 (114th Congress).

framework, developing new technologies, and competing with other existing sources of seafood.

According to many stakeholders and researchers, the lack of a governance system for regulating offshore aquaculture hinders the industry's development in the United States. Development of marine offshore aquaculture would likely require a new regulatory framework for establishing offshore aquaculture in federal waters.[252] A regulatory framework potentially could provide the industry with clear requirements for its development while minimizing potential environmental harm. It remains an open question whether legislation could be crafted to achieve a balance between providing the certainty sought by potential commercial developers of aquaculture and satisfying environmental and other concerns of stakeholders such as environmentalists and fishermen.

While a new regulatory framework potentially could provide greater certainty to offshore aquaculture developers, other challenges would remain. For example, offshore aquaculture may involve higher costs and greater risk of losses associated as compared to inshore operations. Lack of experience operating in offshore areas and limited knowledge of culture techniques for many candidate marine species contribute to the financial risk of offshore aquaculture. Some observers expect that offshore aquaculture may occur incrementally as inshore areas are developed and culture techniques are refined. Federal support may be needed for finance, research, extension, market development, and disaster assistance, similar to USDA support of agriculture.

[252] B. Cicin-Sain et al., *An Operational Framework for Offshore Marine Aquaculture in Federal Waters*, Center for Marine Policy, University of Delaware, 2005.

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Chapter 156

AN APPROACH TO DETERMINING ECONOMIC IMPACTS OF U.S. AQUACULTURE*

Doug Lipton, Matt Parker, John DuBerg and Michael Rubino

ABSTRACT

Fisheries Economics of the United States is produced annually by the National Marine Fisheries Service and provides national and state level estimates of the total economic impacts of U.S. seafood landings and imported seafood on the U.S. economy. However, it does not contain an estimate of the impact of U.S. aquaculturally produced seafood. As a demonstration of the potential for incorporating this information into *Fisheries Economics of the United States*, we took estimates of production and value for four aquaculture species: crawfish, salmon, oysters and clams. Using published production cost data and the same input/output model used for *Fisheries Economics of the United States*, we produced estimates of economic impacts. We make recommendations for improving the annual production and value estimates that are used for the input/output model, and for developing standardized industry surveys on production costs so that reliable impact estimates can be developed on an annual basis and included as part of *Fisheries Economics of the United States*.

EXECUTIVE SUMMARY

This report describes initial efforts to develop an estimate of the economic impacts of all U.S. aquaculture (marine and freshwater) that could be integrated into *Fisheries Economics of the United States*, an annual report published by the National Marine Fisheries Service. Our approach was to gather production budgets for several aquaculture species from published reports along with the annual production and value estimates obtained on an annual basis

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through various sources, and using these numbers, calculate the direct, indirect, and induced economic impacts using an input-output model of the U.S. seafood economy.

The U.S. fishing and seafood industries are important components of the U.S. economy. According to *Fisheries Economics of the U.S. 2015*, the seafood industry supported 1.2 million full-and part-time jobs and generated $144.2 billion in sales, $39.7 billion in income, and $60.6 billion in value-added impacts nationwide. These estimates include the impacts from domestic wild harvest products and imports of wild-harvested and foreign aquaculture products, but not the impacts of domestic aquaculture. The omission of domestic aquaculture production from the estimates will become more problematic as domestic aquaculture expands and becomes a greater share of the U.S. seafood market. Domestic aquaculture already represents one fifth of U.S. seafood production by value.

Due to a lack of current, sufficiently detailed and standardized production budgets for major aquaculture species, it is not currently possible to produce a comprehensive estimate of the national economic impact from aquaculture production for all major species. NOAA and USDA in partnership with industry associations and university researchers could work with aquaculture companies to develop representative and updated aquaculture production budgets to be used in the development of annual estimates of aquaculture impacts.

To explore how this could be accomplished, we developed estimates of the economic impacts of four major species: crawfish, salmon, clams and oysters. Crawfish are a freshwater species and have the most reliable production and cost estimates compared to the other three marine species. Oysters, clams and salmon represented 95% of the first sale value of marine aquaculture production in 2015. From *Fisheries of the U.S. 2016*, marine aquaculture production in 2015 had total first sales (farm gate) of about $394 million. By assuming that the 5% of production we have not calculated impacts for create impacts in the same proportion as those we have calculated, then the total impact for marine aquaculture production on the U.S. economy is estimated to be about $5.1 billion, and results in over 53,000 jobs.

We envision what the economic impact would be if a goal of increasing U.S. aquaculture production to times its current level in ten years is met. Depending on assumptions about the species composition of the increase, we estimate the total economic impact would range from $10.7 - $12.8 billion and the number of jobs from 109,500 - 133,400.

The above estimates should be used cautiously given the lack of reliability in the statistics about the current level of aquaculture production, and the production budgets on which the estimates are based. We make several findings and recommendations as to actions needed to produce reliable annual economic impact estimates that are summarized here:

1) *Fisheries Economics of the United States* currently provides useful information to stakeholders and the general public about the economic impact of the fishing and seafood industries, and should include domestic aquaculture impact estimates, particularly as domestic aquaculture increases in importance as a component of U.S. seafood supply.
2) There is insufficient extant cost information and only greatly outdated information on production costs for several major species to develop a reasonable national estimate of economic impacts.

3) A systematic way of collecting annual aquaculture production data from states, industry associations, or directly from producers is essential to ensuring the quality of the estimates that rely on these numbers.
   a) A clear definition of what constitutes aquaculture production, particularly for shellfish, is necessary and will help avoid some double counting in commercial landings that occurs now.
   b) Since there is interest in reporting on marine versus freshwater aquaculture production, classification of what constitutes each will have to be agreed upon.
   c) Protecting confidentiality of firm level data will be an issue when there are a small number of firms constituting the production for a particular species.
4) Systematic collection of production costs via standardized industry surveys will provide the most reliable information for economic impact analysis. The relevant Federal agencies (i.e., USDA and NOAA), in conjunction with state agencies and aquaculture industry organizations, should come together and plan a survey methodology and a way to administer maintain and update it on a regular basis.
   a) Updating industry cost data every five years would allow National Income and Product Accounts data to be updated at the same frequency.
   b) Short of a census, any type of survey sample would have to be designed to capture the heterogeneity of the industry, even for production of the same species.
5) A comprehensive study on the seafood market chain would allow us to more accurately model product flows and increase reliability of the impact analysis.
   a) Interstate product flows need to be quantified
   b) Upstream (i.e., hatchery and nursery) production costs need to be better quantified, particularly for emerging industries.
6) Aquaculture production for other than just seafood markets (e.g., bait fish, pond stocking, etc.) will require additional analysis.

1. INTRODUCTION

Estimating the economic impact contributed by the aquaculture sector is essential information used to inform Federal, state and local policies. An economic impact analysis denotes the relative importance of the sector to the overall economy as well as quantifies the dependencies among different economic sectors on a regional and national level. Each year, the National Marine Fisheries Service (NMFS) publishes *Fisheries Economics of the United States (FEUS)*[1], providing national and state-by-state economic impact estimates. These estimates include commercial fisheries landings impacts on a state- by-state level, as well as state-level and national impacts from imported seafood as it moves through the value chain to the final consumer. NMFS also has developed regional economic impact models which aid in determining impacts for fisheries management regulations on a more detailed level than the

[1] National Marine Fisheries Service (2017a) Available at: http://www.st.nmfs.noaa.gov/economics/publications/feus/fisheries_economics_2015/index.

national model. NMFS's analysis to date has not included domestic or imported aquaculture products, except some domestic product that may be included in domestic wild catch[2].

It is estimated that as much as 90% by value of U.S. seafood consumed by Americans is imported, with about 50% of that produced via aquaculture[3]. Because *FEUS* includes imports, the contribution of imported aquaculture products to the U.S. economy is accounted for in the *FEUS* analysis. Missing from these reports, however, are the economic impacts of domestic aquaculture production. While still a small percentage of domestic fisheries production in pounds (6.5%), U.S. aquaculture production in 2015 was 21% of the first sale value (*Fisheries of the U.S. 2016*). As domestic aquaculture grows in importance, ignoring its role in economic impact estimates will become more problematic in terms of correctly quantifying the impact of fish and seafood production on the U.S. economy. Additionally, a greater understanding of the economic linkages of domestic aquaculture production in the economy will help in predicting and planning for the potential future contribution of aquaculture as it expands to play an even more significant role in the seafood economy.

Without an annual national survey for all aquaculture, NMFS relies on a variety of sources to estimate U.S. aquaculture production and value on an annual basis, and publishes this with an additional year lag in *Fisheries of the United States* (Table 1)[4]. Sources include the USDA Census of Agriculture and the periodic Census of Aquaculture[5] along with state and industry association supplied data. As in wild harvest production, an accurate estimate of annual production and value is essential to producing meaningful economic impact estimates because these numbers form the basis of all subsequent calculations of impacts, as will be discussed below.

1.1. Purpose and Organization of the Study

The original purpose of this study was to demonstrate that we could produce aquaculture economic impact estimates for most species from existing data that could be incorporated on a regular basis into *Fisheries Economics of the U.S.* By attempting to calculate the economic impact of four aquaculture species with currently available cost data and production estimates, we uncover what changes and additional research are necessary to produce future estimates that are equivalent in rigor to those currently published in *Fisheries Economics of the U.S.* for domestic wild caught fish production.

The work was conducted by a team of University of Maryland and NMFS staff. The research team received design guidance from an Advisory Committee of university and private sector economists along with staff at the U.S. Department of Agriculture (USDA). Difficulties gathering reliable production data for several species and advice from our Advisory Committee led us to narrow the study from all U.S. aquaculture to one focused on four species and one that would provide guidance in terms of data collection and analysis for

[2] Some aquaculture production statistics, particularly those for shellfish, may be mixed in with commercial landings data at the individual state level. For example, oyster aquaculture on public bottom may be counted as part of wild harvest landings.

[3] https://www.fisheries.noaa.gov/national/aquaculture/us-aquaculture.

[4] Fisheries of the United States, 2016 (National Marine Fisheries Service, 2017b) is released in 2017, but the aquaculture production estimates that are included are from 2015.

[5] https://www.agcensus.usda.gov/Publications/Census_of_Aquaculture/.

developing future estimates of the economic impact of all of U.S. domestic aquaculture production. The four species are oysters, clams, salmon, and crawfish.

The paper is organized as follows. First we provide a description of the *FEUS* calculation framework and then demonstrate how domestic aquaculture production can enter into the framework. Case studies for several species, where current data was of acceptable quality, are developed to demonstrate the approach. We discuss shortcomings of the existing data for the case study species, and the necessary steps to include all the major aquaculture species in the analysis. We conclude with recommendations for steps necessary to achieve the goal of producing an annual estimate of the economic impact of U.S. aquaculture production.

Table 1. Estimate of 2015 U.S. aquaculture production and value

	Thousand Pounds	Metric Tons	Thousand Dollars
Freshwater			
Catfish	317,445	143,992	347,021
Striped bass	8,111	3,679	30,831
Tilapia	18,999	8,618	42,745
Trout	45,854	20,799	76,748
Crawfish	140,411	63,690	199,350
Total Freshwater	530,820	240,778	696,695
Marine			
Salmon	47,528	21,559	87,743
Clams	9,086	4,121	112,139
Mussels	717	325	10,201
Oysters	35,229	15,980	172,778
Shrimp	3,979	1,805	11,137
Total Marine	96,539	43,790	393,998
Miscellaneous	-	-	302,774
Totals	627,359	284,568	1,393,468

Source: Fisheries of the United States, 2016.

2. INTEGRATING DOMESTIC AQUACULTURE PRODUCTION IMPACTS INTO FISHERIES ECONOMICS OF THE U.S.

Since our goal is to develop a method to integrate domestic aquaculture production into the calculations made for *FEUS*, we briefly describe the current methodology for the calculations for wild harvest and imports. Fish harvesting and seafood production is a major industry and data collected by the Economic Census, Bureau of Labor Statistics, and other sources are used by the Bureau of Economic Analysis as part of the development of national income accounts used to calculate the gross domestic product of the United States. The North American Industry Classification System (NAICS) describes industry code 114111 Finfish Fishing as:

> "This U.S. industry comprises establishments primarily engaged in the commercial catching or taking of finfish (e.g., bluefish, salmon, trout, tuna) from their natural habitat."

Code 114112 is Shellfish Fishing. With only one code for finfish harvesting and one for shellfish, this is a highly aggregated accounting of a diverse industry that is of limited use for decision-making impacting a particular species or region.[6] Additionally, the underlying source of data used by BEA[7] does not capture the great variability of scale and input use in commercial fisheries harvest in the United States. For these reasons, NMFS had developed its own national economic impact model and also has developed regional models with even greater specificity to support the economic analyses required by law for fisheries management plans.

2.1. Wild Domestic Harvest in Fisheries Economics of the U.S.

Kirkley (2009) describes the process used by the National Marine Fisheries Service for developing economic impact estimates for the domestic fishing and seafood industry. The approach is a modification and customization of a basic input/output model of the U.S. economy using IMPLAN[8] software and data. The model is designed to provide a national estimate of impacts and state level estimates for 23 coastal states. Figure 1 demonstrates the linkages. Starting with domestic landings, to develop estimates of the upstream impacts, it is necessary to determine fishing production costs by category. The downstream impacts require knowledge of the percentage of product that flows to each of the downstream sectors, and then the value added by expenditure category by each of those downstream sectors.

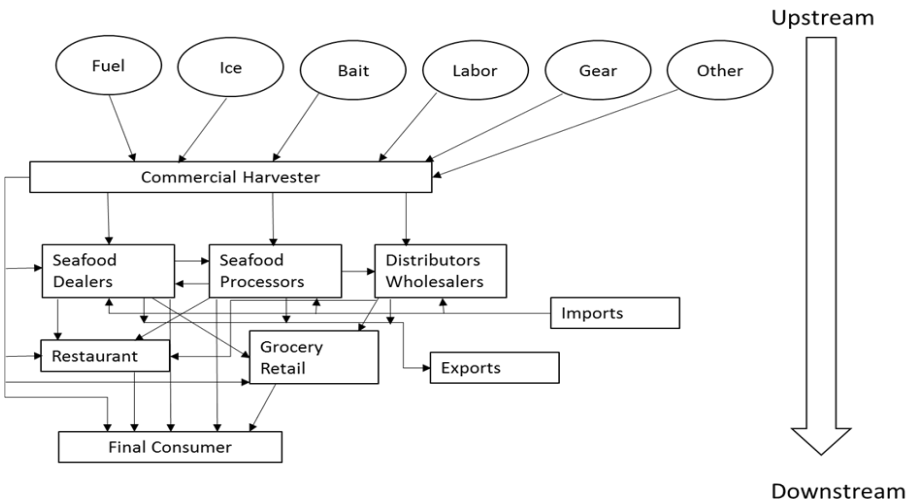

Figure 1. Schematic of the seafood market underlying calculations of economic impacts for Fisheries Economics of the U.S.

[6] There is some greater species specificity in the underlying data that helps to generate the input-output tables in the national accounts. The categories are: Alaska pollock, tuna, salmon, sardines, ground fish (cod, cusk, haddock, hake, Atlantic ocean perch, Atlantic pollock and whiting), flounder, other finfish, shrimp, crabs, oysters, clams, other shellfish, surimi and frozen fish blocks.

[7] See NIPA Handbook: Concepts and Methods of the U.S. National Income and Product Accounts, https://www.bea.gov/methodologies/index.htm.

[8] IMPLAN is a commercial software product commonly used for the calculation of economic impacts of industry sectors, nationally and regionally (www.implan.com).

Table 2. Typical fishing expenditure categories for inclusion in Fisheries Economics of the U.S. calculations (from Kirkley, 2009)

Purchases of goods	Fixed and general expenses
• Fishing gear	• Moorage
• Miscellaneous hardware & supplies	• Dues, fees
• Electronics	• Licenses, permits
Repair & maintenance	• Accounting
• Fishing gear, nets	• Insurance
• Vessel & engine	• Bank fees and services
• Electronics	• Vehicle costs
Trip expenses	• Capital costs, boats
• Groceries, food, & supplies	• Other expenses
• Fuel & lubricants	Taxes
• Ice	Income and profit
• Bait	• Crew & captain shares, other income
	• Profit

Table 3. Species groupings used for impact analysis for Fisheries Economics of the U.S. (from Kirkley, 2009)

Species group	Major species in group
Shrimp	All Shrimp
Crab	All Crab except Blue Crab
Lobster	American Lobster
East Coast Groundfish	Cod, Flounder, Goosefish, Haddock, Hake, Plaice, Pollock, Shark (Dogfish)
Highly Migratory Species	Shark (other than Dogfish), Swordfish, Tuna
Reef Fish	Gag, Grouper, Mackerel (King & Spanish), Snapper, Tilefish
West Coast Groundfish	Cod, Hake, Pollock, Rockfish, Sablefish, Sole, Whiting Halibut All Halibut
Menhaden/Industrial	Alewife, Ladyfish, Menhaden
Salmon	All Salmon
Sea Scallop	All Scallop
Surf Clam/Ocean Quahog	Surf Clam, Ocean Quahog, Quahog
Other Trawl	Anchovies, Croaker, Herring, Mackerel (other than King & Spanish), Mullet, Sardine, Shad, Squid
All Other Finfish	Amberjack, Drum, Hind, Pompano, Porgy, Scad, Sea Bass, Tautog
All Other Shellfish	Clam, Spiny Lobster, Mussel, Oyster, Sea Urchin, Snail (Conch)
Freshwater	Catfish, Crayfish, Perch, Tilapia, Trout
Inshore & Miscellaneous	Bass, Blue Crab, Seaweed, Sponge Bait Worms, Bait fish

Table 2 shows the typical expenditure categories for which data is obtained for domestic fish landings. One decision made early on in developing the process was that it would not be feasible to develop separate expenditure estimates for every species in the landings database. Landings were aggregated into 16 major groupings as shown in Table 3. This aggregation ameliorates the complication that many fisheries are mixed, in that they catch multiple species on the same trip, and it would be difficult to allocate both trip and durable expenditures by category to individual species. Cost data corresponding to the categories shown in Table 2

were obtained from a variety of existing surveys and publications and assigned to each of the harvest groupings. Cost data is periodically updated when new studies and surveys become available. Adjustments are also made on an annual basis for price changes for some volatile sectors such as energy. As can be seen in Table 4, downstream product flows are even more highly aggregated than fish harvesting with only seven harvester categories, three processors and two wholesale/distributor categories. These percentages are updated periodically as new data becomes available.

Once the product flows and expenditures are estimated for the 16 categories of seafood, these are then mapped into North American Industrial Classification System (NAICS) codes, and the direct, indirect and induced impacts can be calculated using an input-output model such as IMPLAN.

Table 4. Downstream product flow for fishing & seafood industries related to domestic harvest (from Kirkley, 2009)

Source of fish, seafood products	Processors	Wholesalers /Distributors	Restaurants/ Food Service	Groceries /Retail Markets	Exports	Final Consumer
Harvesters: non-shrimp, non-bait	40.0%	45.0%	2.5%	7.0%	0.0%	5.5%
Harvesters: shrimp, except as noted	87.5%	12.5%	0.0%	0.0%	0.0%	0.0%
Harvesters: non- bait species in AL, MS	90.0%	5.0%	2.5%	2.5%	0.0%	0.0%
Harvesters: non- bait species in AK	90.0%	5.0%	1.0%	1.0%	0.0%	3.0%
Harvesters: non- bait species in CT, FL, HI, ME, NJ, NY, RI, SC	20.0%	25.0%	5.1%	6.2%	35.0%	8.7%
Harvesters: non- bait species in US	60.7%	27.8%	2.5%	4.0%	5.0%	0.0%
Harvesters: bait	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%
Processors: non-shrimp, non-bait except AK	0.0%	51.7%	17.7%	23.0%	0.0%	7.6%
Processors: shrimp: except AK	0.0%	10.0%	72.0%	17.8%	0.3%	0.0%
Processors: AK	0.0%	5.0%	1.0%	1.0%	93.0%	0.0%
Wholesalers/ distributors: except	0.0%	0.0%	60.0%	30.0%	8.0%	2.0%
Wholesalers/ distributors: AK	0.0%	0.0%	6.0%	3.0%	91.0%	0.0%

2.2. Aquaculture Integration

Similar to wild caught fisheries, the National Accounts include highly aggregated NAICS codes for finfish (112511), shellfish (112512) and other (112519) aquaculture production and hatcheries. The definitions from the 2017 NAICS publication[9] demonstrate the lack of specificity for these industries. For finfish farming and fish hatcheries, the description is *"This U.S. industry comprises establishments primarily engaged in (1) farm raising finfish (e.g., catfish, trout, goldfish, tropical fish, minnows) and/or (2) hatching fish of any kind."* For shellfish farming and hatcheries, the description is, *"This U.S. industry comprises*

[9] https://www.census.gov/eos/www/naics/2017NAICS/2017_NAICS_Manual.pdf (pp. 95-96).

establishments primarily engaged in farm raising shellfish (e.g., crayfish, shrimp, oysters, clams, mollusks).” Finally, for other, the description is, *“This U.S. industry comprises establishments primarily engaged in (1) farm raising of aquatic animals (except finfish and shellfish) and/or (2) farm raising of aquatic plants. Alligator, algae, frog, seaweed, or turtle production is included in this industry.”*

Figure 2 depicts, in general, how a more detailed economic impact analysis can be conducted for domestic aquaculture production. As shown, domestic aquaculture production is assumed to enter the market in a parallel manner to wild caught seafood, and thus can be handled exactly the same for estimating downstream impacts. In the analysis that follows, the aquaculture product is matched with the species groupings in Table 3 and then enters the marketing channels as shown in Table 4. In contrast, for upstream impacts – the upper ovals in Figure 2 – the economics of the production of aquacultured species is treated as fundamentally different from wild-caught fish production. For wild caught fish, all production costs are only associated with harvest. For aquaculture, most of the costs incurred are prior to harvest, with harvesting cost playing a lesser role in the overall cost of production depending on the species and grow-out method. As a result, even if the farm-gate price for an aquacultured species is the same as or similar to the ex-vessel price for a wild caught species, the upstream impacts by expenditure category may be quite different; although the aggregate effect on the economy may be similar. Therefore, it is necessary to develop production expenditure estimates by species and production method for aquaculture production in a manner similar to what was done for wild caught species. A comprehensive literature review revealed a number of studies of aquaculture budgets for different species and production methods that form the basis of the analysis in the ensuing “Case Studies” section of this report.

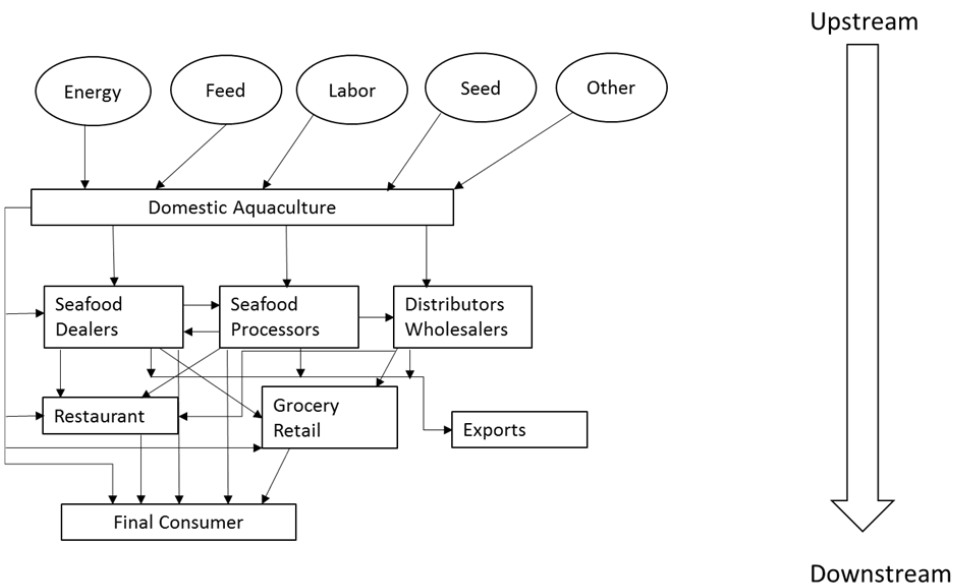

Figure 2. Schematic of the domestic aquaculture seafood market for estimating economic impacts.

3. CASE STUDIES

In this section, we develop case study production budgets for four aquaculture species: crawfish, salmon, clams, and oysters. Although crawfish is not a marine species, we include it here because it has the most complete data. A brief discussion of the data used for each species and its potential shortcomings are discussed, and then we present a table of the calculated economic impact estimates using the NMFS national economic impact model.

3.1. Crawfish Aquaculture

3.1.1. Crawfish Production Data

Production cost and returns data on crawfish was derived from Boucher and Gillespie (2014) which provides estimates for three types of crawfish operations: a single crop crawfish operation and two types of rice-crawfish double crop operations. The report provides significant detail on costs and the volume of production, but we had to reach out to the authors for explanation of some of the costs to properly classify them for the IMPLAN modeling. For example, a significant portion of costs were attributed in the text to "irrig single" and "pond&eq single." These costs were later determined to be associated with the cost of running the pond pump to irrigate in single crop crawfish operations, and the pond and equipment costs, respectively. Another challenge was how to convert detailed data on labor hours required to produce crawfish to a jobs impact in IMPLAN, due to the likelihood of part-time or seasonal employment in crawfish production. For our purposes we assumed that on average, a job in crawfish production is equivalent to 1,200 labor hours per year. Another uncertainty was determining how much of the three different production operations for which costs were obtained contributed to aggregate production. In the absence of additional information, we assumed that the models each represented one-third of aggregate production, and thus were assigned equal weights.

3.1.2. Crawfish Economic Impacts

Given the above qualifications, the estimated national impacts of aquaculture crawfish are presented in Table 5. Estimates of employment impacts (a mix of full-time and part-time jobs), income (both employee compensation and proprietor income), and output are provided for the aquaculture operations (i.e., harvesters) and the other segments in the value-added chain as well as a summary for all industry segments. Estimates include direct effects (the segment itself), indirect effects (those associated with the segment's supply chain), and induced effects (those created by the consumer spending of the directly and indirectly affected workers).

Reading horizontally across Table 5, one can see the impact on the national economy in terms of output, income, and jobs of just the farm level sales. Growers sell $199 million worth of crawfish which leads to a total economic impact of $590 million, due to the indirect and induced effects. Looking at the table vertically at the direct impacts, the farm level sales of $199 million lead to sales to final consumers (grocery and restaurant sales) of $877 million, which is the sum of farm level sales and the value-added for each of the downstream market sectors (i.e., processors, wholesalers, grocers, and restaurants) of $678 million.

Finally, the total economic impact of crawfish aquaculture in 2015 was over $2.6 billion, the summing up of direct, indirect, and induced effects from all segments of the market. Note that the largest contributor to the impact are induced impacts related to restaurant sales ($528 million). We want to emphasize the large contribution that the restaurant final demand sector makes, accounting for 46% of the output impacts and 55% of the jobs.

Table 5. Summary of all impacts for aquaculture: Crawfish

Industry Sector	Direct	Indirect	Induced	Total
Growers				
Employment impacts (jobs)	1,316	979	895	3,190
Income Impacts (000 of dollars)	45,464	58,111	45,141	148,716
Output Impacts (000 of dollars)	199,350	245,922	144,880	590,151
Primary dealers/processors				
Employment impacts (jobs)	862	639	940	2,441
Income Impacts (000 of dollars)	43,275	36,042	47,455	126,772
Output Impacts (000 of dollars)	127,420	107,490	152,033	386,943
Secondary wholesalers/distributors				
Employment impacts (jobs)	1,105	636	702	2,443
Income Impacts (000 of dollars)	78,481	37,691	35,405	151,577
Output Impacts (000 of dollars)	104,921	109,805	113,562	328,289
Grocers				
Employment impacts (jobs)	1,536	163	338	2,037
Income Impacts (000 of dollars)	39,575	10,660	17,074	67,310
Output Impacts (000 of dollars)	45,092	28,817	54,673	128,581
Restaurants				
Employment impacts (jobs)	10,677	1,499	3,265	15,440
Income Impacts (000 of dollars)	224,059	91,118	164,844	480,021
Output Impacts (000 of dollars)	400,106	269,703	527,716	1,197,524
Harvesters and seafood industry				
Employment impacts (jobs)	15,496	3,916	6,140	25,552
Income Impacts (000 of dollars)	430,855	233,623	309,919	974,396
Output Impacts (000 of dollars)	876,888	761,736	992,864	2,631,488

3.2. Oyster Aquaculture

3.2.1. Oyster Production Data

Unlike crawfish aquaculture production, which is concentrated in Louisiana, significant aquaculture production of oysters occurs in many coastal states, including Massachusetts, Maryland, Virginia, North Carolina, Washington, Oregon, and California. The state of Washington, however, dominates production, accounting for nearly half of the total value of production. Production cost data were available for each of these states and were weighted by value of production per state to determine a "national" production cost for aquaculture oyster production.[10]

[10] Data on production costs in Maryland, Virginia, and North Carolina were collected by the authors in consultation with industry experts. Data on Massachusetts were taken from "Massachusetts Shellfish Aquaculture Economic Impact Study," Winter 2015, a report from the University of Massachusetts, Dartmouth. Data for West Coast states was derived from surveys conducted for "The Economic Impact of Shellfish Aquaculture in

Among the significant uncertainties associated with these production costs are the facts that oyster production is often mingled with other shellfish and the wide variation in size of shellfish operations. Reports on production costs in Massachusetts (Augusto and Homes, 2013) and the West Coast states (Northern Economics, 2013) have detailed data on costs, but the data typically address operations that produce two or more shellfish species. Because of this mixing of data on species, it was necessary to dig deeper into the data in order to use it for a national estimate of oyster production costs. For the estimates below, survey data from Washington and California were reviewed, and only those operations that exclusively produced oysters were used to estimate production costs. These "oyster-only" producers were a minority of all operations, accounting for a small fraction of production value, therefore production costs from the West Coast states may be lower in reality than those included in this study since the dominant and potentially lower cost producers grow multiple species of shellfish. Alternatively, a report on Massachusetts shellfish production determined that oyster production accounted for roughly 90 percent of total shellfish production value.

The wide variation in size of operation is also likely to affect estimates of unit costs of production. For example, West Coast production is dominated by two large shellfish companies, which raise multiple species. On the East Coast, where there are about 1,000 shellfish aquaculture companies, three companies in Virginia represent a large share of the production.

As with crawfish, estimating job impacts involved assumptions that introduce uncertainty into the overall estimates. For Maryland, Virginia, and North Carolina, detailed information on either labor hours or labor costs was available. These data made clear that employment involves a mix of full-time and part-time or seasonal work. Accordingly, an average of 1,200 hours per job was assumed in generating an estimate of the number of jobs associated with these operations. Data on employment in Massachusetts, Washington, and California were defined in terms of employees (i.e., jobs) and could be used with less uncertainty to estimate the relationship between value of production and employment.

Other uncertainties include unspecified costs, which made up a significant share of the production data for West Coast states. Guidance from the lead author of that report (Northern Economics, 2013) was used to allocate the unspecified costs to memberships, travel, and marketing. As with other production operations, it was not always clear how costs (e.g., capital or oyster seed/spat) should be modeled. For capital, it was general assumed that trucks or boats were the primary capital cost for small and mid- sized oyster-only operations. Large scale operations also have high capital costs associated with their own hatchery and nursery operations. Seed or spat costs were allocated to an IMPLAN sector that includes fish hatcheries, but may not accurately reflect the economic characteristics of oyster seed production operations.

3.2.2. Oyster Economic Impacts

The preliminary estimates of impacts of aquaculture oysters are presented in Table 6. The 2015 first sale oyster production value was estimated to be $173 million, $26 million less (13%) than crawfish value. Interestingly, the indirect effect of this oyster production was significantly less (60%) than the impact from crawfish, while the induced effect is higher by

Washington, Oregon and California" prepared for the Pacific Shellfish Institute by Northern Economics, April 2013.

23% for oysters. This is due to the fact that labor costs make up a much higher percentage of oyster production compared to crawfish production. The greater labor reliance in oyster production can be seen in the direct employment estimates which equate to 23 jobs per $1 million of production compared to less than 7 jobs per $1 million for crawfish.

Table 6. Summary of all impacts for aquaculture: Oysters

Industry Sector	Direct	Indirect	Induced	Total
Growers				
Employment impacts (jobs)	3,936	553	1,103	5,593
Income Impacts (000 of dollars)	99,748	33,823	55,704	189,274
Output Impacts (000 of dollars)	172,778	98,400	178,358	449,536
Primary dealers/processors				
Employment impacts (jobs)	748	553	815	2,116
Income Impacts (000 of dollars)	37,507	31,238	41,129	109,874
Output Impacts (000 of dollars)	110,435	93,162	131,768	335,366
Secondary wholesalers/distributors				
Employment impacts (jobs)	958	551	608	2,117
Income Impacts (000 of dollars)	68,020	32,667	30,686	131,373
Output Impacts (000 of dollars)	90,936	95,169	98,425	284,530
Grocers				
Employment impacts (jobs)	1,331	142	293	1,766
Income Impacts (000 of dollars)	34,300	9,239	14,798	58,338
Output Impacts (000 of dollars)	39,081	24,976	47,385	111,442
Restaurants				
Employment impacts (jobs)	9,253	1,299	2,830	13,382
Income Impacts (000 of dollars)	194,194	78,972	142,871	416,037
Output Impacts (000 of dollars)	346,775	233,753	457,375	1,037,902
Harvesters and seafood industry				
Employment impacts (jobs)	16,269	3,104	5,661	25,033
Income Impacts (000 of dollars)	433,768	185,940	285,189	904,896
Output Impacts (000 of dollars)	760,005	545,460	913,311	2,218,777

3.3. Clam Aquaculture

3.3.1. Clam Production Data

Washington State is the largest producer of aquaculture clams with 45% of U.S. value. While there are good data on shellfish aquaculture operations in Washington State, most operations produce more than one type of shellfish. Consequently, extracting data on individual species was not practical for this effort.

Data for costs of production and income are from operations in Florida and Virginia as reported by the University of Florida Institute of Food and Agricultural Sciences Shellfish Aquaculture Extension Program and the Virginia Institute of Marine Science, Virginia Sea Grant Extension Program. Data represent statewide conditions and are assumed to be reasonable reflections of the average aquaculture operation in each state. Florida and Virginia are major sources of hard clams, representing 15% and 17% of 2014 value of production, respectively. Connecticut is another significant producer of hard clams with 15% of U.S. production value, but no cost data from Connecticut were available for this analysis.

3.3.2. Clam Economic Impacts

Table 7 provides the summary economic impacts for clam aquaculture. One thing that stands out immediately is that as currently practiced, clam aquaculture is nowhere near as labor intensive as oyster aquaculture; it is more similar in labor utilization to crawfish production. As a result, the induced impacts at the first sale level are more in line with crawfish production.

Table 7. Summary of all impacts for aquaculture: Clams

Industry Sector	Direct	Indirect	Induced	Total
Growers				
Employment impacts (jobs)	1,025	461	565	2,052
Income Impacts (000 of dollars)	44,501	25,948	28,528	98,977
Output Impacts (000 of dollars)	112,139	79,894	91,419	283,452
Primary dealers/processors				
Employment impacts (jobs)	485	359	529	1,373
Income Impacts (000 of dollars)	24,343	20,275	26,694	71,312
Output Impacts (000 of dollars)	71,677	60,465	85,522	217,664
Secondary wholesalers/distributors				
Employment impacts (jobs)	622	358	395	1,374
Income Impacts (000 of dollars)	44,147	21,202	19,916	85,266
Output Impacts (000 of dollars)	59,020	61,768	63,882	184,670
Grocers				
Employment impacts (jobs)	864	92	190	1,146
Income Impacts (000 of dollars)	22,262	5,996	9,605	37,863
Output Impacts (000 of dollars)	25,365	16,210	30,755	72,330
Restaurants				
Employment impacts (jobs)	6,006	843	1,836	8,685
Income Impacts (000 of dollars)	126,039	51,256	92,728	270,023
Output Impacts (000 of dollars)	225,069	151,714	296,852	673,635
Harvesters and seafood industry				
Employment impacts (jobs)	9,013	2,118	3,522	14,653
Income Impacts (000 of dollars)	261,292	124,677	177,472	563,441
Output Impacts (000 of dollars)	493,270	370,052	568,429	1,431,751

3.4. Salmon Aquaculture

3.4.1. Salmon Production Data

Obtaining detailed farm production budgets for salmon aquaculture in the U.S. is difficult because of the limited number of operations. Even when data is available, protecting confidentiality to prevent disclosure of sensitive company financial data is an issue. Atlantic salmon farming in net pens in Maine and Washington State is now almost all owned by one company, Cooke Aquaculture. Pacific Aquaculture has several steelhead net pen farms on the Columbia River with operational features and costs similar to Atlantic salmon. Several small operations growing Atlantic salmon are using land-based recirculating aquaculture facilities and at least four larger land-based Atlantic salmon facilities are in construction or design phases in the U.S. Given these limitations, we examined more readily available production cost data from Canada and Norway to provide cost estimates for this study.

Through discussion with industry experts, we estimate that currently, nearly 100% of U.S. aquaculture salmon production is from open water net pens. We developed an enterprise budget based on Boulet et al. (2010) to determine the percentage cost of production for each input. Due to the geographic proximity and the fact that there is a significant percentage of U.S. net pen salmon operation with Canadian ownership, we determined that these British Columbia-based net pen production estimates were better estimates of U.S. production cost and returns than a set of Norwegian production estimates available in Liu et al. (2016). Cost data in Boulet et al. (2010) was inflated from 2010 U.S. dollars to 2015 U.S. dollars based on the producer price index from the Bureau of Labor Statistics[11].

Once the direct impacts of aquaculture salmon were calculated, the output from growers was treated as the equivalent of commercially harvested wild salmon in the downstream sectors. That is, it was distributed among processors, wholesalers, and retailers as if it were commercially harvested salmon, as calculated in Table 4. However, it is clear from Boulet et al. (2010) that the enterprise budgets represented operations that produced head-on, gutted salmon as the initial product entering the marketing chain. This level of vertical integration is different from the product of commercial fishing and undermines the assumption about how farmed salmon moves through the value-added chain. Compared to commercially harvested salmon, it would seem reasonable to assume large producers process their own product and that a smaller share of aquaculture salmon flows to secondary processors and a larger share to other value-added segments.

Table 8. Summary of all impacts for aquaculture: Salmon

Industry Sector	Direct	Indirect	Induced	Total
Growers				
Employment impacts (jobs)	87	422	476	985
Income Impacts (000 of dollars)	5,630	26,723	24,019	56,372
Output Impacts (000 of dollars)	87,743	121,743	77,042	286,527
Primary dealers/processors				
Employment impacts (jobs)	388	199	437	1,024
Income Impacts (000 of dollars)	22,930	12,276	22,077	57,283
Output Impacts (000 of dollars)	57,360	38,485	70,679	166,523
Secondary wholesalers/distributors				
Employment impacts (jobs)	486	280	309	1,075
Income Impacts (000 of dollars)	34,543	16,590	15,583	66,716
Output Impacts (000 of dollars)	46,180	48,330	49,984	144,495
Grocers				
Employment impacts (jobs)	676	72	149	897
Income Impacts (000 of dollars)	17,419	4,692	7,515	29,626
Output Impacts (000 of dollars)	19,847	12,684	24,064	56,595
Restaurants				
Employment impacts (jobs)	4,699	660	1,437	6,796
Income Impacts (000 of dollars)	98,619	40,105	72,555	211,279
Output Impacts (000 of dollars)	176,105	118,708	232,272	527,085
Harvesters and seafood industry				
Employment impacts (jobs)	6,337	1,633	2,808	10,777
Income Impacts (000 of dollars)	179,140	100,386	141,750	421,276
Output Impacts (000 of dollars)	387,235	339,950	454,040	1,181,225

[11] https://www.bls.gov/ppi/.

3.4.2. Salmon Economic Impacts

Table 8 provides estimates of the 2015 production by U.S. salmon aquaculture operations. Basic input is the *Fisheries of the United States* estimate of the value of this production in 2015:$87.7 million. The direct impacts of salmon aquaculture are based on revenue and expense data for net-pen operations from a feasibility study of British Columbia closed containment aquaculture (Boulet et al. 2010).

4. AGGREGATE SUMMARY AND ANALYSIS

4.1. Overview

The four species for which we provide impact estimates only comprise 41% of the total first sales value of U.S. aquaculture production. Catfish, which we did not include due to a lack of readily available recent cost and returns data, comprises 25% of the total value of production. However, from the marine production perspective, the three marine species analyzed, oysters, clams, and salmon, represent 95% of the first sale value of marine aquaculture production in 2015. Total aquaculture first sale value was $1.3 billion in 2014 and $1.4 billion in 2015. For aquaculture production as a whole, it would be misleading to extrapolate from our measured values in this study to the full impact of U.S. aquaculture, but for marine aquaculture production it is reasonable to extrapolate our estimates from the 95% of production value included in the analysis to an estimate of the full impacts of marine aquaculture production.

Table 9. Summary of all impacts for aquaculture: All marine

Industry Sector	Direct	Indirect	Induced	Total
Growers				
Employment impacts (jobs)	5,337	1,518	2,267	9,122
Income Impacts (000 of dollars)	158,461	91,447	114,449	364,357
Output Impacts (000 of dollars)	393,998	317,217	366,678	1,077,893
Primary dealers/processors				
Employment impacts (jobs)	1,714	1,175	1,883	4,771
Income Impacts (000 of dollars)	89,634	67,441	95,048	252,124
Output Impacts (000 of dollars)	253,184	203,112	304,458	760,754
Secondary wholesalers/distributors				
Employment impacts (jobs)	2,184	1,257	1,387	4,829
Income Impacts (000 of dollars)	155,110	74,493	69,975	299,579
Output Impacts (000 of dollars)	207,367	217,020	224,447	648,834
Grocers				
Employment impacts (jobs)	3,035	324	668	4,027
Income Impacts (000 of dollars)	78,217	21,068	33,746	133,031
Output Impacts (000 of dollars)	89,120	56,955	108,056	254,130
Restaurants				
Employment impacts (jobs)	21,101	2,962	6,452	30,516
Income Impacts (000 of dollars)	442,835	180,086	325,799	948,720
Output Impacts (000 of dollars)	790,776	533,044	1,042,985	2,366,804
Harvesters and seafood industry				
Employment impacts (jobs)	33,429	7,248	12,678	53,355
Income Impacts (000 of dollars)	924,256	434,537	639,019	1,997,811
Output Impacts (000 of dollars)	1,734,444	1,327,349	2,046,621	5,108,413

From *Fisheries of the U.S. 2016*, marine aquaculture production in 2015 had total first sales of about $394 million. By assuming that the 5% of production we have not calculated impacts for creates impacts in the same proportion as those we have calculated, then the total impacts for marine aquaculture production are estimated in Table 9.

The $394 million in first sales of U.S. marine aquaculture production, through processing and distribution, ended up with final sales to consumers of over $1.7 billion. The indirect and induced effects added $1.3 billion and $2.0 billion, respectively, for a total impact on the U.S. economy of $5.1 billion. There were 33,429 jobs directly related to the production, processing, distribution, and final sales of marine aquaculture products. The indirect and induced effects added an additional 19,926 jobs for a total impact of 53,355 jobs.

4.2. Demonstration - Using Analysis for Future Impacts

The NMFS Office of Aquaculture, in consultation with industry leaders, suggests a reasonable target is for there to be a 2.5 times increase in U.S. marine aquaculture production in the next ten years. We adopt that target to demonstrate how the preceding analysis can be used to provide an estimate of the economic impacts from achieving that goal. Even though a 2.5 times increase in marine aquaculture is larger than the recent growth as indicated in *Fisheries of the U.S.*, an even larger expansion is possible. This will depend on changes in U.S. policy (e.g., opening up federal waters to aquaculture), providing access to sites in state waters by overcoming reluctance of coastal landowners to support aquaculture in some states, and via a reduction in production costs in recirculating aquaculture systems (see Knapp and Rubino, 2016).

4.2.1. Estimating Increase in First Sales Value

To estimate the economic impact of an increase in U.S. marine aquaculture production, it is necessary to project what the composition of the increased production will be. Scenario 1 uses a simple and perhaps naïve assumption that production will maintain its current composition of species, prices will not change, and thus result in a simple 2.5 times increase in first sale value to $985 million. It is rather straightforward, then, to estimate the increase in impacts, since the underlying impact model structure is linear, and all the values increase by the same 2.5 times.

Alternatively, for Scenario 2, the Office of Aquaculture, based on interviews with market experts, estimates that a larger percentage, say 75%, of the production volume increase will result from an expansion in finfish production, and this will require a different weighting. We will use salmon value and production costs for this weighting, but it is believed that other species for which we do not yet have reliable production budgets or price projections such as red drum, striped bass, yellowtail, sablefish, and cobia are likely to contribute to this increase. For the assumption of a greater increase in finfish production, we take the same absolute increase in sales volume, but assign 75% of the volume to a price associated with finfish (i.e., salmon) production. This yields a projected first sale value for marine finfish of $288 million, compared with Scenario 1 finfish value of $219 million. The remaining 25% of the increase in volume is split between oysters and clams in proportion to their 2015 production volume.

The total first sale value produced in Scenario 2 is $821 million. The weighting towards more finfish lowers the overall first sales value because current production is more heavily weighted towards high unit value oysters and clams.

4.2.2. Estimating Total Impact and Jobs of First Sales Value

The process of estimating total economic impacts, once the first sale production number is set, proceeds as in the previous examples. Impact estimates are provided in Tables 10 and 11 for Scenarios 1 and 2, respectively. At the producer/grower level, total impacts are $2.7 billion (Scenario 1) or $2.3 billion (Scenario 2). Direct employment associated with production is 13,343 jobs (Scenario 1) or 9,503 jobs (Scenario 2). The difference in scenarios is driven by the fact that the budget we used for salmon production is much less labor intensive than for either shellfish species. Total employment associated with production is estimated at 22,805 jobs (Scenario 1) or 17,468 jobs (Scenario 2). That represents an increase in employment of 13,683 jobs (Scenario 1) or 8,346 jobs (Scenario 2). The increase in jobs associated with production are of particular note, because they potentially represent the difference between producing the increased seafood in the U.S. and importing product produced in overseas aquaculture. The downstream impacts discussed in the next section are generated regardless of where the seafood product is initially grown.

4.2.3. Including Downstream Impacts

The downstream impacts calculated in Tables 10 and 11 are based not only on the assumption that aquaculture products will follow the same distribution and consumption patterns of wild caught domestic products, but also that this pattern will continue into the future. If those assumptions hold, then 2.5 times growth will result in 133,386 jobs (Scenario 1) or 109,515 jobs (Scenario 2) associated with production and final sale of U.S. aquaculture products. This is an increase over the year 2015 associated jobs of 80,031 (Scenario 1) or 56,160 (Scenario 2). The total impact on the U.S. economy will be $12.8 billion (Scenario 1) or $10.7 billion (Scenario 2).

Table 10. Scenario 1 of projected impacts for aquaculture at 2.5 times current: All marine fixed composition

Industry Sector	Direct	Indirect	Induced	Total
Growers				
Employment impacts (jobs)	13,343	3,796	5,667	22,805
Income Impacts (000 of dollars)	396,152	228,616	286,123	910,892
Output Impacts (000 of dollars)	984,995	793,042	916,694	2,694,731
Primary dealers/processors				
Employment impacts (jobs)	4,285	2,937	4,707	11,929
Income Impacts (000 of dollars)	224,086	168,604	237,619	630,309
Output Impacts (000 of dollars)	632,960	507,780	761,145	1,901,885
Secondary wholesalers/distributors				
Employment impacts (jobs)	5,461	3,143	3,468	12,071
Income Impacts (000 of dollars)	387,776	186,234	174,937	748,946
Output Impacts (000 of dollars)	518,416	542,551	561,116	1,622,084
Grocers				
Employment impacts (jobs)	7,588	809	1,670	10,068
Income Impacts (000 of dollars)	195,543	52,670	84,364	332,577

Industry Sector	Direct	Indirect	Induced	Total
Output Impacts (000 of dollars)	222,799	142,386	270,140	635,325
Restaurants				
Employment impacts (jobs)	52,752	7,406	16,131	76,289
Income Impacts (000 of dollars)	1,107,088	450,215	814,497	2,371,799
Output Impacts (000 of dollars)	1,976,940	1,332,609	2,607,462	5,917,011
Harvesters and seafood industry				
Employment impacts (jobs)	83,574	18,119	31,694	133,386
Income Impacts (000 of dollars)	2,310,640	1,086,342	1,597,547	4,994,529
Output Impacts (000 of dollars)	4,336,110	3,318,371	5,116,552	12,771,034

Table 11. Scenario 2 of projected impacts for aquaculture at 2.5 times current: All marine 75% growth in finfish

Industry Sector	Direct	Indirect	Induced	Total
Growers				
Employment impacts (jobs)	9,503	3,285	4,676	17,468
Income Impacts (000 of dollars)	287,364	199,632	236,055	723,050
Output Impacts (000 of dollars)	820,716	733,710	756,411	2,310,833
Primary dealers/processors				
Employment impacts (jobs)	3,579	2,358	3,947	9,884
Income Impacts (000 of dollars)	190,917	136,599	199,277	526,793
Output Impacts (000 of dollars)	528,776	413,535	638,273	1,580,583
Secondary wholesalers/distributors				
Employment impacts (jobs)	4,549	2,619	2,890	10,056
Income Impacts (000 of dollars)	323,102	155,174	145,760	624,038
Output Impacts (000 of dollars)	431,953	452,063	467,532	1,351,552
Grocers				
Employment impacts (jobs)	6,323	674	1,392	8,389
Income Impacts (000 of dollars)	162,930	43,886	70,293	277,111
Output Impacts (000 of dollars)	185,640	118,639	225,086	529,365
Restaurants				
Employment impacts (jobs)	43,954	6,171	13,441	63,566
Income Impacts (000 of dollars)	922,446	375,128	678,653	1,976,226
Output Impacts (000 of dollars)	1,647,222	1,110,354	2,172,585	4,930,159
Harvesters and seafood industry				
Employment impacts (jobs)	68,012	15,127	26,381	109,515
Income Impacts (000 of dollars)	1,886,754	910,420	1,330,045	4,127,217
Output Impacts (000 of dollars)	3,614,308	2,828,303	4,259,882	10,702,494

CONCLUSION AND RECOMMENDATIONS

Fisheries Economics of the United States currently provides useful information to stakeholders and the general public about the importance of the fishing and seafood industries, and demonstrates year over year changes and trends in economic impacts. Adding domestic aquaculture production estimates will increase the utility of this information and provide a greater understanding of the entire seafood industry, particularly as domestic aquaculture increases in importance as a component of U.S. supply.

When we started on this study, the original intent was to develop an estimate of the total economic impact from U.S. aquaculture production for most major species as a demonstration

of how this could be incorporated into *FEUS*. We discovered that there is insufficient extant cost information and only greatly outdated information on production for several major species to develop a reasonable national estimate of economic impacts. Many of the published budgets were out of date, missing key pieces of information, did not include newer production methods, or were only relevant to a single production method in a specific geographic area so that it might not be representative of the industry as a whole. As a result, the project morphed into a data and methodological gap analysis focused on four species as an initial trial analysis of economic impact.

We began the impact analysis with an estimate of annual aquaculture production value by major species as published in *Fisheries of the U.S.* These figures are based on a combination of state reports, industry reports and estimates, USDA's Census of Agriculture and Census of Aquaculture (a survey done once every five years), USDA's regular collection of catfish and trout production data, information from selected companies, and NMFS and USDA staff estimates based on professional knowledge about the industry. Production numbers from some states are not available on an annual basis. Some states gather production data as part of lease or permit requirements but are unwilling to make the data available to a federal agency. A systematic way of collecting this data from states, industry associations or directly from producers is essential to ensuring the quality of the estimates that rely on these numbers.

There are other issues that arise when developing annual aquaculture production estimates. A clear definition of what constitutes aquaculture production, particularly for shellfish[12], is necessary and will help avoid some double counting in commercial landings that occurs now. Close coordination with reporting states is essential for determining this data. Responsibility for aquaculture production figures may be housed in different state agencies or different parts of the same agency than that with which NMFS typically coordinates in reporting fish landings. Since there is interest in reporting on marine versus freshwater aquaculture production, classification of what constitutes each will have to be agreed upon. For example, land-based recirculating aquaculture systems produce both freshwater and marine species. Is a marine species grown in a land-based system considered marine aquaculture production? The annual industry survey-based report produced by the Virginia Sea Grant Marine Extension Program (Hudson, 2018) is an excellent example of the type and quality of production data needed for all aquaculture species. Another challenge in reporting production by species will be the requirement of protecting confidentiality of firm level data, which may be raised as an issue when there are a small number of firms constituting the production for a particular species. Atlantic salmon farming in net pens in Maine and Washington State is currently all owned by one company and shellfish farming is dominated by two companies on the West Coast and three in Virginia.

Once we determined production levels and farm gate values, our next step was to allocate production value to different cost categories. Even when there were recent comprehensive studies of aquaculture production costs for a species, it was sometimes difficult to place costs in an appropriate category. Multiple studies may classify costs differently. As a result, relying on one-off production cost studies that appear in the literature from time to time is an unreliable way of developing representative industry budgets. Systematic collection of

[12] There is a long history of state-supported preparation of shellfish bottom, planting shell and placing wild or hatchery produced seed oysters subsequently harvested by a limited access fishery and reported as part of total fish landings. Essentially, the same production methods are used in privately held leased bottom, which may be reported separately.

production costs via standardized industry surveys will provide the most reliable information. The relevant Federal agencies (i.e., USDA and NOAA), in conjunction with state agencies and aquaculture industry organizations, should come together and plan a survey methodology and a way to administer, maintain, and update it on a regular basis.

Two of the major items to be determined in planning an aquaculture industry production cost survey are the survey frequency and the target sample size. The Bureau of Economic Analysis, in producing National Income Accounts, opens up their underlying model to major revisions every five years, so it would make sense for the same level of updating for an aquaculture survey. The need for a particular industry sample size will depend on the ultimate use of the survey data and will be higher when there is a need for stratification when the industry is diverse in its production technologies. As mentioned above, some of the published cost data from specialized surveys were not representative of the industry. An industry census would ameliorate the concern about representativeness, but short of a census, any type of survey sample would have to be designed with this concern in mind. The aggregate species groupings for commercial landings (Table 3) demonstrates the tradeoffs necessary in developing representative cost estimates.

Our analysis used a very simple assumption about downstream impacts of aquaculture fish. We used the same product flows as shown in Table 4. We are aware that even for currently produced aquaculture species like Atlantic salmon, these product flows may be inaccurate. Emerging aquaculture production may also follow very different routes through the marketing chain. A comprehensive study on the seafood market chain would allow us to relax this assumption and provide insight on how inaccurate or not the impact estimates are as a result. Some publicly available data (e.g., What We Eat in America[13]) and private industry data should be researched to get a better understanding of the geographic distribution of final purchase of seafood. This will be particularly important to eventually produce state level economic impact estimates.

We also need to be concerned about measuring upstream impacts from aquaculture production. Budgets should be developed for finfish and shellfish hatcheries and nurseries for the key aquaculture species as an improvement over the current NAICS data.

Not all aquaculture production is for seafood. Some freshwater aquaculture is for pond stocking for recreational fishing, and there is a substantial market for aquacultured baitfish (Senten and Engle, 2017). Marine algae production will likely be used for both direct consumption and as additives to foods and other products. These other markets will have to be examined and quantified as they develop.

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[13] https://data.nal.usda.gov/dataset/what-we-eat-america-wweia-database.

The views expressed herein are those of the authors and do not necessarily reflect the current views of the National Oceanic and Atmospheric Administration's National Marine Fisheries Service.

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Chapter 157

SALTONSTALL-KENNEDY ACT: BACKGROUND AND ISSUES*

Harold F. Upton

ABSTRACT

The Saltonstall-Kennedy (S-K) Act of 1954 (15 U.S.C. §713c-3) established a program to provide financial support for research and development of commercial fisheries. The S-K Act created a fund (known as the S-K fund) that is financed by a permanent appropriation of a portion of import duties on marine products. S-K funds are distributed by the Secretary of Commerce as grants and cooperative agreements to address needs of the U.S. fishing industry, including but not limited to harvesting, processing, marketing, and associated infrastructure. However, Congress allocates most funding to the National Marine Fisheries Service (NMFS) to fund agency activities related to marine fisheries research and management. Some have questioned whether the allocation of S-K funds reflects the original intent of the S-K Act and whether the S-K Grant Program addresses the needs and priorities of the fishing industry.

Since its creation, the S-K fund's authorizing language and priorities have evolved with changes to the fishing industry, new or amended federal laws governing fisheries management, and changing federal agency responsibilities. In 1980, the American Fisheries Promotion Act (AFPA) amended the S-K Act to authorize a competitive grant program, known as the Saltonstall-Kennedy Grant Program(S-K Grant Program) and the National Program to support fishing industry research and development projects. Both programs are administered by NMFS, part of the National Oceanic and Atmospheric Administration (NOAA). In the 1980s, the S-K Grant Program focused on fisheries development, but in subsequent years, as U.S. fisheries became fully or overexploited, priorities generally shifted to resource conservation and management. The S-K Grant Program has supported a variety of different projects, such as gear technology research, seafood marketing, aquaculture, andothers.

The S-K Grant Program is funded by a permanent appropriation of 30% of the previous calendar year's customs receipts from imports of fish and fish products. These funds are transferred into NOAA's Promote and Develop American Fisheries Products and

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Research Pertaining to American Fisheries Fund (P&D account). Transfers of revenue into the P&D account have grown steadily from$26.7 million in 1980 to $182.8 million in 2020. Congress subsequently transfers most funds into the Operations, Research, and Facilities (ORF) account within NOAA. Congress has directed NMFS to use funds allocated to the ORF account for specific activities including stock assessments, fishing information networks, survey and monitoring projects, cooperative research, and interjurisdictional fisheries. The remaining funds are available for supporting the annual competitive S-K Grant Program and in some cases the National Program.

Since the early 1980s, Congress has transferred most P&D account funds into the ORF discretionary account, sometimes leaving little or no funding for the specified purposes of the S-K Act. Some critics have questioned whether funds from the P&D account could be used more effectively by targeting fishing industry needs, as Congress originally intended. For example, in the 112th, 113th, and 114th Congresses, bills were introduced that would have used most S-K funds to establish a regional fisheries grant program. By contrast, some have expressed concerns that if significant funding is shifted away from NMFS fisheries management programs, additional funds would need to be appropriated or activities such as data collection and fish population assessments could be compromised. These NMFS activities provide information and analyses used to manage and conserve fish populations.

Some also have questioned whether the S-K Grant Program could be modified to provide the fishing industry with more direct input into the S-K grant process. Currently, NMFS, in consultation with the fishing industry, identifies S-K Grant Program priorities and selects the recipients of S-K grants. Over the last several Congresses, bills have been introduced that would change the procedure for screening, evaluating, and awarding S-K grants. In the 116th Congress, the American Fisheries Advisory Committee Act (H.R. 1218 and S. 494) would establish an industry advisory committee to identify the needs of the fishing industry, develop requests for proposals, review grant applications, and select grant applications for approval. S. 494 was reported on August 16, 2019, by the Senate Committee on Commerce, Science, and Transportation; on June 6, 2020, H.R. 1218 was reportedby the House Committee on Natural Resources.

INTRODUCTION

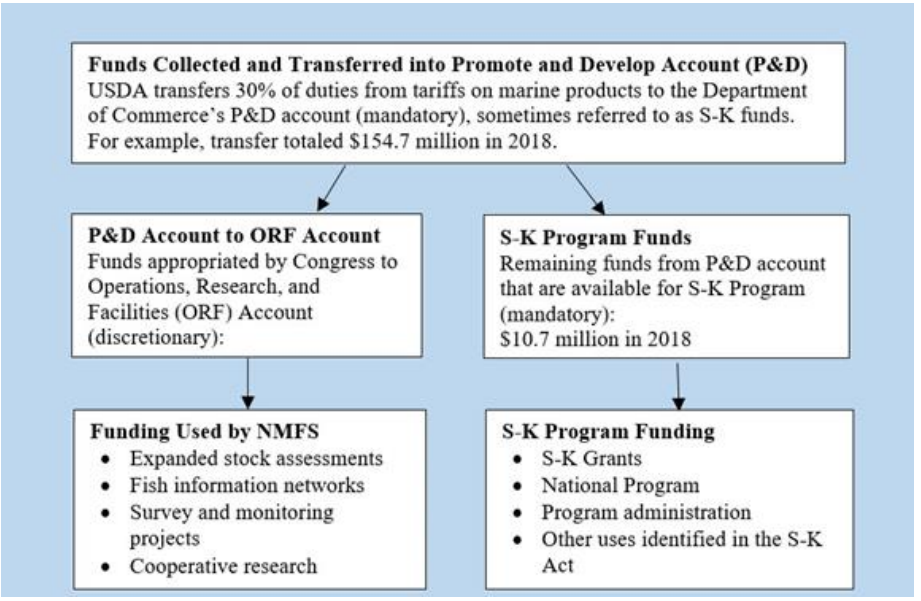
The Saltonstall-Kennedy (S-K) Act of 1954 (15 U.S.C. §713c-3) established a fund (known as the S-K fund) to support U.S. fisheries development and research. Funding originates from a transfer by the Secretary of Agriculture into the Promote and Develop American Fisheries Products and Research Pertaining to American Fisheries Fund (P&D account). The P&D account is administered by the National Marine Fisheries Service (NMFS) of the National Oceanic and Atmospheric Administration (NOAA) in the Department of Commerce.[1] Transfers of revenue into the P&D account have grown steadily from $26.7 million in 1980 to $182.8 million in2020.

Currently, the bulk of P&D account revenue is transferred into the Operations, Research, and Faculties (ORF) account, which supports fisheries science and management administered by NMFS.[2] The remaining funds support the Saltonstall-Kennedy Grant Program (S-K Grant

[1] NMFS is also known as National Oceanic and Atmospheric Administration (NOAA) Fisheries.

[2] he ORF account, NOAA's largest, funds a portion of all of NOAA's line office budgets, including the National Weather Service; National Ocean Service; Oceanic and At mospheric Research; NMFS; National Environmental Satellite, Data, and Information Service; and the Office of Marine and Aviation Operations.

Program) and sometimes the National Program, which focus on fishing industry research and development projects.



Source: Adapted from NOAA Fisheries, Saltonstall-Kennedy Research Program, Presentation provided to New England Fishery Management Council, December 6, 2018, at https://s3.amazonaws.com/nefmc.org/7_NE-Council- Presentation_ Feedback-Sessions-Final.pdf.

Notes: NMFS = National Marine Fisheries Service; Saltonstall-Kennedy (S-K) Act of 1954 (15 U.S.C. §713c-3).

Figure 1. Flow and use of Saltonstall-Kennedy Act (S-K) Funds.

Historically, the use of the S-K fund has evolved with changing fisheries management institutions and changing needs of U.S. fisheries.[3] Congress is continuing to consider whether current funding from the P&D account meets the needs of U.S. fisheries and the U.S. fishing industry. Some have questioned whether the U.S. commercial fishing industry receives sufficient opportunities to provide input into the S-K competitive grant process.[4] Due in part to what they perceive as a lack of industry input, some critics assert that NMFS has not distributed funding in accordance with the primary purposes of the S-KAct, such as supporting projects related to the marketing of fish.[5] Another concern is the allocation of funds, and specifically whether there is a need for more financial support of S-K competitive grants than for funding NMFS fisheries science and management activities in the ORF

[3] The Magnuson-Stevens Fishery Conservation and Management Act (P.L. 94-265) defines fishery as "(A) one or more stocks of fish which can be treated as a unit for purposes of conservation and management and which are identified on the basis of geographical, scientific, technical, recreational, and economic characteristics; and (B) any fishing for such stocks."

[4] Senator Dan Sullivan, "Senators Pass Bill Out of Committee to Give Fishermen Voice in Grant Process, Boost U.S. Seafood," press release, June 29, 2016.

[5] U.S. Congress, Senate Committee on Commerce, Science, and Transportation, American Fisheries Advisory Committee Act, committee print, prepared by Committee on Commerce, Science, and Transportation, 115 th Cong., 1st sess., December 11, 2017, p. 2.

account.[6] However, if funding were reallocated to provide greater support of the S-K Grant Program, Congress may need to consider implications of the likely decrease in funds that would be transferred to ORF from the P&D account to support NMFS fishery research and management activities. *Figure 1* summarizes the flow of funding from the P&D account into NOAA and the S-K program.

THE SALTONSTALL-KENNEDY ACT

Current Provisions

The S-K Act requires the Secretary of Agriculture to transfer 30% of duties on marine products collected under the so-called Section 32 Program to the Secretary of Commerce.[7] These funds are transferred into the P&D account and made available to NMFS. Currently, the uses of S-K funds as specified in 15 U.S.C. 713c-3 include the following:[8]

- providing grants in support of fisheries research and development projects under subsection (c),
- implementing a national fisheries research and development program under subsection (d),
- implementing the Northwest Atlantic Ocean Fisheries Reinvestment Program, and
- funding the federal share of a fisheries capacity reduction fund.

The S-K Act requires the Secretary of Commerce to use no less than 60% of funds to make direct industry-assistance grants pursuant to subsection (c). Subsection (c) refers to topics that may be addressed by research and development grants, including but not limited to harvesting, processing, marketing, and associated infrastructures. Subsection (c) also identifies the terms and conditions of grant awards.

The S-K Act requires the balance of S-K funds to be allocated to finance NMFS activities that support development of U.S. fisheries pursuant to subsection (d). Subsection (d) refers to a national fisheries research and development program (including but not limited to harvesting, processing, marketing, and associated infrastructures), if not adequately covered by projects assisted under subsection (c) of this section or as the Secretary deems appropriate.

History of the Saltonstall-Kennedy Act

In 1935, Congress passed legislation to provide financial support for domestic agricultural commodity markets. Section 32 of the Act of August 24, 1935, provided a permanent

[6] "Senators Kerry and Snowe Will Introduce Bill to Restore Intent of Saltonstall-Kennedy Act," Saving Seafood, March 9, 2012.

[7] The program's name is from the section that established the program, Section 32 of the Act of August 24, 1935, Chapter 641, §32; 7 U.S.C. §612c.

[8] 15 U.S.C. §713c-3(c) and (d).

appropriation equal to 30% of gross receipts from all duties collected under customs laws.[9] The act authorized the Secretary of Agriculture to use these funds to support exports and domestic consumption of agricultural commodities. The Act of August 11, 1939, authorized the Secretary of Agriculture to transfer up to $1.5 million from funds collected under Section 32 to support the fishing industry. Funds were transferred to the Federal Surplus Commodities Corporation to purchase and distribute surplus fishery products and to the Secretary of the Interior to promote markets for fishery products of domestic origin.[10] *Table 1* provides a history of legislative changes to the S-KAct.

In 1954, the S-KAct amended the Act of August 11, 1939, to provide additional funding from Section 32 funds to support the U.S. fishing industry.[11] The S-KAct authorized the transfer from the Secretary of Agriculture to the Secretary of the Interior, from the larger Section 32 account's funding, an amount equal to 30% of gross receipts from duties collected on fishery products.[12] These funds were maintained in a separate account for use by the Secretary of the Interior to support the flow of fishery products in commerce, develop and increase markets for fishery products, and conduct research. Annual expenditures from the fund were limited to $3 million, and the balance of the fund was not allowed to exceed $5 million at the end of any year. In 1956, the S-K Act was amended to remove the limit on annual expenditures from the fund. The S-KAct also authorized the Secretary of the Interior to appoint a fishing industry advisory committee to provide guidance on the formulation of policy, rules, and regulations pertaining to requests for assistance, and other matters.[13]

In 1976, the Fishery Conservation and Management Act (FCMA; P.L. 94-265) established a 200- nautical mile fishery conservation zone (FCZ) and brought marine fisheries within the FCZ under domestic control.[14] Foreign fishing was allowed to continue in the FCZ, but the domestic fishing industry was granted priority fishing rights under the FCMA.[15] In the following years, U.S. policy emphasized development of domestic fisheries and replacement of foreign fishing with domestic fishing in the FCZ.[16] According to the Government Accountability Office, until 1979, NMFS used nearly all S-K funds to support fisheries management and development activities; it granted only small amounts to the fishing industry for development projects.[17] In 1979, likely because of growing industry support of domestic fisheries development, NMFS made available approximately $5.3 million of S-K funds to

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[9] The Act of August 24, 1935, Chapter 641, §32; 7 U.S.C. §612c. See CRS Report RL34081, *Farm and Food Support Under USDA's Section 32 Program* , coordinated by Jim Monke.

[10] Act of August 11, 1939, Chapter 696; 15 U.S.C. §713c-2. The Act of 1939 authorized the Secretary of Agriculture to transfer these funds to the Federal Surplus Commodities Corporation under the Section 32 program.

[11] Act of July 1, 1954, Chapter 447; 15 U.S.C. §713c-3.

[12] Products included fish, shellfish, mollusks, crustaceans, aquatic plants and animals, and any products thereof, including processed and manufactured products.

[13] Act of July 1, 1954, Chapter 447, §2(c).

[14] On March 10, 1983, President Reagan issued Proclamation 5030, which established the 200-nautical mile exclusive economic zone (EEZ). The EEZ provided sovereign rights over the natural resources in the zone, including fisheries, and replaced the fishery conservation zone established by the Fishery Conservation and Management Act (P.L. 94-265) in 1976.

[15] Foreign fishing was allocated the surplus after U.S. domestic fishing needs were met. Allocations to foreign operations were terminated when the surplus was completely utilized by U.S. domestic fishing.

[16] The capacity of U.S. domestic fishing fleets increased during the 1980s; by 1990, domestic fleets had replaced nearly all foreign fishing fleets operating in the U.S. EEZ.

[17] U.S. Government Accountability Office (GAO), *Uses of Saltonstall-Kennedy Fisheries Development Funds*, August 30, 1985, at http://www.gao.gov/assets/150/143275.pdf. GAO was called the General Accounting Office when the report was written in 1985. Hereinafter cited as GAO, 1985.

regional fisheries development foundations, universities, private industry, and state and local governments.[18]

Table 1. History of legislation related to the Saltonstall-Kennedy Act

Year	Act	Brief Description
1935	Act of August 24, 1935, Chapter 641, Section 32	Established a permanent appropriation (§32) to set aside 30% of annual customs receipts; supported the farm sector by purchasing surplus commodities and funding a variety of other activities.
1939	Act of August 11, 1939 (P.L. 76-392), Chapter 696, Section 1	Authorized the purchase and distribution of surplus fishery products with funding of up to $1.5 million per year.
1954	Act of July 1, 1954 (Saltonstall-Kennedy Act), Chapter 447	Required U.S. Department of Agriculture to transfer to the Department of Commerce 30% of duties on marine products to fund U.S. fisheries and limited expenditures to no more than $3 million per year.
1956	Fish and Wildlife Act of 1956, Act of August 8, 1956, Chapter 1036, Section 12(b)	Removed limitation on annual expenditures.
1980	American Fisheries Act (P.L. 96-561), Title II, Section 210	Authorized competitive grants and national fisheries research and development programs; directed that at least 50% of funds be used for competitive grant program, with the balance for the National Program.
1983	Highway Improvement Act of 1982 (P.L. 97-424), Title IV, Section 423(a)	Increased competitive grant share to at least 60% of funds; stipulated that S-K funds were to be used exclusively for promoting U.S. fisheries.
1986	Fish and Seafood Promotion Act of 1986 (P.L. 99-659), Title II, Section 209(e)	Expanded authorized uses of S-K funds to include the Fisheries Promotion Fund for several years.
1992	National Oceanic and Atmospheric Administration Authorization Act of 1992 (P.L. 102-567), Title IX, Section 902(c)	Expanded authorized uses of S-K funds to include implementation of the Northwest Atlantic Ocean Fisheries Reinvestment Program.
1996	Sustainable Fisheries Act (P.L. 104-297), Title I, Section 116(c)	Expanded authorized uses of S-K funds to include the federal share of a fishing capacity reduction program.
2013	Consolidated and Further Continuing Appropriations Act, 2013 (P.L. 113-6), Title I	Restricted the use of the Promote and Develop Fisheries Products funds transferred to the Operations, Research, and Facilities account to cooperative research, annual stock assessments, data collection, interjurisdictional fisheries grants, and fisheries information networks. Subsequent appropriations acts have adopted similar language.

Source: CRS.

In 1980, Congress formally authorized the current competitive S-K Grant Program in Section 210 of the American Fisheries Promotion Act (AFPA; P.L. 96-561). The AFPA directed the Secretary of Commerce to use at least 50% of S-K funds for the S-K Grant Program and the balance of funds for a National Program. Both programs supported research and development efforts to address areas such as harvesting, processing, marketing, and related infrastructures. By 1980, the transfer from the U.S. Department of Agriculture (USDA) had grown to $26.7 million (*Table A- 1*). The AFPA also formally transferred responsibility for administering the fund from the Secretary of the Interior to the Secretary of Commerce. The House committee report accompanying the AFPA noted that the definition of *fishery* includes

[18] GAO, 1985.

recreational fishing and that recreational projects would be eligible for grants.[19] The AFPA also removed a section that established the S-K fishing industry advisory committee; the advisory committee had been previously terminated pursuant to the Federal Advisory Committee Act (P.L. 92-463).[20]

In subsequent years, Congress made additional changes to the allocation and use of the S-K fund (*Table 1*). The Highway Improvement Act of 1982 (P.L. 97-424) increased the share of funds used for the competitive grant program from 50% to 60%. In the following years, potential uses of the fund were broadened to include the Fisheries Promotion Fund (P.L. 99-659), the Northwest Atlantic Ocean Fisheries Reinvestment Fund (P.L. 102-567), and the federal share of a fishing capacity reduction program (P.L. 104-297). Congress established the Fisheries Promotion Fund to support domestic and international markets for domestically produced seafood. A portion of S-K funds was transferred to the fund from FY1987 to FY1991 for this purpose (*Table A-1*).[21]

Revenue

The revenues that are transferred into the P&D account from USDA are derived from duties on fishery products, "including fish, shellfish, mollusks, crustaceans, aquatic plants and animals, and any products thereof, including processed and manufactured products."[22] The P&D account is a mandatory fund that requires no periodic reauthorization or appropriation.[23] Transfers from USDA to NOAA's P&D account have steadily increased from $26.7 million in 1980 to $182.8 million in 2020 (*Figure 2*).[24] In CY2017, approximately 77% of revenues were from duties collected on imports of nonedible marine products, including jewelry, ink, various chemicals, and skins.[25] The remaining 23% of revenues were from duties on imports of edible seafood products. Tariffs on edible fish products have been reduced or eliminated for many seafood products, and most remaining duties are collected on canned products such as tuna or processed products such as fish sticks. In CY2017, most duties were collected on imports from India ($89.9 million), China ($86.2 million), Thailand ($79.8 million), Italy ($53.2 million), and France ($36.2 million).[26]

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[19] U.S. Congress, House Committee on Merchant Marine and Fisheries, *American Fisheries Promotion Act*, to accompany H.R. 7039, 96th Cong., 2nd sess., June 26, 1980, H. Rept. 96-1138, p. 39.

[20] 5 U.S.C. App., Section 14 of Federal Advisory Committee Act (P.L. 92 -463) terminated advisory committees within two years of the law's enactment (January 5, 1973) unless the committee was renewed within that two-year period or, in the case of a committee established by Congress, its duration is otherwise provided by law.

[21] Funding levels included $750,000 in FY1987, $2.6 million in FY1988, $3 million in FY1989, $2 million in FY1990, and $2 million in FY1991.

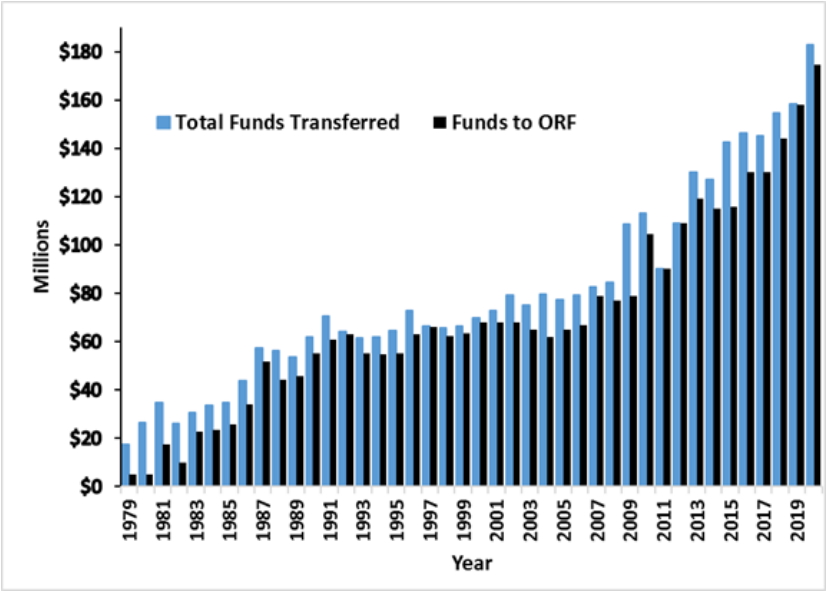
[22] Duties are collected by calendar year but not appropriated for use until the subsequent fiscal year (i.e., collect ions from CY2012 would be appropriated in FY2014).

[23] When funds from the P&D account are used to offset ORF funding, the funding is considered to be discretionary because the ORF is a discretionary account. However, P&D funds used for the S-K Grant Program or the National Program are identified in law and used by NOAA as mandatory funding.

[24] According to NOAA's FY2021budget request, a legislative proposal is being developed to appropriate mandatory funding to the Department of Commerce directly rather than as a transfer from USDA. This change would be part of a broader reform of the USDA Section 32 program.

[25] NOAA Ocean and Coastal Budget Formulation and Communication, NOAA Budget Office, July 12, 2018. For more information on tariffs on fish products, see Chapters 3 and 16 of the Harmonized Tariff Schedule, at https://hts.usitc.gov/current.

[26] NOAA Ocean and Coastal Budget Formulation and Communication, NOAA Budget Office, July 12, 2018.



Source: National Oceanic and Atmospheric Administration (NOAA), Budget Office, Email, January 20, 2020; National Marine Fisheries Service (NMFS), Saltonstall-Kennedy Grant Program: Fisheries Research and Development Reports 2008, 2001, 1991-1992, 1987-1990, and 1982-1986.

Notes: USDA = U.S. Department of Agriculture. For a detailed account of funding, see Table A-1.

Figure 2. Total Funds Transferred From USDA and Funds Transferred to Operations, Research, and Facilities (ORF). (FY1979 to FY2019)

Use of Funds

Operations, Research, and Facilities Account

Congress has allocated a growing portion of revenue in the P&D account to the ORF account rather than funding the S-K Grant Program as prescribed by the S-KAct. The transfer to the ORF account has ranged from $5 million, or 29% of the P&D account in 1979, to over $130 million in the five most recent years (FY2016-FY2020), which is more than 90% of the annual transfer into the P&D account (*Table 2*). ORF funds are used "to support fisheries research and management activities including the analysis and decision-making that supports ecosystem approaches to management."[27] Often the allocation of most funds to the OFR account limits the funding that is available for the specified purposes of the S-KAct.

In the last three fiscal years (FY2018-FY2020), the NOAA budget request proposed that all P&D account funding be transferred to the ORF account in support of NMFS activities. However, the Consolidated and Further Continuing Appropriations Act, 2013 (P.L. 113-6), restricted the use of P&D funds that are transferred into the ORF account. It limited this funding to fisheries activities related to cooperative research, annual stock assessments, survey and monitoring projects, interjurisdictional fisheries grants, and fish information networks. In subsequent years, agency budget requests have reflected this intent by identifying similar areas,

[27] NOAA, NMFS, The Saltonstall-Kennedy Grant Program: Fisheries Research and Development Report, 2011.

and Congress has continued to include similar language in appropriations laws and accompanying Senate committee reports.[28]

Remaining Funding

In most years, the majority of the funds that remain in the P&D account after the transfer into the ORF account have been used for the competitive S-K Grant Program as described in subsection of the S-KAct and the National Program as described in subsection (d) (Table 2).[29] The amount of remaining funding for the S-K Grant Program has varied considerably from year to year, ranging from no funding in FY2011 and FY2012, when Congress did not leave any remaining funding for S-K program, to its highest level of $29.5 million in FY2009 (*Table 2*). The S-K Act directs the Secretary of Commerce to use no less than 60% of funds for fisheries research and development grants pursuant to subsection (c). The Secretary also is required to use the remaining funds to finance NMFS activities directly related to U.S. fisheries development, as outlined in subsection (d). Since 1982, S-K grant funding has been less than 30% of total transfers from USDA, and it has been significantly lower in most years. In many years, Congress did not fund the National Program or provided a small portion of the remaining funds for that purpose.

Historically, financial support also was provided for the Fisheries Promotion Fund, which was funded between $750,000 and $3 million from FY1987 to FY1990. (*Table A-1*). No funding has been provided for the Fisheries Promotion Fund since 1991. From FY2003 to FY2006, most funding remaining after the ORF transfer was used for congressionally directed projects that supported several regional seafood marketing initiatives (*Table A-1*).[30] Annual S-K reports and other sources indicate that S-K funds have not been used for either the Northwest Atlantic Ocean Fisheries Reinvestment fund or the fishing capacity reduction program.

Saltonstall-Kennedy Grant Program

According to NMFS, the S-K program's general goals are to fund projects that address the needs of fishing communities, optimize economic benefits by building and maintaining sustainable fisheries, and increase other opportunities to keep working waterfronts viable. Historically, examples of areas funded by the S-K Grant Program have included enhancing markets for fishery products, examining fishery management options, and developing more efficient and selective fishing gear. Projects often have focused on both state and federal marine commercial fisheries, but other sectors—such as aquaculture and recreational fishing—also have been eligible for and received support.

NMFS solicits proposals as a federal funding opportunity on the federal grants website, which includes funding priorities, application requirements, and proposal evaluation criteria. Funding priorities are developed in coordination with regional fishery management councils, interstate fishery commissions, NMFS science centers, and NMFS regional offices. For

[28] P.L. 113-76, P.L. 113-235, P.L. 114-113, and P.L. 115-31 added the S-K Grant Program to the list of fishery-related activities that should be funded from the P&D account. Fishery-related activities identified in P.L. 115-141, P.L. 116-6, and P.L. 116-93 included the S-K Grant Program, fishery data collection, surveys and assessments, and interjurisdictional fisheries.

[29] 15 U.S.C. §713c-3(c) and (d). A portion of the remainder also has been used to administer the use of S-K funds for the S-K Grant Program and the National Program.

[30] S-K funds also have supported congressionally directed projects focused generally on regional marketing initiatives, including $10 million in FY2003, $17.5 million in FY2004, $12 million in FY2005, and $12 million in FY2006.

example in 2020, S-K program priorities are seafood promotion, development, and marketing, and science or technology that promotes sustainable U.S. seafood production and harvesting.[31]

The review process includes (1) pre-proposal review, (2) technical review and ranking, (3) panel review and ranking, and (4) grant selection. Pre-proposals undergo an administrative review by NOAA staff, a review by subject matter experts, and S-K program evaluation. Full review includes administrative screening; technical review by federal, public, and private sector experts; and funding recommendations by program and NMFS leadership. NMFS also may solicit comments and evaluation from a constituent review panel composed of three or more representatives chosen by the NMFS assistant administrator of fisheries.[32]

Table 2. Requested and Enacted ORF Transfers and S-K Grant Funding (funding in thousands of dollars)

Year	Request ORF Transfer	Request S-K Grant Funding	Enacted ORF Transfer	Remaining Funding for S-K Program[a]
2007	77,000	2,283	79,000	3,816
2008	77,000	5,816	77,000	7,594
2009	79,000	5,594	79,001	29,510
2010	104,600	9,400	104,600	8,771
2011	104,600	8,771	90,239	0
2012	66,200	5,000	109,098	0
2013	119,064	5,000	119,064	11,172
2014	123,164	8,208	115,000	12,187
2015	123,164	8,208	116,000	26,615
2016	130,164	13,574	130,164	16,225
2017	130,164	15,647	130,164	14,909
2018	154,199	0	144,000	10,664
2019	154,868	0	157,980	426
2020	158,407	0	174,774	8,009

Sources: NOAA, Budget Office, Email, January 20, 2020; NOAA, Budget Office, Email, December 2, 2019.

[a] Includes the S-K Grant Program, National Program, and NMFS administrative costs.

Funding of proposals is recommended by the S-K program manager; constituent panel ranking (if applicable); and input from NMFS regional directors, science center directors, and office directors. The agency selecting official, the NMFS assistant administrator, determines which proposals will be funded. The decision is based on the order of the proposals' ranking and other considerations, such as availability of funding, balance and distribution of funds, and duplication.[33] Recently, NMFS has been considering whether the program and fishing industry would benefit from placing greater emphasis on monitoring approved projects and disseminating results. During 2019, feedback sessions were arranged with regional fishery

[31] NOAA Fisheries, "Saltonstall-Kennedy Grant Program: Funding Opportunities," December 2019, at https://www.fisheries.noaa.gov/grant/saltonstall-kennedy-grant-program.

[32] Panelists are chosen from the fishing industry, state government, nongovernmental organizations, and others.

[33] NMFS, "FY20 Saltonstall-Kennedy Competition," May 31, 2019, at https://www.grants. gov/web/grants/search-grants.html?keywords=saltonstall (search in "Archived" status).

management councils to solicit constituents' views on how to improve the dissemination and use of results from funded projects.[34]

ISSUES FOR CONGRESS

Some fishing industry representatives have questioned whether the U.S. commercial fishing industry and fishing communities could benefit from greater direct support from S-K funding. Two of the main concerns have been whether the competitive grant process should include greater fishing industry input and whether a greater portion of P&D funds should be allocated to the annual S-K Grant Program. Some assert that NMFS decides by its own criteria which programs receive grants and that in some cases the fishing industry's priorities do not match those of NMFS.[35] They contend that broader, more direct fishing industry participation is needed to inform the process of identifying the needs and priorities of grant funding.

Another concern has been whether a greater portion of P&D funding should be allocated to the S- K Grant Program.[36] Some contend that Congress, as reflected in statute, intended to provide at least 60% of funds to the S-K Grant Program and remaining funding to the National Program for fishing industry research and development.[37] However, shifting significant funding from current NMFS activities may prompt questions about whether additional discretionary funding would be forthcoming to support other NMFS functions, such as data collection and fish population assessments.

Congressional Actions

Funding Allocation

Several bills were introduced during the 112th, 113th, and 114th Congresses that would have significantly changed the allocation of P&D funding. Similar versions of the Fisheries Investment and Regulatory Relief Act in each of these Congresses would have allocated funding to fisheries management regions and would have established a regional fisheries grant program.[38] Under these bills, each regional fishery management council would have established a fishery investment committee, which would focus resources on strengthening regional fisheries management.[39] Each fishery investment committee would have

[34] Mid-Atlantic Fishery Management Council, "Saltonstall-Kennedy Grant Program - Public Feedback Webinar," press release, June 14, 2019, at http://www.mafmc.org/council-events/2019/saltonstall-kennedy-grant-program-public- feedback-webinar.

[35] Senator Dan Sullivan, "Sullivan Applauds Senate Passage of American Fisheries Advisory Committee Act," press release, July 28, 2018.

[36] Leslie Taylor, "Opinion: Don't Be Fooled by NOAA Grants Increase," October 24, 2014.

[37] Senator Kerry, "Introduction of S. 2184," *Congressional Record*, daily edition, March 12, 2012, p. S1579.

[38] The Fisheries Investment and Regulatory Relief Act of 2012 (H.R. 4208 and S. 2184) was introduced during the 112th Congress; the Fisheries Disaster Relief and Research Investment Act (H.R. 799) was introduced during the 113th Congress; and the Fisheries Investment and Regulatory Relief Act of 2015 (H.R. 2106) was introduced in the 114th Congress. No further action was taken following introduction of any of these bills.

[39] Regional fishery management councils were established under the Fishery Conservation and Management Act (currently known as the Magnuson-Stevens Act) to develop fishery management plans that conserve and manage fisheries in federal waters.

- developed a regional fishery investment plan;
- reviewed grant applications and projects to implement regional fishery investment plans; and
- made recommendations on grant applications.

The regional fishery investment plans would have identified research, conservation, and management needs, as well as corresponding actions to rebuild and maintain fish populations and associated fisheries. Each regional investment plan would have been required to include topics related to

- supporting stock surveys, stock assessments, and cooperative fishery research;
- improving the collection and accuracy of recreational and commercial data;
- analyzing social and economic impacts of fishery management decisions;
- providing financial assistance and investment for fishermen and fishing communities;
- developing methods or technologies to improve the quality and value of landings;
- researching and developing conservation engineering technologies; and
- restoring and protecting fish habitat.[40]

Investment plans would have been reviewed by the Secretary of Commerce to ensure consistency with the Magnuson-Stevens Fishery Conservation and Management Act (16 U.S.C. §§1801 et seq.). Limited funding also would have been provided for administrative costs of the grant program and for the development and implementation of investment plans.

Under these versions of the Fisheries Investment and Regulatory Relief Act, the Secretary of Commerce also would have established a regional fisheries grant program to provide funds to advance the regional priorities identified in the regional fishery investment plans. The Secretary would have awarded grants only to projects that would implement regional fishery investment plans and to projects recommended by respective regional fishery investment committees and approved by each regional fishery management council. The Secretary would have been required to allocate 70% of funds from the P&D account to the eight council regions. Half of this funding would have been allocated equally among the councils, and half would have been distributed according to the combined economic impact of recreational and commercial fisheries in each region.

The Secretary also would have been required to allocate 20% of funds for a national fisheries investment program that would support rebuilding and maintaining fish populations and promote sustainable fisheries. Funding would have been divided equally among five general areas: (1) regional fisheries commissions; (2) seafood promotion; (3) fisheries management; (4) fisheries disasters; and (5) other needs, including highly migratory species and international fisheries. Each of the bills would have limited the transfer of ORF funding from the P&D account to 10% of receipts. The legislation also included a provision to provide funding to review regulations and procedures used to implement management under the Magnuson-Stevens Fishery Conservation and Management Act and to make recommendations to streamline regulations and incorporate new information into the management process.

[40] Research priority plans are developed by regional fishery management councils according to 16 U.S.C. §1852(h)(7).

Stopping the Transfer to the Operations, Research, and Facilities Account

In the 113th Congress, a section of the Magnuson-Stevens Fishery Conservation and Management Reauthorization Act of 2014 (S. 2991) would have attempted to stop the transfer of P&D funds to the ORF account. According to Section 205 of S. 2991, it would not be in order in the Senate or in the House of Representatives to consider any bill, resolution, amendment, or conference report that would reduce any amount in the fund (P&D account).[41] This change in the Senate and House rules would have allowed any Senator or Representative to stop the transfer of P&D funds to the ORF discretionary account by making a point of order that a rule is being violated. No further action was taken following the introduction of S. 2991.

American Fisheries Advisory Committee Act

In the 116th Congress, identical versions of the American Fisheries Advisory Committee Act (S. 494 and H.R. 1218) were reported from the committees of jurisdiction in the Senate and the House.[42] The bills would establish an American fisheries advisory committee and would change the process for awarding S-K competitive grants. The committee would

- identify the needs of the seafood industry;
- develop requests for proposals;
- review grant applications; and
- select grant applications for approval.

Currently, NMFS is responsible for these functions, and NMFS considers industry input during the selection process. Both bills also would expand the specified purposes of fisheries research and development projects by explicitly including projects that focus on fisheries science and recreational fishing.[43]

The committee would be composed of representatives from six geographic regions of the United States.[44] The Secretary of Commerce would appoint three members from each region, including (1) an individual with experience as a seafood harvester or processor, (2) an individual with experience in recreational or commercial fishing or growing seafood, and (3) an individual who represents the fisheries science community or the relevant regional fishery

[41] A point of order is a claim from the floor made by a Senator or Representative that a rule of the House of Representatives or Senate is being violated. If the chair sustains the point of order, the action in violation of the rule is not permitted.

[42] S. 494 was reported by the Senate Committee on Commerce, Science, and Transportation on August 16, 2019, and H.R. 1218 was reported by the House Committee on Natural Resources on June 4, 2020. Similar versions also were introduced in previous Congresses, including S. 3087, reported in the 114th Congress; S. 1322, passed the Senate in the 115th Congress; and H.R. 5775, introduced in the 114th Congress; and H.R. 214, introduced in the 115th Congress.

[43] Projects related to recreational fisheries and science historically have been included, but these projects are not explicitly considered in the statute.

[44] Region 1 would include Alaska and the Western Pacific, including Hawaii, the Commonwealth of the Northern Mariana Islands, and the territories of Guam and American Samoa. Region 2 would include Connecticut, Rhode Island, Massachusetts, New Hampshire, and Maine. Region 3 would include Texas, Louisiana, Mississippi, Alabama, Florida, Arkansas, Puerto Rico, and the Territory of the U.S. Virgin Islands. Region 4 would include California, Oregon, Washington, and Idaho. Region 5 would include New York, New Jersey, Delaware, Maryland, Virginia, North Carolina, South Carolina, and Georgia. Region 6 would include Michigan, Minnesota, Wisconsin, Illinois, Indiana, Ohio, and Pennsylvania.

management council. The Secretary also would appoint four at-large members, including (1) an individual who has experience in food distribution, marketing, retail, or service; (2) an individual with experience in the recreational fishing industry supply chain; (3) an individual with experience in the commercial fishing industry supply chain; and (4) an individual who is an employee of NMFS with expertise in fisheries research.[45] The committee members would meet twice annually, and meetings would rotate among the sixregions.

The Secretary of Commerce would identify three or more experts to undertake technical review of grant applications, which would occur prior to committee review. The Secretary also would be required to develop guidance related to technical review, including criteria for elimination of applications that fail to meet a minimum level of technical merit. A grant would not be approved unless the Secretary was satisfied with the applicant's technical and financial capability. Based on the committee's recommendations, the Secretary would evaluate the proposed project according to listed criteria and other criteria the Secretary may require. If the Secretary fails to provide funds to a grant selected by the committee, the Secretary would be required to send a written document to the committee justifying the decision.

APPENDIX. HISTORY OF FINANCING UNDER THE SALTONSTALL-KENNEDY ACT

Table A-1. Financing history of the Saltonstall-Kennedy Program (in thousands of dollars)

Fiscal Year	Transfer to P&D[a] (nominal $)	Transfer to P&D (real $[b])	NMFS ORF Offset	Fisheries Promotion Fund	Directed by Congress[c]	Remainder[d]
1979	17,436	51,373	5,000	0	0	12.436
1980	26,679	72,265	5,000	0	0	21,697
1981	35,000	86,368	17,500	0	0	17,500
1982	26,200	60,433	10,000	0	0	16,200
1983	30,600	67,635	22,600	0	0	8,000
1984	33,600	71,705	23,600	0	0	10,000
1985	34,900	72,066	25,900	0	0	9,000
1986	43,700	88,241	34,100	0	0	9,600
1987	57,400	113,413	51,600	750	0	5,050
1988	56,300	107,701	44,400	2,600	0	9,300
1989	53,600	98,594	45,600	3,000	0	5,000
1990	61,900	109,813	55,000	2,000	0	4,900
1991	70,800	121,286	60,900	2,000	0	7,900
1992	64,100	107,116	63,100	0	0	1,000
1993	61,400	100,264	55,000	0	0	6,400
1994	61,944	98,985	54,800	0	0	7,144
1995	64,765	101,344	55,500	0	0	9,265
1996	72,893	111,947	63,000	0	0	9,893
1997	66,381	100,168	66,000	0	0	381
1998	65,730	97,967	62,380	0	0	3.350

[45] Individuals with experience in recreational and commercial supply chains are defined as fishermen, manufacturers, retailers, and distributers.

Fiscal Year	Transfer to P&D[a] (nominal $)	Transfer to P&D (real $[b])	NMFS ORF Offset	Fisheries Promotion Fund	Directed by Congress[c]	Remainder[d]
1999	66,430	97,731	63,380	0	0	3,050
2000	69,920	100,807	68,000	0	0	1,920
2001	72,828	102,629	68,000	0	0	4,828
2002	79,127	109,709	68,000	0	0	11,127
2003	75,220	102,438	65,000	0	10,000	220
2004	79,724	106,016	62,000	0	17,250	474
2005	77,539	100,048	65,000	0	12,000	539
2006	79,284	99,143	67,000	0	12,000	284
2007	82,816	100,836	79,000	0	0	3,816
2008	84,594	100,884	77,000	0	0	7,594
2009	108,511	127,897	79,001	0	0	29,510
2010	113,371	132,514	104,600	0	0	8,771
2011	90,239	103,425	90,239	0	0	0
2012	109,098	122,701	109,098	0	0	0
2013	130,236	143,849	119,064	0	0	11,172
2014	127,187	137,862	115,000	0	0	12,187
2015	142,615	152,731	116,000	0	0	26,615
2016	146,389	155,377	130,164	0	0	16,225
2017	145,073	151,359	130,164	0	0	14,909
2018	154,664	157,646	144,000	0	0	10,664
2019	158,406	158,406	157,980	0	0	426
2020	182,783	182,783	174,774	0	0	8,009

Sources: National Oceanic and Atmospheric Administration (NOAA), Budget Office, 2020, January 20, 2020; NOAA, Budget Office, FY2007–FY2019, December 2, 2019; NOAA; National Marine Fisheries Service (NMFS), The Saltonstall-Kennedy Grant Program: Fisheries Research and Development, Report 2008, August 1, 2008, p. 4; NOAA, NMFS, The Saltonstall-Kennedy Grant Program, Fisheries Research and Development, Report 2001, August 1, 2001, p. 8; NOAA, NMFS, The Saltonstall-Kennedy Grant Program: Fisheries Research and Development, Report 1991-1992, 1992, p. 3; NOAA, NMFS, The Saltonstall-Kennedy Grant Program: Fisheries Research and Development, Report 1987-1990, 1990, p. 3; NOAA, NMFS, The Saltonstall-Kennedy Grant Program, Fisheries Research and Development, Report 1982-1986, 1986, p. 2.

[a] Funds transferred from the U.S. Department of Agriculture to NOAA.

[b] Real dollars calculated from Bureau of Economic Analysis, GDP deflator, Table 1.1.7.

[c] Funding appropriated by Congress to seafood marketing boards and programs in FY2003, P.L. 108-7; FY2004, P.L. 108-199; FY2005, P.L. 108-447, and FY2006, P.L. 109-108.

[d] Remainder includes funds used for the Saltonstall-Kennedy Grant Program, the National Program, and administrative costs.

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Chapter 158

AMERICAN FISHERIES ADVISORY COMMITTEE ACT*

The Committee on Natural Resources, to whom was referred the bill (H.R. 1218) to establish the American Fisheries Advisory Committee to assist in the awarding of fisheries research and development grants, and for other purposes, having considered the same, reports favorably thereon with an amendment and recommends that the bill as amended do pass.

The amendment is as follows:

Strike all after the enacting clause and insert the following:

SECTION 1. SHORT TITLE

This Act may be cited as the "American Fisheries Advisory Committee Act."

SECTION 2. AMERICAN FISHERIES ADVISORY COMMITTEE

(a) ESTABLISHMENT.—Section 2 of the Act of August 11, 1939 (15 U.S.C. 713c–3), is amended—

(1) by redesignating subsection (e) as subsection (f); and

(2) by inserting after subsection (d) the following:

"(e) AMERICAN FISHERIES ADVISORY COMMITTEE.—

"(1) DEFINITIONS.—In this subsection:

"(A) COMMITTEE.—The term 'Committee' means the American Fisheries Advisory Committee established under paragraph (2).

"(B) FISHING COMMUNITY.—The term 'fishing community' means harvesters, marketers, growers, processors, recreational fishermen, charter fishermen, and persons providing them with goods and services.

"(C) MARKETING AND PROMOTION.—The term 'marketing and promotion' means an activity aimed at encouraging the consumption of seafood or expanding or maintaining commercial markets for seafood.

* This is an edited, reformatted and augmented version of House of Representatives Report , Publication No. 116–428, dated June 4, 2020.

"(D) PROCESSOR.—The term 'processor' means any person in the business of preparing or packaging seafood (including seafood of the processor's own harvesting) for sale.

"(E) SEAFOOD.—The term 'seafood' means farm-raised and wildcaught fish, shellfish, or marine algae harvested in the United States or by a United States flagged vessel for human consumption.

"(2) ESTABLISHMENT.—Not later than 90 days after the date of the enactment of the American Fisheries Advisory Committee Act, the Secretary shall establish 6 regions within the American Fisheries Advisory Committee as follows:

"(A) Region 1 shall consist of Alaska, Hawaii, the Commonwealth of the Northern Mariana Islands, and the Territories of Guam and American Samoa.

"(B) Region 2 shall consist of Maine, New Hampshire, Massachusetts, Rhode Island, and Connecticut.

"(C) Region 3 shall consist of Texas, Alabama, Louisiana, Mississippi, Florida, Arkansas, Puerto Rico, and the Territory of the Virgin Islands of the United States.

"(D) Region 4 shall consist of California, Washington, Oregon, and Idaho.

"(E) Region 5 shall consist of New Jersey, New York, Delaware, Maryland, Virginia, North Carolina, South Carolina, and Georgia.

"(F) Region 6 shall consist of Michigan, Minnesota, Wisconsin, Illinois, Indiana, Ohio, and Pennsylvania.

"(3) MEMBERSHIP.—The Committee shall be composed of the following members:

"(A) REGIONAL REPRESENTATION.—Each of the regions listed in subparagraphs (A) through (F) of paragraph (2) shall be represented on the Committee by 3 members—

"(i) who are appointed by the Secretary;

"(ii) who reside in a State or territory in the region that the member will represent;

"(iii) of which—

" (I) one shall have experience as a seafood harvester or processor;

"(II) one shall have experience as recreational or commercial fisher or have experience growing seafood; and

"(III) one shall be an individual who represents the fisheries science community or the relevant Regional Fishery Management Council; and

"(iv) that are selected so that the members of the Committee have experience or expertise with as many seafood species as practicable.

"(B) AT-LARGE MEMBERS.—The Secretary shall appoint to the Committee at-large members as follows:

"(i) One individual with experience in food distribution, marketing, retail, or food service.

"(ii) One individual with experience in the recreational fishing industry supply chain, such as fishermen, manufacturers, retailers, and distributors.

"(iii) One individual with experience in the commercial fishing industry supply chain, such as fishermen, manufacturers, retailers, and distributors.

"(iv) One individual who is an employee of the National Marine Fisheries Service with expertise in fisheries research.

"(C) BALANCED REPRESENTATION.—In selecting the members described in subparagraphs (A) and (B), the Secretary shall seek to maximize on the Committee, to the

extent practicable, a balanced representation of expertise in United States fisheries, seafood production, and science.

"(4) MEMBER TERMS.—The term for a member of the Committee shall be 3 years, except that the Secretary shall designate staggered terms for the members initially appointed to the Committee.

"(5) RESPONSIBILITIES.—The Committee shall be responsible for—

"(A) identifying needs of the fishing community that may be addressed by a project funded with a grant under subsection (c);

"(B) developing the request for proposals for such grants;

"(C) reviewing applications for such grants; and

"(D) selecting applications for approval under subsection (c)(2)(B).

"(6) CHAIR.—The Committee shall elect a chair by a majority of those voting, if a quorum is present.

"(7) QUORUM.—A simple majority of members of the Committee shall constitute a quorum, but a lesser number may hold hearings.

"(8) MEETINGS.—

"(A) FREQUENCY.—The Committee shall meet not more than 2 times each year.

"(B) LOCATION.—The meetings of the Committee shall rotate between the geographic regions described under paragraph (2).

"(C) MINIMIZING COSTS.—The Committee shall seek to minimize the operational costs associated with meetings, hearings, or other business of the Committee, including through the use of video or teleconference.

"(9) DESIGNATION OF STAFF MEMBER.—The Secretary shall designate a staff member to coordinate the activities of the Committee and to assist with administrative and other functions as requested by the Committee.

"(10) PER DIEM AND EXPENSES AND FUNDING.—

"(A) IN GENERAL.—A member of the Committee shall serve without compensation, but shall be reimbursed in accordance with section 5703 of title 5, United States Code, for reasonable travel costs and expenses incurred in performing duties as a member of the Committee.

"(B) FUNDING.—The costs of reimbursements under subparagraph (A) and the other costs associated with the Committee shall be paid from funds made available to carry out this section (which may include funds described in subsection (f)(1)(B)), except that no funds allocated for grants under sub-section (f)(1)(A) shall be expended for any purpose under this subsection.

"(11) CONFLICT OF INTEREST.—The conflict of interest and recusal provisions set out in section 302(j) of the Magnuson-Stevens Fishery Conservation and Management Act (16 U.S.C. 1852(j)) shall apply to any decision by the Committee and to all members of the Committee as if each member of the Committee is an affected individual within the meaning of such section 302(j), except that in addition to the disclosure requirements of section 302(j)(2)(C) of such Act (16 U.S.C. 1852(j)(2)(C)), each member of the Committee shall disclose any financial interest or relationship in an organization or with an individual that is applying for a grant under subsection (c) held by the member of the Committee, including an interest as an officer, director, trustee, partner, employee, contractor, agent, or other representative.

"(12) TECHNICAL REVIEW OF APPLICATIONS.—

"(A) IN GENERAL.—Prior to review of an application for a grant under subsection (c) by the Committee, the Secretary shall obtain an independent written technical evaluation from 3 or more appropriate Federal, private, or public sector experts (such as industry, academia, or governmental experts) who—

"(i) have subject matter expertise to determine the technical merit of the proposal in the application;

"(ii) shall independently evaluate each such proposal; and

"(iii) shall certify that the expert does not have a conflict of interest concerning the application that the expert is reviewing.

"(B) GUIDANCE.—Not later than 180 days after the date of enactment of the American Fisheries Advisory Committee Act, the Secretary shall issue guidance related to carrying out the technical evaluations under subparagraph (A). Such guidance shall include criteria for the elimination by the National Oceanic and Atmospheric Administration of applications that fail to meet a minimum level of technical merit as determined by the review described in subparagraph (A)."

(b) ROLE IN APPROVAL OF GRANTS.—Section 2(c)(3) of the Act of August 11, 1939 (15 U.S.C. 713c–3(c)(3)), is amended to read as follows:

"(3)(A) No application for a grant under this subsection may be approved unless the Secretary—

"(i) is satisfied that the applicant has the requisite technical and financial capability to carry out the project; and

"(ii) based on the recommendations of the American Fisheries Advisory Committee established in subsection (e), evaluates the proposed project as to—

"(I) soundness of design;

"(II) the possibilities of securing productive results;

"(III) minimization of duplication with other fisheries research and development projects;

"(IV) the organization and management of the project;

"(V) methods proposed for monitoring and evaluating the success or failure of the project; and

"(VI) such other criteria as the Secretary may require.

"(B) If the Secretary fails to provide funds to a grant selected by the American Fisheries Advisory Committee, the Secretary shall provide a written document to the Committee justifying the decision.".

SECTION 3. EXPANSION OF SPECIFIED PURPOSES OF FISHERIES RESEARCH AND DEVELOPMENT PROJECTS GRANTS PROGRAM TO INCLUDE FISHERIES RESEARCH AND DEVELOPMENT PROJECTS

Section 2(c)(1) of the Act of August 11, 1939 (15 U.S.C. 713c–3(c)(1)) is amended by inserting "fisheries science, recreational fishing," before "harvesting".

SECTION 4. PUBLIC AVAILABILITY OF GRANTS PROPOSALS

Section 2(c) of the Act of August 11, 1939 (15 U.S.C. 713c–3(c)), is amended by adding at the end the following:

"(6) Any person awarded a grant under this subsection shall make publicly available a title and abstract of the project to be carried out by the grant funds that serves as the public justification for funding the project that includes a statement describing how the project serves to enhance United States fisheries, including harvesting, processing, marketing, and associated infrastructures, if applicable."

PURPOSE OF THE BILL

The purpose of H.R. 1218 is to establish the American Fisheries Advisory Committee to assist in the awarding of fisheries research and development grants, and for other purposes.

BACKGROUND AND NEED FOR LEGISLATION

The Saltonstall-Kennedy Grant Program (S–K Grant Program), established by the 1939 Saltonstall-Kennedy Act [1] (S–K Act), provides a mechanism to fund research and development projects involving seafood harvesting, processing, marketing, associated infrastructure, and more,[2] funded through tariffs paid to the Department of Agriculture on imported seafood products and other fisheries products.[3] This fund was originally overseen by an advisory committee, which was eliminated under the 1972 Federal Advisory Committee Act.[4] The National Marine Fisheries Service (NMFS) has since managed the grant program to "address the needs of fishing communities, optimize economic benefits by building and maintaining sustainable fisheries, and increase other opportunities to keep working waterfronts viable."[5]

Lawmakers and stakeholders have raised concerns over the years that NMFS does not administer the program fairly and in accordance with statute. For example, NMFS has arguably underfunded seafood marketing projects in favor of internal priorities.[6] Indeed, much of the statutorily dedicated tariff revenue has been diverted from the grant program to fund NOAA operations over the years,[7] although in some cases at Congress's direction.[8] For Fiscal Year 2019, NMFS recommended awarding five projects using S–K funds for almost

[1] Act of Aug. 11, 1939, 53 Stat. 1411.
[2] 15 U.S.C. § 713c–3(c).
[3] Fish and Wildlife Act of 1956, § 12(a), Pub. L. No. 84–1024, 70 Stat. 1119, 1124 (codified as 15 U.S.C. § 713c–3 note).
[4] See Pub. L. No. 92–463, § 14, 86 Stat. 770, 776 (1972).
[5] Saltonstall-Kennedy Grant Program, NOAA FISHERIES, https://www.fisheries.noaa.gov/grant/ saltonstall-kennedy-grant-program (last visited Oct. 9, 2019).
[6] See, e.g., S. REP. NO. 116–77, at 2 (2019); S. REP. NO. 115–193, at 2 (2017); S. REP. NO. 114–379, at 2 (2016).
[7] Richard Gaines, Feds Ignore Law on Fishing Fund, GLOUCESTER TIMES, Mar. 28, 2011, https://www.gloucestertimes.com/news/localnews/feds-ignore-law-on-fishing-fund/article062b6ce8-2551-5536-ad29-af879a2f73bf.html.
[8] Eugene H. Buck, Cong. Research Serv., RS21799, SALTONSTALL-KENNEDY ACT 3–5, 6, 7, 3 n.12 (2012).

$1.3 million: three projects for marine aquaculture; one for promotion, development, and marketing; and one in support of science that maximizes fishing opportunities, revenue, and jobs in U.S. fisheries.[9]

The purpose of this bill is to create an advisory committee comprised of representatives from the seafood industry to develop grant proposal requests and to review and select grant applications.

COMMITTEE ACTION

H.R. 1218 was introduced on February 13, 2019, by Representative Don Young (R–AK). The bill was referred solely to the Committee on Natural Resources, and within the Committee to the Subcommittee on Water, Oceans, and Wildlife. On May 8, 2019, the Subcommittee held a hearing on the bill. On September 18, 2019, the Natural Resources Committee met to consider the bill. The Subcommittee was discharged by unanimous consent. Chair Rau´ l Grijalva (D–AZ) filed an amendment in the nature of a substitute. By unanimous consent, the sponsor of the amendment in the nature of a substitute was changed from Chair Grijalva to Representative Joe Cunningham (D–SC) and Representative Garret Graves (R–LA). The amendment in the nature of a substitute was agreed to by unanimous consent. The bill, as amended, was adopted and ordered favorably reported to the House of Representatives by unanimous consent.

HEARINGS

For the purposes of section 103(i) of H. Res. 6 of the 116th Congress—the following hearing was used to develop or consider H.R. 1218: legislative hearing by the Subcommittee on Water, Oceans, and Wildlife held on May 8, 2019.

SECTION-BY-SECTION ANALYSIS

Section 1. Short Title

This section provides the short title of the bill, the "American Fisheries Advisory Committee Act."

Section 2. American Fisheries Advisory Committee

This section establishes the American Fisheries Advisory Committee, which manages the administration of grant proposal requests and provides recommendations to the Secretary of

[9] FY19 Successful Saltonstall-Kennedy Grant Applicants, NOAA FISHERIES, https:// www.fisheries.noaa.gov/ content/fy19-successful-saltonstall-kennedy-grant-applicants (last up- dated Sept. 18, 2019).

Commerce for fisheries research and development grants that fund projects meeting the needs of the fishing community. This committee consists of representatives from six geographic regions who are from the seafood harvesting and processing industries; the fishing or seafood-growing communities; and the fisheries science community or the relevant Regional Fishery Management Council. In addition, the committee consists of at-large members with experience in seafood distribution, marketing, retail, or food service; the recreational fishing industry supply chain; the commercial fishing industry supply chain; and an employee of NMFS.

Section 3. Expansion of Specified Purposes of Fisheries Research and Development Projects Grants Program to Include Fisheries Research and Development Projects

This section includes fisheries science and recreational fishing projects among the research and development project grants eligible for funding by the Secretary of Commerce.

Section 4. Public Availability of Grants Proposals

This section requires grantees to make publicly available a title and abstract of each project as public justification for funding, including a description of how the project enhances U.S. fisheries.

COMMITTEE OVERSIGHT FINDINGS AND RECOMMENDATIONS

Regarding clause 2(b)(1) of rule X and clause 3(c)(1) of rule XIII of the Rules of the House of Representatives, the Committee on Natural Resources' oversight findings and recommendations are reflected in the body of this report.

COMPLIANCE WITH HOUSE RULE XIII AND CONGRESSIONAL BUDGET ACT

(1) Cost of Legislation and the Congressional Budget Act. With respect to the requirements of clause 3(c)(2) and (3) of rule XIII of the Rules of the House of Representatives and sections 308(a) and 402 of the Congressional Budget Act of 1974, the Committee has received the following estimate for the bill from the Director of the Congressional Budget Office:

H.R. 1218 would establish the American Fisheries Advisory Committee within the Department of Commerce (DOC). The committee would consist of representatives from each of six geographic regions and at-large members with experience in the seafood and fisheries industries, as selected by DOC. Under the bill, the committee would be responsible for identifying the needs of fishing communities, developing requests for proposals for research

and development grants to meet those needs, reviewing grant applications, and selecting applicants to receive funding.

H.R. 1218, American Fisheries Advisory Committee Act
As ordered reported by the House Committee on Natural Resources on September 18, 2019

By Fiscal Year, Millions of Dollars	2019	2019-2024	2019-2029
Direct Spending (Outlays)	0	0	0
Revenues	0	0	0
Increase or Decrease (-) in the Deficit	0	0	0
Spending Subject to Appropriation (Outlays)	0	0	not estimated

		Mandate Effects	
Statutory pay-as-you-go procedures apply?	No		
Increases on-budget deficits in any of the four consecutive 10-year periods beginning in 2030?	No	Contains intergovernmental mandate?	No
		Contains private-sector mandate?	No

The National Oceanic and Atmospheric Administration currently administers the fishery research and development grant program described above. That program's funding is derived from DOC's permanent statutory authority to spend 30 percent of customs receipts collected from imported fishery products. In 2018 the agency spent $16 million for those purposes. H.R. 1218 would shift the authority to administer that grant program to the newly established committee. The bill also would expand eligibility for grant applicants to include projects related to fisheries science and recreational fishing.

H.R. 1218 would not change the total amount of authorized spending of custom receipts (although the composition of that spending might change). Thus, CBO estimates that enacting H.R. 1218 would not affect the federal budget.

On April 18, 2019, CBO transmitted a cost estimate for S. 494, the American Fisheries Advisory Committee Act, as ordered reported by the Senate Committee on Commerce, Science, and Transportation on April 3, 2019. H.R. 1218 is similar to S. 494 and CBO's estimates of the budgetary effects are the same for both pieces of legislation.

The CBO staff contact for this estimate is David Hughes. The estimate was reviewed by H. Samuel Papenfuss, Deputy Assistant Director for Budget Analysis.

1. General Performance Goals and Objectives. As required by clause 3(c)(4) of rule XIII, the general performance goals and objectives of this bill are to establish the American Fisheries Advisory Committee to assist in the awarding of fisheries research and development grants.

EARMARK STATEMENT

This bill does not contain any Congressional earmarks, limited tax benefits, or limited tariff benefits as defined under clause 9(e), 9(f), and 9(g) of rule XXI of the Rules of the House of Representatives.

UNFUNDED MANDATES REFORM ACT STATEMENT

This bill contains no unfunded mandates.

FEDERAL ADVISORY COMMITTEE ACT STATEMENT

The functions of the American Fisheries Advisory Committee required to be established by this bill are currently being performed, in part, by the Secretary of Commerce acting through the National Marine Fisheries Service and previously by an advisory committee eliminated by the Federal Advisory Committee Act. In reporting the bill favorably to the House of Representatives, the Committee on Natural Resources finds that these functions would be better performed by the proposed advisory committee than by one or more agencies or another existing advisory committee.

EXISTING PROGRAMS

This bill does not establish or reauthorize a program of the federal government known to be duplicative of another program. Such program was not included in any report from the Government Accountability Office to Congress pursuant to section 21 of Public Law 111–139. The Fisheries Development and Utilization Research and Development Grants and Cooperative Agreements Program (CFDA No. 11.427) expanded by this bill is related and complementary to, but not duplicative of, the following programs identified in the most recent Catalog of Federal Domestic Assistance published pursuant to 31 U.S.C. § 6104: Interjurisdictional Fisheries Act of 1986 (CFDA No. 11.407) and Sea Grant Support (CFDA No. 11.417).

APPLICABILITY TO LEGISLATIVE BRANCH

The Committee finds that the legislation does not relate to the terms and conditions of employment or access to public services or accommodations within the meaning of section 102(b)(3) of the Congressional Accountability Act.

PREEMPTION OF STATE, LOCAL, OR TRIBAL LAW

Any preemptive effect of this bill over state, local, or tribal law is intended to be consistent with the bill's purposes and text and the Supremacy Clause of Article VI of the U.S. Constitution.

CHANGES IN EXISTING LAW MADE BY THE BILL, AS REPORTED

In compliance with clause 3(e) of rule XIII of the Rules of the House of Representatives, changes in existing law made by the bill, as reported, are shown as follows (existing law proposed to be omitted is enclosed in black brackets, new matter is printed in italics, and existing law in which no change is proposed is shown in roman):

ACT OF AUGUST 11, 1939

AN ACT To authorize the Federal Surplus Commodities Corporation to purchase and distribute surplus products of the fishing industry.

SEC. 2. (a) DEFINITIONS.—As used in this section—

(2) The term "person" means—

(A) any individual who is a citizen or national of the United States or a citizen of the Northern Mariana Islands;

(B) any fishery development foundation or other private nonprofit corporation in Alaska; and

(C) any corporation, partnership, association, or other entity (including, but not limited to, any fishery development foundation or other private nonprofit corporation not located in Alaska), nonprofit or otherwise, if such entity is a citizen of the United States within the meaning of section 2 of the Shipping Act, 1916 (46 U.S.C. 802) and for purposes of applying such section 2 with respect to this section—

(i) the term "State" as used therein includes any State referred to in paragraph (3),

(ii) citizens of the United States must own not less than 75 percent of the interest in the entity or, in the case of a nonprofit entity, exercise control in the entity that is determined by the Secretary to be the equivalent of such ownership, and

(iii) nationals of the United States and citizens of the Northern Mariana Islands shall be treated as citizens of the United States in meeting the ownership and control requirements referred to in clause (ii).

(2) The term "Secretary" means the Secretary of Commerce.

(3) The term "State" means any State, the District of Columbia, the Commonwealth of Puerto Rico, American Samoa, the Virgin Islands of the United States, Guam, the Northern Mariana Islands, and any other Commonwealth, territory, or possession of the United States.

(4) The term "United States fishery" means any fishery, including any tuna fishery, that is, or may be, engaged in by citizens or nationals of the United States or citizens of the Northern Mariana Islands.

(5) The term "citizen of the Northern Mariana Islands" means—

(A) an individual who qualifies as such under section 8 of the Schedule on Transitional Matters attached to the Constitution of the Northern Mariana Islands; or

(B) a corporation, partnership, association, or other entity organized or existing under the laws of the Northern Mariana Islands, not less than 75 percent of the interest in which is owned by individuals referred to in subparagraph (A) or citizens or nationals of the United States, in cases in which "owned" is used in the same sense as in section 2 of the Shipping Act, 1916 (46 U.S.C. 802).

(b) FUND.—(1) The Secretary of Agriculture shall transfer to the Secretary each fiscal year, beginning with the fiscal year commencing July 1, 1954, and ending on June 30, 1957, from moneys made available to carry out the provisions of section 32 of such Act of August 24, 1935, an amount equal to 30 per centum of the gross receipts from duties collected under the customs laws on fishery products (including fish, shellfish, mollusks, crustacea, aquatic plants and animals, and any products thereof, including processed and manufactured products), which shall be maintained in a separate fund only for—

(A) use by the Secretary—

(i) to provide financial assistance for the purpose of carrying out fisheries research and development projects approved under subsection (c);

(ii) to implement the national fisheries research and development program provided for under subsection (d);

(iii) to implement the Northwest Atlantic Ocean Fisheries Reinvestment Program established under section 314 of the Magnuson Fishery Conservation and Management Act; and

(iv) to fund the Federal share of a fishing capacity reduction program established under section 312 of the Magnuson Fishery Conservation and Management Act; and

(B) the provision of moneys, subject to paragraph (2), to carry out the purposes of the Fisheries Promotion Fund established under section 208(a) of the Fish and Seafood Promotion Act of 1986.

(2) There are transferred from the fund established under paragraph (1) to the Fisheries Promotion Fund referred to in paragraph (1)(B) $750,000 in fiscal year 1987, $3,000,000 in each of fiscal years 1988 and 1989, and $2,000,000 in each of fiscal years 1990 and 1991.

(c) FISHERIES RESEARCH AND DEVELOPMENT PROJECTS.—(1) The Secretary shall make grants from the fund established under sub- section (b) to assist persons in carrying out research and development projects addressed to any aspect of United States fisheries, including, but not limited to, *fisheries science, recreational fishing*, harvesting, processing, marketing, and associated infrastructures.

(2) The Secretary shall—

(A) at least once each fiscal year, receive, during a 60-day period specified by him, applications for grants under this subsection;

(B) prescribe the form and manner in which applications for grants under this subsection must be made, including, but not limited to, the specification of the information which must accompany applications to ensure that the proposed projects comply with Federal law and can be evaluated in accordance with paragraph (3)(B); and

(C) approve or disapprove each such application before the close of the 120th day after the last day of the 60-day period (specified under subparagraph (A)) in which the application was received.

ø(3) No application for a grant under this subsection may be approved unless the Secretary—

ø(A) is satisfied that the applicant has the requisite technical and financial capability to carry out the project; and

ø(B) evaluates the proposed project as to—

ø(i) soundness of design,

ø(ii) the possibilities of securing productive results,

ø(iii) minimization of duplication with other fisheries research and development projects,

ø(iv) the organization and management of the project,

ø(v) methods proposed for monitoring and evaluating the success or failure of the project, and

ø(vi) such other criteria as the Secretary may require.¿

(3)(A) No application for a grant under this subsection may be approved unless the Secretary—

(i)is satisfied that the applicant has the requisite technical and financial capability to carry out the project; and

(ii) based on the recommendations of the American Fisheries Advisory Committee established in subsection (e), evaluates the proposed project as to—

(I) soundness of design;

(II) the possibilities of securing productive results;

(III) minimization of duplication with other fisheries research and development projects;

(IV) the organization and management of the project;

(V) methods proposed for monitoring and evaluating the success or failure of the project; and

(VI) such other criteria as the Secretary may require.

(B) If the Secretary fails to provide funds to a grant selected by the American Fisheries Advisory Committee, the Secretary shall provide a written document to the Committee justifying the decision.

(4) Each grant made under this subsection shall be subject to such terms and conditions as the Secretary may require to protect the interests of the United States, including, but not limited to, the following:

(A) The recipient of the grant must keep such records as the Secretary shall require as being necessary or appropriate for disclosing the use made of grant funds and shall allow the Secretary and the Comptroller General of the United States, or any of their authorized representatives, access to such records for purposes of audit and examination.

(B) The amount of a grant may not be less than 50 percent of the estimated cost of the project.

(C) The recipient of the grant must submit to the Secretary periodic project status reports.

(5)(A) If the cost of a project will be shared by the grant recipient, the Secretary shall accept, as a part of all of that share, the value of inkind contributions made by the recipient, or made available to, and applied by, the recipient, with respect to the project.

(B) For purposes of subparagraph (A), inkind contributions may be in the form of, but are not limited to, personal services rendered in carrying out functions related to, and permission to use real or personal property owned by others (for which consideration is not required) in carrying out the project. The Secretary shall establish

(i) the training, experience, and other qualifications which shall be required in order for services to be considered as inkind contributions; and (ii) the standards under which the Secretary will determine the value of inkind contributions for purposes of subparagraph (A).

(C) Any valuation determination made by the Secretary for purposes of this paragraph shall be conclusive.

(6) Any person awarded a grant under this subsection shall make publicly available a title and abstract of the project to be carried out by the grant funds that serves as the public justification for funding the project that includes a statement describing how the project

serves to enhance United States fisheries, including harvesting, processing, marketing, and associated infrastructures, if applicable.

(d) NATIONAL FISHERIES RESEARCH AND DEVELOPMENT PROGRAM.—(1) The Secretary shall carry out a national program of research and development addressed to such aspects of United States fisheries (including, but not limited to, harvesting, processing, marketing, and associated infrastructures), if not adequately covered by projects assisted under subsection (c), as the Secretary deems appropriate.

(2) The Secretary shall, after consultation with appropriate representatives of the fishing industry, submit to the Committee on Commerce, Science, and Transportation of the Senate and the Committee on Merchant Marine and Fisheries of the House of Representatives, an annual report, that must be submitted not later than 60 days before the close of each fiscal year, containing—

(A) the fisheries development goals and funding priorities under paragraph (1) for the next fiscal year;

(B) a description of all pending projects assisted under subsection (c) or carried out under paragraph (1), in addition to—

(i) a list of those applications approved and those disapproved under subsection (c), and the total amount of grants made, for the current fiscal year, and

(ii) a statement of the extent to which available funds were not obligated or expended by the Secretary for grants under subsection (c) during the current fiscal year; and

(C) an assessment of each project assisted under subsection

(c) or carried out under paragraph (1) that was completed in the preceding fiscal year regarding the extent to which (i) the objectives of the project were attained, and (ii) the project contributed to fishery development.

(e) AMERICAN FISHERIES ADVISORY COMMITTEE.—

(1) DEFINITIONS.—In this subsection:

(A) COMMITTEE.—The term "Committee" means the American Fisheries Advisory Committee established under paragraph (2).

(B) FISHING COMMUNITY.—The term "fishing community" means harvesters, marketers, growers, processors, recreational fishermen, charter fishermen, and persons providing them with goods and services.

(C) MARKETING AND PROMOTION.—The term "marketing and promotion" means an activity aimed at encouraging the consumption of seafood or expanding or maintaining commercial markets for seafood.

(D) PROCESSOR.—The term "processor" means any person in the business of preparing or packaging seafood (including seafood of the processor's own harvesting) for sale.

(E) SEAFOOD.—The term "seafood" means farm-raised and wild-caught fish, shellfish, or marine algae harvested in the United States or by a United States flagged vessel for human consumption.

(2) ESTABLISHMENT.—Not later than 90 days after the date of the enactment of the American Fisheries Advisory Committee Act, the Secretary shall establish 6 regions within the American Fisheries Advisory Committee as follows:

(A) Region 1 shall consist of Alaska, Hawaii, the Commonwealth of the Northern Mariana Islands, and the Territories of Guam and American Samoa.

(B) Region 2 shall consist of Maine, New Hampshire, Massachusetts, Rhode Island, and Connecticut.

(C) Region 3 shall consist of Texas, Alabama, Louisiana, Mississippi, Florida, Arkansas, Puerto Rico, and the Territory of the Virgin Islands of the United States.

(D) Region 4 shall consist of California, Washington, Oregon, and Idaho.

(E) Region 5 shall consist of New Jersey, New York, Delaware, Maryland, Virginia, North Carolina, South Carolina, and Georgia.

(F) Region 6 shall consist of Michigan, Minnesota, Wisconsin, Illinois, Indiana, Ohio, and Pennsylvania.

(3) MEMBERSHIP.—The Committee shall be composed of the following members:

(A) REGIONAL REPRESENTATION.—Each of the regions listed in subparagraphs (A) through (F) of paragraph (2) shall be represented on the Committee by 3 members—

(i) who are appointed by the Secretary;

(ii) who reside in a State or territory in the region that the member will represent;

(iii) of which—

(I) one shall have experience as a seafood harvester or processor;

(II) one shall have experience as recreational or commercial fisher or have experience growing seafood; and

(III) one shall be an individual who represents the fisheries science community or the relevant Regional Fishery Management Council; and

(iv) that are selected so that the members of the Committee have experience or expertise with as many seafood species as practicable.

(B) AT-LARGE MEMBERS.—The Secretary shall appoint to the Committee at-large members as follows:

(i) One individual with experience in food distribution, marketing, retail, or food service.

(ii) One individual with experience in the recreational fishing industry supply chain, such as fishermen, manufacturers, retailers, and distributors.

(iii) One individual with experience in the commercial fishing industry supply chain, such as fishermen, manufacturers, retailers, and distributors.

(iv) One individual who is an employee of the National Marine Fisheries Service with expertise in fisheries research.

(C) BALANCED REPRESENTATION.—In selecting the members described in subparagraphs (A) and (B), the Secretary shall seek to maximize on the Committee, to the extent practicable, a balanced representation of expertise in United States fisheries, seafood production, and science.

(4) MEMBER TERMS.—The term for a member of the Committee shall be 3 years, except that the Secretary shall designate staggered terms for the members initially appointed to the Committee.

(5) RESPONSIBILITIES.—The Committee shall be responsible for—

(A) identifying needs of the fishing community that may be addressed by a project funded with a grant under subsection (c);

(B) developing the request for proposals for such grants;

(C) reviewing applications for such grants; and

(D) selecting applications for approval under subsection (c)(2)(B).

(6) CHAIR.—The Committee shall elect a chair by a majority of those voting, if a quorum is present.

(7) QUORUM.—A simple majority of members of the Committee shall constitute a quorum, but a lesser number may hold hearings.

(8) MEETINGS.—(A) FREQUENCY.—The Committee shall meet not more than 2 times each year.

(B) LOCATION.—The meetings of the Committee shall rotate between the geographic regions described under paragraph (2).

(C) MINIMIZING COSTS.—The Committee shall seek to minimize the operational costs associated with meetings, hearings, or other business of the Committee, including through the use of video or teleconference.

(9) DESIGNATION OF STAFF MEMBER.—The Secretary shall designate a staff member to coordinate the activities of the Committee and to assist with administrative and other functions as requested by the Committee.

(10) PER DIEM AND EXPENSES AND FUNDING.—

(A) IN GENERAL.—A member of the Committee shall serve without compensation, but shall be reimbursed in accordance with section 5703 of title 5, United States Code, for reasonable travel costs and expenses incurred in performing duties as a member of the Committee.

(B) FUNDING.—The costs of reimbursements under sub-paragraph (A) and the other costs associated with the Committee shall be paid from funds made available to carry out this section (which may include funds described in subsection (f)(1)(B)), except that no funds allocated for grants under subsection (f)(1)(A) shall be expended for any purpose under this subsection.

(11) CONFLICT OF INTEREST.—The conflict of interest and recusal provisions set out in section 302(j) of the Magnuson-Stevens Fishery Conservation and Management Act (16 U.S.C. 1852(j)) shall apply to any decision by the Committee and to all members of the Committee as if each member of the Committee is an affected individual within the meaning of such section 302(j), except that in addition to the disclosure requirements of section 302(j)(2)(C) of such Act (16 U.S.C. 1852(j)(2)(C)), each member of the Committee shall disclose any financial interest or relationship in an organization or with an individual that is applying for a grant under subsection (c) held by the member of the Committee, including an interest as an officer, director, trustee, partner, employee, contractor, agent, or other representative.

(12) TECHNICAL REVIEW OF APPLICATIONS.—

(A) IN GENERAL.—Prior to review of an application for a grant under subsection (c) by the Committee, the Secretary shall obtain an independent written technical evaluation from 3 or more appropriate Federal, private, or public sector experts (such as industry, academia, or governmental experts) who—

(i) have subject matter expertise to determine the technical merit of the proposal in the application;

(ii) shall independently evaluate each such proposal; and

(iii) shall certify that the expert does not have a conflict of interest concerning the application that the expert is reviewing.

(B) GUIDANCE.—Not later than 180 days after the date of enactment of the American Fisheries Advisory Committee Act, the Secretary shall issue guidance related to carrying out the technical evaluations under subparagraph (A). Such guidance shall include criteria for the elimination by the National Oceanic and Atmospheric Administration of applications that

fail to meet a minimum level of technical merit as determined by the review described in subparagraph (A).

ø(e)¿ *(f)* ALLOCATION OF FUND MONEYS.—(1) Notwithstanding any other provisions of law, all moneys in the fund shall be used exclusively for the purpose of promoting United States fisheries in accordance with the provisions of this section, and no such moneys shall be transferred from the fund for any other purpose. With respect to any fiscal year, all moneys in the fund, including the sum of all unexpended moneys carried over into that fiscal year and all moneys transferred to the fund under subsection (b) or any other provision of law with respect to that fiscal year, shall be allocated as follows:

(A) the Secretary shall use no less than 60 per centum of such moneys to make direct industry assistance grants to develop the United States fisheries and to expand domestic and foreign markets for United States fishery products pursuant to subsection (c) of this section; and

(B) the Secretary shall use the balance of the moneys in the fund to finance those activities of the National Marine Fisheries Service which are directly related to development of the United States fisheries pursuant to subsection (d) of this section.

(2) The Secretary shall, consistent with the number of meritorious applications received with respect to any fiscal year, obligate or expend all of the moneys in the fund described in paragraph (1). Any such moneys which are not expended in a given fiscal year shall remain available for expenditure in accordance with this section without fiscal year limitation, except that the Secretary shall not obligate such moneys at a rate less than that necessary to prevent the balance of moneys in the fund from exceeding $3,000,000 at the end of any fiscal year.

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SUPPLEMENTAL, MINORITY, ADDITIONAL, OR DISSENTING VIEWS

None.

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Chapter 159

FISHERY DISASTER ASSISTANCE (UPDATED)*

Harold F. Upton

ABSTRACT

The Secretary of Commerce is authorized to provide disaster assistance to the fishing industry when fish populations decline or other disruptions cause economic losses. The criteria for determining whether a commercial fishery failure or fishery resource disaster has occurred are provided in Section 308(b) and Section 308(d) of the Interjurisdictional Fisheries Act (IFA; 16 U.S.C. §4107), and in Sections 312(a) and 315 of the Magnuson-Stevens Fishery Conservation and Management Act (MSA; 16 U.S.C. §1861(a) and §1864).

The governor of a state, the Secretary of Commerce, or a representative of a fishing community may initiate a request for assistance. The National Marine Fisheries Service (NMFS), state agencies, and fishing communities compile information needed to make a determination. When all necessary information has been obtained and reviewed, the Secretary of Commerce determines whether a fishery failure or fishery disaster has occurred. In most cases, Congress has appropriated funds to support the fishing industry following the Secretary's determination. NMFS, states, regional commissions, and industry representatives often work together to plan how assistance will be distributed to the fishing industry and allocated among potential projects.

Oceanic conditions, climate, and weather events can affect fishery resources and commercial infrastructure, such as boats, shoreside processing, and ports. Since 1990, the Secretary of Commerce has made 72 fishery disaster determinations and Congress has appropriated nearly $1.15 billion for fishery disaster relief. Recent fishery disaster determinations have been made for salmon fisheries in the Pacific Northwest and Alaska, Dungeness crab fisheries in the Pacific Northwest, the West Coast sardine fishery, and fisheries affected by several hurricanes.

Direct federal financial assistance has been provided to fishermen and fishing communities in the form of grants, job retraining, and low-interest loans. Assistance also supported efforts to prevent or lessen the effects of future disruptions to fisheries. These efforts included fishery data collection, habitat restoration, research, and fishing capacity reduction programs. Whereas some observers support efforts to provide assistance, others

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contend that disaster assistance programs sometimes fall short of expectations when funds are not disbursed in a timely manner, relief is not integrated with long-term fishery management objectives, and funds do not reach the people who may be in the greatest need of assistance.

In the 116th Congress, similar versions of the Fishery Failures: Urgently Needed Disaster Declarations Act (S. 2346 and H.R. 5548) have been introduced that would make extensive changes to the current law. Both bills would amend Section 312 of the MSA, repeal Section 315 of the MSA, and repeal Section 308 of the IFA. Generally, the legislation would consolidate and clarify fishery disaster provisions in the MSA and the IFA and incorporate some parts of the NMFS agency directive on fishery disasters. Both bills would define terms frequently used when making fishery disaster determinations, clarify procedures and criteria for making fishery disaster determinations, provide guidance for allocating and disbursing funds, and place additional conditions on assistance for fishing capacity reduction programs. On November 13, 2019, S. 2346 was marked up by the Committee on Commerce, Science, and Transportation, and on January 14, 2020, the House Natural Resources Subcommittee on Water, Oceans, and Wildlife held a hearing that included testimony related to H.R. 5548.

Several additional bills related to fishery disaster assistance have been introduced. The Strengthening Fishing Communities and Increasing Flexibility in Fisheries Management Act (H.R. 3697) includes a section that would require the Secretary of Commerce to make a fishery disaster determination within 90 days of receiving an estimate of the economic impact of the disaster from the entity making the request. H.R. 3697 also would require the Secretary to publish the estimated cost of recovery from a fishery resource disaster within 30 days of making a determination. Two identical bills (H.R. 3514 and S. 1984) would add the effects of certain duties on seafood markets as a potential cause of a commercial fishery failure under the MSA. The Commercial Fishing and Aquaculture Protection Act of 2019 (S. 2209) would provide assistance to eligible commercial fishermen and aquaculture producers who suffer losses in revenue. No further actions have been taken on any of these bills.

INTRODUCTION

The productivity and profitability of marine fisheries may vary significantly due to natural and anthropogenic causes, such as oceanic conditions, climate, pollution, and weather events. These factors can cause fishery resource declines and fishery closures and can damage commercial infrastructure such as boats, shoreside processing, and ports. Fishery disasters occur when fishermen endure economic hardships resulting from fish population declines or other disruptions to the fishery. The federal government may provide disaster relief to assist the fishing industry when it has been harmed by a fishery disaster.

A governor of a state, the Secretary of Commerce (Secretary), or a representative of a fishing community initiates a request for assistance. The National Marine Fisheries Service (NMFS) at the National Oceanic and Atmospheric Administration (NOAA), state agencies, and fishing communities compile information needed to make a determination. When all necessary information has been obtained, the Secretary makes a determination of whether a fishery disaster or failure has occurred. In most cases, Congress appropriates funds to support the fishing industry following the Secretary's disaster determination. Congress generally appropriates funding in supplemental or annual appropriations as needs arise rather than in anticipation of future needs.

Statutory Authorities and Different Types of Fishery Disasters[1]

The Secretary of Commerce (Secretary) is authorized to determine that a fishery disaster has occurred under the Magnuson-Stevens Fishery Conservation and Management Act (MSA; 16 U.S.C. §1861(a) and 16 U.S.C. §1864) and the Interjurisdictional Fisheries Act (IFA; 16 U.S.C. §4107). Secretarial determinations are similar but vary according to the underlying cause of the disruption to a fishery.[2]
Commercial Fishery Failure—A commercial fishery failure occurs when revenues from commerce in the fishery decrease due to a fishery resource disaster, such that the decrease causes fishermen to suffer economic hardship.
Fishery Resource Disaster—A fishery resource disaster is a sudden, unexpected, large decrease in fish stock biomass or other change that results in significant loss of access to the fishery resource.
Section 312(a) of the MSA authorizes the Secretary to determine whether a *commercial fishery failure* has occurred due to a *fishery resource disaster*.

- Section 315 of the MSA authorizes the Secretary to determine whether a catastrophic regional disaster has occurred. The cause of the catastrophic regional disaster may be a *commercial fishery failure* under Section 312(a) of the MSA or a *fishery resource disaster* under Section 308(d) of the IFA.
- Section 308(b) of the IFA authorizes the Secretary to provide assistance when a fishery has been affected by a *commercial fishery failure* or serious disruption to future production due to a *fishery resource disaster* arising from natural or undetermined causes.
- Section 308(d) of the IFA authorizes the Secretary to initiate projects to alleviate harm determined by the Secretary to have been incurred as a direct result of a *fishery resource disaster* arising from a hurricane or other natural disaster.

NMFS, states, regional commissions, and industry representatives often work together to distribute assistance to the fishing industry and to allocate funding among potential projects.

The federal government and its partners have allocated fishing disaster funds to fisheries of the North Pacific, Western Pacific, Pacific Northwest, Gulf of Mexico, Southeast, and the Northeast regions. Fisheries with multiple commercial fishery failure determinations include the West Coast salmon troll fishery, Fraser River sockeye salmon fishery, the Northeast multispecies fishery, Gulf fisheries following hurricanes, New England shellfish fisheries, Alaska salmon fisheries, and the Bering Sea snow crab fishery.

Under the fishing disaster authorities, the federal government has provided direct financial assistance to fishermen and fishing communities in the form of grants, job retraining, and low- interest loans. The federal government has also provided indirect assistance that has included fishery data collection, resource restoration, research, and fishing capacity reduction programs to prevent or lessen the effects of future disruptions to fisheries. Whereas most observers recognize that disaster assistance has provided much-needed assistance to the fishing industry, others contend that disaster assistance programs sometimes fall short of expectations because funds may not be appropriated or disbursed in a timely manner, relief may not be integrated with long-term fishery management objectives, economic estimates of fishery disasters are inconsistent, and funds may not reach the people in the greatest need of assistance.

[1] National Marine Fisheries Service (NMFS), *Policy on Disaster Assistance under the Magnuson-Stevens Act 312(a) and 315 and Interjurisdictional Fisheries Act 308(b) and 308(d)*, National Marine Fisheries Service Policy 01-122, June 16, 2011. Hereinafter cited as NMFS, *Policy*.

[2] The term *fishery disaster* is used in parts of this report to make general references to commercial fishery failures and fishery resource disasters.

DISASTER REQUIREMENTS AND PROCEDURES

The Department of Commerce provides fishery disaster assistance under the Magnuson-Stevens Fishery Conservation and Management Act (MSA; 16 U.S.C. §1861(a) and 16 U.S.C. §1864) and the Interjurisdictional Fisheries Act (IFA; 16 U.S.C. §4107) (Table 1). Assistance may be provided to fisheries managed by states, such as blue crab, and to fisheries under federal management, such as the Northeast multispecies fishery.[3] Differences exist under each law with regard to the allowable causes of a commercial fishery failure or fishery resource disaster and the use of funds. Often fishery disasters have been declared under both laws, which may provide managers with greater latitude when matching relief with different needs of the fishery and its participants.

Table 1. Fishery disaster causes, types of assistance, and use of funds

Section	Causes of Commercial Fishery Disruption	Types of Assistance and Use
Section 312(a) of MSA	Commercial fishery failure (due to a fishery resource disaster) as a result of— (1) natural causes (2) man-made causes beyond the control of fishery managers to mitigate through conservation and management measures, including regulatory restrictions imposed to protect human health or the marine environment (3) undetermined causes	(1) assessment of the social and economic effects of the failure (2) assistance to the community and fishermen (3) projects to restore the fishery or prevent reoccurrence of a similar failure (4) federal share of assistance cannot be greater than 75%
Section 308(b) of IFA	Commercial fishery failure or a fishery resource disaster arising from— (1) natural causes (2) undetermined causes	(1) restore a fishery affected by a fishery failure (2) prevent a future fishery failure (3) federal share of funding is limited to 75% of costs
Section 308(d) of IFA	Fishery resource disaster arising from— (1) natural disasters such as a hurricane or other natural disaster	(1) direct assistance to fishermen (2) indirect assistance through state agencies, local government, and nonprofit organizations (3) no limit on the federal share of costs
Section 315 of MSA	Catastrophic regional fishery disaster— (1) results in economic losses to coastal or fishing communities (2) affects more than one state or a major fishery managed by a Council or interstate fishery commission (3) is determined by the Secretary to be a commercial fishery failure under §312(a) of MSA or a fishery resource disaster under §308(d) of IFA	(1) activities authorized under either MSA or IFA (2) the Secretary may waive matching requirements if no reasonable means are available for meeting the match and the probable benefit of federal financing outweighs the public interest in imposing the match

Source: National Marine Fisheries Service (NMFS), Policy on Disaster Assistance under the Magnuson-Stevens Act 312(a) and 315 and Interjurisdictional Fisheries Act 308(b) and 308(d), National Marine Fisheries Service Policy 01-122, June 16, 2011.

Notes: MSA = Magnuson-Stevens Fishery Conservation and Management Act (16 U.S.C. §1861(a) and 16 U.S.C. §1864). IFA = Interjurisdictional Fisheries Act (16 U.S.C. §4107).

[3] Fisheries under state jurisdiction generally occur in state waters that include internal waters such as Chesapeake Bay or from 0-3 nautical miles (nm) from shore. State jurisdiction off the west coast of Florida and Texas extend to 9 nm from shore. Fisheries under federal jurisdiction generally occur from 3 to 200 nm from shore.

Magnuson-Stevens Fishery Conservation and Management Act

In 1996, Section 312(a) was added to the MSA to provide fishery disaster relief when a commercial fishery failure occurs as the result of a fishery resource disaster.[4] A *fishery resource disaster* is "a sudden unexpected, large decrease in fish stock biomass or other change that results in a significant loss of access to the fishery resource."[5]

In 2007, NMFS developed a policy directive to provide guidance for the disaster relief process.[6] The process begins at the discretion of the Secretary of Commerce, following the request of the governor of an affected state or at the request of a fishing community representative.[7] The Secretary determines whether a commercial fishery failure has occurred, depending on three factors.

First, there must be a fishery resource disaster resulting from a decrease in fish population biomass or the loss of access to the fishery resource.[8] Second, under the MSA, the cause of the fishery resource disaster must be one of the following:

- natural causes;
- man-made causes beyond the control of fishery managers to mitigate through conservation and management measures, including regulatory restrictions imposed to protect human health or the marine environment; or
- undetermined causes.

Finally, there must be an economic harm resulting from the commercial fishery disaster.

Requests for a commercial fishery failure determination usually contain information describing how the fishery and its users were harmed. The Secretary typically directs the appropriate Regional Administrator for NMFS to collect and analyze information such as landings, stock assessments, number of participants, and revenues. These data are used to determine the magnitude of the disaster and the relationship between underlying causes and the alleged fishery disaster. The magnitude of the disaster may be measured by the percentage decline in landings and revenues, the number of fishermen affected, loss of habitat, and lost or restricted fishing time. Depending on the circumstances, the analysis usually is conducted in consultation with the state(s) and typically includes information and data that the state(s) provide. According to NMFS, a reasonably predictable, foreseeable, and recurrent fishery resource cycle of variations in species distribution or stock abundance does not constitute a fishery resource disaster.[9]

Once it is concluded that a fishery resource disaster has occurred and its cause(s) is covered under the MSA, economic data are reviewed to determine whether a commercial fishery failure exists. The final decision depends on whether a significant number of people engaged in the fishery have suffered economic hardship as a result of the fishery resource disaster. NMFS has developed policy guidance to clarify and interpret the fishery disaster

[4] Section 312(a) was added by the Sustainable Fisheries Act of 1996 (P.L. 104-297).
[5] NMFS, *Policy*.
[6] NMFS, *Policy*.
[7] According to NMFS, a fishing community representative may include a tribal representative, city manager, or county executive.
[8] Loss of access to the resource may result from damages to fishing vessels, gear and related infrastructure or fishery closures because of oil spills or toxic algal blooms.
[9] NMFS, *Policy*.

assistance provisions of the MSA and the IFA.[10] The guidance specifies the following thresholds based on the loss of annual revenue compared to average annual revenue over the most recent five-year period:

- Revenue losses greater than 80% will result in the determination of a commercial fishery failure.
- Revenue losses between 35% and 80% will be further evaluated to determine the severity of losses.
- Revenue losses less than 35% will not be eligible for determination of a commercial fishery failure, except where the Secretary determines there are special and unique circumstances that may justify considering and using a lower threshold in making the determination.[11]

Once it is determined that a commercial fishery failure exists, Congress may use the authorization in the MSA to appropriate funds for financial assistance to harvesters and other affected parties.

After funds are appropriated, the affected state, community, or group must develop a spending plan that is evaluated by NMFS regional offices. Funding under the MSA may be used to address a broad variety of needs, including an assessment of the social and economic effects of the failure, assistance to the community, and projects to restore the fishery or prevent reoccurrence of a similar failure. Before releasing funds, the Secretary must also determine that activities would not expand the size and scope of the failure in that fishery or other fisheries, or affect fisheries in other geographic regions. The federal share of assistance carried out under Section 312(a) of the MSA cannot be greater than 75% of the cost of relief activities, while the other 25% is usually provided by the state or other local entity. In some cases, regional fishery commissions administer claims and disburse funds to fishing communities.[12]

MSA Regional Coastal Disaster Assistance

In 2006, the MSA was amended by adding Section 315—the Regional Coastal Disaster Assistance, Transition, and Recovery Program. A *catastrophic regional fishery disaster* is defined as a natural disaster, such as a hurricane or tsunami, or a regulatory closure to protect human health or the marine environment. A catastrophic regional fishery disaster is an event that

- results in economic losses to the coastal or fishing communities;
- affects more than one state or a major fishery managed by a council or interstate fishery commission;[13] and

[10] NMFS, *Policy*.
[11] NMFS, *Policy*.
[12] For example, the Pacific States Marine Fisheries Commission disbursed funds to fishermen following the Pacific Salmon commercial fishery failure in 2009-2011.
[13] Eight regional Fishery Management Councils are created by the MSA. Council members are appointed by the Secretary of Commerce from lists of candidates knowledgeable of fishery resources, provided by state

- is determined by the Secretary to be a commercial fishery failure under Section 312(a) of MSA or a fishery resource disaster under Section 308(d) of IFA of 1986.

Within two months after a catastrophic regional fishery disaster, the Secretary is required to provide the governor of each participating state with a comprehensive economic and socioeconomic evaluation of the region's fisheries. The evaluation assesses the current and future economic viability of affected fisheries, including the economic impact of foreign fish imports and direct, indirect, or environmental impacts of the disaster on the fishery and coastal communities. Subject to the availability of appropriations, the program may provide funds for infrastructure needs, job training assistance, fishing capacity reduction, and other activities authorized under either MSA or IFA. Various fishing groups in the region may be eligible for disaster assistance, including fishermen, charter fishing operators, U.S. fish processors, and owners of related fishery infrastructure.[14] Under the Regional Coastal Disaster Assistance, Transition, and Recovery Program, the Secretary may waive the matching requirements if no reasonable means are available for meeting the match and the probable benefit of 100% federal financing outweighs the public interest in imposing the match. Since it was added to the MSA, determinations under Section 315 have been made only for Hurricane Sandy in 2012; Hurricanes Irma and Maria in 2017; and Gulf of Mexico freshwater flooding in Louisiana, Mississippi, and Alabama in 2019.

Interjurisdictional Fisheries Act

The IFA was enacted in 1986 to provide federal support to states for developing interstate fishery research programs. Under IFA, funds are authorized to provide assistance for a commercial fishery failure (§308(b)) or for a fishery resource disaster (§308(d)). Under Section 308(b), a *commercial fishery failure* is a serious disruption to future production due to a fishery resource disaster arising from natural or undetermined causes. The process of collecting information and determining whether a commercial fishery failure has occurred under Section 308(b) of the IFA is similar to requirements of Section 312(a) of the MSA.

In Section 308(d), *fishery resource disasters* are referred to as natural disasters. Instead of assessing the occurrence of a commercial fishery failure, Section 308(d) of the IFA requires demonstration of harm. *Harm* is defined as uninsured damage to fishing vessels, fishing gear, processing facilities, marketability, habitat, or infrastructure. The same thresholds used for MSA fishery failure determinations are used for IFA determinations.[15]

IFA funding under Section 308(b) may be used by states alone or by the Secretary in cooperation with the states. Funding may be provided for any purpose the Secretary

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governors. The councils prepare fishery management plans for those fisheries that occur primarily within the federal waters of the Exclusive Economic Zone (3-200 nautical miles from shore). Links to Council websites are at http://www.nmfs.noaa.gov/ole/fishery_mgmt.html.

The three interstate fisheries commissions include the Atlantic States Marine Fisheries Commission, http://www.asmfc.org/; the Gulf States Marine Fisheries Commission, http://www.gsmfc.org/#:links@1; and the Pacific States Marine Fisheries Commission, http://www.psmfc.org/.

[14] Businesses supported by recreational fisheries may be eligible of fishery disaster assistance under Section 312(a) of the MSA if they are part of the affected fishing community. Recreational charter fishing businesses are mentioned explicitly in Section 315 of the MSA.

[15] NMFS, *Policy*.

determines appropriate to restore a fishery affected by a commercial fishery failure or to prevent a future fishery failure.

Under Section 308(b), funds may not be used to charter fishing vessels, and the federal share of funding is limited to 75% of costs. Funding under Section 308(d) of IFA may be used to provide direct assistance to fishermen or to provide assistance indirectly through state agencies, local government, and nonprofit organizations. In contrast to the MSA and Section 308(b) of IFA, there is no limit on the federal share of costs under Section 308(d). Section 308(d) also outlines the conditions under which funding may be used for other activities such as fishing capacity reduction programs. These programs include fishing vessel buybacks, gear reduction, or fishing permit retirement.

Other Potential Sources of Assistance

When businesses suffer economic injuries from a disaster, the Small Business Administration (SBA) may also determine whether a disaster declaration is warranted.[16] For example, when red tide required closure of the Maine shellfish fishery in 2005, SBA evaluated the impact on small businesses and determined a disaster declaration was justified. The declaration makes affected businesses eligible for Economic Injury Disaster Loans.[17] The purpose of the loan program is to provide working capital at low interest rates to assist recovery of businesses harmed by a disaster.

The Economic Development Administration (EDA) provides community grants and revolving loan funds to help distressed communities.[18] EDA has assisted fishing communities through its Public Works Program by funding port and harbor improvements. EDA's Economic Adjustment Program helps communities adjust to economic disruptions through support of business development, planning, and market research. Industries that have been adversely affected by increased imports of similar or competitive goods can seek technical assistance under EDA's Trade Adjustment Assistance Program.

SECRETARIAL DISASTER DETERMINATIONS

Since 1990, the Secretary of Commerce has made 72 different fishery disaster determinations, of which 58 were original determinations and 14 were extensions to existing determinations.[19] In 27 cases, the determination of a fishery failure or a fishery disaster was made under both the MSA and the IFA. During this period, Congress has appropriated nearly $1.15 billion for fishery disaster relief. Funds for disaster assistance have been used for a wide variety of purposes and may include direct assistance to fishermen, such as

[16] For Small Business Administration purposes, disasters also may be declared by the President, state governor, Secretary of Agriculture, or Secretary of Commerce.

[17] CRS Report RL33243, *Small Business Administration: A Primer on Programs and Funding*, by Robert Jay Dilger and Sean Lowry.

[18] For information on Economic Development Administration programs, see https://www.eda.gov/about/.

[19] National Oceanic and Atmospheric Administration (NOAA) Fisheries, Funding, and Financial Services, *Fishery Disaster Determinations*, at https://www.fisheries.noaa.gov/national/funding-and-financial-services/fishery-disaster- determinations.

- compensation;
- community grants;
- training;
- loans and debt refinancing; and
- employment on fishery related projects.

Other forms of indirect fishery-related assistance have included fishing capacity reduction (vessel, permit, and gear buybacks), formation of a fisheries research trust, economic planning grants, and research grants.

Fishery failures are diverse with respect to their causes and scope. Most declarations have resulted from natural events such as hurricanes, floods, changes in ocean conditions, or algal blooms such as red tide. In coastal areas hurricanes may damage fishing industry infrastructure such as vessels, docks, fish houses, and related businesses. Even if the resource remains abundant, harvesting, processing, and transport to markets may not be possible until repairs are undertaken and basic services are restored. In addition to the costs of repairs and the replacement of equipment and gear, lost fishing time also can be costly. Hurricanes also may damage natural resources such as oyster beds by depositing silt and debris. Algal blooms such as red tide are another type of natural event that can render seafood toxic and result in fishery closures. Under these conditions, fishermen may be completely shut down for months until toxin levels in shellfish decline to acceptable levels.

Declines in fishery resource abundance are often caused by several factors, such as natural environmental variations, human effects on the environment (e.g., pollution), and overfishing. For example, salmon fisheries are sensitive to natural changes in oceanic conditions. However, salmon abundance has also been affected where dams, irrigation, grazing, mining, and forestry practices have degraded salmon habitat in the Pacific Northwest. Overfishing by itself may not be used to qualify for a fishery failure determination, because it is usually within the control of fishery managers.[20] However, a fishery failure caused by natural or undetermined causes, criteria that may be considered by the Secretary of Commerce, may be exacerbated by overfishing. In these cases assistance may include efforts to rationalize (decrease) fishing capacity. For example, overfishing contributed to fish population declines in several resource disaster cases, such as the New England multispecies fishery and the Pacific groundfish fishery. In these cases, fish abundance decreased significantly and stock rebuilding has required substantial decreases in harvest. However, it was determined that other factors beyond the control of fishery managers played a role in the fishery failure.[21]

State Role

States are frequently active partners throughout the fishery disaster process, from requesting the Secretary to declare a fishery failure and providing related data to disbursing relief to fishermen and related businesses. The disaster request typically includes a spending

[20] NMFS, *Policy*.
[21] Letter from Rebecca M. Blank, Acting Secretary of Commerce, to Honorable Deval L. Patrick, Governor of Massachusetts, September 13, 2012.

plan that addresses the causes of the disaster. Relief funding is often provided directly to states or through regional commissions, such as the Pacific States Marine Fisheries Commission. For example, in 2007, distribution of Oregon salmon troll fishery relief was planned and coordinated by the state's department of agriculture in cooperation with related agencies and nonprofit organizations such as the Oregon Salmon Commission. In addition to matching funds, state governments may also provide funding when federal funds are not available, although historically such funding has been limited.

Fishing Capacity Reduction Programs

Historically, many U.S. fisheries have been overcapitalized—investments in fishing capacity became greater than that needed to harvest the fishery resource on a sustainable basis. Fishing capacity reduction, often referred to as buyback programs, has been a prominent feature of several disaster relief programs.[22] Capacity reduction is usually accomplished through the direct purchase and permanent retirement of fishing vessels, gear, and/or fishing permits. Programs may be funded by the federal government, by fishermen who remain in the fishery, or by a combination of both. The general objectives of buyback programs are to provide immediate relief to fishermen, decrease the level of fishing effort to improve the profitability of the remaining fishing fleet, and conserve the resource.

The effectiveness of buyback programs in reducing fishing capacity depends on whether the remaining fishermen have the incentive to continue investing in boats and gear. Often there is also "latent" fishing effort—boats and gear with permits to fish that are inactive or only marginally utilized in the fishery. The exit of some vessels may encourage this latent fishing effort (vessels) to reenter the fishery, resulting in little or no net reduction in fishing capacity. Furthermore, the first to accept buybacks may be the least efficient vessels in the fleet. This results in fleet reductions that are relatively modest yet expensive, because only the oldest and least efficient units are taken out of production.

Although capacity reduction programs attempt to provide long-term benefits to those who decide to remain in the fishery, poorly crafted programs may result in little or no benefit at the expense of taxpayers. Although capacity reduction can be a means to ease financial hardship caused by a fishing disaster, lasting benefits may depend on better recognition of the motivations of vessel owners and fishermen.

Selected Fishery Failure Cases

West Coast Salmon Ocean Troll Fishery (Sacramento)

On April 10, 2008, the Pacific Fishery Management Council adopted a complete closure of commercial and sport fisheries off California and most of Oregon in response to the collapse of the Sacramento River fall Chinook salmon run. The minimum conservation goal for Sacramento fall Chinook is 122,000 to 180,000 spawning salmon,[23] while as recently as 2002, 769,868 adults returned to spawn.[24] Even with ocean fishery closures, the 2008 returns

[22] Capacity reduction is referred to in Section 312(b) of the MSA and Section 308(d) of the IFA.
[23] The number of salmon needed to return to the river to sustain this salmon population.
[24] For Pacific salmon fishery management information, see http://www.pcouncil.org/.

of Sacramento fall Chinook were projected to be 59,000 fish and actual returns totaled 65,364 fish.[25] In March 2009, NMFS released a report on the causes of the decline of Sacramento fall Chinook. The report identified unfavorable ocean conditions as the primary factor that led to poor survival of juvenile salmon when they entered the ocean in 2005 and 2006. It also found that the stock was more susceptible to poor ocean conditions because of habitat degradation in the freshwater portion of its range.

On May 1, 2008, in response to requests by the governors of California, Oregon, and Washington, the Secretary of Commerce declared a commercial fishery failure for the West Coast salmon troll fishery. Congress provided $170 million in disaster funds in the Food, Conservation, and Energy Act of 2008 (P.L. 110-246) for commercial and recreational members of fishing communities who were affected by the fishery failure. In September 2008, $100 million was released to the Pacific States Marine Fishery Commission for distribution to commercial fishermen, processors, charter boat operators, recreational guides, and other businesses dependent on fishing. The declaration also allowed the SBA to make economic injury loans available to businesses affected by the fishery failure. On April 30, 2009, the Secretary of Commerce notified the governors of California and Oregon that the fishery failure would continue in 2009. Returns of Sacramento fall Chinook salmon remained below levels required for a fishery and the 2009 commercial salmon troll fishery was closed for most of Oregon and all of California. The ocean recreational fisheries were also limited in both states, especially California. The extension of the disaster declaration ensured release of the remaining unspent funds from the original $170 million.

In 2010, revenue from commercial salmon landings in California remained significantly lower than the 2003-2005 average. On September 2, 2010, the Secretary of Commerce continued the fishery failure for California and Oregon commercial salmon fisheries under Section 308(d) of the IFA and 312(a) of the MSA. The availability of SBA economic injury loans was continued, but additional disaster relief was not appropriated by Congress. In 2012, PFMC reported that a total of nearly 286,000 fall Chinook salmon returned to the Sacramento River. This was the first year since 2007 that commercial and recreational ocean landings returned to historical levels off California.

New England Red Tide

Red tide has been a reoccurring problem for shellfish fisheries in northern New England. Blooms of the algae *Alexandrium fundyense*, commonly referred to as red tide, produce a toxin that is ingested and concentrated by shellfish such as clams, mussels, and oysters. When the concentration of the algae is high, shellfish beds must be closed because shellfish may cause paralytic shellfish poisoning, which can be toxic to humans.[26] In 2005, shellfish beds were closed from Canada to Martha's Vineyard, Massachusetts. On June 23, 2005, NOAA announced a commercial fishery failure determination for the region's shellfish fishery. In 2006, $5 million was appropriated in the Emergency Supplemental Appropriations Act for Defense, the Global War on Terror, and Hurricane Recovery, 2006 (P.L. 109-234), to assist fishermen who were affected by the red tide bloom.

[25] Pacific Fishery Management Council, *Stock Assessment and Fishery Evaluation (SAFE) Documents: Review of the 2016 Ocean Salmon Fisheries*, February 2017, p. 203, at http://www.pcouncil.org/salmon/stock-assessment-and-fishery-evaluation-safe-documents/review-of-2016-ocean-salmon-fisheries/.

[26] In extreme cases, paralytic shellfish poisoning (PSP) can be fatal to humans. PSP also has been implicated in the mortality of certain species of marine mammals.

During 2008, red tide was also widespread in ocean waters off New England. On November 14, 2008, the Secretary of Commerce determined a commercial fishery failure had occurred because the bloom triggered closures of shellfish fisheries. The Consolidated Security, Disaster Assistance, and Continuing Appropriations Act, 2009 (P.L. 110-329), provided up to $5 million to assist the fishing industry and for research and monitoring related to red tide events. On December 22, 2010, the Secretary of Commerce determined that red tide caused another fishery failure in the Maine shellfish fishery during the 2009 season, but Congress did not appropriate funding for this event.

Gulf of Mexico Fisheries (Hurricanes Katrina and Rita)

In the wake of Hurricanes Katrina and Rita, Gulf of Mexico harvesting and shoreside fishery infrastructure were damaged or in some cases destroyed. On September 9, 2005, the Secretary determined that a fishery failure in the Gulf of Mexico had occurred. On October 4, 2005, the Secretary announced a formal determination of an additional fishery failure in Louisiana and Texas due to the effects of Hurricane Rita.

The immediate effects of the fishery failure were difficult to discern because of the broad geographic area affected by the hurricanes and the substantial damage to infrastructure such as ports, processing, and general access to markets. In 2004, Gulf of Mexico annual landings of major fisheries, including shrimp, finfish, and oysters, totaled 1.476 billion pounds with a dockside value of $669 million.[27] In the areas initially affected by Katrina there were 15 major fishing ports, 177 seafood processing facilities, 1,816 federally permitted fishing vessels, and more than 13,000 state-permitted fishing vessels. Private recreational fishing boats, charter boats, and related infrastructure were also extensively damaged.

In July 2007, NMFS released its *Report to Congress on the Impacts of Hurricanes Katrina, Rita, and Wilma on Alabama, Louisiana, Florida, Mississippi, and Texas Fisheries*. This report described fishery conditions before and after the 2005 hurricane season and also described other factors that affect the fishing industry, such as rising costs and seafood imports. A second report, *Economic Damages to Infrastructure Incurred by Louisiana Fishing Industries Due to Hurricanes Katrina and Rita in 2005*, was also released in July 2007. This report estimated losses to the fishing industry of $582 million in Louisiana and $988 million for the entire Gulf of Mexico.[28] Both reports stressed that estimates should be conditioned on data and methods used in each state, factors influencing fisheries, and uncertainties related to the rate of recovery from storm damage.

On June 15, 2006, the Emergency Supplemental Appropriations Act for Defense, the Global War on Terror, and Hurricane Recovery, 2006 (P.L. 109-234), was enacted. It allocated $128 million to the National Oceanic and Atmospheric Administration (NOAA) "Operations, Research, and Facilities" account for expenses related to Hurricane Katrina.[29] On May 25, 2007, the U.S. Troop Readiness, Veterans' Care, Katrina Recovery, and Iraq Accountability Appropriations Act, 2007 (P.L. 110-28), was enacted. Additional funding was allocated to the NOAA "Operations, Research, and Facilities" account totaling $110 million

[27] U.S. Department of Commerce, National Marine Fisheries Service, *Fisheries of the United States, 2005*, Current Fishery Statistics No. 2005 (Washington, DC: February 2007), p. 6.

[28] R. H. Caffey et al., *Economic Damages to Infrastructure Incurred by Louisiana Fishing Industries Due to Hurricanes Katrina and Rita in 2005*, Report to the U.S. Department of Commerce National Oceanic and Atmospheric Administration, July 2007, pp. 86-88.

[29] The measure included $90 million plus a $38 million transfer from the U.S. Department of Agriculture that was to be used for improving oyster grounds.

for impacts of Hurricanes Katrina and Rita on the shrimp and fishing industries. The Gulf States Marine Fisheries Commission, through a cooperative agreement with NOAA, administered and coordinated funding of recovery programs through grant agreements with each of the Gulf states. Funds appropriated in 2006 were used to restore damaged oyster beds, remove debris, restore fishery habitat, and support cooperative research.[30] Funds appropriated in 2007 were used to assist individual commercial fishermen, other fishing industry businesses, and seafood promotion of Gulf fishery products.[31]

California Dungeness and Rock Crab Fishery

In early November 2015, the California Dungeness crab and rock crab fisheries were closed due to a harmful algal bloom along the California coast. The California Office of Environmental Health Hazard Assessment and California Department of Health determined that there were unsafe levels of domoic acid in crab tissue. Domoic acid is a neurotoxin, and when ingested by people it can cause nausea, diarrhea, vomiting, memory loss, seizures, and sometimes death. In response, the California Department of Fish and Game closed commercial and recreational crab fisheries in the affected areas. The closure occurred during the peak months of the fishery from December through January and was persistent, as many areas remained closed through May.

The initial estimate of economic impact based on average commercial landings over the previous five years was $48.3 million for Dungeness crab and $376,000 for rock crab.[32] On February 9, 2016, the California governor requested a commercial fishery failure determination under Section 312(a) of the MSA and a fishery resource disaster under Section 308(d) of the IFA.[33] On January 18, 2017, the Secretary of Commerce found that the Dungeness crab and rock crab fisheries met requirements for a determination under both laws.

In the 114th and 115th Congresses, several bills were introduced in the House and Senate to fund the Dungeness and rock crab fishery failures but no bills were enacted. On February 9, 2018, Congress included $200 million in the Bipartisan Budget Act of 2018 (P.L. 115-123) for fishery resource disasters declared by the Secretary in 2017. Of the total, $25.8 million was allocated to provide assistance to the California Dungeness crab and rock crab fisheries. In August 2018, the draft crab disaster relief spending plan was finalized, dividing total funding among mitigation of future disasters (10%), direct payments (89%), and administration (1%).[34] On May 22, 2019, the Pacific States Marine Fisheries Commission announced that it had received funds to be disbursed to crab fishermen.

[30] Gulf States Marine Fisheries Commission, *Emergency Disaster Recovery Program I*, at http://www.gsmfc.org/edrp- i.php.

[31] Gulf States Marine Fisheries Commission, *Emergency Disaster Recovery Program II*, at http://www.gsmfc.org/edrp- ii.php.

[32] Letter from Edmund G. Brown Jr., Governor of California, to Honorable Penny Pritzker, Secretary, U.S. Department of Commerce, February 9, 2016. Hereinafter cited as Brown, 2016.

[33] Brown, 2016.

[34] California Department of Fish and Wildlife, *Crab Disaster Relief Spending Plan: Building Resilience*, August 29, 2018, at https://nrm.dfg.ca.gov/FileHandler.ashx?DocumentID=161007&inline.

POTENTIAL ISSUES FOR CONGRESS

Commercial fisheries are strongly influenced by environmental conditions that affect the abundance and distribution of fishery resources and fishing infrastructure. These changes often take place suddenly with little or no warning, as in the cases of hurricanes, oil spills, and harmful algal blooms. Disaster relief programs may help businesses that have been harmed by these events and can address these disruptions to fisheries by providing assistance until conditions return to "normal." As Congress continues to debate and respond to fishery disasters, several issues have emerged related to the nature of commercial fisheries and disaster relief programs, including (1) timing relief to meet crucial needs, (2) relating disaster relief to long-term fisheries management, (3) defining a fishery failure, (4) determining who benefits from relief, and (5) considering other related sectors.

Timing of Relief

The delivery of disaster relief depends on the determination by the Secretary that a fishery failure has occurred and on the appropriation of relief funding by Congress. Historically, approximately half of fishery failure determinations have been made within six months of the initial request and over two-thirds have been made within one year.[35] Information related to the scope of the disaster usually needs to be compiled by the fishing industry, state and local governments, and NMFS. Difficulties in concluding this task can be compounded by the lack of data and readily available economic studies. In cases such as Hurricane Katrina (2005), it was immediately clear that a disaster had occurred and the Secretary made a determination within two weeks of when the hurricane made landfall.[36] In other cases, such as the Long Island, NY, hard shell clam fishery (2009), Northern Mariana Islands fisheries following a super typhoon (2003), and the Florida shark fishery (2008), it took two to three years before the Secretary made a determination (in these cases a determination to deny the requests).

After a fishery failure is declared, funding is dependent on appropriations by Congress. In most cases, Congress has provided funding for declared fishery failures, but the timing of appropriations has varied considerably. For some approved determinations, such as the Dungeness crab and rock crab fisheries, pink salmon in Alaska, Fraser River Sockeye salmon, and Washington coastal salmon, Congress appropriated funds over two years after the time of the Secretary's fishery failure determination.[37] Hurricanes Katrina and Rita fishery disaster funding was appropriated in June 2006, more than nine months after the Gulf fishery failure

[35] NOAA Fisheries, *Fisheries Disaster Determinations*, at https://www.fisheries.noaa.gov/national/funding-and-financial-services/fishery-disaster-determinations.

[36] The Secretary of Commerce made the fishery failure determination before the actual request for a fishery failure was made later in 2006.

[37] Fraser River sockeye salmon fishery failure requests were made by several different tribes in 1999-2000, 2007, 2009, 2013, and 2015 and approved by the Secretary of Commerce. Congress did not appropriate funds for the first determination in 2002. A request for a continuation of the fishery failure in 2007 and 2008 was approved and funded in 2008. A request for a continuation of a fishery failure was made in 2009 and approved in 2011 but not funded. A new fishery failure request in 2013 was approved in 2014 and funded in 2018. An additional fishery failure request in 2015 was approved in 2017 and funded in 2018. Another request was made in 2019, but as of March 2020 the Secretary had not made a fishery failure determination.

was declared in September 2005. Many in the industry believed the greatest need occurred immediately after the hurricanes, when fishermen lost fishing opportunities because of damaged infrastructure, vessels, and gear and disrupted markets.[38] Although the full dimensions of the disaster and the level and scope of resource needs remained uncertain, some fishermen thought some basic aid should have been provided to members of the fishing industry immediately after the disaster.

For immediate needs following a fishery failure, some have advocated establishing a disaster fund with annual appropriations that could provide assistance on short notice.[39] For example, the Robert T. Stafford Disaster Relief and Emergency Assistance Act (P.L. 93-288) provides disaster assistance to state and local governments. The funds are provided by the Federal Emergency Management Agency in various forms through its Disaster Relief Fund (DRF). The DRF is funded through regular appropriations acts using a formula that includes several factors, including historical disaster costs. Fishery disaster assistance is sometimes included in NOAA's annual appropriations, such as the FY2018 and FY2019 Consolidated Appropriations Acts, but in most years disaster assistance is not included in the agency's appropriations requests or annual appropriations acts passed by Congress.

Others have considered the use of existing agriculture programs to supplement existing fishery disaster assistance. For example, during the 112th Congress, the Senate approved an amendment to S. 3240, the Agriculture Reform, Food, and Jobs Act of 2012 (the 2012 farm bill), which would have made commercial fishermen eligible for emergency loans that are currently available to farmers. Emergency loans assist farmers who have suffered physical or production losses in disaster areas that are declared by the President.[40] However, the amendment was not included in the Agricultural Act of 2014 (P.L. 113-79) when it was passed by Congress.

Long-Term Management Approaches

Often, direct or indirect assistance to the fishing industry is part of a relief program. Some have criticized federal assistance because it can delay the readjustment that may be needed for fisheries with excess harvesting capacity. These critics argue that climatic and/or environmental conditions are often blamed for fish population declines caused by overfishing.

Features of several programs, such as buybacks and training for fishermen in other vocations, focus on concerns related to the need to decrease fishing fleet size. Yet, when relief is provided, even when it includes a buyback program, greater numbers of fishermen and effort may often remain in the fishery than might be sustainable in the long run.[41] Many fisheries managers agree that relief such as vessel buybacks needs to be more closely

[38] William E. Gibson, "Gulf Coast Fishermen Need Federal Aid, Official Says Hurricanes Have Wiped Out Boats, Docks," *South Florida Sun-Sentinel*, December 16, 2005, p. A-4.

[39] Tim Sloane, *Fulfilling the Promise of the Magnuson-Stevens Act*, Pacific Coast Federation of Fishermen's Associations, March 2016, at http://pcffa.org/wp-content/uploads/2016/05/FN0316_PCFFA.pdf.

[40] U.S. Department of Agriculture, Farm Service Agency, "Farm Loans," fact sheet, at http://www.fsa.usda.gov/Internet/FSA_File/loans11.pdf.

[41] U.S. Government Accountability Office, *Commercial Fisheries*, GAO/RCED-11-120, June 2000, at https://www.gao.gov/assets/240/230376.pdf.

integrated with ongoing fisheries management objectives.[42] Some have proposed that long-term measures and disaster planning should occur before disasters occur. In this way, more deliberate approaches to build resiliency may be considered and potentially enacted instead of emergency measures that fill short-run needs. Other types of assistance that may provide long-term fishery benefits include habitat restoration and enhancement, marketing and promotion programs, and cooperative research.

Defining Fishery Failures

The general causes of fishery resource disasters that result in determination of a commercial fishery failure are defined by the MSA and IFA. However, specific characteristics of a fishery resource disaster, such as scale, timing, and extent, are not defined in statute. Since Congress did not fully define a fishery failure or fishery resource disaster, the Secretary of Commerce has a large degree of discretion when determining whether a fishery failure has occurred.

The NOAA policy guidance provides specific revenue thresholds for determining whether a commercial fishery failure has occurred.[43] However, unless the revenue decline is greater than 80%, the request for a commercial fishery failure would still be evaluated on a case-by-case basis. Most fish populations vary over time, and frequently it is difficult to determine the relative importance of the factors that cause these variations. Thus, the factors that are responsible for the decline may include causes that are allowable, such as environmental changes, and not allowable, such as overfishing. It might be questioned whether additional criteria can be developed to make fishery failure determinations more consistent.

Who Benefits?

Who benefits from disaster funding is a recurring point of contention.[44] Participants such as fishermen and fish processors may be difficult to identify and directly associate with a fishery failure. Although it is often possible to contact vessel and processing plant owners, industry- related labor such as crew members and fish processing employees may be difficult to track. In some fisheries, crew members are temporary laborers that follow fishing opportunities. Because of the transient nature of employment in the fishing industry and seasonal movement of fishing vessels among regions, labor statistics regarding the employment of fishermen are either difficult to obtain or may not exist. Similar problems may occur in related fishery processing and distribution sectors.

Economic effects of fishery disasters on the local community and region are also difficult to quantify. Services directly related to fishing, such as boat repairs, dock services, and

[42] Eric Thunberg, Andrew Kitts, and John Walden, "A Case Study of New England Groundfish Fishing Capacity Reduction," in *Fisheries Buybacks*, ed. Rita Curtis and Dale Squires (La Jolla, CA: Blackwell Publishing, 2004), pp. 239-249.

[43] NMFS, *Policy*.

[44] Tom Dempsey, *Dempsey Commentary on Federal Disaster Aid*, Cape Cod Commercial Fishermen's Alliance, June 9, 2014, at http://www.capecodfishermen.org/item/commentary-dempsey-federal-aid.

fishing equipment suppliers, as well as other businesses indirectly related to fishing, are likely to be harmed by losses in the fish harvesting and processing sectors. Although general regional impacts can be estimated using economic models, it is often difficult to identify the level of impacts on these businesses because of their dispersed nature and their indirect relationship to fishing. A broader understanding of these community impacts depends on more deliberate and long-term data collection and planning to link community concerns with marine fisheries management. An open question is whether NOAA's efforts to integrate management with social concerns might be applied to increasing fishing community resilience to fishery failures and to improving assistance programs when disasters occur.[45]

Aquaculture, Subsistence, and Recreational Fisheries

Fishery disasters affect other resource users, such as recreational fishermen, subsistence users, and aquaculture facilities, but there is ambiguity regarding the eligibility of these groups for disaster relief. These groups are not considered explicitly in disaster relief sections of either the MSA or the IFA.

Charter boat operators who take paying customers for fishing trips have been included in previous determinations and have benefited from assistance. However, it is unclear whether and how assistance would be provided to businesses that support recreational fishing, such as bait and tackle shops. Some observers could contend that these businesses should be included because they are dependent on fisheries and a part of the coastal community. Congress also might consider questions related to whether a disaster could be determined for the decline of a species only sought after by recreational fisherman, such as red drum, and how the losses to these businesses would be quantified.

Subsistence users are affected by resource declines and associated losses to household benefits. These impacts are difficult to assess in economic terms; consequently, it may be difficult to determine the form that relief might take. Furthermore, the term *subsistence*, as it relates to fisheries, is not defined in either the MSA or the IFA.[46] Some observers might contend that different approaches may be needed for cases of subsistence disaster relief.

Aquaculture is broadly defined as the propagation and rearing of aquatic species in controlled or selected environments. Aquaculture operations range from extensive farming where there is only minimal control over the organism's environment to intensive systems where complete control is taken at each stage of the organism's life history.[47] Aquaculture is not addressed or defined in the MSA, but according to NMFS, the act's management authority over all fish within the exclusive economic zone (EEZ) and statutory definitions of *fishery* and *fishing* provide a sound basis for regulating aquaculture in the EEZ.[48] NMFS has included marine aquaculture operations in disaster assistance determinations. For red tide

[45] National Marine Fisheries Service, *Human Dimensions*, Office of Science and Technology, at http://www.st.nmfs.noaa.gov/humandimensions/index.

[46] For example, *subsistence* is defined by the state of Alaska as customary and traditional uses of fish and wildlife, and the definition highlights the unique importance of wild resources and the continuing role of subsistence activities in sustaining the way of life in Alaska.

[47] For example, oyster farming may resemble a fishery where the habitat is enhanced by adding substrate (shells) for spat (small oyster) attachment. In other cases, greater control is taken and oysters are raised in cages or trays.

[48] Memorandum from Constance Sathre, Office of the General Counsel, to Lois Schiffer, NOAA General Counsel, June 9, 2011.

fishery failures, oyster farms were included in the request for assistance with wild shellfish fisheries. However, questions remain regarding the eligibility of losses that are specific to aquaculture, such as salmon cage culture or events that affect only aquaculture and not wild fisheries. Further, a recent court decision cast doubt on whether NMFS has authority to regulate aquaculture under the MSA.[49]

Recent Congressional Actions

Fishery Failures: Urgently Needed Disaster Declarations Act

In the 116th Congress, similar versions of the Fishery Failures: Urgently Needed Disaster Declarations Act (S. 2346 and H.R. 5548) have been introduced that would make extensive changes to current law.[50] Both bills would amend Section 312 of the MSA, repeal Section 315 of the MSA, and repeal Section 308 of the IFA. Generally, the legislation would consolidate and clarify many of the fishery disaster provisions in the MSA and the IFA and incorporate parts of the NMFS agency directive on fishery disasters.

The bills cover the main elements of the current fishery disaster program, including

- definitions of terms, including fishery disaster;
- initiation of a fishery resource disaster review;
- the review process, including information required for the review;
- evaluation of requests and criteria for disaster determinations;
- allocation and disbursement of appropriated fishery resource disaster assistance; and
- eligible uses of disaster funds.

The bills also include requirements for the timing of determinations and the disbursement of funds. The marked-up versions of S. 2346 and H.R. 5548 also include subsistence, charter, and recreational users as eligible entities but make no specific references to assistance for aquaculture producers.[51]

Both bills also include sections on the following related topics:

- additional conditions on assistance for fishing capacity reduction programs;
- a report from the Comptroller General on efforts to prepare and adapt U.S. fishery management for the impacts of climate change; and
- reporting and certification under the High Seas Driftnet Fishing Moratorium Protection Act (16 U.S.C. §§1826f and 1826h).

[49] In Gulf Fisherman's Association v. National Marine Fisheries Service, the U.S. District Court for the Eastern District of Louisiana held that NOAA Fisheries exceeded its authority under the MSA when it adopted a regulatory scheme for aquaculture operations in the Gulf of Mexico. The court found that the MSA's grant of authority to regulate "fishing" and "harvesting" did not include aquaculture.

[50] Most sections of the marked up version of S. 2346 and the version of H.R. 5548 that was introduced in the House of Representatives are either identical or similar.

[51] The introduced version of S. 2346 included aquaculture producers.

On November 13, 2019, S. 2346 was marked up by the Committee on Commerce, Science, and Transportation, and on January 14, 2020, a hearing was held by the House Natural Resources Subcommittee on Water, Oceans, and Wildlife that included testimony related to H.R. 5548.

Other Bills Introduced in the 116th Congress

Several additional bills related to fishery disaster assistance have been introduced during the 116th Congress, but no action has been taken on any of them since their introduction. A bill that would reauthorize the MSA, Strengthening Fishing Communities and Increasing Flexibility in Fisheries Management Act (H.R. 3697), would make several changes to fishery disaster provisions of the act.[52] Section 401 of the bill would require the Secretary of Commerce to publish the estimated cost of recovery from a fishery resource disaster no later than 30 days after the Secretary makes the fishery disaster determination. For requests from a state governor, Section 402 would require the Secretary to make a fishery failure determination within 90 days of receiving an estimate of the economic impact from the entity requesting the relief.

The Commercial Fishing and Aquaculture Protection Act of 2019 (S. 2209) would amend the MSA to provide supplemental revenue assistance to eligible commercial fishermen and aquaculture producers.[53] Assistance could be provided when an eligible loss occurs due to an algal bloom, freshwater intrusion, adverse weather, bird depredation, disease, or another condition determined by the Secretary of Commerce. Eligible losses would be calculated as the difference between gross revenue in the calendar year in which losses occurred and 85% of the average gross revenue for the three previous years.[54] The assistance could be provided by the Secretary whether or not a fishery resource disaster determination was made.

Two identical bills (H.R. 3514 and S. 1984) were introduced in response to duties imposed on U.S. seafood products. The bills would add certain duties to the list of potential causes of a commercial fishery failure listed in Section 312 of the MSA. Fishery disaster assistance could be provided if duties are placed by other countries on U.S. seafood products as retaliation for increases in duties imposed by the United States.[55]

Recent Disaster Determinations and Appropriations

Funding of fisheries disaster assistance depends on congressional action, because there is no permanent fund to provide relief after the Secretary of Commerce makes determinations. Usually, funding is appropriated for a number of disasters and allocated among specific fishery disasters by NOAA. For example, P.L. 115-123 funded 10 disasters and P.L. 115-141 funded 7 disasters. In the 116th Congress, fishery disasters have been funded by the Consolidated Appropriations Act, 2019 (P.L. 116-6), and the Additional Supplemental Appropriations for Disaster Relief Act, 2019 (P.L. 116-20). Table 2 provides a list of fishery

[52] H.R. 200, which was introduced in the 115th Congress and passed the House, proposed identical amendments to MSA fishery disaster assistance provisions.

[53] An eligible commercial fisherman and farm-raised fish producer are generally described as an individual or entity that assumes production and market risks associated with harvesting fish (fisherman) or production of fish in a controlled environment (farm-raised fish producer) for commerce. The term *fish* would include shellfish, finfish, and other aquatic organisms harvested with the intent of entering commerce.

[54] Payment could not exceed 85% of the average gross revenue received during the three previous calendar years.

[55] The bill refers to increases in duties imposed by the United States pursuant to Section 232 of the Trade Expansion Act of 1962 (19 U.S.C. §1862) or Section 301 of the Trade Act of 1974 (19 U.S.C. §2411).

disasters that have that been approved or are under consideration for a determination by the Secretary of Commerce during the 116th Congress. Table 3 provides a list of disasters that were approved and funded during the 115th Congress.

Table 2. Fishery disaster requests 116th Congress (Funded, not yet funded, and pending determination)

Fishery Disaster/Failure	Request and Determination	Funding
Washington Fraser River and Skagit River Salmon Fisheries (Swinomish, Lummi, Upper Skagit Tribes), 2019	Request Letter—December 31, 2019, October 31, 2019, March 5, 2019 Determination—Pending	Awaiting Determination
New York Bay Scallop, 2019	Request Letter—December 6, 2019 Determination—Pending	Awaiting Determination
Washington Chehalis River Spring Chinook Salmon Fishery (Confederate Tribes of the Chehalis Reservation), 2019	Request Letter—August 2, 2019 Determination—Pending	Awaiting Determination
Washington Puget Sound Coho Salmon Fishery (Port Gamble S'Klallam Tribe), 2018	Request Letter—July 1, 2019 Determination—Pending	Awaiting Determination
California Pacific Sardine Fishery, 2017-2019	Request Letter—June 18, 2019 Determination—October 30, 2019	$2,218,904
Gulf of Mexico Freshwater Flooding, 2019 (Louisiana, Mississippi, and Alabama)	Request Letters—May 31, 2019, June 13, 2019, July 10, 2019 Determination—October 30, 2019	Awaiting Funding
Florida Red Tide Events 2015-2019	Request Letter—May 24, 2019 Determination—Pending	Awaiting Determination
California Red Sea Urchin Fishery, 2016-2017	Request Letter—February 28, 2019 Determination—October 30, 2019	$3,329,343
Klamath River Fall Chinook Commercial Fishery, 2018 (Yurok Tribe)	Request Letter—February 11, 2019 Determination—October 30, 2019	$2,226,068
Alaska Sockeye Salmon Fishery, 2018	Request Letter—November 28, 2018 Determination—October 30, 2019	$10,327,039
Hurricane Florence, 2018	Request Letter—November 1, 2018 Determination—December 6, 2018	$7,728,000
Hurricane Michael, 2018	Request Letter—October 23, 2018 Determination—October 31, 2018	$7,812,000
Georgia and South Carolina Penaeid Shrimp, 2018	Request Letter—May 18, 2018, May 29, 2018, July 13, 2018, November 16, 2018 Determination—September 25, 2018	$3,442,913 (GA) $3,938,245 (SC)
Gulf of Alaska Pacific Cod, 2018	Request Letter—March 8, 2018 Determination—September 25, 2018	$24,416,440

Source: NOAA Fisheries, Fishery Disaster Determinations, Accessed April, 6, 2020, at https://www.fisheries.noaa.gov/national/funding-and-financial-services/fishery-disaster-determinations.

Notes: Legislation providing funding for fishery failures that was approved in the 116th Congress and enacted into law includes P.L. 116-6 and P.L. 116-20.

Table 3. Funded fishery disaster requests, 115th Congress

Fishery Disaster/Failure	Request and Determination	Legislation/Funding
Washington Ocean Troll Coho and Chinook Salmon Fisheries, 2016 (Makah Tribe)	Request Letter—March 14, 2018 Determination—September 24, 2018	P.L. 115-141—March 23, 2018 Funding—$1,654,000
Hurricane Harvey, 2017	Request Letter—February 14, 2018 Determination—March 20, 2018	P.L. 115-123—February 29, 2018 Funding—$13,945,263
Washington State Coho Salmon Fisheries, 2016 (Quileute Tribe)	Request Letter—May 4, 2017 Determination—September 24, 2018	P.L. 115-141—March 23, 2018 Funding—$970,000
Hurricanes Irma and Maria 2017 (Florida, U.S. Virgin Islands, and Puerto Rico)	Request Letters—October 2, 2017, October 6, 2017, and November 7, 2017 Determination—February 8, 2018	P.L. 115-123—February 29, 2018 Funding—$66,738,161
California Pacific Sardine Fishery, 2015-2016	Request Letter—September 5, 2017 Determination—September 9, 2018	P.L. 115-141—March 23, 2018 Funding—$1,640,000
Oregon and California Klamath River Fall Chinook Salmon Fishery, 2016-2017 (Hoopa Valley, Yurok, Oregon and California)	Request Letters—March 13, 2017 April 25, 2017, and May 24, 2017 Determination—September 24, 2018	P.L. 115-141—March 23, 2018 Funding—$8,886,000
Washington State Coho and Pink Salmon Fisheries, 2015 (Hoh, Stillaguamish, Nooksack, Muckleshoot, Quileute, Upper Skagit, and Suquamish)	Request Letters—Seven letters from July 27, 2016, to March 8, 2018 Determination—September 24, 2018	P.L. 115-141—March 23, 2018 Funding—$3,856,000
Gulf of Alaska Pink Salmon, 2016	Request Letter—September 19, 2016, September 28, 2016, and October 26, 2016 Determination—January 18, 2017	P.L. 115-123—February 29, 2018 Funding—$56, 361,332
Washington Ocean Salmon Troll Fishery, 2016	Request Letter—September 14, 2016 Determination—January 18, 2017	P.L. 115-123—February 29, 2018 Funding—$834,401
California Klamath River Chinook Salmon Fishery, 2016 (Yurok Tribe)	Request Letter—July 20, 2016 Determination—January 18, 2017	P.L. 115-123—February 29, 2018 Funding—$3,864,904
Washington Dungeness Crab Fishery, 2015 (Quileute Tribe)	Request Letter—June 23, 2016 Determination—January 18, 2017	P.L. 115-123—February 29, 2018 Funding—$1,481,199
Washington South Puget Sound Coho, Chinook, and Chum Salmon Fishery, 2015 (Nisqually, Squaxin Island, Port Gamble S'Klallam, and Jamestown S'Klallam Tribes)	Request Letters—May 9, 2016, June 22, 2016, July 5, 2016, and July 31,2016 Determination—January 18, 2017	P.L. 115-123—February 29, 2018 Funding—$13,542,624
California Dungeness Crab and Rock Crab, 2015-2016	Request Letter—February 9, 2016 Determination—January 18, 2017	P.L. 115-123—February 29, 2018 Funding—$25,797,268
Washington Coastal Salmon, 2015 (Quinault and State of Washington)	Request Letter—September 14, 2016 November 23, 2015 Determination—January 18, 2017	P.L. 115-123—February 29, 2018 Funding—$14,606,939
Fraser River Sockeye Salmon, 2014 (Elwha, Makah, and Swinomish Tribes)	Request Letters—January 13, 2015, January 20, 2015, and February 25,2015 Determination—January 18, 2017	P.L. 115-123—February 29, 2018 Funding—$2,827,909
Fraser River Sockeye Salmon, 2013 (Nooksack, Tulalip, Suquamish, Makah, Lower Elwha, Jamestown S'Klallam, Port Gamble S'Klallam, Lummi Nation, and State of Washington)	Request Letter—October 28, 2013 Determination—January 14, 2014	P.L. 115-141—March 28, 2018 Funding—$1,932,000

Source: NOAA Fisheries, Fishery Disaster Determinations, at https://www.fisheries.noaa.gov/national/funding-and-financial-services/fishery-disaster-determinations.

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Chapter 160

MIXED-USE FISHERIES: SOUTH ATLANTIC AND GULF OF MEXICO COUNCILS WOULD BENEFIT FROM DOCUMENTED PROCESSES FOR ALLOCATION REVIEWS*

United States Government Accountability Office

WHY GAO DID THIS STUDY

Commercial and recreational marine fisheries—including those in the South Atlantic and Gulf of Mexico—are critical to the nation's economy, contributing approximately $99.5 billion to the U.S. gross domestic product in 2016, according to the Department of Commerce. NMFS and the councils may allocate fishing privileges for mixed-use fisheries in federal waters, but establishing and revising such allocations can be complex, in part because of concerns about equity.

The Modernizing Recreational Fisheries Management Act of 2018 includes a provision for GAO to review mixed-use fisheries allocations in the South Atlantic and Gulf of Mexico. For these regions, this report examines (1) the extent to which the councils established or revised mixed-use fisheries allocations, (2) key sources of information that may be available for reviewing allocations, and (3) the extent to which the councils have developed processes to help guide such reviews. GAO reviewed NMFS and council policies and other council documents; analyzed information on allocations established and revised; compared council processes to agency guidance and internal control standards; and interviewed NMFS officials, council members and staff, and 46 stakeholders that reflected various interests. Views from these stakeholders are not generalizable.

* This is an edited, reformatted and augmented version of United States Government Accountability Office; Report to Congressional Committees, Publication No. GAO-20-216, dated March 2020.

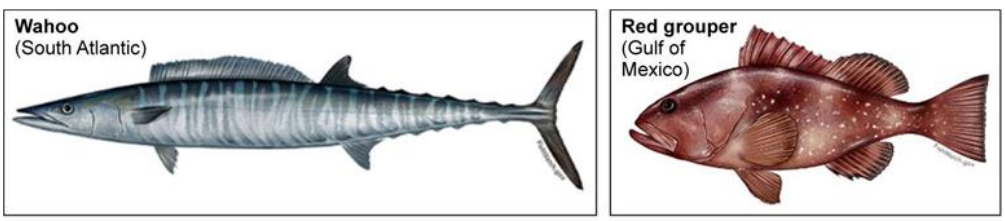
WHAT GAO RECOMMENDS

GAO is making two recommendations, including that NMFS work with the councils to develop documented processes for conducting allocation reviews. The agency agreed with GAO's recommendations.

WHAT GAO FOUND

The South Atlantic and Gulf of Mexico regional fishery management councils, with approval from Department of Commerce's National Marine Fisheries Service (NMFS), established and revised allocations to varying degrees for mixed-use fish stocks—fisheries with a combination of commercial and recreational fishing. Regional councils were created by statute to help manage fisheries in federal waters, including allocating—or distributing—fishing privileges, when warranted. Starting in 1985, the South Atlantic council established allocations, generally a percentage of allowable harvest, for 50 of its 51 mixed-use fish stocks and revised most of those at least once. The Gulf of Mexico council established allocations for nine of its 23 mixed-use fish stocks, revising three of those once. Historically, allocations have been largely based on estimates of the commercial and recreational fishing sectors' past use of the resource, according to NMFS.

Key sources of information that may be available to help NMFS and the councils review allocations include trends in catch and landings (the amount of fish caught or brought to shore); fish stock assessments; and economic analyses. Each source presents some challenges in supporting allocation decisions, however. For example, NMFS works with states to estimate recreational catch, which provides information about demand, but faces difficulties generating reliable estimates. This is in part because of attributes of the recreational fishing sector, including the greater number of recreational anglers compared with commercial fishing participants. NMFS issued guidance in 2019 to promote consistency in estimating recreational catch data to help improve the quality of the information.



Source: National Marine Fisheries Service (images). | GAO-20-216.

Examples of Fish Stocks with Allocation Reviews Underway as of December 2019.

The South Atlantic and Gulf of Mexico councils developed processes for when to initiate fish stock allocation reviews, but not for how to conduct those reviews. A 2012 report for NMFS found that reviews had been done inconsistently, and stakeholders were dissatisfied with allocation decision-making. In response, NMFS developed guidance calling for structured and transparent allocation review processes. Both councils established criteria for initiating reviews, such as time-based triggers, and as of December 2019 they had several

reviews underway (see figure). In April 2019, the Gulf of Mexico council began convening a workgroup to propose a draft allocation review process, but has not indicated what actions it will take, if any, in response to a proposal. The South Atlantic council postponed any discussions until March 2020. As of December 2019, neither council had a documented process. Documented processes for conducting allocation reviews would provide NMFS with better assurance that the councils carry out upcoming reviews in a structured and transparent manner.

ABBREVIATIONS

Councils	Regional Fishery Management Councils
Magnuson-Stevens Act	Fishery Conservation and Management Act of 1976, as amended
NMFS	National Marine Fisheries Service
NOAA	National Oceanic and Atmospheric Administration

March 31, 2020

The Honorable Roger Wicker
Chairman
The Honorable Maria Cantwell
Ranking Member
Committee on Commerce, Science, and Transportation
United States Senate

The Honorable Raúl M. Grijalva
Chairman
The Honorable Rob Bishop
Ranking Member
Committee on Natural Resources
House of Representatives

Commercial and recreational marine fisheries are critical to the nation's economy, contributing approximately $99.5 billion to the U.S. gross domestic product and supporting approximately 1.7 million jobs in 2016, according to the Department of Commerce's National Oceanic and Atmospheric Administration (NOAA).[1] The South Atlantic and Gulf of Mexico regions are each home to multiple fisheries with a combination of commercial and

[1] U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, *Fisheries Economics of the United States, 2016*, Technical Memorandum NMFS-F/SPO-187a (Silver Spring, MD: December 2018). Information on gross domestic product and jobs includes data on commercial seafood harvesters, processors, dealers, wholesalers, distributors, importers, and retailers, as well as recreational fishing trips and fishing equipment. Data for 2016 were the most recent available at the time of our review.

recreational fishing,[2] known as mixed-use fisheries.[3] Commercial fishing in these regions landed nearly 2 billion pounds of seafood in 2016, valued at more than $1 billion dollars.[4] These regions also have the greatest recreational fishing activity in federal waters, according to NOAA, which estimates that recreational anglers in these regions made more than 127 million fishing trips in 2016.[5]

The lead federal agency responsible for managing commercial and recreational marine fisheries is NOAA's National Marine Fisheries Service (NMFS). Under the Fishery Conservation and Management Act of 1976, often referred to as the Magnuson-Stevens Act, as amended,[6] NMFS and eight regional fishery management councils (councils) created by the act are responsible for fisheries management and conservation in federal waters.[7] In particular, NMFS and the councils, including the South Atlantic and Gulf of Mexico councils, are responsible for allocating—or distributing—privileges for catching fish between the commercial and recreational fishing sectors in these two regions when such allocations may be warranted.[8] Allocations are generally a percentage of the fisheries' allowable harvest. Historically, mixed-use fisheries allocations have been predominantly based on estimates of each fishing sector's past use of the resource, according to NOAA.[9]

Allocations between the commercial and recreational fishing sectors can be complex and difficult, in part due to perceptions of fairness that arise in making allocation decisions. Allocation decisions establish the proportional access each sector has to a fishery, which in turn may result in economic and social impacts for participants in the sectors. There may be differences in the economic and social values that participants in each fishing sector place on fishery resources, leading to divergent views on what the allocations should be.

Differences in the management of the commercial and recreational fishing sectors have also led to questions about the equity of allocations. For instance, participants from the commercial fishing sector have raised concerns that fishery management disparities between the two sectors could result in unfair allocations. Specifically, commercial participation in

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[2] The recreational fishing sector comprises anglers accessing fisheries from private boats and for-hire sector business entities, which include charter boats and head boats. A charter boat is usually hired by a group of anglers for a period of time. Head boats are typically large capacity multi-passenger vessels that charge a per angler fee for a fishing trip.

[3] A fishery refers to one or more fish stocks that can be treated as a unit for conservation and management purposes and that are identified on the basis of geographical, scientific, technical, recreational, and economic characteristics. A fish stock refers to a species, subspecies, geographical grouping, or other category of fish capable of management as a unit. A fish stock may be one species or a complex of comparable species.

[4] National Marine Fisheries Service, *Fisheries Economics, 2016*.

[5] U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, *Fisheries of the United States, 2017*, NOAA Current Fishery Statistics No. 2017 (Silver Spring, MD: September 2018). Federal waters of the United States are generally located 3 to 200 nautical miles offshore. However, federal waters in some areas and for the management of some fish begin at 9 nautical miles.

[6] The Fishery Conservation and Management Act of 1976, as amended, among other things, sets forth national standards for federal fisheries conservation and management. Pub. L. No. 94-265, § 301(a), 90 Stat. 331, 346 (1976) (codified as amended at 16 U.S.C. § 1851(a)).

[7] The councils are supported by federal funds and generally comprise NMFS regional administrators, the principal state official with responsibility for marine fishery management in each state within the council's region, and members of the fishing industry and conservation groups appointed by the Secretary of Commerce as voting members. The councils also include nonvoting members, such as officials from other federal agencies.

[8] NMFS defines an allocation of fishing privileges as a direct and deliberate distribution of the opportunity to participate in a fishery among identifiable, discrete user groups or individuals. 50 C.F.R. § 600.325(c)(1).

[9] Morrison, W.E., and T.L. Scott, *Review of Laws, Guidance, Technical Memorandums and Case Studies Related to Fisheries Allocation Decisions*, U.S. Department of Commerce, National Oceanic and Atmospheric Administration Technical Memorandum NMFS-F/SPO-148 (Silver Spring, MD: 2014).

fisheries is generally limited through federal permits, but recreational anglers do not have similar limits, according to commercial sector participants. They also noted that the recreational sector has at times exceeded its allocations for certain fisheries, and that the two sectors are not always held accountable for adhering to their allocations in the same way. In contrast, recreational participants have expressed concerns that recreational interests have been historically underrepresented in allocations. These participants indicated that as coastal populations have increased and fishing technologies such as navigational systems have improved, recreational fishing has become more popular, generating significant economic activity in related sales and jobs, including in the South Atlantic and Gulf of Mexico. They indicated that some allocations may be outdated and called for NMFS and the councils to review those allocations.

In 2016, NMFS issued a policy and guidance to the councils on establishing and reviewing fisheries allocations, which are intended to help the councils and NMFS review and update allocations under the Magnuson-Stevens Act.[10] In particular, the NMFS guidance calls for the councils to identify criteria for triggering allocation reviews and outlines various factors the councils should consider in conducting their allocation reviews and when making allocation decisions. The NMFS guidance calls for the councils to develop a structured and transparent process by which allocation reviews are to be conducted.

The Modernizing Recreational Fisheries Management Act of 2018 includes a provision for us to review mixed-use fisheries allocations in the South Atlantic and Gulf of Mexico regions.[11] This report examines, for the South Atlantic and Gulf of Mexico regions, (1) the extent to which the councils have established or revised mixed-use fisheries allocations, (2) key sources of information that may be available to help NMFS and the councils conduct allocation reviews, and (3) the extent to which the councils have developed processes to help guide their allocation reviews.

To conduct our work, we focused on mixed-use fisheries allocations between the commercial and recreational fishing sectors in the South Atlantic and Gulf of Mexico regions.[12] We reviewed the Magnuson- Stevens Act and policies and guidance related to allocations from NMFS and the councils. We interviewed officials from NMFS, the two relevant councils, and the related interstate fisheries commissions. Specifically, we interviewed the following:

- NMFS officials from the agency's Southeast Regional Office and Southeast Fisheries Science Center;

[10] U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, *Fisheries Allocation Review Policy*, NMFS Policy 01- 119 (effective July 27, 2016 and renewed September 27, 2018); *Criteria for Initiating Fisheries Allocation Reviews Council Coordinating Committee Allocation Workgroup Guidance Document,* NMFS Procedure 01-119-01 (effective July 27, 2016 and renewed October 3, 2018); and *Recommended Practices and Factors to Consider When Reviewing and Making Allocation Decisions*, NMFS Procedure 01-119-02 (effective July 27, 2016 and renewed October 3, 2018).

[11] Pub. L. No. 115-405, § 101, 132 Stat. 5355, 5356 (2018). The act defines a mixed-use fishery as a federal fishery in which two or more of the following occur: (a) recreational fishing, (b) charter fishing, or (c) commercial fishing. *Id.* § 3(4). In our report, we consider for-hire fishing (both charter fishing and head boats) to be part of the recreational fishing sector because the South Atlantic and Gulf of Mexico councils generally manage for-hire fishing as part of the recreational sector, according to council staff.

[12] NMFS and the councils may also establish other types of allocations for the fisheries they manage, such as for the use of different fishing gear types.

- South Atlantic and Gulf of Mexico council members, including members from state fisheries agencies in Alabama, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Texas;
- South Atlantic and Gulf of Mexico council staff, including the two councils' executive directors, economists, and social scientists;
- members of the two councils' socioeconomic panels, which report to the councils' scientific and statistical committees;[13] and
- the executive directors of the Atlantic and Gulf States Marine Fisheries Commissions.[14]

In addition, to inform our work, we interviewed 46 stakeholders from the commercial and recreational fishing sectors, related industries, and conservation organizations to gather their perspectives, as well as any associated information, on allocations. We included a diversity of stakeholders across the council regions. For example, these stakeholders included fishing associations and individual fishing participants from the commercial and recreational sectors (including charter fishing), seafood dealers or retailers, food and lodging industry representatives, and conservation organizations. We met with many of these stakeholders in person when we attended the June 2019 meetings of the South Atlantic and Gulf of Mexico councils in Florida. In advance of the meetings, each council publicized our attendance at the meeting and provided our contact information so that interested stakeholders could contact us to set up a meeting. We interviewed stakeholders that (1) contacted us before or after the meetings, and (2) contacted us on a first-come, first-served basis at the council meetings.[15] During our interviews, we discussed, among other things, how allocation decisions may affect stakeholders and the councils' processes for reviewing allocations. The results of our interviews with NMFS officials; council members, staff, and socioeconomic panels; and stakeholders cannot be generalized to other regions or stakeholders, but provide a range of examples of perspectives on allocations within the South Atlantic and Gulf of Mexico regions.

To determine the extent to which the councils have established or revised mixed-use fisheries allocations, we asked the councils' staff to identify (1) any allocations established or revised for each of the mixed-use fish stocks they manage and what those allocation percentages comprised, and (2) when the councils established or revised those allocations (from 1976, when the Magnuson-Stevens Act was enacted and the councils were established, through December 2019). We analyzed the information to summarize and describe the number of allocations established for mixed-use fish stocks in the two council regions and the

[13] Socioeconomic panels comprise economists and social scientists who provide the councils' scientific and statistical committees with information on potential economic and social implications of fishery management plans. Scientific and statistical committees— which may comprise federal or state officials, academics, or independent experts— evaluate technical aspects of fisheries and advise councils on the scientific adequacy of statistical, biological, economic, and social information as it pertains to fishery management plans.

[14] The Atlantic and Gulf States Marine Fisheries Commissions are interstate compacts that seek to promote better utilization of fisheries, the promotion and protection of such fisheries, and the prevention of physical waste of the fisheries for the Atlantic seaboard and Gulf of Mexico. The Atlantic States Marine Fisheries Commission was formed in 1942 and develops plans to sustain the shared coastal fishery resources of Atlantic coast states from Maine to Florida. The Gulf States Marine Fisheries Commission was established in 1949 and recommends management measures to the governors and legislatures of the five Gulf States (Alabama, Florida, Louisiana, Mississippi, and Texas).

[15] In addition, nine other stakeholders submitted their perspectives on allocations to us in writing.

extent to which those allocations have been revised. To verify the information provided by the councils, we reviewed related documents, including fishery management plans and plan amendments the councils submitted to NMFS that established or revised allocations for specific fish stocks.[16] To clarify any potential discrepancies in their documents on allocations, we also interviewed council staff or reviewed their written responses to our questions. Based on our review of the documents and information from council staff, we determined that the information on allocations the councils provided is sufficiently reliable for describing the allocations for mixed-use fisheries in the South Atlantic and Gulf of Mexico.

To identify key sources of information that may be available to help NMFS and the councils conduct allocation reviews, we reviewed NMFS' 2016 policy and guidance on establishing and reviewing fisheries allocations and interviewed or received written comments from NMFS officials and staff from the two councils. We reviewed documents on key sources of economic, social, ecological, and other information identified by NMFS officials and council staff, including NMFS and other documents on recreational fishing data collection, stock assessments, economic analyses, social indicators, and ecosystem or other ecological models.[17] The information sources we include are key sources identified by NMFS and the councils; other sources of information may also be available to NMFS and the councils that are not reflected in our report. In addition, we interviewed or received written comments from NMFS officials and staff and members from the two councils to obtain their perspectives on any challenges related to such information, and to identify steps NMFS or the councils are taking related to the information or challenges. We also reviewed available documents on those steps.

To determine the extent to which the councils have developed processes to help guide their allocation reviews, we obtained documents on the councils' plans for future reviews of mixed-use fisheries allocations. These documents include their council policies for specific criteria that will trigger reviews and available documents on their plans for when and how they plan to conduct those reviews. We compared this information with criteria in NMFS' allocations policy and guidance, the agency's operational guidelines for processes under the Magnuson-Stevens Act and associated regional operating agreements,[18] and the framework for internal controls established by the Committee of Sponsoring Organizations of the

[16] According to NMFS' website, the councils develop fishery management plans or plan amendments to, among other things, prevent overfishing, allocate fishing quotas to different fishing groups, implement gear restrictions, and protect sensitive habitats. To help ensure transparency and incorporate stakeholder feedback, proposed decisions included in plans or plan amendments are subject to review and comment by scientists, stakeholders, and the public. In this report, we present the dates the councils established or revised allocations based on the dates the councils submitted fishery management plans or plan amendments to NMFS for review and approval.

[17] For example, we reviewed NMFS technical memorandums on economic efficiency analyses the agency had conducted for fisheries in the Gulf of Mexico or South Atlantic regions. These analyses examined the economic efficiency of allocations for red snapper and gag, red, and black grouper in the Gulf of Mexico.

[18] U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, *Operational Guidelines for the Magnuson-Stevens Fishery Conservation and Management Act Fishery Management Process*, NMFS Procedure 01-101-03 (effective October 25, 2017); South Atlantic Fishery Management Council, *Operating Agreement Between the South Atlantic Fishery Management Council, NOAA Fisheries Service Southeast Regional Office, and NOAA Fisheries Service Southeast Fisheries Science Center* (January 2014); and Gulf of Mexico Fishery Management Council, *Regional Operating Agreement Between the Gulf of Mexico Fishery Management Council, NOAA National Marine Fisheries Service Southeast Regional Office, NOAA National Marine Fisheries Service Southeast Fisheries Science Center, and NOAA General Counsel, Southeast Section* (August 2016).

Treadway Commission.[19] This framework is recognized as a leading model for designing, implementing, and conducting internal control and assessing the effectiveness of internal control. In addition, we interviewed or received written comments from NMFS officials and council staff and members to obtain information on how the planned allocation reviews may affect their workloads and priorities.

We conducted this performance audit from April 2019 to March 2020 in accordance with generally accepted government auditing standards. Those standards require that we plan and perform the audit to obtain sufficient, appropriate evidence to provide a reasonable basis for our findings and conclusions based on our audit objectives. We believe that the evidence obtained provides a reasonable basis for our findings and conclusions based on our audit objectives.

BACKGROUND

NMFS and the eight regional fishery management councils are responsible for managing approximately 460 fish stocks in federal waters, as shown in Figure 1.[20]

NMFS has overall responsibility for collecting data on fish stocks and ocean conditions and for generating scientific information for the conservation, management, and use of marine resources.[21] NMFS carries out this responsibility primarily through its five regional offices and six regional fisheries science centers, which are responsible for collecting and analyzing data to conduct stock assessments. Stock assessments consider information about the past and current status of a managed fish stock, including information on fish biology, abundance, and distribution that can be used to inform management decisions.[22] To the extent possible, stock assessments also predict future trends of stock abundance. NMFS provides the results of its stock assessments and other analyses, as appropriate, to the councils for use in implementing their respective fisheries management responsibilities. In the South Atlantic and Gulf of Mexico regions, NMFS provides support to the councils' management efforts through its Southeast Regional Office and the Southeast Fisheries Science Center.

Under the Magnuson-Stevens Act, the councils are responsible for managing the fisheries in their region. This includes developing fishery management plans, subject to NMFS approval, based on the best scientific information available and through collaboration with a range of stakeholders. The councils convene committees and advisory panels to assist them in developing research priorities and selecting fishery management options, in addition to

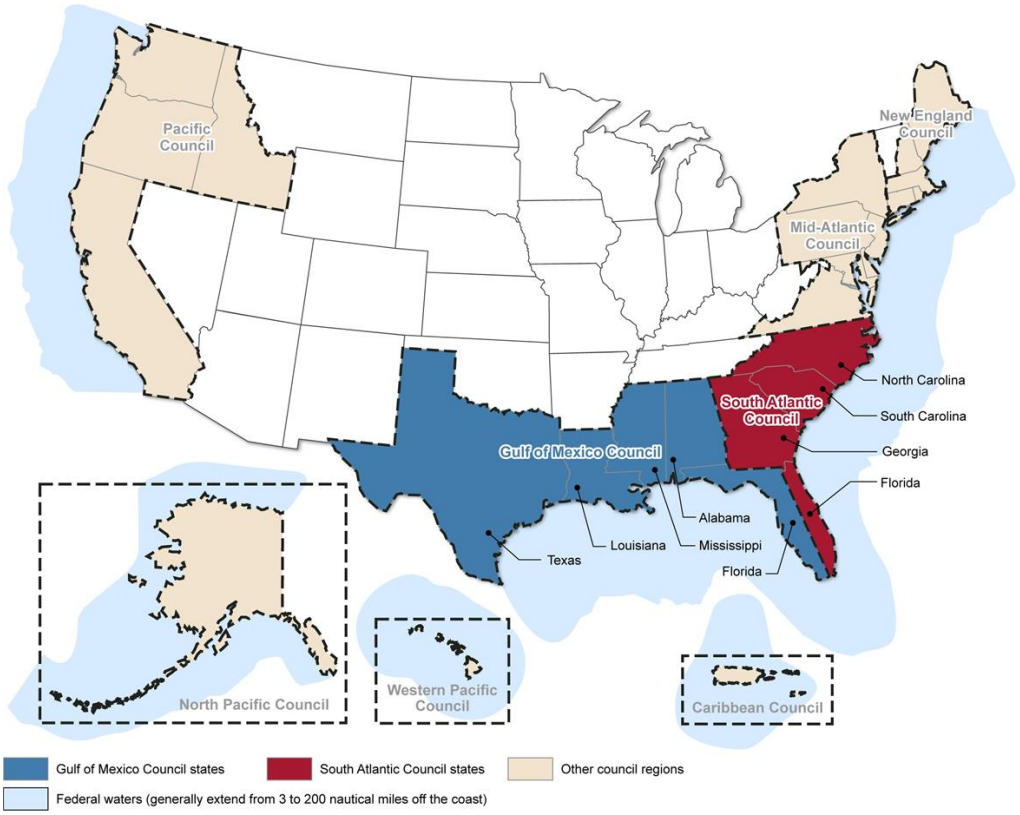
[19] Committee of Sponsoring Organizations of the Treadway Commission, *Internal Control- Integrated Framework* (2013). This framework is a common internal control model against which companies and organizations can evaluate their control systems and provides a means to apply internal control to any type of entity. The framework comprises principles related to the five components of internal control, and the *Standards for Internal Control in the Federal Government*—adapted for a government environment—uses the same components and similar language. See GAO, *Standards for Internal Control in the Federal Government*, GAO-14-704G (Washington, D.C.: September 2014).

[20] The number of fish stocks NMFS manages can vary from year to year, according to NMFS officials. For more information, see https://www.fisheries.noaa.gov/national/population-assessments/fishery-stock-status- updates.

[21] In addition to NMFS' fisheries management responsibilities, the agency is also responsible for, among other things, managing marine species protected under the Endangered Species Act.

[22] We previously reviewed NMFS' fish stock assessment prioritization process. See GAO, *Fish Stock Assessments: Prioritization and Funding*, GAO-14-794R (Washington, D.C.: Sept. 19, 2014).

conducting public meetings. The councils are to comprise members from federal and state agencies, as well as the commercial and recreational fishing sectors (see Figure 2).



Sources: National Marine Fisheries Service officials and *Fisheries of the United States, 2014* (data); Map Resources (map). | GAO-20-216.

Note: Coastal states are generally responsible for managing fisheries in waters that extend approximately 3 nautical miles from their coastlines, and the National Marine Fisheries Service and the councils manage fisheries in federal waters, which generally extend from 3 to 200 nautical miles off the coast of the United States. However, federal waters in some areas and for the management of some fish begin at 9 nautical miles. The Western Pacific council includes the Mariana Islands archipelago, American Samoa, and a range of remote island areas in the central and western Pacific Ocean that are not depicted on this map.

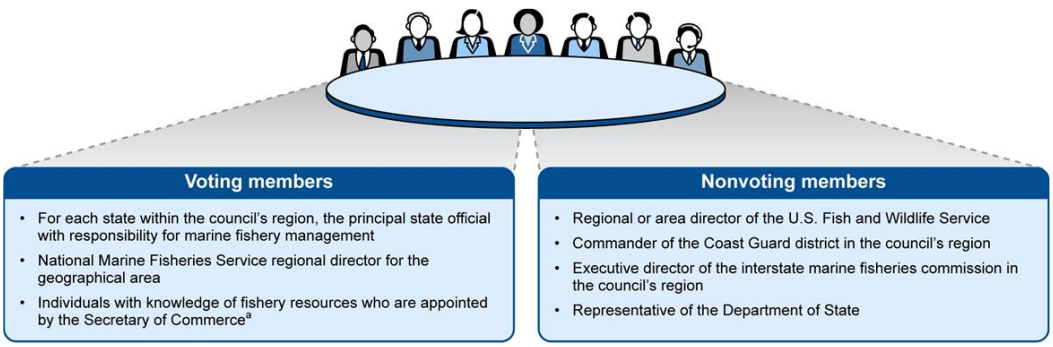
Figure 1. Boundaries of the eight Regional Fishery Management Councils.

The councils—supported by council staff such as biologists, economists, and social scientists—are responsible for preparing proposed fishery management plans or plan amendments for NMFS review. These plans or amendments are to identify, among other things, conservation and management measures to be used to manage a fishery, including determining the maximum size of a fish stock's allowable harvest. This is generally done by developing annual catch limits for each fish stock, that is, the amount of fish that can be harvested in the year.[23] Fishery management plans or amendments also include establishing

[23] The annual catch limit cannot exceed the recommended acceptable biological catch level set by the council's scientific and statistical committee.

or revising any allocations between the commercial and recreational sectors for mixed-use fish stocks where the councils determine it may be warranted.[24] For example, councils may allocate a percentage of a fish stock's annual catch limit between the recreational and commercial fishing sectors. See Figure 3 for an overview of the federal fisheries management process.

Council staff facilitate the fisheries management process by organizing council meetings, preparing and providing analyses for those meetings, and facilitating input from stakeholders and the public on fisheries management issues, among other things.[25] Stakeholders include participants in the commercial and recreational fishing sectors and related industries, such as fishing associations, seafood dealers and processors, food and travel industry representatives, and conservation groups. Once the councils complete proposed fishery management plans or plan amendments, they are to provide them to NMFS for review. NMFS is responsible for determining if the plans or amendments are consistent with the Magnuson-Stevens Act and other applicable laws, and for issuing and enforcing final regulations to implement approved plans.



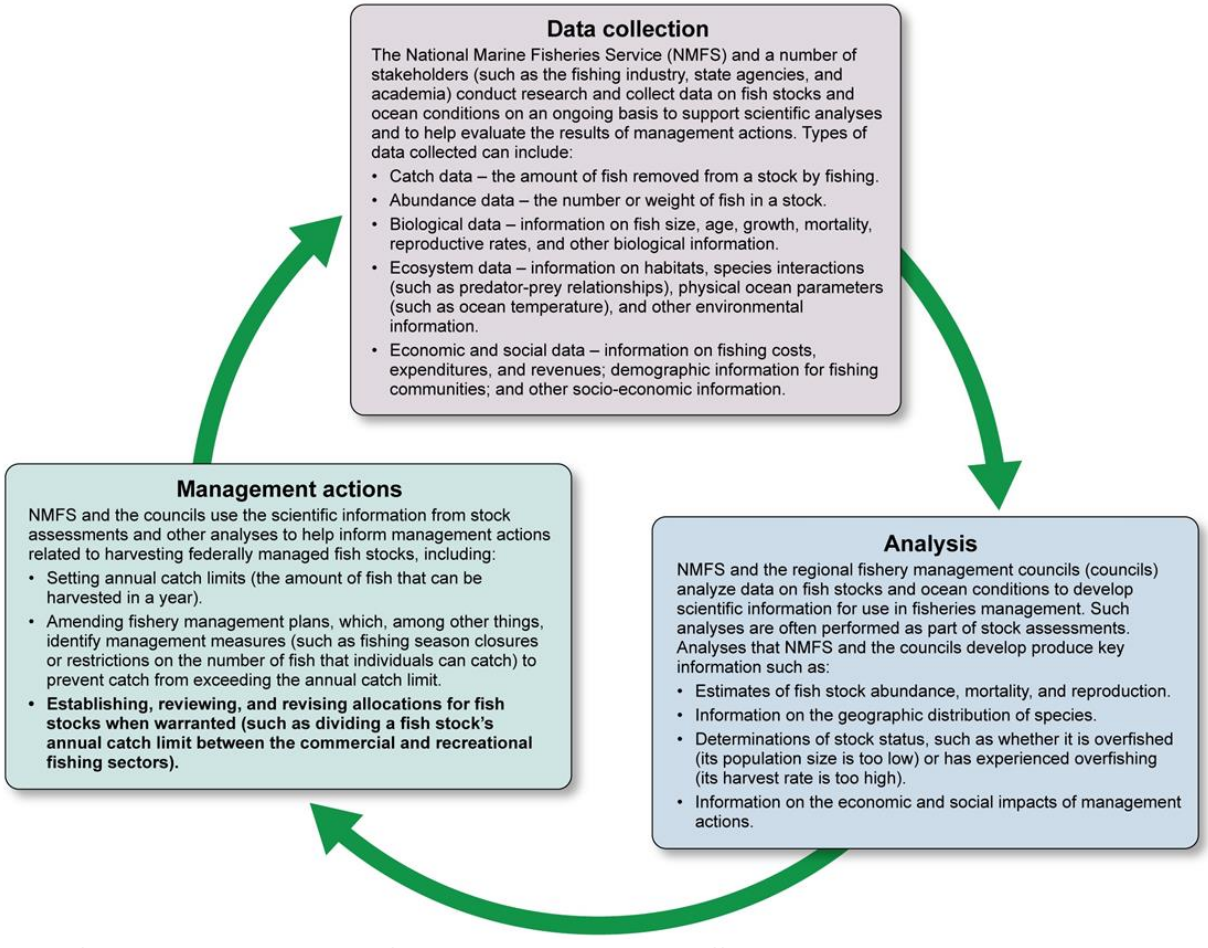
Source: 16 U.S.C. § 1852; GAO analysis. | GAO-20-216.

[a]For members appointed by the Secretary of Commerce, the governors of the states in the council's region submit to the Secretary a list of nominees who are knowledgeable regarding fisheries conservation and management, or the commercial or recreational harvest of fishery resources in the region. When selecting members, the Secretary is required to ensure, to the extent practicable, a balance of participants from the commercial and recreational sectors. In addition, 16 U.S.C. § 1852(b)(2)(D) directed the governors submitting names for appointment to the Gulf of Mexico Regional Fishery Management Council to include: (1) at least one nominee each from the commercial, recreational, and charter fishing sectors, and (2) at least one other individual who is knowledgeable regarding the conservation and management of fisheries resources in the jurisdiction of the council. That requirement expired at the end of fiscal year 2012.

Figure 2. Membership of the Regional Fishery Management Councils.

[24] Fishery management plans also identify other management measures that will be used to manage a fishery, such as fishing equipment restrictions, permitting policies, and restrictions on the timing or location of permissible fishing.

[25] In addition, the councils maintain scientific and statistical committees and socioeconomic panels to receive specialized input to the councils on fishery management plans or amendments.



Source: GAO analysis of NMFS documentation and information provided by NMFS officials. | GAO-20-216.

Figure 3. General steps in the Federal Fisheries Management process.

Table 1. Mixed-use fish stocks managed by the South Atlantic fishery management council

Fish stocks managed individually	Fish stocks managed as complexes	
1) Atlantic spadefish	Deepwater complex:	Shallow-water groupers complex:
2) Bar jack	28) Blackfin snapper	44) Coney
3) Black grouper	29) Misty grouper	45) Graysby
4) Black sea bass	30) Queen snapper	46) Red hind
5) Blueline tilefish	31) Sand tilefish	47) Rock hind
6) Cobia, Gulf group, Florida East Coast Zone[a]	32) Silk snapper	48) Yellowfin grouper
	33) Yellowedge grouper	49) Yellowmouth grouper
7) Dolphin (mahimahi)	Grunts complex:	Snappers complex:
8) Gag grouper	34) Margate	50) Cubera snapper
9) Golden tilefish	35) White grunt	51) Gray snapper
10) Gray triggerfish	36) Jacks complex:	52) Lane snapper
11) Greater amberjack	37) Almaco jack	
12) Hogfish[b]	38) Banded rudderfish	
13) King mackerel, Atlantic group[a]	39) Lesser amberjack	
14) Mutton snapper	Porgy complex:	
15) Red grouper	40) Jolthead porgy	
16) Red porgy	41) Knobbed porgy	
17) Red snapper	42) Saucereye porgy[e]	
18) Scamp	43) Whitebone porgy	
19) Snowy grouper		
20) Spanish mackerel, Atlantic group[a]		
21) Speckled hind[c]		
22) Spiny lobster[d]		
23) Vermilion snapper		
24) Wahoo		
25) Warsaw grouper[c]		
26) Wreckfish		
27) Yellowtail snapper		

Source: GAO analysis of documents from the South Atlantic Fishery Management Council and information from council staff. | GAO-20-216.

Note: Fish stocks listed by complex are managed together as a group. For this report, we count a complex as a single fish stock if the allocation is for the complex, rather than for the individual stock within the complex. If the fish stocks within a complex have their own allocations, as they do in the South Atlantic, we count them as separate stocks for reporting purposes. In addition, the South Atlantic Fishery Management Council manages golden crab and shrimp, which are exclusively fished by the commercial sector, and sailor's choice, tomtate, and scup, which are exclusively fished by the recreational sector, according to council staff. The staff said the council also manages over 130 species of corals but has delegated management of the harvest—which is primarily commercial but not allowed for most species—to the state of Florida.

[a]The South Atlantic and Gulf of Mexico Fishery Management Councils jointly manage cobia, king mackerel, and Spanish mackerel through a single fishery management plan for coastal migratory pelagic resources.

[b]In 2016, the South Atlantic Fishery Management Council split the South Atlantic hogfish stock into two: Georgia-North Carolina hogfish and Florida Keys/East Coast of Florida hogfish.

[c]Harvest is not allowed for speckled hind and warsaw grouper as of December 2019.

[d]The South Atlantic and Gulf of Mexico Fishery Management Councils jointly manage spiny lobster through a single fishery management plan for spiny lobster.

[e]Saucereye porgy in the South Atlantic is, in practice, a recreational fish stock, according to South Atlantic Fishery Management Council staff. Council staff indicated that because the total annual catch limit for the fish stock is low, there is no commercial fishing in practice for the stock.

Tables 1 and 2 highlight the mixed-use fish stocks the South Atlantic and Gulf of Mexico councils manage, respectively.[1]

Table 2. Mixed-use fish stocks managed by the Gulf of Mexico fishery management council

Fish stocks managed individually	Fish stocks managed as complexes	
1) Cobia[a]	19) Deep water grouper aggregate complex (snowy grouper, speckled hind, warsaw grouper, and yellowedge grouper)	21) Mid-water snapper complex (blackfin snapper, queen snapper, silk snapper, and wenchman)
2) Cubera snapper	20) Jacks complex (almaco jack, banded rudderfish, and lesser amberjack)	22) Shallow water grouper aggregate complex (black grouper, scamp, yellowfin grouper, and yellowmouth grouper)
3) Gag grouper		23) Tilefish aggregate complex (blueline tilefish, golden tilefish, and goldface tilefish)
4) Goliath grouper[b]		
5) Gray snapper		
6) Gray triggerfish		
7) Greater amberjack		
8) Hogfish		
9) King mackerel, Gulf group[a]		
10)Lane snapper		
11)Mutton snapper		
12)Red grouper		
13)Red snapper[c]		
14)Spanish mackerel[a]		
15)Spiny lobster[d]		
16)Yellowtail snapper		
17)Vermilion snapper		
18)Corals (more than 140 species)[e]		

Source: GAO analysis of documents from the Gulf of Mexico Fishery Management Council and information from council staff. | GAO-20-216.

Note: Fish stocks listed by complex are managed together as a group. For this report, we count a complex as a single fish stock if the allocation is for the complex, rather than for the individual stock within the complex. If the fish stocks within a complex have their own allocations, we count them as separate fish stocks for reporting purposes. In addition, the Gulf of Mexico Fishery Management Council manages commercial fishing for several types of shrimp. The council also manages recreational fishing for red drum; however, harvest of red drum is not allowed as of December 2019, according to National Marine Fisheries Service officials.

[a]The South Atlantic and Gulf of Mexico Fishery Management Councils jointly manage cobia, king mackerel, and Spanish mackerel through a single fishery management plan for coastal migratory pelagic resources.

[b]Harvest is not allowed for goliath grouper as of December 2019.

[c]In 2019, the Gulf of Mexico Fishery Management Council began taking steps to delegate responsibility for the management of certain recreational fishing for red snapper in federal waters to the coastal states in the Gulf of Mexico. Specifically, following a pilot program in 2018 and 2019, the council completed an amendment to the fishery management plan for reef fish in May 2019 that would, among other things, allow a Gulf of Mexico state with an approved management program to manage private angling for red snapper in federal waters. The Secretary of Commerce approved the amendment on November 5, 2019 and the final rule implementing the amendment was under development as of December 2019, according to National Marine Fisheries Service officials.

[d]The South Atlantic and Gulf of Mexico Fishery Management Councils jointly manage spiny lobster through a single fishery management plan for spiny lobster.

[e]Harvest of stony corals, sea fans (soft corals), and wild live rock is generally prohibited in federal waters of the Gulf of Mexico as of December 2019.

[1] For some fish stocks, the councils manage groups of comparable species as complexes of fish. For this report, we count a complex as a single fish stock if the allocation is for the stock complex, rather than for the individual stock within the complex. If the fish stocks within a complex each have their own allocations, we count them as separate fish stocks for reporting purposes. In addition, the councils manage several fish stocks that are exclusively fished by the commercial or recreational sectors, according to council staff. In the South Atlantic, the staff said that the council manages commercial fishing for golden crab and shrimp, as well as recreational fishing for sailor's choice and tomtate (both part of the grunts complex) and scup (part of the porgy complex). The Gulf of Mexico council manages commercial fishing for several types of shrimp. The council also manages recreational fishing for red drum. However, harvest of red drum is not allowed as of December 2019, according to NMFS officials.

Fisheries Allocations

Under the Magnuson-Stevens Act's national standards for fishery management plans, allocations are to be fair and equitable to all U.S. fishermen; reasonably calculated to promote conservation; and carried out in such manner that no particular individual, corporation, or other entity acquires an excessive share.[2] NMFS guidelines for the national standards further indicate that in making allocations, councils should consider certain factors relevant to the fishery management plan's objectives. These factors include economic and social consequences of the allocations, food production, consumer interest, dependence on the fishery by present participants and coastal communities, efficiency of various types of gear used in the fishery, transferability of effort to and impact on other fisheries, opportunity for new participants to enter the fishery, and enhancement of opportunities for recreational fishing. In reviewing and approving fishery management plans and amendments, NMFS is responsible for ensuring that the councils' allocation decisions comply with the Magnuson-Stevens Act's national standards. In this report, the terms "established" and "revised" allocations refer to allocations established or revised by the councils and subsequently approved by NMFS, unless otherwise stated.

Historically, mixed-use fisheries allocations have been based predominantly on data estimating each fishing sector's past use of the resource, according to NOAA. To collect commercial and recreational data, NMFS works with partners such as coastal states and interstate marine fisheries commissions. In particular, for the commercial fishing sector, NMFS collects data on landings, which include the weight and value of fish stocks sold to seafood dealers using a network of cooperative agreements with states.[3] For recreational fishing, NMFS uses data from its Marine Recreational Information Program, which the agency began implementing in 2008 in place of the Marine Recreational Fisheries Statistics Survey. The Marine Recreational Information Program collects data on private anglers' fishing effort and catch rates and uses these to estimate total recreational fishing catch.[4] NMFS officials said that the program also collects information to estimate recreational landings. The program collects these data through such methods as mail surveys and shore-side interviews of anglers at public access fishing sites.[5]

Recognizing the difficulty in making allocation decisions—in part because allocations may be perceived as unfair by some stakeholders—NMFS commissioned a nationwide study in 2012 to examine allocation issues and gain stakeholders' perspectives from commercial and recreational fishing sectors.[6] The results of the study showed widespread dissatisfaction with how past allocation decisions were made. The study found little consensus on how to

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[2] Pub. L. No. 94-265, § 301(a)(4), 90 Stat. 331, 346 (1976) (codified as amended at 16 U.S.C. § 1851(a)(4)). The national standards are statutory principles that must be followed in any fishery management plan.

[3] Landings are defined as the number or poundage of fish unloaded by commercial fishermen and sold to seafood dealers or brought to shore by private anglers for personal use.

[4] Effort measures the number of angler trips, and catch rates measure the average number and size of fish per trip—by species—that are brought to shore, caught and used as bait, or discarded (i.e., caught but then released alive or dead).

[5] For more information on the Marine Recreational Information Program, see https://www.fisheries.noaa.gov/topic/recreational-fishing-data. We previously reported on recreational fisheries data. See GAO, *Recreational Fisheries Management: The National Marine Fisheries Service Should Develop a Comprehensive Strategy to Guide Its Data Collection Efforts*, GAO-16-131 (Washington, D.C.: Dec. 8, 2015).

[6] Lapointe, George, *Marine Fishery Allocation Issues: Findings, Discussion, and Options* (George Lapointe Consulting LLC, December 2012).

address concerns with allocations. For example, some stakeholders said that some allocations were outdated and that changes over time in human population, seafood demand, and recreational fishing warranted a comprehensive examination of allocations. Other stakeholders expressed concern that a uniform approach to allocation policy could harm fishing sectors, while others noted that it is important for the councils to have the flexibility to make regionally-focused decisions. The study concluded that many stakeholders may continue to view allocations as unbalanced or unfair unless the outcomes align with the positions they seek. The study recommended that NMFS take a number of steps to address allocation issues, including increasing stakeholder engagement in allocation decisions, periodically reviewing allocations, and creating a list of factors to guide allocation decisions.

In response to the 2012 study, NMFS issued a fisheries allocation review policy in 2016 and two guidance documents to the councils,[7] intended to help the councils and NMFS review and update allocations.[8] The objective of the NMFS policy was to describe the fisheries allocation review process, which called for using an adaptive management approach.[9] NMFS policy defined fisheries allocation review as the evaluation that leads to the decision of whether or not the development and evaluation of allocation options is warranted, but the allocation review is not, in and of itself, an implicit trigger to consider alternative allocations.

Through its policy, NMFS established a multi-step process for reviewing and potentially revising fisheries allocations. Specifically, once an allocation review trigger has been met (as described below), the councils are to complete an allocation review. For this review, NMFS policy does not call for in-depth analyses but calls for a clear articulation of how objectives are or are not being met and a clear rationale and documentation on relevant factors considered. Based on the allocation review, the councils may decide to maintain existing allocations, or proceed to evaluate allocation options for a fishery management plan amendment. When proceeding with this next step, the councils are to undertake formal analyses and follow the fishery management plan amendment process to ultimately recommend that an existing allocation either be retained or revised.

To supplement its fisheries allocation review policy, NMFS also issued two guidance documents, as follows:

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[7] NMFS developed the policy and guidance in coordination with the Council Coordination Committee. The reauthorization of the Magnuson-Stevens Act in 2007 permitted the councils to establish a Council Coordination Committee, which consists of the chairs, vice chairs, and executive directors from each council, or other council members or staff, as appropriate. Pub. L. No. 109-479, §103(g), 120 Stat. 3575, 3581 (2007) (codified at 16 U.S.C. § 1852(*l*)). The committee meets twice each year to discuss issues relevant to all councils, including issues related to the implementation of the Magnuson-Stevens Act.

[8] National Marine Fisheries Service, *Fisheries Allocation Review Policy*, *Criteria for Initiating Fisheries Allocation Reviews*, and *Recommended Practices and Factors to Consider When Reviewing and Making Allocation Decisions*. In addition, a 2018 NMFS technical memorandum recommended that councils develop and document a process for making allocation decisions when fish stocks change their distributions. See Karp, M. A., J. Peterson, P. D. Lynch, and R. Griffis (editors), *Accounting for Shifting Distributions and Changing Productivity in the Fishery Management Process: From Detection to Management Action*, U.S. Department of Commerce, National Oceanic and Atmospheric Administration Technical Memorandum NMFS-F/SPO-188 (Silver Spring, MD: 2018).

[9] NMFS policy defined adaptive management as the ongoing process of evaluating if management objectives have been met and adjusting management strategies in response. It stated the process includes periodic re-evaluation and updating of the management goals and objectives to ensure they are relevant to current conditions and needs.

- **Criteria for initiating fisheries allocation reviews.** [10] NMFS guidance recommended that the councils establish criteria for initiating allocation reviews—or allocation review triggers—within 3 years, or as soon as practicable, for all fisheries that have allocations between sectors. The guidance identified three types of potential criteria for allocation review triggers: (1) time-based, which include provisions for periodic allocation reviews at specific time intervals on a regular basis; (2) public interest-based, which provide an opportunity for the public to express interest in allocation reviews; and (3) indicator-based, such as triggers based upon economic or other metrics.
- **Factors to consider when reviewing and making allocation decisions**.[11] NMFS guidance outlined four categories of factors for the councils to consider when making allocation decisions, and noted that there may also be other appropriate factors to consider. These factors are not intended to prescribe particular outcomes with respect to allocations, but rather are intended to provide a framework for analysis, according to the guidance. The four categories of factors include:
  - **Fishery performance and change factors**, to assess the current conditions of a fishery and any changes in those conditions that may indicate a need for updated allocations. Such factors could include historical or current trends in catch or landings, the status of the fish stock (for example, whether it is subject to overfishing, is overfished, or is rebuilding[12]), or changes in the distribution of species within the fishery.
  - **Economic factors**, to consider the monetary consequences of an allocation, such as by analyzing (1) whether the existing or recommended allocation is the most economically efficient, and (2) the economic impacts of the allocation.[13]
  - **Social factors**, to assess the consequences of an allocation on individuals and communities, such as whether an allocation may have disproportionate adverse effects on low income or minority groups or could lead to fishing despite unsafe conditions if access to the fishery is restricted to a limited number of days.
  - **Ecological factors**, to consider the potential ecological impacts of allocations, such as impacts on the habitat or predator-prey dynamics of the fishery or of other fisheries within the ecosystem.

[10] National Marine Fisheries Service, *Criteria for Initiating Fisheries Allocation Reviews*.

[11] National Marine Fisheries Service, *Recommended Practices and Factors to Consider When Reviewing and Making Allocation Decisions.*

[12] A fish stock that is subject to overfishing has a fishing mortality (harvest) rate that is too high to meet long-term sustainable catch level targets under current conditions. Under the Magnuson-Stevens Act, overfished means a rate or level of fishing mortality that jeopardizes the capacity of a fishery to produce the maximum sustainable yield on a continuing basis. 16 U.S.C. § 1802(34). Rebuilding a stock involves taking actions to allow it to grow back to a predefined target level.

[13] According to the guidance, analyses that estimate the monetary value individuals or sectors place on their share of the harvest—their willingness to pay—can inform how allocation changes could improve economic efficiency. Economic impacts may be analyzed using models that include, for example, changes to sales, income, and employment levels.

SOUTH ATLANTIC AND GULF OF MEXICO COUNCILS HAVE ESTABLISHED AND REVISED ALLOCATIONS TO VARYING DEGREES

Since the Magnuson-Stevens Act was passed in 1976, the South Atlantic and Gulf of Mexico councils have established and revised allocations to varying degrees for the mixed-use fish stocks they manage in their regions. The South Atlantic council has established allocations for almost all of its mixed-use fish stocks and the Gulf of Mexico council has done so for certain stocks.

South Atlantic Council Has Established Allocations for Almost All Mixed-Use Fish Stocks and Revised Most of Those Allocations in 2012

Based on documents from the South Atlantic council, we found that the council has established allocations for 50 of the region's 51 mixed-use fish stocks.[14] The council first established an allocation for one fish stock—king mackerel—in 1985. From 1987 through 2010, the council set allocations for eight fish stocks. The council then established most allocations, encompassing 40 of its mixed-use fish stocks, in 2011, with allocations generally based on estimates of each fishing sector's historical landings.[15] The council's most recently established allocation—for a cobia stock—was in 2014, according to council documents. Appendix I provides additional information on the allocations for the mixed-use fisheries in the South Atlantic council region and the years in which the council established and revised allocations.

According to South Atlantic council staff, the council's approach to revising allocations has been to rely on stakeholder input to inform them of allocations that may need revision but to otherwise leave established allocations in place. For example, council staff noted that the allocation for king mackerel—which distributes a percentage of the annual catch limit to each fishing sector—has not changed since 1985 because it is still effective for both the commercial and recreational fishing sectors. Council staff explained that because neither sector has typically caught the amount of king mackerel they have been allocated, the council has not needed to revise the allocation.

[14] The South Atlantic council has not established an allocation for spiny lobster. Council staff said this is because spiny lobster fishing primarily occurs in the waters off Florida, where the state takes the lead in regulating this fishery through a protocol developed with NMFS and the South Atlantic and Gulf of Mexico councils. Outside of these Florida state- managed waters, spiny lobster fishing is subject to a two lobsters-per-person, per-trip catch limit, according to a council document.

[15] For example, the council based allocations for many snapper and grouper stocks on the following formula: 50 percent of each fishing sector's average landings for the period 1986 to 2008, plus 50 percent of each sector's average landings for the period 2006 to 2008, according to a council document.

Table 3. Mixed-use fish stocks with commercial and recreational allocations and subsequent revisions in the South Atlantic Fishery Management Council Region, as of December 2019

Fish stocks with allocations established and not revised	Fish stocks with allocations revised once	Fish stocks with allocations revised more than once
1985 • King mackerel, Atlantic group 2006 • Black sea bass 2008 • Gag grouper • Red porgy • Vermilion snapper 2010 • Golden tilefish 2011 • Black grouper • Greater amberjack • Mutton snapper • Red grouper • Red snapper • Speckled hindb • Warsaw grouperb • Wreckfish • Yellowtail snapper 2014 • Cobia, Gulf group, Florida East Coast Zone	2012 • Atlantic spadefish • Bar jack • Blueline tilefish • Gray triggerfish • Scamp Deepwater complex: • Blackfin snapper • Misty grouper • Queen snapper • Sand tilefish • Silk snapper • Yellowedge grouper Grunts complex: • Margate • White grunt Jacks complex: • Almaco jack • Banded rudderfish • Lesser amberjack Porgy complex: • Jolthead porgy • Knobbed porgy • Saucereye porgyc • Whitebone porgy Shallow-water groupers complex: • Coney • Graysby • Red hind • Rock hind • Yellowfin grouper • Yellowmouth grouper Snappers complex: • Cubera snapper • Gray snapper • Lane snapper 2013 • Wahoo 2014 • Snowy grouper	1989 and 1998 • Spanish mackerel, Atlantic group 2012 and 2016 • Hogfisha 2011, 2013, and 2015 • Dolphin (mahimahi)

Source: GAO analysis of documents from the South Atlantic Fishery Management Council. | GAO-20-216.

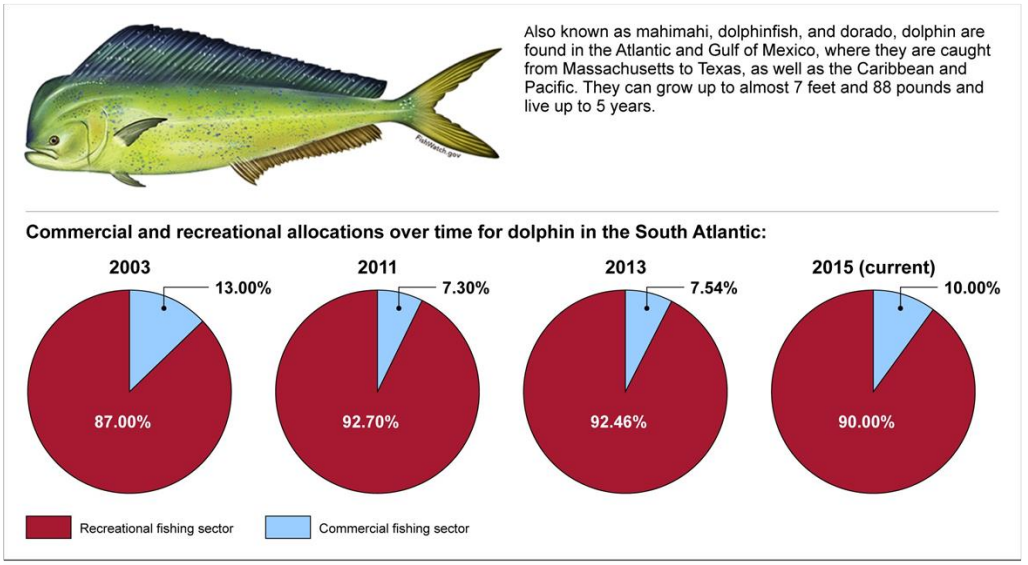
Note: Years shown represent the year that the council completed its fishery management plan amendment and sent it to the National Marine Fisheries Service for review and approval. For allocations that have not been revised, the dates shown are the years the councils established the allocations. For allocations that have been revised, the years shown are the years the councils revised them. The fish stocks listed as part of complexes are managed together as groups. In the South Atlantic region, the fish stocks within these complexes have their own allocations.

[a]In 2016, the South Atlantic Fishery Management Council split the South Atlantic hogfish stock into two and established allocations for Georgia-North Carolina hogfish and Florida Keys/East Coast of Florida hogfish.

[b]Harvest is not allowed for speckled hind and warsaw grouper as of December 2019.

[c]Saucereye porgy in the South Atlantic is, in practice, a recreational fish stock, according to South Atlantic Fishery Management Council staff. The staff said because the total annual catch limit for the stock is low, there is no commercial fishing in practice for the stock.

As of December 2019, the South Atlantic council had revised allocations for most of their mixed-use fish stocks once, according to council documents, as shown in Table 3. The council revised allocations for 30 fish stocks in 2012,[16] based on changes to the source of recreational catch data the council was using in its formulas for calculating allocation percentages.[17]



Source: GAO analysis of documents from the National Marine Fisheries Service and South Atlantic Fishery Management Council. | GAO-29-216.

Note: Years shown represent the year that the South Atlantic Fishery Management Council sent a fishery management plan amendment to the National Marine Fisheries Service for its review and approval. The allocation represents each sector's percentage of the annual catch limit for dolphin, as measured in pounds of whole fish. The council has set allocation percentages to two decimal places, as indicated in the figure.

Figure 4. History of the Commercial and Recreational Allocation for Dolphin in the South Atlantic, as of December 2019.

The South Atlantic council has revised few allocations more than once. Specifically, they revised allocations for two fish stocks twice and for one, dolphin, three times.[18] For example, the council first established an allocation for dolphin (also known as mahimahi, dolphinfish, and dorado) in 2003. It established the allocation to maintain the fishery as predominantly

[16] The 30 fish stocks with allocations revised in 2012 include hogfish, which the council revised in 2012, and then again in 2016.

[17] In 2012, the South Atlantic council changed from using Marine Recreational Fisheries Statistics Survey data to data calculated from NMFS' Marine Recreational Information Program. Specifically, the council adjusted allocation amounts using data from the Marine Recreational Information Program for recreational catch estimates for the years 2004- 2008. The council also based allocation amounts on updated recreational catch estimates for 1986-2003. For these estimates, the council used data developed by a regional working group that developed a regional calibration method to recalculate previous recreational fishing estimates for these years.

[18] In addition, the council began work in March 2016 on a draft fishery management plan amendment that considers alternatives for revising dolphin and wahoo allocations by increasing the recreational sector's allocation. The allocation alternatives under consideration were based on catch and landings data. At the council's December 2019 meeting, the council postponed discussion of the amendment until its June 2020 meeting.

recreational and based the allocation on historical landings, according to the council's fishery management plan (see Figure 4). According to council documents, the council then revised the dolphin allocation three times:

- in 2011, when initially setting annual catch limits for dolphin,
- in 2013, based on changes to the source of recreational catch data used to calculate allocation percentages, and
- in 2015, because the recreational sector had not been catching the amount of fish it was allocated, and the council was concerned that the commercial sector could exceed its allocation in the future.

The extent to which the South Atlantic council may have considered other revisions to allocations is unclear. For example, South Atlantic council staff said that their council had deliberated on revising allocations for some fish stocks at council meetings, but they do not have records of the deliberations because the council decided not to make revisions and did not initiate related fishery management plan amendments. South Atlantic council staff explained that they document all allocation revisions through fishery management plan amendments, but they have not otherwise formally documented reviews that did not result in revisions.[19] Council staff said they recognize the need to better document such reviews in the future; however, the council did not identify how it plans to do so, as discussed later in this report.

Gulf of Mexico Council Has Established Allocations for Certain Mixed-Use Fish Stocks and Revised Three of Those Allocations in 2008

The Gulf of Mexico council established commercial and recreational allocations for nine of the region's 23 mixed-use fish stocks, according to documents from the council (see app. I for allocations for the mixed-use fisheries in the Gulf of Mexico council region). Council staff said most of the council's allocations were made based on estimates of each sector's historical landings. The council has not established allocations for most mixed-use fish stocks in the region because allocations for these stocks have not been warranted, according to council staff.[20] Council staff said the council generally considers establishing allocations when stakeholders identify issues, or if new information such as a stock assessment becomes available and indicates that allocations may be needed to help manage a fish stock. In the absence of such information, the Gulf of Mexico council manages the fish stocks with other methods— for example, with seasonal closures or trip or bag limits, which establish the number of fish that can be legally taken in a specified period.

[19] South Atlantic council staff said that in 2019 the council began to convert its historical meeting minutes and final documents into a searchable format that will improve the council's ability to track past discussions of allocations. They said that this project will take several years to complete.

[20] Specifically, the council has not established allocations for the following mixed-use fish stocks: (1) cobia; (2) corals; (3) cubera, (4) gray, (5) lane, (6) mutton, (7) vermillion, and (8) yellowtail snapper; (9) goliath grouper; (10) hogfish; (11) Spanish mackerel; (12) spiny lobster; (13) the Jacks complex (almaco jack, banded rudderfish, and lesser amberjack); and (14) the mid-water snapper complex (blackfin snapper, queen snapper, silk snapper, and wenchman).

As of December 2019, the Gulf of Mexico council had revised allocations for three mixed-use fish stocks, as shown in Table 4. For example, the council revised the allocation for red grouper in 2008 to increase the recreational sector's allocation after a stock assessment indicated the fishery had recovered from overfishing, according to a council document. In 2008, the council also revised the gag grouper allocation to increase the commercial sector's allocation. In addition, the Gulf of Mexico council completed a fishery management plan amendment in 2015 that revised the red snapper allocation by increasing the recreational sector's percentage. However, after the Secretary of Commerce approved the amendment in 2016, a U.S. District Court vacated the amendment in 2017, and the council returned to the initial allocation established for red snapper.[21]

Table 4. Mixed-use fish stocks with commercial and recreational allocations and subsequent revisions in the Gulf of Mexico Fishery Management Council Region, as of December 2019

Fish stocks with allocations established and not revised	Fish stocks with allocations revised once
1985 • King mackerel, Gulf group 1989 • Red snapper[a] 2008 • Gray triggerfish 2011 • Deep water grouper aggregate complex (snowy grouper, speckled hind, warsaw grouper, and yellowedge grouper)[b] • Shallow water grouper aggregate complex (black grouper, scamp, yellowfin grouper, and yellowmouth grouper)[b] • Tilefish aggregate complex • (blueline tilefish, golden tilefish, and goldface tilefish)[b]	2008 • Gag grouper • Greater amberjack[c] • Red grouper

Source: GAO analysis of documents from the Gulf of Mexico Fishery Management Council. | GAO-20-216.

Note: Years shown represent the year that the council completed its fishery management plan amendment and sent it to the National Marine Fisheries Service (NMFS) for review and approval. For allocations that have not been revised, the dates shown are the years the councils established the allocations. For allocations that have been revised, the years shown are the years the councils revised them. The fish stocks listed as part of complexes are managed together as groups. In the Gulf of Mexico region, the fish stocks within these complexes do not have their own allocations.

[a]In 2015, the Gulf of Mexico Fishery Management Council completed a fishery management plan amendment that revised the red snapper allocation. However, after the Secretary of Commerce approved the amendment in 2016, a U.S. District Court vacated the amendment in 2017 and the council returned to the initial allocation established for red snapper. *See Guindon v. Pritzker*, 240 F. Supp. 3d 181 (D.D.C. 2017).

[b]According to NMFS officials, the Gulf of Mexico Fishery Management Council established allocation percentages for each complex as a whole, based on quotas for commercial fishing established for these complexes. Recreational allocation percentages for the complexes represent the remainder of allowable harvest, after factoring in quota amounts.

[c]For greater amberjack, the council did not revise the allocation directly; instead, the council indirectly revised the commercial and recreational allocations by establishing harvest reductions that were applied unequally to these fishing sectors, according to a 2008 fishery management plan amendment.

[21] *Guindon v. Pritzker*, 240 F. Supp. 3d 181 (D.D.C. 2017). The court held that the revised allocation was not fair and equitable and therefore violated National Standard 4. The council also began work in January 2018 on a separate fishery management plan amendment to consider revising red snapper allocations. As of August 2019, the council decided to postpone further work on the amendment until 2020, to review our report and further progress in calibrating estimates of recreational fishing for red snapper through the Marine Recreational Information Program, according to council documents.

Gulf of Mexico council staff said the council has not identified a need to revise allocations for the other mixed-use fish stocks in the region with allocations. For instance, for the deep water grouper and tilefish complexes, council staff said there has been limited competition between the recreational and commercial fishing sectors and the council has not needed to revise the allocations initially established for those fish stocks in 2011.

When the Gulf of Mexico council has considered revising allocations, it has done so through fishery management plan amendments, according to council staff. For example, in a 2016 fishery management plan amendment, the council considered revising the allocation for king mackerel because estimates indicated that the recreational sector had not been landing the amount of fish it was allocated. However, the council decided not to revise the allocation, citing the potential for increased recreational fishing for king mackerel in the future.[22]

VARIOUS SOURCES OF INFORMATION MAY BE AVAILABLE TO HELP NMFS AND THE COUNCILS CONDUCT ALLOCATION REVIEWS

Through our review of agency documents and interviews with NMFS and South Atlantic and Gulf of Mexico council staff, we found that various sources of information may be available to help NMFS and the councils review allocations, but each source presents some challenges to councils for supporting allocation decisions. Councils can use these sources of information to consider the factors NMFS' 2016 guidance calls for— including fishery performance and change, economic, social, and ecological factors—when reviewing allocations.[23] Five key sources of information that NMFS and the councils identified are trends in catch and landings, stock assessments, economic analyses, social indicators, and ecosystem models. NMFS officials said that the councils would like to incorporate these key sources into their allocation reviews, and use such information in supporting future allocation decisions. However, they said the availability, specificity, or quality of information can present challenges to using some of the information. In particular, they noted that available information other than landings is often sparse and uncertain for many fish stocks. As a result, the officials said it may be difficult for the councils to use such information as the basis for allocation decisions. NMFS is taking some steps to improve the information available, as discussed below.

Trends in Catch and Landings

NMFS' 2016 guidance states that changes in the performance or conditions of a fishery may indicate the need for updated allocations. Fishery performance and change factors include trends in catch or landings. Data on historical and current catch and landings can

[22] For example, the amendment noted that an increase in the recreational bag limit and recent short recreational seasons for other popular fish could result in more fishing effort shifting to king mackerel. However, the amendment also noted it seemed unlikely that recreational fishing for king mackerel would increase substantially in the near future, even with an increase in the bag limit.

[23] The guidance states that the factors are intended to provide a framework for analyses, but that the priority and weight afforded each factor may vary depending on such things as the fishery, the objectives of the fishery management plan or the allocation, and overarching council goals.

provide the councils with important information about demand, according to NMFS guidance, including whether a fishing sector may be catching above or below its allocation. Generally, NMFS collects landings data for commercial fisheries from state fisheries agencies, who obtain landings data from monthly reports submitted by seafood dealers on the weight and value of fish sold at the dock. NMFS collects data to estimate recreational catch and landings through survey and interview methods through its Marine Recreational Information Program.

However, recreational catch estimates present some limitations. A 2017 National Academies study noted that obtaining reliable data on recreational catch can be challenging because of several attributes of the recreational fishing sector.[24] For example, the greater number of recreational anglers compared with the number of participants in the commercial fishing sector, and the greater number of access and landing points available to recreational anglers, make it difficult to obtain reliable data on the extent of recreational fishing, according to the study.

In 2018, the Marine Recreational Information Program updated how NMFS estimates recreational catch based on a change in the survey methodology used to collect data from anglers on the Atlantic and Gulf of Mexico coasts.[25] According to NMFS documents, updated recreational catch estimates for many fish stocks are several times higher than previous estimates because of the change in methodology.[26] However, any implications these updated estimates may have for allocations in the South Atlantic and Gulf of Mexico may not be fully understood until NMFS incorporates the estimates into stock assessments, which were scheduled for completion between 2019 and 2021, according to NMFS documents.

Further, in the Gulf of Mexico, states collect recreational catch data through their own programs, which supplement NMFS' Marine Recreational Information Program data. The states' programs use different methodologies, however, which Gulf of Mexico council staff said make it difficult to reconcile the states' recreational fisheries data with NMFS' data on catch estimates. According to an NMFS document, some of the different methodologies the states use to design surveys have produced different estimates in years when two or more surveys were conducted side by side, making it difficult to determine the best estimates of recreational catch in the Gulf of Mexico.[27]

NMFS is taking steps to improve its recreational catch estimates. For instance, in September 2019 NMFS issued procedural guidance to help ensure that survey estimates from the Marine Recreational Information Program are based upon the best scientific information available and to promote nationwide consistency in collecting data and estimating

[24] National Academies of Sciences, Engineering, and Medicine, *Review of the Marine Recreational Information Program* (Washington, D.C.: The National Academies Press, 2017). We also reported on this topic in 2015. See GAO-16-131.

[25] In 2018, the Marine Recreational Information Program completed its transition from using its Coastal Household Telephone Survey to its mail-based Fishing Effort Survey for shore and private boat fishing in the Atlantic and Gulf of Mexico. Because these surveys used two different methodologies to collect data, and because fishery managers need consistent data that can be compared over time, NMFS has been working to calibrate data from the telephone survey to the mail survey. From 2015 to 2018, NMFS worked to calibrate the data using a model it developed to adjust historic estimates so that those estimates can be compared with new estimates. In July 2018, the program released revised estimates of recreational catch and effort for 1981 through 2017.

[26] According to an agency document, NMFS determined that the higher estimates resulted from improved methods for estimating fishing activity and not a sudden rise in fishing.

[27] U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, *Recommended Use of the Current Gulf of Mexico Surveys of Marine Recreational Fishing in Stock Assessments* (July 2019).

recreational catch.[28] NMFS is also working with Gulf of Mexico states to evaluate the critical assumptions made by each state's data collection program and to help ensure that the states' recreational catch estimates are comparable across years and with other states. As part of this effort, NMFS is calibrating recreational catch estimates from Gulf of Mexico states with data from the Marine Recreational Information Program. According to an agency official, NMFS anticipates completing this effort in May 2020.

Stock Assessments

Stock assessments are a key source of information the councils can use to review allocations given the information they provide on the status of fish stocks, according to NMFS documents. Stock assessments can range in complexity from a simple description of historical trends in catch and landings to complex assessment models that incorporate spatial and seasonal analyses in addition to ecosystem or multispecies considerations.[29] Stock assessments are not available for all fish stocks with allocations, however. In the South Atlantic, 32 of the 50 mixed-use fish stocks with allocations do not have stock assessments, according to council staff.[30] Of these fish stocks, NMFS plans to complete stock assessments for three—gray triggerfish, scamp, and white grunt—by 2024, according to South Atlantic council staff. In the Gulf of Mexico, stock assessments are available for the mixed-use fish stocks with allocations, with the exception of the shallow and deep water grouper aggregate complexes.[31]

Stock assessments can provide maps of the spatial distributions of fish stocks and may show changes in those distributions over time, according to NMFS officials. Changes in a fish stock's distribution may lead to allocation disputes, and basing allocations on historical catch may not be appropriate in such situations, according to an NMFS document. NMFS' 2016 guidance states that the councils may need to update allocations if the distributions of fish stocks change over time for reasons such as climate change or natural fluctuations in abundance.[32] However, NMFS officials noted that few stock assessments incorporate spatial

[28] U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, *Implementing Recreational Fishery Catch and Effort Survey Design Changes: Guidance and Procedures for the MRIP Certification Process*, NMFS Procedure 04-114-02 (Sept. 4, 2019).

[29] According to an NMFS report, the level of complexity of a stock assessment has a large impact on the amount of data and effort needed to complete the assessment, as well as on the extensiveness of review needed of assessment results. Some fish stocks necessitate frequent and complex assessments because they have high importance to the fishery, play an important ecosystem role, or are vulnerable to overexploitation. However, other stocks do not need such comprehensive monitoring, according to the NMFS report.

[30] South Atlantic council staff cited the following as reasons why stock assessments may not be conducted: existing data are insufficient for the analyses needed; NMFS staff or funding are not available to perform the analyses; and some species are encountered infrequently during fishing. For fish stocks that do not have stock assessments, the council's scientific and statistical committee has formulated rules for determining acceptable biological catch using proxy measures of landings and the council sets annual catch limits based on these, according to council staff.

[31] A stock assessment is available for black grouper, which is within the shallow water grouper aggregate complex.

[32] A 2018 NMFS technical memorandum recommended that councils develop and document a process for making allocation decisions when fish stocks change their distributions. See Karp, M. A., J. Peterson, P. D. Lynch, and R. Griffis (editors), *Accounting for Shifting Distributions and Changing Productivity in the Fishery Management Process: From Detection to Management Action*, U.S. Department of Commerce, National Oceanic and Atmospheric Administration Technical Memorandum NMFS-F/SPO-188 (Silver Spring, MD: 2018).

models that would allow forecasts of future spatial distributions.[33] To help improve the availability of such information, NMFS is conducting evaluations that will, among other things, assess changes in the distribution of fish stocks in the Gulf of Mexico and South Atlantic in response to regional climate change impacts. NMFS officials said they anticipate completion of these evaluations in 2020, which will help them forecast future spatial distributions for some fish stocks going forward.

In addition, stock assessments are one source of information that the councils can use to assess each fishing sector's expected ecological impacts, according to NMFS officials. For example, NMFS officials said that stock assessments commonly provide information on each sector's discards—fish intentionally thrown back. Discards may be caught as bycatch—that is, incidentally to the harvest of the primary fish stock targeted. NMFS' 2016 guidance states that councils can consider the expected impacts of each fishing sector's allocation on bycatch and bycatch mortality.[34] However, the availability and certainty of bycatch and discard information can vary, according to NMFS officials.

NMFS is taking steps to improve information on bycatch and discards. For instance, beginning in 2020, the for-hire component of the recreational fishing sector is to use an electronic system to report its bycatch and discards in the South Atlantic and Gulf of Mexico, according to NMFS officials. The officials said that the commercial fishing sector will begin using this system by 2023. NMFS officials said that the agency is also developing a model that will, among other things, estimate the number of released fish caught by the recreational fishing sector in the South Atlantic and Gulf of Mexico.[35] The officials said that the first version of the model is focused on gag grouper in the Gulf of Mexico, but that the model could be customized to any fish stock with the necessary data available. As of December 2019, NMFS officials anticipated completion of the model by late 2020 and estimated that the model would be ready to incorporate into stock assessments in fiscal year 2021 or later.

Economic Analyses

Economic analyses can provide information on the economic consequences of allocations, according to NMFS documents. NMFS' 2016 guidance notes that councils should consider if the current or preferred allocation results in the most economically efficient use of the fishery resource.[36] According to the guidance and NMFS officials, economic efficiency

[33] In addition, in 2016, we found that NMFS and the councils have limited stock-specific information about the magnitude and timing of climate change effects—such as changes in distribution—for federally managed fish stocks. See GAO, *Federal Fisheries Management: Additional Actions Could Advance Efforts to Incorporate Climate Information into Management Decisions*, GAO-16-827 (Washington, D.C.: Sept. 28, 2016).

[34] According to a 2019 Gulf of Mexico council report, mortality from discards in the recreational fishing sector is a problem in the region. Fish may be discarded because they are under a minimum size limit or out of season, anglers have already retained their bag limit, or they may be voluntarily discarded because anglers prefer to catch and release, according to the report. In October 2019, the Gulf of Mexico council hosted a discard mortality symposium with the goal of reducing discard mortality from recreational fishing efforts. See Gulf of Mexico Fishery Management Council, *Release Mortality Symposium Summary Report* (October 2019).

[35] According to NMFS officials, this model is based on work originally done for groundfish in the Northeast. See Lee, Min-Yang, Scott Steinback, and Kristy Wallmo, *Applying a Bioeconomic Model to Recreational Fisheries Management: Groundfish in the Northeast United States*, Marine Resource Economics, volume 32, number 2 (Chicago, IL: Feb. 3, 2017).

[36] Under the Magnuson-Stevens Act, National Standard 5 provides that fishery management plan measures shall, where practicable, consider efficiency in the utilization of fishery resources; except that no such measure shall

refers to how well scarce resources are used in production and consumption, and is achieved when all resources are allocated to their most valuable productive use. In principle, an allocation is most economically efficient when the net economic benefits to the commercial and recreational fishing sectors in total are maximized.[37] If net economic benefits are not maximized, then modifying the allocation may increase economic efficiency and economic benefits to the nation. NMFS officials said the agency focuses on conducting economic efficiency analyses to help guide allocation reviews.[38] Economic efficiency analyses can help NMFS and the councils analyze whether a proposed change in an allocation would generate greater net economic benefits for society (that is, improve economic efficiency), compared with the current allocation, according to NMFS officials.

We found the councils face challenges in using economic efficiency analyses in allocation decisions. According to NMFS officials and the agency's published research, reliable data for estimating economic values associated with recreational fishing may not be readily available. This is because no market prices for fish caught by private anglers are available and thus, non-market valuation techniques must be used to estimate the marginal value of fish to recreational anglers.[39] For example, a 2014 NMFS study on the economic efficiency of allocations for gag, red, and black grouper found that there are insufficient data on the recreational harvest by grouper species to generate statistically reliable estimates of economic value for each fish stock.[40]

In addition, it is difficult to estimate the economic value associated with one fish stock because recreational anglers may be willing to catch other species of fish if fishery managers limit anglers' access to a particular stock, according to members of both councils' socioeconomic panels. This transfer of effort from one fish stock to another makes it difficult to determine which fish stock drives the economic value that anglers associate with fishing. Further, a 2014 NMFS study on the economic efficiency of red snapper allocations indicated that a relevant market price that could be used as a benchmark for the recreational estimates is unavailable. The study found that in prior work the agency attempted to use charter fishing trip prices to address this concern, but no current data on charter prices existed to update that

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have economic allocation as its sole purpose. 16 U.S.C. § 1851(a)(5). National Standard 5 Guidelines state that this standard prohibits only those measures that distribute fishery resources on the basis of economic factors alone and that have economic allocation as their only purpose. 50 C.F.R. § 600.330(e). The guidelines also explain that given a set of objectives for the fishery, a fishery management plan should contain management measures that result in as efficient a fishery as is practicable or desirable. 50 C.F.R. § 600.330(b)(1).

[37] More specifically, net economic benefits are maximized at the allocation where the marginal values are equalized across the commercial and recreational sectors. In principle, net benefits are measured in terms of changes in consumer and producer surplus. Consumer surplus is the difference between the amounts consumers are willing to pay for goods and services, and the amounts they actually pay. Producer surplus is the difference between the amounts producers actually receive for providing goods and services, and the economic cost producers incur in doing so.

[38] According to NMFS' 2016 guidance, the councils should only use certain data points as measured by economic impact analyses to understand the potential short-term distributive effects of allocation decisions on the affected communities. For example, a change in an allocation may increase seafood sales and business income in one community but decrease them in another. However, NMFS officials said they discourage using economic impact analyses when considering allocations because economic impact analyses do not measure changes in economic welfare.

[39] Agar, Juan J. and David W. Carter, *Is the 2012 Allocation of Red Snapper in the Gulf of Mexico Economically Efficient?*, National Oceanic and Atmospheric Administration Technical Memorandum NMFS-SEFSC-659 (Miami, FL: June 2014).

[40] Agar, Juan J. and David W. Carter, *Are the 2012 Allocations of Gag, Red, and Black Grouper in the Gulf of Mexico Economically Efficient?*, National Oceanic and Atmospheric Administration Technical Memorandum NMFS-SEFSC-660 (Miami, FL: June 2014).

analysis.[41] As a result, the study cautioned against comparing estimates of recreational value to that in the commercial sector, which is a key aspect of determining an economically efficient allocation.

Moreover, two 2014 NMFS studies found that there are also methodological and data challenges related to obtaining economic information from the commercial fishing sector.[42] For example, the studies raised questions about the quality of some of the price data that were used in developing estimates of economic values for the commercial sector.[43] In addition, the studies' estimates of the economic value of commercial fishing did not include the potential net value derived from other components of the commercial seafood supply chain, such as the processing, distribution, and sale of the fish to the end consumers, according to the NMFS studies and agency officials (see Figure 5).[44] These NMFS studies noted that data for estimating the values from these other components are not readily available. Council staff and members, socioeconomic panel members, and fishery stakeholders we interviewed noted the importance of including the value of fish to the end consumers when considering the economic value of commercial fishing. To estimate the values of these other components of the commercial seafood supply chain, NMFS would need information about the consumer demand for fish as a function of domestic and international production, as well as information on changes in the price of the fish as they move from the dockside to retail markets, according to a separate NMFS study.[45]

NMFS officials said they are taking some steps related to improving economic analyses that the councils could consider in allocation reviews. For example, the agency is developing a manual of best practices for NMFS and council staff responsible for conducting economic analyses. NMFS officials said that they anticipate completing the manual by the end of fiscal year 2020. According to NMFS officials, the manual is intended to help (1) achieve consistency in analyses across the councils and regions, (2) establish an understanding of why economic analyses of allocations are important to fisheries management decisions, as well as their role in complying with various legal requirements and NMFS' policy, and (3) establish an understanding of the basic concepts and tools used in these analyses and how they are expected to be applied in practice. In addition, NMFS conducted a study on the economics of the for-hire fishing sector in federal waters of the South Atlantic and Gulf of Mexico and completed a report on the study at the end of 2019.[46] Among other things, agency officials said the study provides data sufficient to estimate producer surplus for the for-hire sector. This information could help inform future allocation decisions, according to NMFS officials.

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[41] Agar and Carter, *Is the 2012 Allocation of Red Snapper in the Gulf of Mexico Economically Efficient?*.

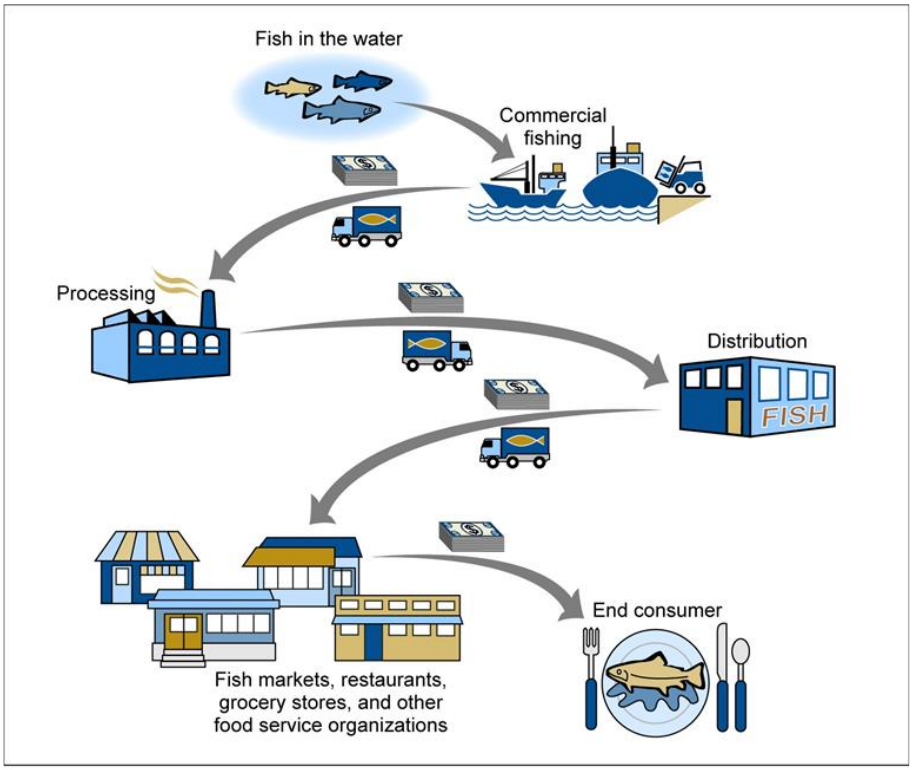
[42] Agar and Carter, *Is the 2012 Allocation of Red Snapper in the Gulf of Mexico Economically Efficient?* and *Are the 2012 Allocations of Gag, Red, and Black Grouper in the Gulf of Mexico Economically Efficient?*.

[43] Specifically, the 2014 NMFS studies used lease prices for commercial fishing quotas and other information to derive willingness to pay estimates for the commercial sector. However, the studies stated that many of the lease prices were low and that it is vital to ensure that correct prices are reported if the data are to be used in an economic analysis.

[44] Specifically, NMFS' 2014 studies indicated that the estimates of the economic value of commercial fishing did not include consumer surplus—the difference between the price that consumers pay and the price they are willing to pay for a service or product.

[45] Carter, David W., Juan J. Agar, and James R. Waters, *Economic Framework for Fishery Allocation Decisions with an Application to Gulf of Mexico Red Grouper*, National Oceanic and Atmospheric Administration Technical Memorandum NMFS-SEFSC-576 (Miami, FL: 2008).

[46] Souza, Philip M., Jr. and Christopher Liese, *Economics of the Federal For-Hire Fleet in the Southeast - 2017,* National Oceanic and Atmospheric Administration Technical Memorandum NMFS-SEFSC-740 (Miami, FL: November 2019).



Source: GAO. | GAO-20-216.

Figure 5. Simplified diagram of a seafood supply chain for commercial fishing.

Social Indicators

NMFS has developed social indicators to characterize community well- being for coastal communities engaged in fishing activities, which the councils could consider in reviewing allocations, according to NMFS officials. NMFS' 2016 guidance states that the councils could consider individual, local, and regional fishing dependence and engagement, and that such analyses should include potential impacts on commercial, for- hire, private angler, and subsistence fishing, as well as fishing-related industries if data are available. NMFS' social indicators are numerical measures that describe the well-being of fishing communities in coastal counties across the United States and their level of dependence on commercial and recreational fishing.[47] For example, one indicator describes the vulnerability of fishing communities to disruptive events, such as a change to a fishing sector's access to a fishery. Communities that are dependent on commercial fishing can be more socially vulnerable than other communities to changes, according to an NMFS document.

[47] For more information on NMFS' social indicators, see https://www.st.nmfs.noaa.gov/humandimensions/social-indicators/. See also Jepson, Michael and Lisa L. Colburn, *Development of Social Indicators of Fishing Community Vulnerability and Resilience in the U.S. Southeast and Northeast Regions*, U.S. Department of Commerce, National Oceanic and Atmospheric Administration Technical Memorandum NMFS-F/SPO-129 (St. Petersburg, FL: 2013).

However, NMFS' social indicators on communities' reliance on and engagement in commercial and recreational fishing are not specific to particular fish stocks. NMFS officials said this makes it challenging for councils to incorporate the information into their allocation reviews for specific fish stocks. The officials said that given current resource limitations and limited data available, it would be difficult to generate social indicators that are specific to fish stocks. In some instances, NMFS has some stock-specific information at the community level for the commercial fishing sector.[48] But NMFS officials said that comparable information is not available for the recreational sector at the community level, making it difficult to develop fish stock-specific social indicators.

NMFS officials said that the agency continues to work to update and improve social indicators relevant to recreational and commercial fisheries and is exploring other sources to provide better social data for fisheries management decisions. However, NMFS officials did not identify specific steps they plan to take to improve social indicators—such as developing information specific to particular fish stocks—so that the councils could more easily incorporate such information into their allocation reviews.

Ecosystem Models

NMFS' 2016 guidance calls for the councils to consider the potential ecological impacts of allocation alternatives in determining the allocation between different sectors or groups. However, NMFS officials said there are few ecosystem models that incorporate ecological information that could be considered in reviewing allocations, in part because limited quantifiable ecological information is available. They said that it will be difficult to use ecosystem models in allocation decisions until such models are more fully developed.

NMFS officials said they are taking some steps to enhance the use of ecological and ecosystem-based information. For instance, they noted that in 2016, NMFS released a policy to, among other things, establish a framework of guiding principles to enhance and accelerate the implementation of ecosystem-based fisheries management.[49] Ecosystem- based fisheries management is a systematic approach to fisheries management in a geographically specified area that: contributes to the resilience and sustainability of the ecosystem; recognizes the physical, biological, economic, and social interactions among the affected fishery- related components of the ecosystem, including humans; and seeks to optimize benefits among a diverse set of societal goals, according to the policy. Among other things, this approach can help communicate the potential consequences of management decisions—including allocations—across fish stocks and improve the understanding of the potential benefits and effectiveness of management decisions, according to the policy. In 2019, NMFS issued plans for implementing ecosystem- based fisheries management in the South Atlantic and Gulf of Mexico.[50]

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[48] For example, NMFS has indicators of communities' engagement in and reliance on commercial fishing for certain fish stocks.

[49] U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, *Ecosystem-Based Fisheries Management Policy*, NMFS Policy 01-120 (effective May 23, 2016 and renewed September 27, 2018).

[50] U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, *Ecosystem Based Fisheries Management Implementation Plan for the South Atlantic* (May 2019) and *2019 Gulf of Mexico Ecosystem Based Fisheries Management Implementation Plan* (2019).

SOUTH ATLANTIC AND GULF OF MEXICO COUNCILS DEVELOPED CRITERIA FOR INITIATING ALLOCATION REVIEWS, BUT NOT PROCESSES FOR CONDUCTING OR DOCUMENTING THEM

The South Atlantic and Gulf of Mexico councils each established criteria for initiating allocation reviews in response to NMFS' 2016 guidance, but neither council has developed processes to guide how they will conduct or document their allocation reviews. The Gulf of Mexico council has taken initial steps to develop a process for how it will review allocations, and staff from both councils said they are waiting for our report to inform their next steps on developing processes for conducting allocation reviews in the future.

Both Councils Established Criteria for Initiating Allocation Reviews

The South Atlantic and Gulf of Mexico councils each developed policies that established criteria for initiating allocation reviews. The South Atlantic council's July 2019 policy established certain conditions as the primary criteria for triggering allocation reviews. Specifically, the policy states that the council is to initiate an allocation review for a particular fish stock if any of the following conditions are met:[51]

- the commercial or recreational fishing sector exceeds its annual catch limit or closes prior to the end of its fishing year in 3 out of 5 consecutive years;
- the commercial or recreational fishing sector under harvests its annual catch limit or optimum yield by at least 50 percent in 3 out of 5 consecutive years;[52]
- the council's scientific and statistical committee approves a stock assessment and presents it to the council; or
- the council reviews a fishery performance report.[53]

The South Atlantic council's policy also established time-based triggers as secondary criteria for initiating allocation reviews.[54] Its policy states that the council will review

[51] The South Atlantic council's policy also states that the council's allowance of harvest of speckled hind or warsaw grouper, which have annual catch limits of zero, would trigger an allocation review for those fish stocks.

[52] The Magnuson-Stevens Act defines optimum, with respect to the yield from a fishery, as the amount of fish that (1) will provide the greatest overall benefit to the nation, particularly with respect to food production and recreational opportunities and taking into account the protection of marine ecosystems; (2) is prescribed on the basis of the maximum sustainable yield from the fishery, as reduced by any relevant social, economic, or ecological factor; and (3) in the case of an overfished fishery, provides for rebuilding to a level consistent with producing the maximum sustainable yield in such fishery. 16 U.S.C. § 1802(33).

[53] South Atlantic council staff said they began in 2017 to develop fishery performance reports using information provided by the council's advisory panels—panels that include representatives from the recreational and commercial fishing sectors and conservation groups that may provide information about trends in fisheries, environmental concerns, and the impacts of any allocation changes. Each fishery performance report is to focus on a specific species and provide insights into regional differences, catch, and regulatory concerns, among other things, according to the council's website.

[54] The 2016 NMFS guidance also identified, as a third option, the potential for public interest-based triggers, to provide an opportunity for the public to express interest in allocation reviews. The South Atlantic council did not select public interest as an allocation review trigger because, according to the council's policy, the council provides sufficient opportunity for public input on allocations and receives substantial and regular comments from the public through scoping and public hearing sessions, general public comment periods held at every

allocations not less than every 7 years if one of the conditions identified in the policy has not already triggered a review. The policy also states that once a review occurs, the next one will be automatically scheduled for 7 years later.

Regional Fishery Management Councils That Have Established Allocation Review Criteria

Of the eight regional fishery management councils (councils), six developed policies by August 2019 establishing specific criteria for initiating allocation reviews. In addition to the South Atlantic and Gulf of Mexico councils, the other four councils' plans include the following:

- The New England council plans to review certain allocations 8 to 10 years after initial implementation.
- The Mid-Atlantic council plans to review Atlantic mackerel allocations at least every 3 years, spiny dogfish allocations at least every 5 years, and certain other allocations every 10 years.
- The North Pacific council plans to review allocations every 10 years.
- The Pacific council plans to review certain allocations every 1 to 2 years.

The four councils also identified public input as a potential allocation review trigger, but they did not specify what threshold of public interest would trigger a review.

The remaining two councils—the Western Pacific and Caribbean—do not have allocations subject to National Marine Fisheries Service (NMFS) policy requiring councils to establish allocation review criteria, according to NMFS officials.

Source: GAO analysis of council documents and information from NMFS officials. | GAO-20-216.

In contrast, the Gulf of Mexico council's April 2019 policy established time-based triggers as its primary criteria for initiating allocation reviews. Specifically, its policy indicates time intervals of 4 to 7 years for reviewing allocations, depending on the particular fish stock, and identifies the planned month and year for beginning each review. The council's policy also identified public interest as a secondary allocation review trigger but did not specify thresholds for the level or type of public input that would trigger an allocation review. According to the policy, the council is to consider relevant social, economic, and ecological conditions as an intermediate step before determining whether public interest will trigger a review.

According to NMFS' 2016 guidance, periodic review of allocations on a set schedule is in several respects the most simple and straightforward criterion for such a review—it is unambiguous and less vulnerable to political and council dynamics. The guidance also states that time-based triggers for initiating allocation reviews might be most suitable for fisheries where the conflict among sectors or stakeholder groups makes the decision to simply initiate a review so contentious that use of alternative criteria is infeasible. In such a situation, a fixed schedule ensures that periodic reviews occur regardless of political dynamics or specific fishery outcomes, according to the guidance. However, the guidance also indicates that, compared with alternative approaches, adherence to a fixed schedule may be less sensitive to other council priorities and the availability of time and resources to conduct such reviews, which could potentially lead to significant expenditures. Therefore, given the inflexible nature

council meeting, the public comment form on the council's website, and through other more informal channels.

of time-based triggers, the guidance recommends that they be used only in those situations where the benefit of certainty outweighs the costs of inflexibility.

The South Atlantic and Gulf of Mexico councils' policies laid out planned schedules for their respective allocation reviews, which both councils adjusted after issuing their policies. Table 5 shows both councils' plans for allocation reviews as of December 2019. For example, the Gulf of Mexico council's policy states that it plans to review the red grouper allocation in 2026. However, in response to the completion of an updated stock assessment for red grouper in July 2019, the council directed its staff in October 2019 to begin work on a fishery management plan amendment to update the red grouper allocation, according to a council document.[55] The stock assessment for red grouper included the Marine Recreational Information Program's updated estimates for recreational landings. The updated estimates approximately doubled previous estimates of recreational landings, according to a council newsletter. Council staff said that applying these updated estimates to the time series the council had used to establish the red grouper allocation could result in a percentage shift of the allocation to the recreational fishing sector.[56] As a result, the council decided to begin review of the red grouper allocation sooner than the policy's scheduled 2026 time frame, according to the staff.

In addition, we found that the councils' planned allocation review schedules may affect their workload and other priorities, but it is not clear to what extent. NMFS' 2016 allocation guidance states that the councils' allocation review processes should include consideration of current council priorities, other actions under deliberation, and available resources. NMFS officials and council staff expressed concern that the councils' planned schedules—as identified in their April and July 2019 policies—may negatively affect the workloads and other priorities of NMFS' social scientists, economists, and data analysts and council staff. For instance, staff from both councils said the planned allocation review schedules will increase their workloads and, depending on the nature and substance of how those reviews are conducted, could take resources away from other council activities and lead them to reprioritize or delay those activities. One council's staff also noted that the council members have a difficult time keeping up with existing workloads.

Further, NMFS officials stated the councils' accelerated schedules as of December 2019, as shown in Table 5, will exacerbate the concerns. These schedules include starting reviews for 50 allocations in the South Atlantic between 2019 and 2026, assuming no conditions trigger earlier reviews, and reviews for 10 allocations in the Gulf of Mexico between 2019 and 2026.[57] One NMFS official said that any additional workload for economists and social scientists in the Southeast Fisheries Science Center is difficult to anticipate because it will depend on the type of information the councils would like to use for the reviews and whether additional studies may be needed or data collected. Another NMFS official stated that the regional office will shift priorities from less important tasks and gain efficiencies where possible to accommodate the planned allocation reviews.

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[55] The proposed amendment 53 to the fishery management plan for reef fish in the Gulf of Mexico addressed options for red grouper allocations.

[56] According to NMFS and council documents, if the council used the same allocation formula that it used in establishing the allocation for red grouper, but applied the updated Marine Recreational Information Program's estimates from July 2019, then the allocation for the recreational fishing sector would shift from 24 percent to 41 percent, and the allocation for the commercial sector would shift from 76 percent to 59 percent.

[57] In addition, the Gulf of Mexico council plans to review some allocations between gear types, zones, states, or councils, according to the council's policy.

Table 5. South Atlantic and Gulf of Mexico Fishery Management Councils' planned schedules for reviewing mixed-use fish stock allocations, as of December 2019

Year	South Atlantic fish stocks		Gulf of Mexico fish stocks
2019	• Dolphin (mahimahi) • Wahoo • Wreckfish		• Red grouper
2020	• Atlantic spadefish • Bar jack • Black grouper • Blackfin snapper • Blueline tilefish • Golden tilefish • Gray triggerfish • Greater amberjack • Hogfish (Georgia-North Carolina stock)	Grunts complex: • Margate • White grunt Jacks complex: • Almaco jack • Banded rudderfish • Lesser amberjack Porgy complex: • Jolthead porgy • Knobbed porgy • Saucereye porgy	—
2020	• King mackerel, Atlantic group • Red porgy • Scamp • Snowy grouper • Yellowtail snapper Deepwater complex: • Blackfin snapper • Misty grouper • Queen snapper • Sand tilefish • Silk snapper • Yellowedge grouper	• Whitebone porgy Shallow-water groupers complex: • Coney • Graysby • Red hind • Rock hind • Yellowfin grouper • Yellowmouth grouper Snappers complex: • Cubera snapper • Lane snapper	
2021	• Cobia, Gulf group, Florida East Coast Zone • Vermilion snapper		—
2022	• Gag grouper • Spanish mackerel, Atlantic group		—
2023	• Black sea bass • Hogfish (Florida Keys/East Coast Florida stock) • Mutton snapper • Red grouper		• Red snapper (allocations between the private angling and for-hire components of the recreational allocation)
2024	• Red snapper		—
2025	Snappers complex: • Gray snapper		• Gray triggerfish • Greater amberjack • King mackerel
2026			• Gag grouper • Red snapper • Deep water grouper aggregate complex (snowy grouper, speckled hind, warsaw grouper, and yellowedge grouper) • Shallow water grouper aggregate complex (black grouper, scamp, yellowfin grouper, and yellowmouth grouper) • Tilefish aggregate complex (blueline tilefish, golden tilefish, and goldface tilefish)

Legend: — = not applicable. Source: GAO analysis of South Atlantic and Gulf of Mexico Fishery Management Council documents and information from council staff. | GAO-20-216.

Note: The years shown represent the years in which the councils plan to begin—not necessarily complete—their allocation reviews. This table only includes reviews of allocations between the commercial and recreational fishing sectors or within the recreational sector. The Gulf of Mexico Fishery Management Council also plans to review red snapper allocations among the Gulf of Mexico states in 2024; allocations of king mackerel between zones and between gear types in 2025; and allocations between the Gulf of Mexico and South Atlantic Fishery Management Councils for black grouper, mutton snapper, and yellowtail snapper in 2026. According to South Atlantic Fishery Management Council staff, the council plans to use a single fishery management plan amendment in 2020 to review allocations for Atlantic spadefish; bar jack; black grouper; blackfin snapper; gray triggerfish; hogfish (Georgia-North Carolina stock); scamp; and the deepwater, grunts, jacks, porgy, shallow-water groupers, and snappers complexes.

NMFS' and the Councils' Costs of Establishing, Reviewing, or Revising Allocations

Establishing, reviewing, or revising allocations may involve a variety of costs, according to National Marine Fisheries Service (NMFS) officials and South Atlantic and Gulf of Mexico Regional Fishery Management Council (council) staff, such as:

- NMFS and council staff time for collecting data, conducting analyses, and developing recommendations and proposed revisions;
- council staff time for conducting scoping meetings, public workshops, and hearings; and
- travel, document preparation and review, and information dissemination.

NMFS officials and council staff said that factors that may affect these types of costs include the complexity of the analyses, the number of NMFS or council staff involved in the process, and the degree of public interest. Fishery management plan amendments that establish or revise allocations can be controversial, and will likely have more public hearings and opportunity for public comment than other types of amendments, according to NMFS officials and council staff.

NMFS officials and South Atlantic and Gulf of Mexico council staff said they have not tracked costs of establishing, reviewing, or revising allocations. The councils often make allocation decisions concurrently with other management actions, making it difficult to isolate costs.

Source: GAO analysis of information from NMFS officials and staff from the South Atlantic and Gulf of Mexico councils. | GAO-20-216.

Neither Council Has Developed a Process for How to Conduct or Document Allocation Reviews, Although the Gulf of Mexico Council Began Taking Steps to Develop One

The South Atlantic and Gulf of Mexico councils have not developed processes for how they will conduct or document their allocation reviews to implement NMFS' 2016 policy and related guidance, although the Gulf of Mexico council has begun taking steps to do so. As noted, NMFS policy calls for a multi-step process for reviewing and potentially revising fisheries allocations. Specifically, once an allocation review trigger has been met, NMFS policy calls for an allocation review, after which the councils may maintain existing allocations or evaluate allocation options through a fishery management plan amendment. NMFS guidance states that the councils should develop a structured and transparent process for conducting allocation reviews, including consideration of current council priorities, other actions under deliberation, and available resources.

In April 2019, the Gulf of Mexico council began taking steps to develop an allocation review process, according to council documents. Specifically, the Gulf of Mexico council convened an allocation review workgroup consisting of staff from the council and from NMFS' Southeast Regional Office and Southeast Fisheries Science Center. The council expects the workgroup to propose draft allocation review procedures, including identifying data sources that would be needed to conduct allocation reviews, according to a council document. The workgroup met in June and July 2019 and discussed these topics and other potential proposals, such as establishing a tiered system for allocation reviews that would involve different levels of analysis for different tiers of reviews, according to council

documents. Council staff said the workgroup plans to next meet after the issuance of our report to finalize a proposal for developing an allocation review process for the council to consider. However, the council has not indicated what actions it will take, if any, regarding the workgroup's proposal; instead, the council will determine its course of action after reviewing this report, according to council staff.

The South Atlantic council postponed discussion of defining or documenting its allocation review process until March 2020, according to council staff and members, to review our report before deciding any next steps. At the council's June 2019 meeting, the council chair questioned the need for developing an allocation review process through policy. For instance, the chair cited concerns that the council may be continuously developing exceptions to such a policy to accommodate fishery-specific issues or other unique circumstances. The chair also stated that aside from establishing criteria for initiating allocation reviews, NMFS' guidance does not require the councils to take other actions related to developing allocation review processes.

NMFS officials said that the agency's 2016 guidance recommending that the councils develop a structured and transparent process was not intended to require the councils to develop a separate policy or documented process for conducting allocation reviews. NMFS officials said that the agency's operational guidelines for processes under the Magnuson-Stevens Act and associated regional operating agreements with the councils lay out the key requirements and processes guiding development, review, and implementation of fishery management plans and plan amendments, which would include actions related to allocations.[58] The officials further explained that in developing the 2016 allocation policy, they intended that allocation reviews be conducted through the processes identified in the agency's operational guidelines and regional operating agreements with the councils, which allow the councils flexibility to factor in their own needs.

However, the operational guidelines and regional operating agreements for the South Atlantic and Gulf of Mexico councils apply to the fishery management plan and amendment process overall, and they do not specifically address allocations. The goals of the operational guidelines include promoting a timely, effective, and transparent public process for development and implementation of fishery management measures, and the guidelines note that the regional operating agreements are meant to make council procedures and processes transparent. The guidelines and agreements, however, do not lay out processes the councils are to follow in reviewing allocations apart from developing fishery management plans or plan amendments. As noted in NMFS' 2016 policy and guidance, the councils may conduct allocation reviews separate from the fishery management plan amendment process. Moreover, the regional operating agreements are not intended to limit or prevent the councils' use of additional processes in response to specific management needs, according to these documents and the operational guidelines, and the Gulf of Mexico council has taken initial steps in developing an allocation review process as previously described.

Based on the framework for internal controls established by the Committee of Sponsoring Organizations of the Treadway Commission, documented policies and processes can be more difficult to circumvent, less costly to an organization if there is turnover in personnel, and

[58] National Marine Fisheries Service, *Operational Guidelines*; South Atlantic Fishery Management Council, *Operating Agreement*; and Gulf of Mexico Fishery Management Council, *Regional Operating Agreement*.

increase accountability.[59] The framework also states that when subject to external party review, policies and processes would be expected to be formally documented. Among other things, documented processes— according to the framework—promote consistency; assist in communicating the who, what, when, where, and why of internal control execution; enable proper monitoring; and provide a means to retain organizational knowledge and mitigate the risk of having the knowledge within the minds of a limited number of individuals.

The 2012 report commissioned by NMFS to review fisheries allocation issues found that allocation reviews had not been done in a regular, consistent manner and stated that this makes it harder for stakeholders to understand the reviews as well as the process for conducting them.[60] Similarly, stakeholders we interviewed indicated that a clear process for conducting allocation reviews is needed and would increase their confidence in or understanding of the councils' decisions, regardless of specific outcomes.[61] Other stakeholders stressed the need for predictability and certainty to be able to plan critical business decisions, such as securing loans from local banks or other lenders. Such uncertainty may cause participants in the commercial sector to leave the fishery because they cannot secure loans or meet other business requirements, according to one stakeholder, or it may create instability that could affect the market price of fish, according to another stakeholder. By working with the councils to develop documented allocation review processes, NMFS would have better assurance that the councils carry out their upcoming allocation reviews in a structured and transparent manner, consistent with the agency's 2016 guidance.

Further, it is unclear whether or how the councils plan to document each allocation review, such as the basis for their allocation decisions, whether fishery management plan objectives are being met, and what factors were considered in each review.[62] NMFS' operational guidelines state that fishery management decisions must be supported by a record providing the basis for the decision. In addition, NMFS' 2016 policy and guidance call for the councils to clearly articulate in their allocation reviews how fishery management plan objectives are or are not being met, as well as to document their rationale for determining whether any factors are unimportant or not applicable in making an allocation decision.

NMFS officials and council staff said that any allocation revisions would be documented through fishery management plan amendments. However, the councils may conduct allocation reviews separate from the fishery management plan amendment process, and it is not clear whether or how the councils will document those reviews. For example, as previously noted, in the past the South Atlantic council has not formally documented the results of allocation reviews that did not lead to fishery management plan amendments that

[59] Committee of Sponsoring Organizations of the Treadway Commission, *Internal Control- Integrated Framework*.
[60] Lapointe, *Marine Fishery Allocation Issues*.
[61] These stakeholder views are consistent with our past work on effective stakeholder participation in fisheries management, in which we found that using a clearly defined decision-making process helps provide transparency and gives stakeholders clear expectations about how decisions will be made, enhancing understanding and trust in the organization's decisions. See GAO, *Fisheries Management: Core Principles and a Strategic Approach Would Enhance Stakeholder Participation in Developing Quota-Based Programs*, GAO-06-289 (Washington, D.C.: Feb. 23, 2006).
[62] According to South Atlantic council staff, the council has begun using a spreadsheet to track allocation discussions and plans to develop an electronic tracking system. The spreadsheet tracks allocation percentages and dates of associated fishery management plan amendments, and it will capture future discussions of potential allocation revisions, according to council staff. However, the spreadsheet does not contain information on the basis for the council's decisions, whether fishery management plan objectives are being met, and what factors were considered in reviewing the allocations.

revised the allocations. By working with the councils to specify how they plan to document their allocation reviews, NMFS could help ensure that the councils provide a clear record of the basis for their decisions, whether fishery management plan objectives are being met, and applicable factors considered. Clear records could also help increase transparency and stakeholder understanding of the councils' decisions, particularly in those instances when reviews are separate from the fishery management plan amendment process.

CONCLUSION

Making allocation decisions between the commercial and recreational fishing sectors can be complex and difficult, and the outcomes of those decisions may have important economic and social implications for stakeholders in each of the sectors. The South Atlantic and Gulf of Mexico councils have taken an important step in developing policies outlining criteria for initiating allocation reviews, in accordance with NMFS guidance. The Gulf of Mexico council has also taken initial steps to define how it will conduct its allocation reviews. However, neither council has developed a process for how they will conduct such reviews. By working with the councils to develop documented allocation review processes, NMFS would have better assurance that the councils carry out their upcoming allocation reviews in a structured and transparent manner, consistent with the agency's 2016 guidance. Moreover, by working with the councils to also specify how they plan to document their allocation reviews, NMFS could help ensure that the councils provide a clear record of the basis for their decisions, whether fishery management plan objectives are being met, and applicable factors considered.

RECOMMENDATIONS FOR EXECUTIVE ACTION

We are making the following two recommendations to the NMFS Assistant Administrator for Fisheries:

The NMFS Assistant Administrator for Fisheries should work with the South Atlantic and Gulf of Mexico councils, and other councils as appropriate, to develop documented processes for conducting allocation reviews. (Recommendation 1)

The NMFS Assistant Administrator for Fisheries should work with the South Atlantic and Gulf of Mexico councils, and other councils as appropriate, to specify how the councils will document their allocation reviews, including the basis for their allocation decisions, whether fishery management plan objectives are being met, and what factors were considered in the reviews. (Recommendation 2)

AGENCY COMMENTS AND OUR EVALUATION

We provided a draft of this report to the Department of Commerce for review and comment. In written comments (reproduced in app. II), Commerce and NOAA agreed with our recommendations and stated that NOAA's NMFS will work to implement them to the

extent possible. NOAA stated that the report accurately describes the extent to which the councils established and revised allocations for mixed-use fisheries, the key sources of information that may be available for reviewing allocations, and the extent to which the councils have developed processes to help guide such reviews. NOAA also highlighted the delicate balance that councils seek to achieve in deciding what fishery management approaches to implement to comply with the Magnuson-Stevens Act and its 10 national standards.

In addition, Commerce and NOAA stated that NMFS does not have the legal authority to direct the councils to take the actions included in our two recommendations, stating that such actions are outside of legal requirements that guide council fishery management actions. In response, we revised the wording of our two recommendations to state that the NMFS Assistant Administrator for Fisheries should "work with," rather than "direct," the councils to take the recommended actions.

In response to our first recommendation, NOAA stated that it would build on the recommendations in its allocation policy by working with the South Atlantic and Gulf of Mexico councils, and other councils as appropriate, to develop documented processes for conducting allocation reviews. In response to our second recommendation on specifying how the councils will document their allocation reviews, NOAA stated that it will work with the councils on consistent documentation of allocation reviews. NOAA noted that transparency in the allocation process improves with a documented process for conducting allocation reviews, and that consistent documentation of those reviews will create further transparency in the allocation process and could improve stakeholders' understanding of the councils' decisions. NOAA also provided technical comments, which we incorporated as appropriate.

Anne-Marie Fennell
Director, Natural Resources and Environment

APPENDIX I: MIXED-USE FISHERIES ALLOCATIONS IN THE SOUTH ATLANTIC AND GULF OF MEXICO FISHERY MANAGEMENT COUNCIL REGIONS

Tables 6 and 7 provide information on mixed-use fisheries allocations— privileges for catching fish between the commercial and recreational fishing sectors—in the South Atlantic and Gulf of Mexico Fishery Management Council (council) regions, respectively.[63] Not all mixed-use fish stocks in these regions have allocations. In the South Atlantic council region,

[63] The National Marine Fisheries Service (NMFS) defines an allocation of fishing privileges as a direct and deliberate distribution of the opportunity to participate in a fishery among identifiable, discrete user groups or individuals. 50 C.F.R. § 600.325(c)(1). In our report, we consider for-hire fishing (both charter fishing and head boats) to be part of the recreational fishing sector because the South Atlantic and Gulf of Mexico councils generally manage for-hire fishing as part of the recreational sector, according to council staff. A fishery refers to one or more fish stocks that can be treated as a unit for conservation and management purposes and that are identified on the basis of geographical, scientific, technical, recreational, and economic characteristics. A fish stock refers to a species, subspecies, geographical grouping, or other category of fish capable of management as a unit. A fish stock may be one species or a complex of comparable species.

spiny lobster does not have an allocation.[64] In the Gulf of Mexico council region, 14 of 23 mixed-use fish stocks do not have allocations.[65]

Table 6. Mixed-use fish stock allocations in the South Atlantic Fishery Management Council Region, as of December 2019

		Initial allocations			Revised allocations	
Fish stock	Year	Commercial (percent)	Recreational (percent)	Year	Commercial (percent)	Recreational (percent)
Atlantic spadefish	2011	12.90	87.10	2012	18.53	81.47
Bar jack	2011	32.58	67.42	2012	21.25	78.75
Black grouper[a]	2011	36.88	63.12	—	—	—
Black sea bass	2006	43.00	57.00	—	—	—
Blueline tilefish	2011	47.39	52.61	2012	50.07	49.93
Cobia, Gulf group, Florida East Coast Zone	2014	8.00	92.00	—	—	—
Dolphin (mahimahi)	2003	13.00	87.00	2011 2013 2015	7.30 7.54 10.00	92.70 92.46 90.00
Gag grouper	2008	51.00	49.00	—	—	—
Golden tilefish	2010	97.00	3.00	—	—	—
Gray triggerfish	2011	45.39	54.61	2012	43.56	56.44
Greater amberjack	2011	40.66	59.34	—	—	—
Hogfish[b]	2011	33.03	66.97	2012 2016[c] 2016[d]	36.69 69.13[c] 9.63[d]	63.31 30.87[c] 90.37[d]
King mackerel, Atlantic group	1985	37.10	62.90	—	—	—
Mutton snapper	2011	17.02	82.98	—	—	—
Red grouper	2011	44.00	56.00	—	—	—
Red porgy	2008	50.00	50.00	—	—	—
Red snapper	2011	28.07	71.93	—	—	—
Scamp	2011	69.36	30.64	2012	65.34	34.66
Snowy grouper	2008	95.00	5.00	2014	83.00	17.00
Spanish mackerel, Atlantic group	1987	76.00	24.00	1989 1998	50.00 55.00	50.00 45.00
Speckled hind[e]	2011	65.59	34.41	—	—	—
Vermilion snapper	2008	68.00	32.00	—	—	—
Wahoo	2011	4.30	95.70	2013	3.93	96.07
Warsaw grouper[e]	2011	17.79	82.21	—	—	—
Wreckfish	2011	95.00	5.00	—	—	—
Yellowtail snapper	2011	52.56	47.44	—	—	—
Fish stocks managed as part of a complex						
Deepwater complex						
Blackfin snapper	2011	31.68	68.32	2012	29.91	70.09
Misty grouper	2011	70.91	29.09	2012	83.42	16.58

[64] South Atlantic council staff said spiny lobster does not have an allocation because fishing primarily occurs in the waters off Florida, where the state takes the lead in regulating this fishery through a protocol developed with NMFS and the South Atlantic and Gulf of Mexico councils. Outside of these Florida state-managed waters, spiny lobster fishing is subject to a two lobsters-per-person, per-trip catch limit, according to a council document.

[65] Specifically, the Gulf of Mexico council has not established allocations for the following mixed-use fish stocks: (1) cobia; (2) corals; (3) cubera, (4) gray, (5) lane, (6) mutton, (7) vermillion, and (8) yellowtail snapper; (9) goliath grouper; (10) hogfish; (11) Spanish mackerel; (12) spiny lobster; (13) the Jacks complex (almaco jack, banded rudderfish, and lesser amberjack); and (14) the mid-water snapper complex (blackfin snapper, queen snapper, silk snapper, and wenchman).

Table 6. (Continued)

		Initial allocations			Revised allocations	
Fish stock	Year	Commercial (percent)	Recreational (percent)	Year	Commercial (percent)	Recreational (percent)
Queen snapper	2011	93.12	6.88	2012	92.50	7.50
Sand tilefish	2011	16.22	83.78	2012	22.17	77.83
Silk snapper	2011	73.14	26.86	2012	73.95	26.05
Yellowedge grouper	2011	96.19	3.81	2012	90.77	9.23
Grunts complex						
Margate	2011	19.83	80.17	2012	18.88	81.12
White grunt	2011	32.67	67.33	2012	31.59	68.41
Jacks complex						
Almaco jack	2011	51.53	48.47	2012	48.70	51.30
Banded rudderfish	2011	25.25	74.75	2012	26.01	73.99
Lesser amberjack	2011	46.62	53.38	2012	46.07	53.93
Porgy complex						
Jolthead porgy	2011	4.05	95.95	2012	4.15	95.85
Knobbed porgy	2011	54.12	45.88	2012	51.18	48.82
Saucereye porgy[f]	2011	0.01	99.99	2012	0.01	99.99
Whitebone porgy	2011	0.96	99.04	2012	1.05	98.95
Shallow-water groupers complex						
Coney	2011	23.26	76.74	2012	24.45	75.55
Graysby	2011	14.48	85.52	2012	15.74	84.26
Red hind	2011	73.28	26.72	2012	73.60	26.40
Rock hind	2011	62.54	37.46	2012	60.90	39.10
Yellowfin grouper	2011	40.78	59.22	2012	52.70	47.30
Yellowmouth grouper	2011	1.35	98.65	2012	1.10	98.90
Snappers complex						
Cubera snapper	2011	19.75	80.25	2012	19.57	80.43
Gray snapper	2011	20.00	80.00	2012	24.23	75.77
Lane snapper	2011	12.21	87.79	2012	14.75	85.25

Legend: — = not applicable.

Source: GAO analysis of South Atlantic Fishery Management Council documents and information from council staff. | GAO-20-216.

Note: This table includes fish stocks that have allocations between the commercial and recreational fishing sectors. Fish stocks listed by complex are managed together as a group. For this report, we count a complex as a single fish stock if the allocation is for the stock complex, rather than for the individual stock within the complex. If the fish stocks within a complex each have their own allocations, we count them as separate fish stocks for reporting purposes. The years shown represent the year the council completed a fishery management plan amendment and sent it to the National Marine Fisheries Service (NMFS) for review and approval. The South Atlantic Fishery Management Council has set allocation percentages to two decimal places, as indicated in this table.

[a]Prior to the initial allocations shown in this table, NMFS and the South Atlantic Fishery Management Council managed black grouper, red grouper, and gag grouper as a complex, including establishing one combined allocation for the three fish stocks.

[b]In 2016, the South Atlantic Fishery Management Council split the South Atlantic hogfish fish stock into two and established allocations for Georgia-North Carolina hogfish and Florida Keys/East Coast of Florida hogfish.

[c]Allocation shown is for the Georgia-North Carolina hogfish stock.

[d]Allocation shown is for the Florida Keys/East Coast of Florida hogfish stock.

[e]Harvest is not allowed for speckled hind and warsaw grouper as of December 2019.

[f]Saucereye porgy in the South Atlantic is, in practice, a recreational fish stock, according to South Atlantic Fishery Management Council staff. Council staff indicated that because the total annual catch limit for the fish stock is low, there is no commercial fishing in practice for the stock.

Table 7. Mixed-use fish stock allocations in the Gulf of Mexico Fishery Management Council Region, as of December 2019

		Initial allocations			Revised allocations	
Fish stock	Year	Commercial (percent)	Fish stock	Year	Commercial (percent)	Fish stock
Gag grouper	1989	35	65	2008	39	61
Gray triggerfish	2008	21	79	—	—	—
Greater amberjack	1989	16	84	2008[a]	27[a]	73[a]
King mackerel, Gulf group	1985	32	68	—	—	—
Red grouper	1989	77	23	2008	76	24
Red snapper[b]	1989	51	49	[c]	[c]	[c]
Fish stocks managed as part of a complex[d]						
Deep water grouper aggregate complex (snowy grouper, speckled hind, warsaw grouper, and yellowedge grouper)	2011	96.4	3.6	—	—	—
Shallow water grouper aggregate complex (black grouper, scamp, yellowfin grouper, and yellowmouth grouper)	2011	77.0	23.0	—	—	—
Tilefish aggregate complex (blueline tilefish, golden tilefish, and goldface tilefish)	2011	99.7	0.3	—	—	—

Legend: — = not applicable.

Source: GAO analysis of Gulf of Mexico Fishery Management Council documents and information from council staff and National Marine Fisheries Service (NMFS) officials. | GAO-20-216.

Note: This table includes fish stocks that have allocations between the commercial and recreational fishing sectors. Fish stocks listed by complex are managed together as a group. For this report, we count a complex as a single fish stock if the allocation is for the stock complex, rather than for the individual stock within the complex. If the fish stocks within a complex each have their own allocations, we count them as separate fish stocks for reporting purposes. The years shown represent the year the council completed a fishery management plan amendment and sent it to the National Marine Fisheries Service for review and approval.

[a]For greater amberjack, the council did not revise the allocation directly; instead, the council indirectly revised the commercial and recreational allocations by establishing harvest reductions that were applied unequally to these fishing sectors, according to a 2008 fishery management plan amendment.

[b]In addition, in 2014 the Gulf of Mexico Fishery Management Council finalized a fishery management plan amendment that established an allocation between the private angling and for-hire components of the recreational allocation for red snapper. This resulted in a private angling allocation of 57.7 percent of the recreational allocation and a for-hire fishing allocation of 42.3 percent of the recreational allocation.

[c]In 2015, the Gulf of Mexico Fishery Management Council completed a fishery management plan amendment that revised the red snapper allocation to 48.5 percent commercial and 51.5 percent recreational. However, after the Secretary of Commerce approved the amendment in 2016, a U.S. District Court vacated the amendment in 2017 and the council returned to the initial allocation established for red snapper. *See Guindon v. Pritzker*, 240 F. Supp. 3d 181 (D.D.C. 2017).

[d]Fish stocks managed as part of a complex in the Gulf of Mexico do not have individual allocation percentages for each fish stock. Instead, the Gulf of Mexico Fishery Management Council established allocation percentages for each complex as a whole, based on quotas for commercial fishing established for these complexes. Recreational allocation percentages for the complexes represent the remainder of allowable harvest, after factoring in quota amounts, according to NMFS officials. Allocation percentages for these complexes are presented to one decimal point to reflect percentages provided by the Gulf of Mexico Fishery Management Council.

APPENDIX II: COMMENTS FROM THE DEPARTMENT OF COMMERCE

UNITED STATES DEPARTMENT OF COMMERCE
The Secretary of Commerce
Washington, D.C. 20230

March 17, 2020

Ms. Anne-Marie Fennell
Director
Natural Resources and Environment
U.S. Government Accountability Office
441 G Street, NW
Washington, DC 20548

Dear Ms. Fennell:

Thank you for the opportunity to review and comment on the Government Accountability Office's (GAO) draft report *Mixed-Use Fisheries: South Atlantic and Gulf of Mexico Councils Would Benefit from Documented Processes for Allocation Reviews* (GAO-20-216).

The Department of Commerce agrees with GAO's two recommendations regarding the National Oceanic and Atmospheric Administration (NOAA) and will work to implement them to the extent possible. Specifically, NOAA's National Marine Fisheries Service (NMFS) will work with the fishery management councils on these recommendations; however, neither the Department nor NMFS has the authority to direct councils to take action. Enclosed is our response and recommended technical changes to the draft report.

If you have any further questions, please contact MaryAnn Mausser, GAO Liaison, at (202) 482-8120 or mmausser@doc.gov.

Sincerely,

Wilbur Ross

Wilbur Ross

Enclosure

Department of Commerce
National Oceanic and Atmospheric Administration
Response to the GAO Draft Report Entitled
Mixed-Use Fisheries: South Atlantic and Gulf of Mexico Councils Would Benefit from Documented Processes for Allocation Reviews
(GAO-20-216, March 2020)

General Comments

The Department of Commerce's National Oceanic and Atmospheric Administration (NOAA) appreciates the opportunity to review the Government Accountability Office's (GAO) report. GAO does a thorough job in reviewing allocations for mixed-use fisheries in the South Atlantic and Gulf of Mexico. The report accurately describes the extent to which the fishery management councils (councils) established and revised allocations to mixed-use fisheries, the key sources of information that may be available for reviewing allocations, and the extent to which the councils have developed processes to help guide such reviews.

We appreciate the report's acknowledgment of the National Marine Fisheries Service's (NMFS) 2016 Allocation Policy, which required councils to identify allocation triggers for all fisheries with an allocation, including allocations to mixed-use fisheries. The recommendations from this report (see below) reiterate the importance of this policy and request documentation of the process and results consistent with the policy. We also appreciate the report's discussion of the various data challenges, in particular for economic analyses, as well as acknowledgment of NMFS efforts to address these challenges such as our upcoming best practices manual on conducting economic analyses for use in allocation decisions.

We would like to highlight the delicate balance that councils seek to achieve in deciding what fishery management approaches to implement so that there is compliance with all ten national standards and other Magnuson-Stevens Fishery Conservation and Management Act (MSA) requirements. When considering the different means by which the conservation goals of the MSA can be achieved, the councils can consider the potential trade-offs between the national standards. For example, maintaining employment may be in conflict with improving economic efficiency. Similarly, long-term goals related to rebuilding stocks may also be in conflict with short-term goals of minimizing impacts on fishery-dependent communities. Updated and measurable objectives help clarify decisions about these trade-offs within and between fishery management plans (FMP).

NOAA Response to GAO Recommendations

The draft GAO report made two recommendations:

Recommendation 1: "The NMFS Assistant Administrator for Fisheries should direct the South Atlantic and Gulf of Mexico councils, and other councils as appropriate, to develop documented processes for conducting allocation reviews."

NOAA Response: Building on recommendations in the Allocation Policy, we will work with the South Atlantic and Gulf of Mexico Councils, and other councils as appropriate, to develop documented processes for conducting allocation reviews. However, NOAA lacks authority to "direct" the council to take such action as it is outside of legal requirements that guide council fishery management actions. We suggest that GAO reword the recommendation to say, "The NMFS Assistant Administrator for Fisheries should work with…" instead of "should direct."

Note that a fishery allocation review is the initial evaluation that leads to the decision of whether or not to maintain current allocations or develop alternative allocation options, as described in the Allocation Policy. It does not require in-depth data and analyses and is not, in and of itself, an implicit trigger to consider alternative allocations. Because they may or may not lead to a fishery management action, these initial reviews are not currently documented in a consistent way and could benefit from established or documented processes. If following an initial allocation review, a council proceeds with a formal analysis to consider alternative allocations, this would occur through the fishery management plan process. These processes are well established and documented through the agency's Operational Guidelines and associated Regional Operating Agreements.

Transparency in the allocation process improves with a documented process for conducting allocation reviews. NOAA looks forward to working with the councils in implementing these recommendations.

Recommendation 2: "The NMFS Assistant Administrator for Fisheries should direct the South Atlantic and Gulf of Mexico councils, and other councils as appropriate, to specify how the councils will document their allocation reviews, including the basis for their allocation decisions, whether fishery management plan objectives are being met, and what factors were considered in the reviews."

NOAA Response: We will work with the South Atlantic and Gulf of Mexico Councils, and other councils as appropriate, on consistent documentation of allocation reviews. However, NOAA lacks authority to "direct" the council to take such action as it is outside of legal requirements that guide council fishery management actions. We suggest that GAO reword the recommendation to say, "The NMFS Assistant Administrator for Fisheries should work with…" instead of "should direct."

Consistent documentation of allocation reviews, including timing, factors considered, summaries of how the fishery is or is not meeting its objectives, and the basis of final decisions to keep status quo or proceed to the FMP amendment process will create further transparency in the allocation process. Clear records could also improve stakeholder understanding of the councils' decisions, particularly in those instances when reviews do not result in pursuit of alternative allocations through the FMP amendment process. NOAA looks forward to working with the councils in implementing these recommendations.

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